OARAC | OFFICE OF AIDS RESEARCH ADVISORY COUNCIL

FORTY-SECOND MEETING | APRIL 7, 2016

U.S. Department of Health and Human Services
National Institutes of Health

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
NATIONAL INSTITUTES OF HEALTH
OFFICE OF AIDS RESEARCH

OFFICE OF AIDS RESEARCH ADVISORY COUNCIL MEETING

April 07, 2016

TABLE OF CONTENTS

Agenda

Roster and Seating Chart

Minutes from November 2015 Meeting

Introduction to OARAC Topic

- The Controlled Clinical Trial: Theory and Practice
- Blocking HIV-1 Transmission in the Female Reproductive Tract: From Microbicide Development to Exploring Local Antiviral Responses
- Pre-exposure Prophylaxis for Human Immunodeficiency Virus
- The Blind Leading the Blind: Use and Misuse of Blinding in Randomized Controlled Trials

Background Materials for Morning Presentations

Microbicides and PrEP Studies: What Have We Learned

- Pre-Exposure Chemoprophylaxis for HIV Prevention in Men Who Have Sex With Men
- Antiretroviral Prophylaxis for HIV Prevention in Heterosexual Men and Women
- Rectal Pre-Exposure Prophylaxis (PrEP)
- Use of a Vaginal Ring Containing Dapivirine for HIV-1 Prevention in Women

How Good is Good Enough: Efficacy vs. Effectiveness

- The "Efficacy-Effectiveness Gap": Historical Background and Current Conceptualization
- Apples and Oranges? Interpreting Success in HIV Prevention Trials
- Statistical Issues in Trials of Pre-Exposure Prophylaxis

Designing Products That People Will Use: If We Build It, Will They Come?

- Beyond "Getting Drugs Into Bodies": Social Science Perspectives on Pre-Exposure Prophylaxis for HIV
- Combination HIV Prevention Interventions: The Potential of Integrated Behavioral and Biomedical Approaches
- Patient Preferences for Injectable Treatments for Multiple Sclerosis in the United States: A Discrete-Choice Experiment

Current Challenges in Biomedical Prevention Research

Pharmacokinetic-Pharmacodynamic Relationships in HIV Prevention: Where Does The Drug Need To Be

- Pharmacokinetics of Antiretrovirals in Mucosal Tissue
- Drug Transporters in Tissues and Cells Relevant to Sexual Transmission of HIV: Implications for Drug Delivery
- A Translational Pharmacology Approach to Predicting HIV Pre-Exposure Prophylaxis Outcomes in Men and Women Using Tenofovir Disoproxil Fumarate=Emtricitabine
- Pharmacokinetics and Pharmacodynamics in HIV Prevention; Current Status and Future Directions: A Summary of the DAIDS and BMGF Sponsored Think Tank on Pharmacokinetics (PK)/Pharmacodynamics (PD) in HIV Prevention

Background Materials for Afternoon Presentations

Moving Forward In HIV Prevention

Non-ARV-Based Products for HIV Prevention

- Prevention of Vaginal SHIV Transmission in Macaques by a Live Recombinant Lactobacillus
- Broadly Neutralizing Anti-HIV Antibodies Prevent HIV Infection of Mucosal Tissue Ex Vivo
- Investigation of Griffithsin's Interactions With Human Cells Confirms Its Outstanding Safety and Efficacy Profile as a Microbicide Candidate

Long Acting ARVs

- Drug Delivery Strategies and Systems for HIV/AIDS Pre-Exposure Prophylaxis and Treatment
- Physiologically Based Pharmacokinetic Modelling to Inform Development of Intramuscular Long-Acting Nanoformulations for HIV
- Formulation and Pharmacology of Long-Acting Cabotegravir
- New Approaches to Antiretroviral Drug Delivery: Challenges and Opportunities Associated with the Use of Long-Acting Injectable Agents
- The Promise and Pitfalls of Long-Acting Injectable Agents for HIV Prevention

Topical Formulations: Options & Challenges

- Formulation and Delivery of Microbicides
- Microbicide Vaginal Rings: Technological Challenges and Clinical Development

Nano-Formulations for Local and Injectable Use

- Nanoparticle-Based ARV Drug Combinations for Synergistic Inhibition of Cell-Free and Cell-Cell HIV Transmission
- A Review of Nanotechnological Approaches for the Prophylaxis of HIV/AIDS
- Nanotech-Derived Topical Microbicides for HIV Prevention: The Road to Clinical Development

Novel Approaches to Measuring Adherence

- Strong Correlation Between Concentrations of Tenofovir (TFV) Emtricitabine (FTC) in Hair
- Strong Relationship Between Oral Dose and Tenofovir Hair Levels in a Randomized Trial: Hair as a Potential Adherence Measure for Pre-Exposure Prophylaxis (PrEP)
- Emtricitabine-Tenofovir Exposure and Pre-Exposure Prophylaxis Efficacy in Men Who Have Sex With Men
- Pre-Exposure Prophylaxis (PrEP) for HIV Infection: How Antiretroviral Pharmacology Helps to Monitor and Improve Adherence

Trial Design in the Era of PrEP and TasP

- Study Design Considerations for Evaluating Efficacy of Systemic Preexposure Prophylaxis Interventions
- Statistical Issues in Trials of Preexposure Prophylaxis

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
NATIONAL INSTITUTES OF HEALTH
OFFICE OF AIDS RESEARCH

OFFICE OF AIDS RESEARCH ADVISORY COUNCIL MEETING

April 7, 2016
5601 Fishers Lane Conference Center
Rockville, Maryland

DRAFT AGENDA

MICROBICIDES AND PREP: THE NEXT STEPS

OPEN TO THE PUBLIC

MORNING

8:30 – 8:40 AM **Welcome and Meeting Overview**
Dr. Roy M. Gulick, Weill Medical College of Cornell University

8:40 – 8:50 AM **Report from the OAR Acting Director**
Dr. Robert W. Eisinger, Office of AIDS Research

8:50 – 9:05 AM **Update on OARAC Working Groups for Treatment and Prevention Guidelines**
Dr. Jonathan E. Kaplan, Centers for Disease Control and Prevention

9:05 – 9:15 AM **Introduction to OARAC Topic**
Dr. Gina Brown, Office of AIDS Research

9:15 – 9:45 AM **Microbicides and PrEP Studies: What Have We Learned?**
Dr. Sharon L. Hillier, University of Pittsburgh

9:45 – 10:15 AM **How Good is Good Enough? Efficacy vs. Effectiveness**
Dr. Elizabeth R. Brown, Fred Hutchinson Cancer Research Center, University of Washington

10:15 – 10:30 AM ***Discussion***

10:30 – 10:45 AM *Break*

10:45 – 11:15 AM **Designing Products That People Will Use: If We Build It, Will They Come?**
Dr. Cynthia Grossman, FasterCures

CURRENT CHALLENGES IN BIOMEDICAL PREVENTION RESEARCH

11:15 – 11:45 AM **Pharmacokinetic-Pharmacodynamic Relationships in HIV Prevention: Where Does The Drug Need To Be?**
Dr. Angela Kashuba, University of North Carolina at Chapel Hill

11:45 – 12:00 PM ***Discussion***

12:00 – 1:00 PM *Lunch*

MOVING FORWARD IN HIV PREVENTION

1:00 – 1:30 PM **Non-ARV-Based Products for HIV Prevention**
Dr. James A. Turpin, National Institute of Allergy and Infectious Diseases

1:30 – 2:00 PM **Long Acting ARVs**
Dr. Charles W. Flexner, Johns Hopkins University

2:00 – 2:30 PM **Topical Formulations: Options & Challenges**
Dr. Lisa C. Rohan, University of Pittsburgh

2:30 – 3:00 PM **Nano-Formulations for Local and Injectable Use**
Dr. Kimberly A. Woodrow, University of Washington

3:00 – 3:15 PM *Break*

3:15 – 3:45 PM **Novel Approaches to Measuring Adherence**
Dr. Monica Gandhi, University of California, San Francisco

3:45 – 4:15 PM **Trial Design in the Era of PrEP and TasP**
Dr. Deborah J. Donnell, Fred Hutchinson Cancer Research Center

4:15 – 4:30 PM ***Discussion***

4:30 – 4:40 PM *Public Comments*

4:40 – 5:00 PM **Summary of Discussion and Next Steps**
Dr. Roy M. Gulick, Weill Medical College of Cornell University

5:00 PM *Adjourn*

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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OFFICE OF AIDS RESEARCH

OFFICE OF AIDS RESEARCH ADVISORY COUNCIL

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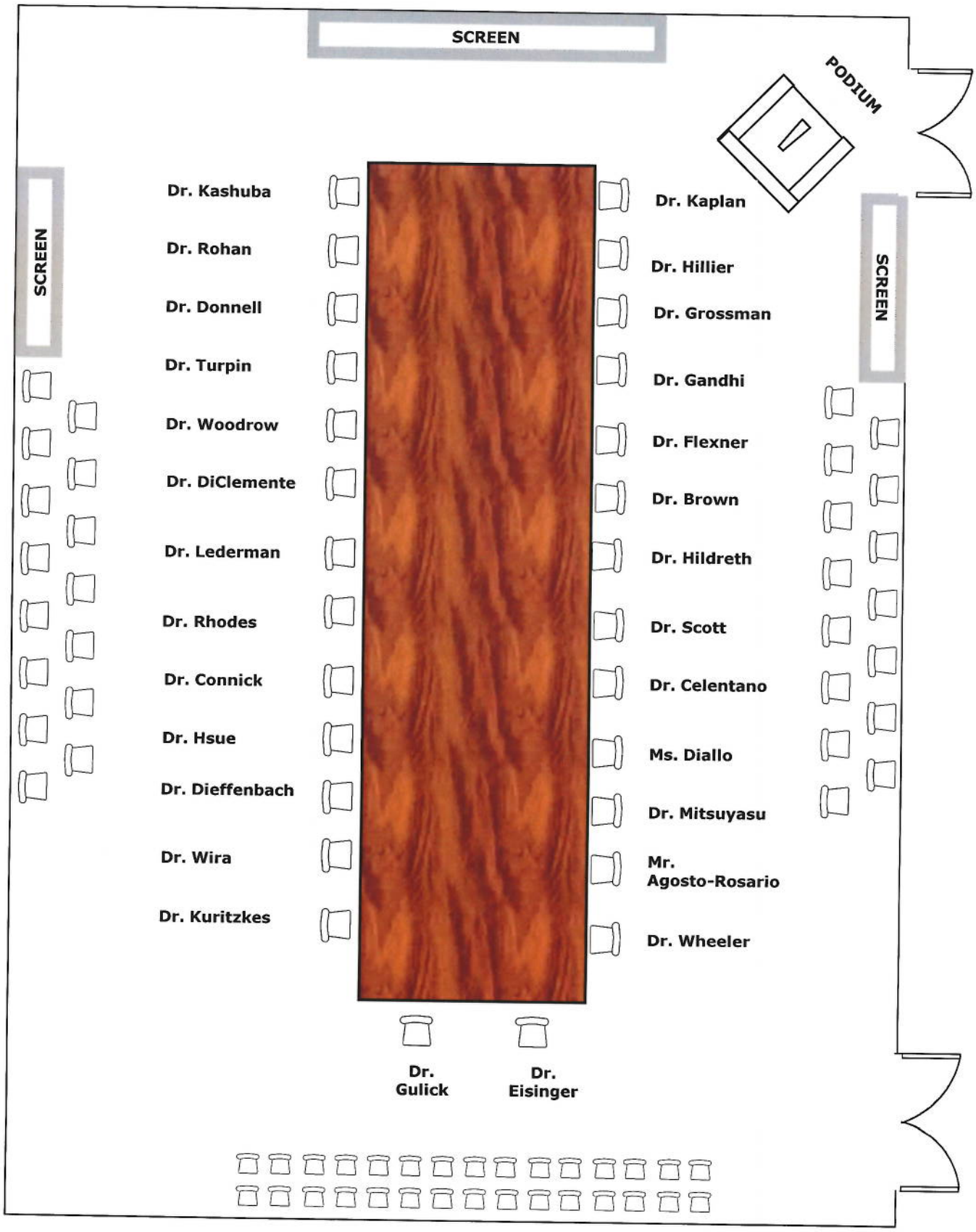

Introduction to OARAC Topic

Gina Brown, M.D.
Office of AIDS Research
National Institutes of Health

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Original Communications

THE CONTROLLED CLINICAL TRIAL: THEORY AND PRACTICE*

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BALTIMORE, MD.

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(Received for publication Nov. 3, 1954.)

The doctor of today is under constant bombardment with claims as to the efficacy of drugs, old and new. It is difficult, if not impossible, to read a journal, attend a medical meeting, or open the morning mail without encountering a new report on the success or failure of some medication. The clinician who would avoid nihilistic rejection or trusting acceptance of all such claims, or capricious decisions as to their merits, is well advised to adopt a yardstick, a set of criteria, that will improve his chances of making sound evaluations. The investigator is equally well advised to do so. The present review will present one such set of guiding principles. In so doing it will pursue two main lines of emphasis. On the one hand, fundamental principles will be accented, not only to provide a framework for subsequent discussion, but to distinguish clearly between the important primary considerations and less essential embellishments and refinements of technique. On the other hand, these same principles will

*Presented on Oct. 30, 1954, at a Fourth Year Therapeutic Conference, The Johns Hopkins University School of Medicine, Baltimore.

be scrutinized in detail and the complexities in their application in actual experiments described. This second line of emphasis is needed to avoid painting a picture of deceptive simplicity and to support the thesis that a doctrinaire insistence on literal and complete adherence to the principles *under all circumstances* can be achieved only by a cavalier dismissal of useful data or by the practice of self-delusion.

It is possible to summarize the fundamentals of the clinical trial[1-4] in the form of five principles. These are: (1) the cooperation of individuals with appropriate skills at all temporal levels of planning and execution; (2) randomization; (3) the "double blind" control; (4) statistical treatment of data; and (5) cautious generalization. Let us consider each principle separately.

A. THE JOINT NATURE OF THE CLINICAL TRIAL

Ideally, the clinical trial represents a fusion of talents, with different individuals contributing to the efficiency and quality of the experiment. This is merely the old principle of employing specialists when needed. Abuse of this principle is inadvisable at most times, but is especially foolhardy when an extensive, expensive, time-consuming project is planned. Although the make-up of such a team should obviously be appropriate to the experiment, a typical group might include a clinician, a pharmacologist, and a statistician (or multiples thereof). There are certain functions performed by the entire group, such as formulating a precise statement of the purpose of the clinical trial. Let us examine some of the particular functions of each member, however. It is currently fashionable in some circles to consider the clinician member of the team as some sort of minor excrescence, "a fifth cousin about to be removed." Such an attitude is arrant nonsense. The clinician is still in most such investigations the prime mover. It is generally he who is aware of the diseases requiring better therapy, of the symptoms inadequately treated. The clinician is also responsible usually for delineating the criteria for success or failure, and for devising methods for the gathering of pertinent data. It is the clinician who provides the all-important background for the clinical trial—knowledge, accumulated over the years, concerning the process under study. An example of the usefulness of this function may help to clarify the point. If one considers the field of antihypertensive drugs, it is apparent how helpful the body of knowledge on hypertension can be in setting up a clinical trial. How much more difficult the task would be, for example, did we not know that elevated blood pressure is a nonspecific manifestation of many disease processes; that even within the field of "essential" hypertension the prognosis varies from decades of unimpaired well-being to the brief downhill course of "malignant" hypertension; that the crucial body areas in this disease are the heart, the brain, and the kidneys. All this information enables the members of the team to focus on the appropriate goals of therapy, and points up the importance of such factors as patient selection and duration of study.

In addition, the clinician is at times in the enviable position of being able to make a real contribution to therapeutics without much outside assistance. I have in mind such disease processes as tuberculous meningitis, or acute staphy-

lococcal septicemia, or acute leukemia, where past experience indicates a uniformly poor prognosis in the untreated case. If, then, a physician uses Drug A on a patient known to have such a disease and the patient is quickly and completely restored to permanent good health, the event is one of major importance. To criticize such a felicitous physician, who calls the case to the attention of his colleagues, because of a lack of placebo controls, or for not having a large series of similar cases, represents a naive display of pharmacologic Philistinism.*

The clinician should also bear the primary responsibility for reminding his associates of the ethical considerations involved in a proposed clinical trial. Let us suppose, for example, that two antibiotics are available, and that the laboratory evidence is conflicting as to whether the two drugs, when used together, are synergistic or antagonistic. In the case of an infectious disease for which there is no effective therapy, it is easy to justify a trial of the combination; the patients have nothing to lose and everything to gain. If no such conditions obtain, there may still be a disease process for which therapy is available but by no means optimal. Pneumococcal meningitis might be considered such a disease; subacute bacterial endocarditis another. Here one may be willing to entertain the risk of some loss of potency in a combination of drugs on the chance that a substantial synergism between the drugs might be demonstrated, with lifesaving effect for some patients. Let us take, however, a disease which is 100 per cent (or essentially 100 per cent) curable with presently available antibiotics. The trial of a new antibiotic on such patients, desirable as it may be from a variety of standpoints (e.g., a search for a cheaper or less toxic drug), must take into account the fact that the patients to be so treated are not likely to appreciate the importance of the data contributed by their own misfortunes to the welfare of unborn generations. Such patients have a good deal to lose by participation in such studies and very little to gain. If it is decided that the points at issue are of such importance that the trial has to be performed, adequate safeguards must be written into the clinical trial so that any patient who seems to be doing poorly on a particular regimen be switched without delay to standard forms of therapy. Such provisions have a good deal of nuisance value for investigators, and have at times ruined whole experiments, but only individuals devoid of empathy can argue that they are never necessary.

The contributions of the other members of the team should not be minimized. The pharmacologist can be most helpful in deciding whether the available data on the pharmacologic action or the toxicity of a compound in animals are such as to warrant its trial in human beings. He can also utilize his specialized talents to analyze what is known about the absorption, distribution, excretion, and fate of the drug, so that rational dosage schedules can be set up. If data on human beings are lacking, he can be invaluable in planning the cautious preliminary work that is so essential.

The statistician's aid is frequently indispensable. It is he who is most attuned to the need for safeguards against bias, for devising an experiment which is

*This is not to deny, however, that happy is the physician who can confirm his own work and twice happy is he who is confirmed by others.

free from systematic errors of allocation or assessment. He can comment critically on how pertinent the intended measurements are to the objective of the trial. An expert statistician can frequently increase the efficiency of a trial, so that the same amount of information can be elicited from a smaller number of patients or procedures. In evaluating data at the end of the experiment he can ensure that the appropriate analytic techniques be applied. Failure to utilize the aid of such specialists can be disastrous, as evidenced by a fiasco of some years ago. An ambitious polio vaccination program was responsible for the vaccination of some 450 children, while 680 in the same community remained unvaccinated as controls. An epidemic visited the area that year, but there was not a single recognized case of polio in either group. This was entirely consistent with the records of previous years, which indicated that only two cases could be expected in such a total number of children. A few simple calculations done (alas!) in retrospect revealed that meaningful data could have been obtained only by having 7,500 to 25,000 children in each of the groups.

Having stressed the *cooperative* nature of the trial, one should also emphasize the phrase "at all temporal levels of planning and execution." Too often, for example, the statistician is called in at the end of a trial, in the hope that the chanting of a few mathematical formulas or Greek symbols over the corpse of an ill-planned experiment will restore the breath of life to the unfortunate victim. One eminent statistician has advised his fellows to eschew any part of a clinical trial in which they have not been active participants from its very inception. Such a remark may be a little extreme, but its point is well taken. In addition to participation at beginning and end, the members of the team should consult with each other during the progress of the trial. It is often difficult or impossible to foresee all possible contingencies, and it may be wiser to scrap an experimental design part way along, if serious errors in planning or execution manifest themselves, than to continue doggedly to the bitter end and find that the therapeutic egg which the investigators were so tenderly hatching had been in reality a billiard ball all the while.

Having said all this, it is only fair to add that the ideal conditions described previously are not always achievable, even with the best of intentions. "Full use of a biostatistician," for example, is easier said than done. Such individuals are relatively few in number and their time is limited even if funds are available to pay their salaries. The outlook is not as bleak as it may seem, however. It is perfectly feasible for a clinician with a working knowledge of statistics, and an appreciation of the principles to be described, to plan and execute a sound clinical trial, and to analyze the data satisfactorily. He may be somewhat inefficient in his methods but is not so likely to commit a basic error fatal to the entire enterprise.

B. THE PRINCIPLE OF RANDOMIZATION

It is difficult to conceive of a rule more important for the clinical investigator than that of avoiding bias in the allocation of cases to different treatment groups. If two drugs (or two procedures, or two surgical techniques) are to be compared,

it is obvious that the elimination of all variables other than the treatments in question is a prime goal. The conscious or unconscious distribution of one type of case (the "sickest" ones, for example) to a particular treatment group will introduce another variable into the design which will usually result in a completely useless experiment.* Such a procedure presents potentially different therapeutic challenges to the two treatments. Since it is difficult or impossible to gauge the degree of bias so introduced, or even its direction at times, the result of the study is usually to leave the experimenter no better off than when he began. The best way to avoid such bias is to assign cases by some technique which eliminates the possibility of prejudice. One may employ a randomized sequence (prepared in advance), or flip coins, or use even or odd last numbers on hospital admissions, or alternate cases. The important consideration is not which method is employed so much as the degree to which it succeeds in achieving an unbiased distribution. Thus alternate assignment of admissions to two groups is satisfactory only if the person in charge of admissions is unable to funnel cases selectively into one or another treatment group by the order of admitting them.

It is unfortunate but true that the easiest way to allocate cases in more complicated situations is invariably the least desirable way. For example, if two hospitals are to compare the efficacy of two treatments it is much simpler, administratively, to give all the patients in Hospital Y one drug, and all the patients in Hospital Z the other drug. Such a plan may be disastrous, however, because of the excellent chance that the type of patients, the nursing, the medical staff will differ enough in the two institutions as to affect seriously the results *exclusive* of any possible difference between drugs. During the wartime evaluation of motion sickness preventives it was found that merely the amount of ballast carried by a troopship could affect the incidence of nausea and vomiting in the passengers. In addition, it was established that the section of the ship is an important determinant of the incidence of motion sickness. Thus, not only is it vital to compare medications on soldiers on the *same ship*, but it is imperative that all the treatments under study be allocated at random to the persons *within any given compartment* on the ship.

This principle of randomization is abused with such frequency that examples are easily found. One type of error is illustrated in a certain study of the effectiveness of anticoagulant therapy in the management of patients with cardiac infarction. Patients in this trial were assigned to the drug or nondrug groups on the basis of the day on which they were admitted to the hospital. Thus all patients admitted on odd days were allocated to the anticoagulant group, whereas those admitted on even days were given no anticoagulants. Since this plan is easily recognized by referring physicians, it becomes ridiculously simple to send in patients on a day when the type of therapy is such as the physician considers most appropriate for his patients. Thus, a physician who considers anticoagulants a dangerous and unnecessary measure could avoid sending his patients

*A classic example is the Lanarkshire milk experiment, where one-half the children got milk, but the teachers were permitted to assign the neediest ones to the milk group.[5]

in on odd days, so long as their clinical situation did not demand immediate hospitalization. Another physician who might be "sold" on anticoagulants could adopt just the opposite procedure, or at least attempt to get his "sickest patients" in on "anticoagulant days." The potentialities for bias are obvious. In this study 589 patients were treated and 442 patients were not. Thus, patients were probably not randomly selected since the excess of treated cases is greater than should be expected in a completely random selection of cases. One possibility is that physicians were in general eager to have their patients receive the "advantage" of anticoagulant treatment, and tried to refer patients in on treatment days. Since a critically ill patient would probably be hospitalized as rapidly as possible, it is also conceivable that there may have been a higher percentage of sicker patients in the untreated group.

Another type of error is to use as "controls" all patients who refuse a particular medication or procedure. Thus the postoperative history of a large series of hypertensive patients subjected to sympathectomy has been compared with the course of a large series of patients to whom the operation was tendered but who refused it. Even if one accepts the author's conclusion that the subsequent life history of the operated group was more favorable than that of the unoperated group, it is impossible to say whether the "improvement" is actually due to the surgery, or to the fact that the course of patients who refuse sympathectomy (for whatever reason) is basically less sanguine than that of their more pliable fellow sufferers.

Although the principle of randomization has thus far been discussed only in terms of cases allocated to different treatment groups, it is equally important for the order of administration of treatments to the same patient. For example, for each patient who, serving as his own control, takes Drug W for a period, then Drug Z for a similar period, it is essential for another patient to have this order of drugs reversed. The importance of the order of administration may be illustrated by a study of the effects of hypnotic drugs on psychomotor performance, on the morning after drug administration. One half of the subjects received the placebo on the first night of study, and pentobarbital on the second night, while the other half had this order reversed. The reason for this precaution was not only that the attitude of a subject might change on repetition of the experiment, or that he might become accustomed to the new environment, but that it is well known that most psychomotor tests show a significant "learning effect" with repetition. In order, then, not to underestimate or overestimate any difference between drug and placebo, no one treatment should be allowed to be always first or always second. In this situation, the "learning effect" was appreciated in advance. Regardless of whether the order of administration has been previously demonstrated (or can be reasonably expected) to be important in determining results, however, the wisest procedure is to assume that it will and to proceed accordingly.

A second example can be culled from the literature on coronary vasodilators. One group of investigators observed that outpatients suffering from angina pectoris usually improved during their first few weeks in the clinic regardless of

what they were given. Although these investigators did not pursue this observation to its logical conclusion (they always gave the placebo first), it is clear that randomization of order of treatments would be the preferred procedure.

The use of the principle of randomization eliminates the need for separate listing of another principle—that of concurrent comparison. This latter concept is designed to eliminate differences between groups arising from the fact that they were studied at different periods of time. It is very unwise, for example, to compare the results of treatment with veratrum in a group of hypertensives studied in 1954 with an untreated group studied in 1934. Even if no specific measures against hypertension were available now, it is possible that a group of patients treated today would do better in any event because of other advances in therapy since 1934. (Obviously a patient with hypertension is less likely to die of infections today than he was twenty years ago. Similarly, if he goes into shock for any reason today, he is in a much better position, because of the widespread availability of blood and blood substitutes, than he was in 1934.) If the error is compounded by utilizing the 1934 data of another clinic in a different part of the country or the world for comparison, the additional possibility of differences in diagnostic criteria or patient selection (already somewhat present in the first situation) is introduced to wreak further havoc with the validity of the investigator's comparisons.

C. THE "DOUBLE BLIND" TEST

The third major principle is directed to the elimination of "errors of assessment." It is commonly referred to as the "double blind" or "double blindfold" test and consists of attempts to keep both patient and observer unaware of the nature of the medication being given. Thus, if one group of patients is to receive a potent drug and another group is to receive placebos, the patients and observers are kept in ignorance as to which group is which. If the same patient is to receive, on different occasions, a drug and a placebo, neither he nor the person responsible for evaluating the results of therapy is informed of which is the control period and which is the drug period. The bias which these precautions are primarily designed to eliminate is that attributable to "patient enthusiasm" or "investigator enthusiasm." Most patients with disease want to get better, and most investigators are anxious to come up with successful results. Both patient and investigator, therefore, may be tempted (on a conscious or unconscious level) to record improvement in symptoms under treatment. This enthusiasm must therefore be handled by allowing it to diffuse itself out as equally as possible over the active and inactive medications.

There are some situations where prejudice of this sort is very easy to introduce, and others where it is very difficult. For example, pain is known to be present or absent in a patient only if he tells us so. Pain is also composed in part of a psychologic ("emotional") reaction to certain neurophysiologic stimuli. In such a situation, subjective reaction is all-important, and it is easy to see how the results of medication can be affected by suggestion or wishful thinking. On the other hand, if the end point in a clinical trial is survival or

death, it is unlikely that there will be much understatement or overstatement on the part of the patient or observer.

The usual way of avoiding errors of assessment is to use placebos. Such pharmacologically inert substances (which some prefer to call "dummy" tablets or injections) are usually described as "indistinguishable" from the medication under study. By this is usually meant that the two medications look, smell, and taste "alike." These active and inactive medications are then designated by code letters or names and their true nature is known only to certain individuals not directly concerned with the accumulation of data. These codes are preferably changed at frequent intervals so that the results of previous trials cannot influence subsequent data.

So far, so good. Let us examine the placebo somewhat more critically, however, since it and "double blind" have reached the status of fetishes in our thinking and literature. The Automatic Aura of Respectability, Infallibility, and Scientific Savoir-faire which they possess for many can be easily shown to be undeserved in certain instances.

First of all, there is a mistaken notion that placebos merely control "suggestion" or "suggestibility." There is no question that this is partly their function, although it is not true that symptoms can be improved by a placebo only if they are of psychologic (nonpathologic) origin. But placebos control something else. They also are used to control naturally occurring, that is, spontaneous, changes in the course of disease. Many processes improve sans therapy of any sort. In a recent study of hypnotics in preoperative patients, it was found that 70 per cent of placebo-treated individuals fell asleep satisfactorily. This superficially implies a rather suggestible group. Quite the contrary seemed to be true, however, for the percentage of patients falling asleep successfully in a similar group *receiving no medication at all* (drug or placebo) was almost identical with that in the placebo group. The placebo rate in this instance was thus primarily a reflection of the ability of a group of such patients to fall asleep under certain specific conditions regardless of medication. Such a situation can be appreciated only if one contrives an experiment so that there are control periods (or groups) when nothing of any sort is given and which may be compared with a placebo-treated period (or group). Such a design involves more subjects, time, and effort, and may not be justified by the interests of the experimenter, but it behooves the latter to be cautious in his interpretations of "placebo effects" if he does not include an "untreated" group.

A second misconception about placebos is the belief that they always fool the patient or the observer. With a medication which produces no effects, subjective or objective, other than the one under study, this is possible. Many drugs, however, *do* produce side effects. A good-sized nitroglycerine tablet, for example, will hardly be confused with a lactose tablet by a reasonably alert person once he has had it under his tongue for a few minutes. Most subjects who have never previously received an injection of morphine can quickly tell 15 mg. of this drug from an injection of saline solution. They may not experience euphoria, but they will very likely be dizzy, or nauseated, or sleepy. (Indeed an experimental population which could not distinguish between the two would

be useless for many purposes.) Therefore, if such a subject can voluntarily affect some measurement being studied, it is a simple matter to bias the results regardless of "placebo controls." It should be stated, however, that there are all degrees of recognition of medications. Thus a novice to narcotics can distinguish morphine from saline solution, but a postaddict can also recognize that it *is* morphine, which complicates matters still further. The perfect placebo, therefore, would be one which would mimic exactly all qualities and effects of the drug under study *except* for the effect in question. Obviously, in many cases the achievement of the perfect placebo is an actual impossibility.

The placebo can also fail miserably in eliminating "negative" bias, that is, tendencies on the part of a patient or investigator to deny any effect of a drug, or to deny differences between drug and placebo. Thus, a patient receiving morphine and placebo alternately for pain can, by the simple expedient of calling every dose of medication effective (or ineffective), mask the differences between the most potent and least potent analgesics available. Similarly, let us suppose that a neurologist is convinced that the injection of the stellate ganglion with procaine is a ridiculous treatment for cerebrovascular accidents. Regardless of the fact that someone else sees to it that alternate cases are treated with saline solution and procaine, and that the neurologist is unaware which patient got which, if the neurologist in question is in charge of evaluating the therapeutic results, he can bias the data beautifully by merely writing "no significant improvement" in all cases. The net result will be no difference between drug and placebo. The safeguards against this latter kind of "negative bias" are first of all the predominant tendency, previously mentioned, toward "positive" bias, and second the fact that a failure to demonstrate differences between treatments can only be interpreted as "no difference was demonstrated" ("not proved"), rather than "no difference exists" ("not guilty").

A word should also be said about certain difficulties arising from the use of placebos because of either practical or ethical considerations. If one is trying to evaluate an analgesic drug, for example, the inclusion of placebos in the experimental design is difficult to rationalize from the patient's standpoint, because drugs of established potency are available which will do a reasonably good job of relieving symptoms. Fortunately, this fact can be utilized in the design in a way which will at least partly solve the problem of controls. One can use a standard drug (morphine, for example) and compare the new drug with it at several dose levels. The answer will thus be to the question, "How good is the drug in comparison with morphine?" rather than to the question, "How much better than a placebo is this new drug?" In such a study, every other dose can be of the standard medication so that even if the new drug is ineffective, the timing of doses can be such that a patient does not go very long without getting an effective medication. Such a procedure not only provides comfort for the patient, but also maintains rapport with the medical and nursing staffs responsible for the patients, who soon become aware of difficulties if multiple placebos are given. With a disease like tuberculosis, one of a variety of available drugs can be employed as similar "standards" to serve as yardsticks instead of placebos.

Such techniques are not perfect solutions because it is often extremely interesting and important to know whether a drug is any better than a placebo. In addition, the presence of considerable numbers of "placebo reactors" may result in an underestimation of the optimal dose for the population at large. Further, a number of reports now indicate that the separate handling of data from "placebo reactors" and "nonreactors" may bring out differences between drugs not obvious in the unselected data.[6,7] It has been suggested that a possible solution to the latter problem may lie in the focusing on patients with symptoms of severe degree, since there is evidence that the patients with more severe pain, for example, are less likely to get relief from a placebo than those with less severe pain, and it seems reasonable that severity of other symptoms may likewise be inversely related to placebo success rates.[8] (The extreme of this approach has already been discussed, and is exemplified by such diseases as tuberculous meningitis.) There are dangers in this approach, however. If one studies a new diuretic drug in patients with "intractable" congestive failure which is refractory to the usual therapeutic procedures, a successful outcome is most impressive. A negative result is less easily interpreted. One cannot say that the new drug is completely without effect, because such patients are by definition refractory to digitalis and mercurials, which would thus also qualify as "inactive drugs" in these same patients. Therefore, a negative result in such a difficult therapeutic situation should usually be followed up by studies in less severely ill patients. It is rare to come up with a superdrug, and it would be unfortunate to miss a compound of moderate potency.

Finally, an example should be given of a trial that was conducted without the benefit of a "double blind" control of all aspects, but which nevertheless yielded useful data. The study by the Medical Research Council of Great Britain on the treatment of pulmonary tuberculosis with streptomycin was performed by allocating patients at random to two groups, one group of which received streptomycin, while the other group received only bed rest. This second group was not given placebos, and the physicians in charge of the two groups obviously knew which patients were receiving the drug and which were not. The major safeguard in this study was the reliance on objective criteria, such as fever, sedimentation rate, weight, x-ray changes, and survival rate. As already mentioned, the latter criterion seems hardly liable to biased recording. As an added precaution in studying the x-ray changes, however, the roentgenograms were interpreted by physicians who were unaware of the names and treatments of the patients. Since both the x-ray results and the mortality were strikingly different in the treated and untreated groups, there was little question that a new therapeutic agent was at hand. A purist might say that it is possible that the mere suggestion of potent therapy involved in being stuck four times a day for four months might have produced the beneficial results. To interpret a decrease in mortality from 27 to 7 per cent as a placebo effect in this situation seems a trifle captious, however.*

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*Dr. Paul Meier suggests that unfortunately this is just the sort of remark often made to justify a poor experiment. He is, of course, entirely correct, which points up how difficult life can be.

D. STATISTICAL TREATMENT OF DATA

The fourth major principle refers to the analysis of data in a sophisticated fashion so that one can answer such important questions as "What are the chances that the observed differences between treatments may have been due to chance alone?" or "How reliable are my estimates of the potency of these drugs?" Let it be emphasized that a flagrant disregard of one or more of the principles previously described usually renders it unnecessary to apply *any* statistical techniques to the data. For example, if a series of patients is subjected to portacaval shunts, and the first fifty are performed under ether and the next fifty under cyclopropane, it is unsophisticated to make a detailed analysis of the results in the two groups with the notion of comparing the relative safety of the two anesthetics. There are obviously other variables involved with the passage of time (increasing experience and skill of the surgical team, differences in patient selection, and so forth) that would render it impossible to state that any significant differences between groups were actually due to the anesthetic agents. In such a situation it is sounder merely to tabulate the incidence of deaths, complications, therapeutic results, and so forth, and present the results as "our experience during the first two years" versus "our experience during the last two years."

For those of us who are not professional statisticians, it is always most reassuring to have expert help with the final analysis of data. Frequently, one reads papers in which the use of inappropriate statistical techniques has either failed to show significance where such exists or has "demonstrated" significance where none actually exists.

As an example of the latter type of error, one can cite the failure of investigators to make an over-all chi square test or analysis of variance on the total data to find whether individual comparisons of pairs of treatments are warranted. Even if one compares treatments *of equal effectiveness*, there will be a spread of "cure rates" which will vary over a range the size of which is partly determined by the number of treatments. It has been calculated that if one compares the highest and lowest "cure rates" in such an experiment, "significant" differences "at the 5 per cent level" will be "demonstrated" 13 per cent of the time if 3 treatments are used, 40 per cent if 6, and 90 per cent if 20 treatments are run! Obviously, then, to study 5 drugs and 2 placebos and then say that the "best" drug differs from the placebo "at the 5 per cent level" really means little unless one runs an over-all chi square first.*

An example of the inappropriate evaluation of data where just the opposite effect is obtained has been given by Mosteller.[7] If 100 patients each receive 2 drugs at different times, and one analyzes the following incidence of nausea by an ordinary 2 by 2 contingency table:

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*As in all areas of human endeavor, there is disagreement among statisticians as to the preferred method of attacking problems. The performance of an "over-all" test followed by t tests is a time-honored and popular procedure, but recent workers (Scheffé, Tukey, and so forth) have proposed substitute tests which seem more reasonable or sophisticated to segments of the profession.

	NAUSEA	NOT NAUSEA	TOTAL
Drug A	18	82	100
Drug B	10	90	100
Total	28	172	200

the results are not statistically significant. Such an analysis, however, is not only predicated on independent samples, and therefore incorrect in these matched data, but is also inefficient since it does not make use of the fact that each person who is nauseated after one drug is more likely to be nauseated after the second drug. Actually, we are not interested in people who are either nauseated with both, or with neither. The only information on differences between the drugs is derived from those individuals who have nausea with one but not with the other. Analyzing the data from this standpoint, we find that 9 of the patients had nausea with both drugs, 81 with neither, 9 after Drug A but not Drug B, and 1 after Drug B but not Drug A. A new table can thus be set up, as follows:

		DRUG A		
		NAUSEA	NOT NAUSEA	TOTAL
DRUG B	NAUSEA	9	1	10
	NOT NAUSEA	9	81	90
	TOTAL	18	82	100

Analysis of these figures by a simple formula gives highly significant differences.

A word should be said about two diametrically opposed tendencies which I think are equally mistaken. Both are the result of an overzealous worship of the magic "p = 0.05." This latter figure means that the differences would have been observed only 5 times out of 100 due to chance alone, and that there are 95 chances in 100 that the observed differences are real ones. This common standard has been chosen because it seems like a reasonable compromise between the error of saying something is significantly different when it is not and of saying that something is not significantly different when it is. Let us examine some ways in which this value can be abused. Let us say that as a result of hundreds of observations, one drug is found to be a few per cent more effective than a placebo in relieving cough. The p value for this difference may be < 0.05, but what real meaning does this have? How important is it to know that a drug is "microscopically" better than no drug at all? On the other hand, suppose that one makes a small study on two antitussives and both turn out to be significantly better than a placebo, but in comparing the two drugs against each other, Drug A just misses being significantly better than Drug B (p of 0.06, for example). To dismiss this difference as completely meaningless seems rash. If possible, and if it were important enough, the experiment should be repeated

with larger numbers. If the experiment cannot be repeated, and there are no other data available for coming to a conclusion on the relative merits of the two medications, one would be justified in using the *apparently* better of the two.

Statistical techniques are essential in problems of "estimation." If an experiment is run on a diuretic drug, it is one thing to know that it can cause patients to lose fluid, but it is equally or more important to be able to state *how much* fluid can be expected to be lost by, let us say, 95 per cent of patients. For such an estimate we are concerned with putting limits ("confidence limits," so-called) on our estimate of the fluid loss in the population studied. Obviously, a drug which will cause almost all patients to lose 2.5 to 3.5 pounds differs from one which may produce responses varying from 0 to 20 pounds. Predictability of response is usually of considerable importance in the evaluation and use of a drug, and statistical analysis provides a measure of predictability, whether one is concerned with the magnitude of effect of a given drug, or the magnitude of the difference between two drugs.*

E. CAUTIOUS GENERALIZATION

The final principle to be discussed is the principle of cautious generalization. The problem of transposition of data and conclusions from one experimental setup to others is a fundamental one, of course, for many areas of scientific endeavor. The action of morphine in patients with increased intracranial pressure or severe pulmonary disease may be at least quantitatively different from its effects in normal individuals. The action of digitalis in patients with congestive heart failure is hardly the same as its effects in a normal individual. The pain of childbirth is different from the pain of chronic headache. Many such examples could be given to illustrate this point. Reports of the efficacy or inefficacy of a medication should always be qualified with phrases such as "under the conditions of the experiment," "with these dosages," "in these patients," and so forth. There are many instances of disagreement in the medical literature on results obtained with various drugs. Some of these differences are easily explained by experimental technique, dose, duration of study, and so forth. Many are not, however, and it would seem appropriate to be generous in such instances, assume the honesty of the investigators, and look elsewhere for explanations.

One possible source of error which has been emphasized recently[9] is the frequent use of volunteers as subjects for studies, the results of which are ultimately applied to more general situations. It is quite apparent that volunteers may differ quite markedly from the nonvolunteers in their own group, let alone from individuals in other groups. This is not to say that such studies are useless—indeed, they are the only studies possible in many circumstances. One must, however, be careful to use such data for what they are worth and rely on subsequent confirmation in other situations before generalizing broadly.

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*For example, an experiment may show two drugs to differ in mean effectiveness by 20 per cent, but it is most helpful to state that from the data one is almost certain that the "true" difference is not less than 10 or more than 30 per cent.

One other source of error is the use of fractions of an experimental population chosen because of certain characteristics which make an experimental study easier for the investigator than if a random sample were chosen. An example is the choice of 52 patients of a group of 3,000 patients with angina pectoris for a study on vasodilator drugs. This small sample was chosen because its members responded consistently (from an electrocardiographic standpoint) to exercise and because they showed favorable response to nitroglycerine. Making a study on such patients is perfectly permissible, but it may not tell much about the average patient with angina pectoris, who obviously did not qualify for the study. In addition, the method of selection made it inevitable that no drug studied would outperform nitroglycerine, since the best that any drug could do was to equal its performance.

Obviously, the most satisfactory situation with regard to a drug is to have its performance tested carefully by a variety of independent observers in different clinics with different types of patients under different conditions. If such studies are in general agreement as to the drug's efficacy, there is little room for doubt. On the other hand, a single negative or positive study, no matter how carefully performed, must always leave some question unanswered.

FINAL COMMENTS

These principles, then, seem the crucial ones for conducting clinical trials or evaluating their results. It is hoped that the picture drawn seems neither too confusing nor overly simple. The principles are simple but their application is frequently not. As in so many fields, a middle course is actually the wisest one. In the field of the clinical trial, the Golden Mean is not "a virtue flanked by two vices" but the only rational approach. I should like to discuss briefly some additional personal sources of humility in this field.

First, there is the fact that frequently a careful clinical trial merely substantiates what has been previously determined by uncontrolled experiments. This is not always so, but many instances can be cited. For example, the carefully done analgesic studies of the group at the Harvard Medical School Anesthesia Laboratory have for the most part defined equianalgesic doses of drugs which are in agreement with those already determined by clinicians at the bedside. Such valid observations obtained by clinicians in an uncontrolled way are particularly likely to be made if a drug is exceptionally potent or toxic, if the disease process being studied is one not likely to improve spontaneously, or if a potent drug is already available for comparison. Although once again one can cite exceptions, it thus behooves investigators who come up with completely negative studies on a time-honored drug to be exceptionally careful in dismissing the medication as inactive. Too frequently the next investigator devises a satisfactory testing situation and corroborates past generations of maligned clinicians.

Finally, for those who like to think that satisfactory clinical trials are new and a part of the pharmacology of the last decade or so, I should like to quote the following, abstracted from an article by Gaddum.[3]

" . . . I took twelve patients . . . (with) scurvy. . . . Their cases were as similar as I could have them. . . . They lay together in one place and had one diet common to all. Two of these were ordered each a quart of cyder a day. Two others took twenty-five drops of elixir of vitriol three times a day upon an empty stomach. Two others took two spoonfuls of vinegar three times a day. . . . Two of the worst patients were put upon a course of sea-water. Of this they drank half a pint every day. Two others had each two oranges and one lemon given them every day. The two remaining patients took an electuary recommended by a hospital surgeon made of garlic, mustard, balsam of Peru and myrrh. The consequence was that the most sudden and visible good effects were perceived from the use of oranges and lemons; one of those who had taken them being at the end of six days fit for duty. The other was the best recovered of any in his condition and was appointed nurse to the rest of the sick."

This experiment was started by James Lind on May 20, 1747.

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REVIEW

Blocking HIV-1 transmission in the female reproductive tract: from microbicide development to exploring local antiviral responses

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The majority of new HIV-1 infections are transmitted sexually by penetrating the mucosal barrier to infect target cells. The development of microbicides to restrain heterosexual HIV-1 transmission in the past two decades has proven to be a challenging endeavor. Therefore, better understanding of the tissue environment in the female reproductive tract may assist in the development of the next generation of microbicides to prevent HIV-1 transmission. In this review, we highlight the important factors involved in the heterosexual transmission of HIV-1, provide an update on microbicides' clinical trials, and discuss how different delivery platforms and local immunity may empower the development of next generation of microbicide to block HIV-1 transmission in the female reproductive tract.
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HIV-1/AIDS has been a public health priority for the past three decades. In 2013, an estimated of 35 million people were living with HIV/AIDS worldwide while women accounted for 16 million people, which represent 46% of the HIV infected population.[1] HIV-1 sexual transmission remains the main mechanism for its spread worldwide. The infection rates of HIV are higher in women than in men, and that could be because of less selection bias and a more permissive environment in the female reproductive tract.[2] Because of gender inequality, women (particularly in developing countries) have limited power to implement HIV-1 prevention options. The reliance on the male sexual partner in HIV prevention practices often make women more susceptible to acquire the virus infection 5–7 years earlier and eightfold higher than men.[3] Finding an effective method to reduce these high infection rates among women is vital to control HIV-1 epidemic.

To curb the new HIV-1 infections, extensive efforts have been made to develop HIV prevention methods involving anti-retroviral drugs (ARVs) and microbicides with varying mechanisms.[4] Since 1987 there has been substantial development of anti-retroviral therapies, whereas a combination of anti-retrovirals are used to lower HIV-1 viremia to undetectable levels ($\leqslant$50 copies/ml).[5] Achievement of persistent low-level viremia is dependent on drug adherence and compliance with the medication dosing. Individuals failing to adhere to drug regimen increased the probability of generation of drug-resistant mutants and viral shedding in the genital compartment thus raising the risk of new HIV-1 infection.[6] Currently, the US Food and Drug Administration has approved 25 anti-retroviral agents in the treatment against HIV-1. These anti-retroviral agents can be categorized based on their mechanism of action into (i) nucleoside analog reverse transcriptase inhibitors (NRTIs), (ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (iii) protease inhibitors (PI), (iv) entry inhibitors (such as CCR5 co-receptor antagonists and fusion inhibitors) and (v) integrase strand transfer inhibitors. Timing the start of an anti-retroviral therapy is vital in reducing HIV-1 transmission. It has been suggested that early treatment against HIV-1 may expose the patients to the cytotoxic effect of the drugs (for example, rash, renal and hepatic abnormalities),[7,8] although some of these concerns have subsided with improved understanding of the pharmacokinetics and pharmacodynamics of these anti-retroviral agents. The potential development of drug resistance has also been a concern when monotherapy was used in treatment. Amongst all limitations, poor adherence is likely to be a major factor in the development of drug resistance HIV.[9]

The updated guidelines (April 2015) for first-line of anti-HIV regimen often consist of two NRTIs in combination with either an integrase strand transfer inhibitors, or a PI, or a NNRTI. Patients failing with the first-line anti-retrovirals regimen will often be given an alternative of either a NNRTI-based regimen, or a PI-based regimen, or an integrase strand transfer inhibitors-based regimen, or all combined. The choice of drugs to be included in the second-line of anti-retroviral regimen is based on the patient's treatment history, and drug-resistance testing.[10] The burden of the HIV/AIDS related disease has been greatest in the poorest countries where the switch to the second-line ARV regimen is not always an option because of: high cost, inaccessibility to the drugs, and inadequate health care infrastructures.[11] The implementation of the aforementioned ARVs

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Table 1 First- and second-generation microbicides clinical trials summary (modified from Cottrell and Kashuba[12] and D'cruz and Uckun[108])

Candidate microbicide	*Category*	*Formulation*	*Clinical trial phase*	*Outcome*
Nonoxynol-9	Surfactant	1000 mg Sponge gel	II	No success and vaginal epithelial disruption
		70 mg Film	II	No success and 30% increase of vaginal irritation
		3.5% Gel	II/III	Failed; higher rate of HIV acquisition associated with frequency of use plus epithelial disruption
C31G (SAVVY)	Surfactant	1% Gel	III	Failed (early termination) in preventing HIV and was associated with genital irritations
Carraguard (sulfate polysaccharide)	Blocker	3% Gel	II	Failed to provide protection against HIV
Celllose sulfate	Blocker	6% Gel	III	Failed to provide protection against HIV and possible harm
PRO2000	Blocker	0.5–2% Gel	IIb/III	No statistical difference and uselessness in preventing HIV
BufferGel (polyacrylic acid)	Buffer	Gel	IIb	No statistical difference with trend toward increase in HIV incidence
Tenofovir (TFV)	HIV replication inhibitor (NtRTI)	1% Gel	II (CAPRISA 004)	39% reduction of HIV acquisition to 54% in high adherer's women
TDF	HIV replication inhibitor (NtRTI)	1% Gel	III (VOICE)	Failed to show efficacy in preventing HIV
Stampidine	HIV replication inhibitor (NRTI)	5–25 mg kg^{-1} Capsule	I	Clinically safe; no toxicity
Dapivirine	HIV replication inhibitor (NNRTI)	Intravaginal ring (IVR 25 mg or IVR 200 mg)	I	Considered safe and tolerated by prticipants
Dapivirine+Maraviroc	NNRTI+CCR5 antagonist	IVR (25 +100 mg)	I	Completed but results have not been published yet
GSK'744	Integrase strand-inhibitor	Monthly injection	I	Tolerated by the participants

Abbreviations: NtRTI, nucleotide reverse transcriptase inhibitor; TDF, TFV-disoproxil fumarate.

regimen often provides an undetectable viral load and lower transmission rate. Although combinational ARV treatment is unable to remove the last trace of HIV-1 virus from the infected patient, loweing the cost of ARVs and making them universally accessible would significantly impair the spread of HIV in a global scale. In the last two decades, development of microbicides has emerged to be an alternative strategy to reduce the incidence of new HIV infection.

In this review, we will discuss the unique relationship between HIV-1, type-I interferons (IFNs) and mucosal immunity. In addition, we will discuss the recent advances in microbicides development, and the lessons learnt from these experiences for future design and generation of effective microbicides against HIV.

MICROBICIDES PAST AND CURRENT CHALLENGES

Microbicides, by definition, act as a barrier and prevent acquisition of HIV-1 via sexual transmission. Microbicides can roughly be divided into two groups: (1) the non-anti-retroviral candidate microbicides; and (2) the anti-retroviral based candidate microbicides. These two groups of microbicides are sometimes grouped as 'first generation' and 'second generation' of microbicide, respectively.[12] One of the latest microbicide developments consists of combination of anti-retroviral agents with distinct mechanisms of action against HIV-1 in an attempt to achieve greater level of protection. There is also strong inclination to combine non-hormonal contraceptives with the candidate microbicides as a preventive measure against HIV-1 transmission and unwanted pregnancy.

A number of advances have been made to deliver microbicide candidates into the female reproductive tract. These delivery systems can be divided into coitally dependent (gels, films, tablets and diaphragms) and coitally independent (rings and fibers) means. Ideally, delivery of microbicides should not cause disruption to the vaginal epithelium and to the innate microflora (complex vaginal microbiome), as this might induce adverse effects. For example, alteration of microflora profiles was reported in clinical trials upon usage of 1% Tenofovir gel, whereas 3% of participants developed asymptomatic vaginal candidiasis and sometimes bacterial vaginosis.[13] As the association of bacterial vaginosis and high risk of HIV-1 transmission/susceptibility has been confirmed,[14,15] Pyles *et al.*[16] highlighted the importance of screening vaginally applied compounds (including microbicides), for impact on vaginal microbiome, using colonized epithelial multilayer cultures method to detect unwanted alterations in bacterial community profiles.[16] Other considerations include the timing of the product application and menses, as they are imperative to maintain the effective dosage of microbicide needed to block the transmission of HIV-1 and success in protection. In addition, sustaining vaginal pH ($pH \leqslant 4.5$) level, mainly via the presence of L-lactic acid, is a critical determinant to having a broad-spectrum HIV virucidal activity.[17] Ultimately, an effective microbicide against HIV-1 should offer protection against HIV-1 without compromising the existing delicate balance in the female reproductive tract to prevent pathogen invasions.

NON-ANTI-RETROVIRAL BASED MICROBICIDE CANDIDATES

Only a small number of candidate microbicides have successfully gone through the preclinical pipeline (high efficacy and minimal toxicity, both *in vitro* and in animal models) to clinical trial testing in humans. First-generation microbicides include surfactants, blockers and buffers (Table 1). Among the surfactants group, two products (nonoxynol-9 and C31G) were clinically investigated. They act by disrupting the viral envelopes (solubilizing lipids or denaturing proteins). Nonoxynol-9 (N-9) is a known spermicide that went through clinical trials between 1992–2002 based on different formulations (gel, vaginal film and

suppositories). The clinical trials showed an inverse association between HIV-1 incidence and frequency of suppository use; whereas an increase of the administered dose to twice and four times daily has led to epithelial disruption.[18] The N-9 vaginal film formula showed no benefit but possible harm, and 30% increase of vaginal irritation.[19] Two studies using the gel formulation (COL-1492) demonstrated a higher rate of HIV-1 acquisition and positively associated with frequency of use, plus epithelial disruption.[20] Like N-9, C31G (SAVVY) showed disappointing results (studied in four trials for safety evaluation and two trials for effectiveness between 2004 and 2008) in preventing HIV-1 transmission, and was associated with genital irritations.[21,22] The next class of candidate microbicides assessed was the 'blockers' (cellulose sulfate gel, Carraguard and PRO 2000). They act by interfering with HIV-1 attachment to the host cells. The first product assessed was Carraguard (sulfate polysaccharide derived from seaweed). Perotti *et al.*[23] showed effectiveness of carrageenan in blocking HIV-1 infection of cervical epithelial cells and preventing macrophages trafficking from the vagina to lymph nodes. Although Carraguard (gel) was safe, it failed to provide protection against HIV-1 in the efficacy trial[24] (Table 1). The second 'blocker' investigated was cellulose sulfate (vaginal gel). Two phase III clinical trials of 6% cellulose sulfate gel showed ineffectiveness in preventing HIV-1 infection.[25,26] Later on, Mesquita *et al.*[27] showed that disruption of epithelial cells tight junctions by cellulose sulfate reduced epithelial barrier and activated proinflammatory signaling pathway, thus facilitating HIV-1 infection. The last product in this category is PRO 2000 vaginal gel (naphthalene sulfonate). PRO 2000 vaginal gel was evaluated in two trials and proven ineffective in preventing HIV infection in women.[28,29]

In the category of 'Buffers', the last in the first-generation microbicides, BufferGel (polyacrylic acid) was checked in efficacy trial and demonstrated no benefit in protection against HIV-1[28] (Table 1). The underlined principle for this category of microbicide is to preserve the acidic/low pH of the vaginal milieu that can be elevated by the presence of semen or reduction of lactobacilli (vaginal flora bacteria).

As an alternative to the abovementioned non-anti-retroviral candidates, LACTIN-V (*Lactobacuillus crispatus*) and a live recombinant *Lactobacillus jensenii* (producing cyanovirin-N lectin which has high affinity recognition of gp120 carbohydrate moieties) was shown to be safe, tolerated among women, reduced SIV (63%) in animal trials and may proceed in the microbicides development pipeline.[30,31]

ANTI-RETROVIRAL BASED MICOBICIDE CANDIDATE

Anti-retroviral based microbicide candidates comprise HIV replication inhibitors (Tenofovir, Dapivirine, and Stampidine), HIV fusion inhibitors (Maraviroc) and HIV integrase inhibitor (GSK'744), which were evaluated in clinical trials, in addition to promising candidates in preclinical studies (MIV-150, HI-443, Rilpivirine and PSC-RANTES). Tenofovir (TFV) is a Food and Drug Administration-approved drug for HIV-1 treatment. TFV is a nucleotide reverse transcriptase inhibitor (NtRTI), and it blocks the synthesis of viral cDNA during infection. TFV was formulated as a vaginal gel by containing the TFV or TFV- disoproxil fumarate (TDF) (Table 1). Application of TFV gel (1%) before and after sex was included in a phase II clinical trial (CAPRISA 004). The CAPRISA 004 trial showed 39% reduction of HIV-1 acquisition but two seroconverters were super-infected with HIV subtype C.[32] However, HIV-1 incidence was reduced to 54% in high adherer' women.[32]

The VOICE trial (phase IIb) dealt with evaluation of daily dosage of 1% TDF gel, oral TDF and TFV+FTC (entricitabine) or Truvada. The VOICE trial failed to show efficacy in preventing HIV-1 transmission in women.[33] It is thought that the lack of protection against HIV-1 was attributed to low adherence of the participants. In addition, a number of ongoing trials will provide invaluable insight for the future development of this microbicide. For example, FACTS 001 (phase III) is a trial that has been completed in April 2015. This study will reveal the effectiveness of TDF gel in preventing the transmission of HIV-1 and HSV-2,[34] whereas CAPRISA 009 trial was recently completed in April 2015, and will determine whether exposure to TFV gel alters the therapeutic responses to future TFV containing ARVs regimen.[35] Other ongoing trial, such as CAPRISA 008 (phase III), is the continuation of CAPRISA 004 trial. It is aimed to assess the effectiveness and safety of TFV gel provision through family planning services.[36] Recently, two safety studies have been completed by investigating the effect of tenofovir gel (1%) application on pregnancy and lactation (MTN-008), plus the effect of contraception and menstrual cycle on the gel use and effectiveness in protection (A10–114).[37,38]

Volk *et al.*[39] provided analyses on data collected between July 2013 and February 2015 from pre-exposure prophylaxis—PrEP (tenofovir/enticitabine) initiators and non-initiators among members of the Kaiser Permanente Medical Center in San Francisco. The authors observed high rates of sexually transmitted infection (STIs) (50%) after 12 months use of PrEP. However, there was no HIV diagnosis among PrEP initiators and was associated with 41% decrease in the reported use of condoms. Despite the limitation of this study, the authors' recommendations were to increase understanding of 'risk compensation' and how sexual risk behavior may impact PrEP users,[39] In addition, the risks from prolonged exposure to ARV-based microbicide candidates, such as kidney toxicity, mitochondrial toxicity, osteoporosis and emergence of drug resistance HIV-1 mutants because of poor adherence, still require more attention and careful evaluation. However, a number of barriers and challenges remain including behavioral disinhibition/risk compensation, societal stigmatization against HIV and healthy individuals' reluctance of using anti-HIV-1 drug as preventive strategy. Recently, Biswas *et al.*[40] assessed the effect of TFV on epithelial cells, fibroblasts, $CD4^+$ T-cells and $CD14^+$ cells isolated from the female reproductive tract (endometrium, endocervix and ectocervix). They found that TFV induces proinflammatory responses in the female reproductive tract (site-specific) by upregulating Interleukin (IL)-8, tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein 3α (MIP-3α), which may potentially increase the risk of HIV-1 acquisition. Adding up, TFV modulation of proinflammatory cytokines (TNF-α, IL-2, IL-7, and IL-12p70) and activation of natural killer cells were associated with HIV-1 acquisition in women showing high systemic innate immune activation before infection.[41]

Aside from TFV, a distinct new HIV anti-retroviral, stampidine, was evaluated recently in phase I clinical trial, and showed no toxicity at dosage ranging from 5 to 25 mg kg^{-1} when taken daily as capsules,[42] which offers an alternative option of using anti-retrovirals as microbicide strategy. To overcome barriers to adherence, intravaginal ring (IVR 25 mg or IVR 200 mg) formulated with a non-nucleoside RT inhibitor (Dapivirine) has been developed. Intravaginal ring was demonstrated to be safe and tolerated in phase I trials (Table 1),[43,44] which is likely to help maintain adherence for future clinical application.

Several anti-retroviral agents (for example, NNRTIs: MIV-150, Rilpivirine and HI-443; CCR5 antagonist: PSC-RANTES) are being considered as potential candidate microbicides. These compounds have good records in preclinical studies and are being considered for different formulations for delivery. One of the current focuses is on

the development of combination microbicides targeting different steps in the HIV-1 life cycle in parallel with improving the efficacy of the delivery systems (for example, gels, rings). Lehman *et al.*[45] have showed that selected drug resistance mutations can be detected in patients who acquired HIV-1 after participating in microbicide clinical trials. Drug resistance remains a major concern when ARV is being used as part of the microbicide strategy. Furthermore, the rise of the drug-resistant virus transmission (5–15%) in areas where ARVs have been used for long period of time is an important public health concern,[46] which can negatively impact on the effectiveness of ARV-based microbicides. In addition, the lack of comprehensive knowledge of the long-term effects of these ARV based microbicides on the female reproductive tract (such as vaginal cells and tissues, epithelial integrity, inflammatory responses and vaginal microbiota) is also a concern. It is therefore important to consider an alternative strategy to develop a non-ARV-based microbicide to empower the natural host defenses within the female reproductive tract to prevent HIV-1 infection.

FEMALE REPRODUCTIVE TRACT IMMUNOBIOLOGY AND HIV-1 HETEROSEXUAL TRANSMISSION

Understanding the female genital tract immunobiology is important in designing any preventive methods to HIV-1 spread. The female reproductive tract represents a unique site where a balance must be maintained to ensure successful pregnancy and at the same time to offer protection against sexually transmitted diseases. The female reproductive tract consists of two different types of mucosal surface: type I mucosa (endocervix, endometrium, and the fallopian tubes) and type II mucosa (vagina and ectocervix). Type I mucosal surface includes a monolayer of columnar epithelial cells with tight junction contrary to type II mucosal surface, which is covered with multilayer of squamous epithelial cells[47] (Figure 1). It is well known that the multilayer of squamous epithelial cells and the tight junctions between columnar epithelial cells present good barrier protection in upper and lower female reproductive tract. The transition between the squamous and columnar epithelial cells occurs at the cervical transformation zone (Figure 1) and it is believed to provide a more susceptibility site for HIV-1 penetration and transmission. However, the actual anatomical site(s) of initial HIV-1 infection establishment is still highly debated. It was demonstrated that hysterectomized women (lacking a cervix) and hysterectomized macaques (lacking a cervix) can be infected with HIV-I and SIV (simian immunodeficiency virus), respectively, by crossing the vaginal mucosa.[48,49] This could be explained either by infection of mucosa cells (epithelial cells) or that the virus can cross an intact mucosal barrier. Micsenyi *et al.*[50] reported infection of epithelial cells with HIV-1 leading to *de novo* infection of underlying $CD4^+$ T-cells in a contact-dependent manner. On the other hand, SIV infection of macaques showed that the virus can cross intact mucosal barrier, and infect intraepithelial Langerhans cells, that can extend through epithelial cells into the vaginal lumen.[51] In addition, resident immature dendritic cells (DC) can trap HIV-1 through C-type lectin DC-SIGN, and migrate to secondary lymphoid tissue and undergo maturation where they can subsequently infect neighboring $CD4^+$ T-cells via *trans*-infection.[52] Recently, it was shown that *trans*-infection is strongly enhanced via sialic acid binding Immunoglobulin-like lectin 1 (Siglec-1), which is a type-I interferon (IFN) inducible gene product, and highly expressed on mature DC.[53,54] This data shows that, while many IFN-stimulated genes (ISGs) are expressed in the early stages of HIV infection to suppress viral infection,[55,56] HIV-1 has evolved ways to recruit host IFN-induced genes to help establish infection *In vivo*.

It is well known that the vaginal submucosa area is heavily populated with a spectrum of HIV-1 target immune cells including mainly $CD4^+$ T-lymphocytes cells, antigen-presenting cells (DC and Langerhans cells), natural killer cells, neutrophils and macrophages.[57,58] Therefore, understanding the biological impacts of candidate microbicides on this target population of immune cells is crucial for the development of an effective microbicide formulation.

Whether cell-free or cell-associated HIV-1 virus particles are responsible of initiating infection; HIV-1 heterosexual transmission is initiated by a presumably fitter genetic variant (founder virus) in the vaginal mucosal compartment.[2] Founder virus requires cell surface receptors (CD4) and co-receptors (CCR5 rather than CXCR4) for attachment and entry. Infection will eventually expand locally then spread via lymphatic drainage to establish in the draining lymph nodes. Shortly thereafter, HIV-1 viral RNA is detected in the circulation around 21–28 days after sexual mucosal exposure at which time mucosal (for example, genitorectal and gastrointestinal tracts) $CD4^+$ T-cells undergo severe depletion related to viral cytopathogenicity and failing of CD4+ memory T-cell homeostasis.[59] This depletion of T-cells can be restored slowly or incompletely under combined anti-retroviral therapy.[60,61] In addition, increased risk of HIV-1 transmission has been found to be associated with sexually transmitted infections including herpes simplex type-2,[62] bacterial vaginosis,[15] pregnancy,[63] as well as hormonal contraception options.[64–66]

HOW SEX HORMONES AND HIV-1 WINDOW OF VULNERABILITY ARE RELATED

The host cell responses in the female reproductive tract are affected by sex hormones and the continuous variation of the ratio between the levels of progesterone and those of estrogen. It has been suggested that a 'window of vulnerability' of HIV-1 infection coincides with the high levels of progesterone in the secretory phase.[67] This vulnerability may be due to thinning of the vaginal epithelium during the secretory phase resulting in increased susceptibility to HIV-1 by reaching the underlying target immune cells as showed in macaque experiments.[68–70] Others have demonstrated increased frequency of $CCR5^+CD4^+$ T-cells (HIV-I target cells) and α4β7 expression on DCs (its expression influences preferential trafficking to gut lamina propria and associated lymphoid tissues) in the vaginal tissue and endocervix tissue respectively in progesterone treated macaques.[71]

On the other hand, the female reproductive tract epithelial cells act as a barrier and contribute to protection by secreting factors that display potent antimicrobial activity (that is, HD5, HBD1–4, Elafin, and SLP1), chemokines/cytokines (that is, IL-8, CCL20, RANTES).[72] More recently, a novel cytokine IFN-epsilon (IFN-ε) with potent antimicrobial activity has been shown to be expressed in the luminal and glandular epithelial cells of the endometrium.[73] However, these factors are also regulated by sex hormones and dependent on the anatomic site within the female reproductive tract. Therefore, it is important to study the anti-HIV-1 activity of candidate microbicides throughout the menstrual cycle.

HIV-1 TRANSMISSION—CYTOKINES AND ROLES OF TYPE-I IFNS

Exposure to HIV-1 and activation of innate immunity can result in rapid upregulation and release of cytokines, chemokines and chemokine receptors leading to recruitment of additional DCs and T-cells. In addition, epithelial cells, fibroblasts and immune cells in the female reproductive tract contribute to this pool of cytokines and chemokines through an autocrine or paracrine loop. For instance, epithelial cells secrete proinflammatory cytokines (IL-6, IL-8 and MIF), IFN-β,

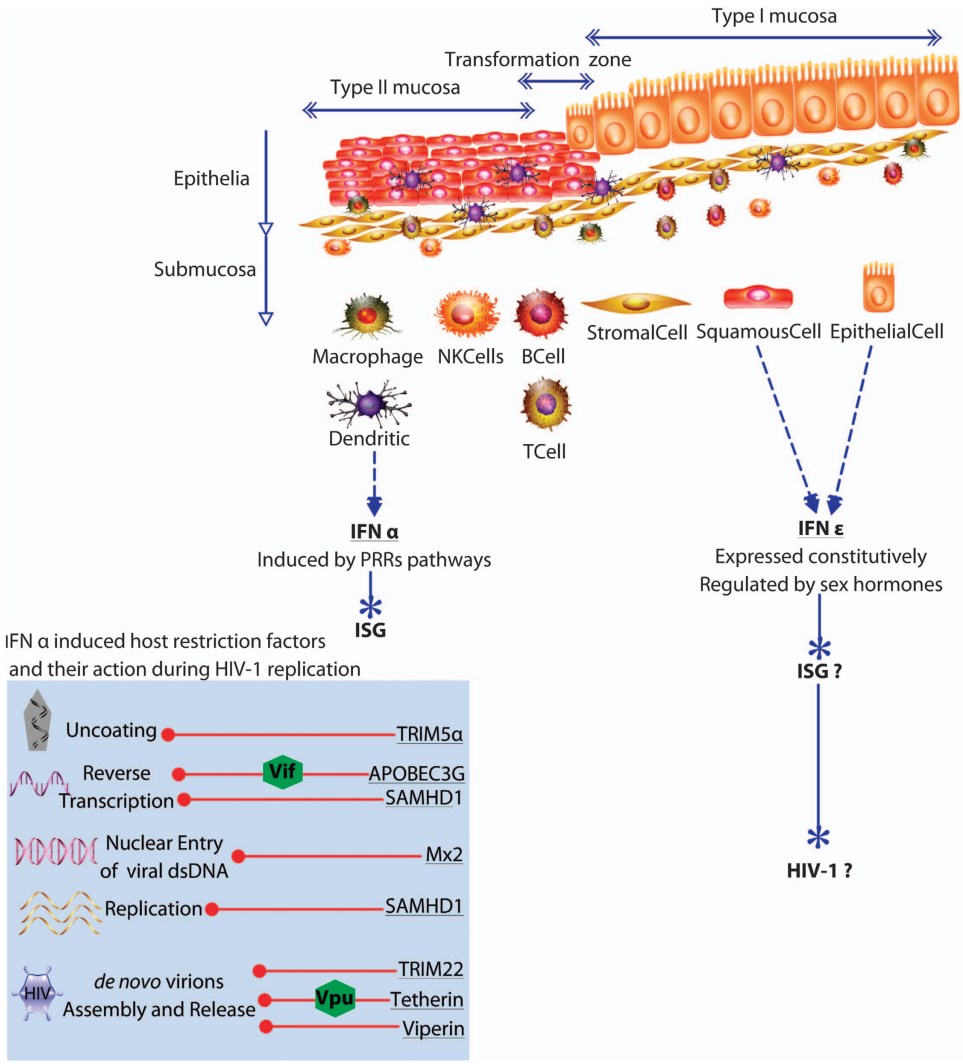

Figure 1 Schematic representation of the human female reproductive tract mucosa surveyed by innate and adaptive immune cells and antiviral responses (generated using ePath3D: http://www.epath3d.com). First part: type I mucosa, type II mucosa, transformation zone and spectrum of resident immune cells. Second part: secretion of IFNα by dendritic cells occurs upon infection while IFNε by epithelial cells is constitutively expressed and hormonally regulated. Target stages of IFNα-Stimulated genes within HIV-1 life cycle: Red connectors with round head represent inhibition of particular stage of HIV-1 life cycle by the designated cellular restriction factor(s) and the green hexagons represent the viral counterpartners. Question marks (?) represent unresolved questions.

defensins (antimicrobial peptides) and MIP3-α/CCL20 (recruits CCR6^{+} plasmacytoid DCs), whereas resident macrophages and DCs secrete antiviral factors such as type-I IFNs and cytokines.[74] Furthermore, migrating cells were shown to secrete IFN-α, IFN-β, MIP-1α, MIP-1β, and CCL5 or RANTES (CCR5^{+} chemoattractant chemokines), which recruit additional HIV-1 target cells and participate in expanding the infection.[75] HIV-1 founder viruses have been shown to be less sensitive to IFN-α[76,77] thus avoiding this cytokine storm, despite a high level production of IFN-α by plasmacytoid DCs, by counteracting IRF-3 and modulating NF-κB, thus disrupting the innate signaling pathways in infected cells.[78,79]

IFN-α THROUGHOUT THE COURSE OF HIV-I DISEASE

IFN-α is produced and secreted following cellular detection of pathogen-associated molecular patterns by pattern-recognition receptors to pilot the upregulation of ISGs.[80] ISGs that exhibit restriction to HIV-1 infection will be briefly introduced here by order of their obstruction with HIV-1 life cycle from entry to *de novo* virions production and the correspondent antagonist HIV-1 proteins (Figure 1).

Shortly after HIV-1 entry, the virus encounters the tripartite motif (TRIM) protein TRIM5α that binds to the virus capsid and presumably accelerates the disassembly of the virus capsid structure before reverse transcription can occur. Therefore, it is thought that TRIM5α block HIV infection via dismantling the virus, although the exact mechanism is still unclear.[81] Another protein of the same family TRIM22 was found to inhibit HIV-1 Gag protein trafficking to host cell membrane. TRIM22 interferes in the very last stage of the virus life cycle and decreases virus particle production.[82] After virus entry and uncoating, apoliprotein B-editing catalytic polypeptide-3G protein, APOBEC3G, exerts its antiviral activity during reverse transcription by driving hypermutation in the newly synthesized viral cDNA, or reducing priming and initiation of viral cDNA synthesis. As a result, this hypermutation fate leads to degradation by a cellular DNA repair mechanism yielding proviruses that are non-functional.[83,84] HIV-1 counteracts APOBEC3G restriction by the viral infectivity factor (Vif).

Vif induces proteasomal degradation of APOBEC3G and blocks its catalytic activity. In addition, Vif reduces APOBEC3G translation and packaging into the viral particle, which ultimately lessen the APOBEC3G mediated antiviral effect.[85,86] The next step in the HIV-1 life cycle is the transportation of the synthesized double-stranded viral cDNA into the cell nucleus. It has been suggested that the murine myxovirus resistance 2 (Mx2) can act at the level of nuclear entry and ultimately obstruct chromosomal integration of the viral cDNA.[87] Other ISG products that may impair retrovirus infection include SAMHD1. The cytoplasm and nucleus localized sterile alpha motif and histidine-aspartate domain containing protein 1 (SAMHD1) reduces the intracellular nucleotide pools and consequently affects retrovirus reverse transcription and replication. SAMHD1 is expressed in monocytes, macrophages, DCs, and resting $CD4^+$ T-cells.[88–90] SAMHD1 has a greater antiviral activity against both HIV-2 and SIV over HIV-1.[91] No anti-SAMHD1 counteracting protein has yet discovered in HIV-1 infected cells and it seems that HIV-1 can tolerate lower levels of nucleotides available in macrophage during cDNA synthesis owing to the enzyme kinetics of HIV-1 RT to support viral replication in low concentrations of substrates.[92] At the late stage of HIV-1 replication cycle, the release of virus particles is barred by the type 2 transmembrane protein 'Tetherin' through physical binding and trapping of HIV-1 particles on the plasma membrane.[93] Tetherin is constitutively expressed in B cells and plasmacytoid DCs.[94] Antagonizing Tetherin function by HIV-1 Viral protein unique (Vpu) includes ubiquitination followed by Tetherin degradation.[95] Another ISG protein that have been shown to have anti-HIV-1 activity is Viperin. It is thought that Viperin block HIV infection via inhibiting farnesyl diphosphate synthase, which alters the plasma membrane fluidity and thus particles' release.[96]

Despite the available of large number of ISGs to suppress HIV-1 infection, once HIV-1 transmission has occurred and infection is established in the host, type-I IFN sensitivity itself is unable to stop the spread of HIV-1 amongst different compartments of the infected host.[97,98] *In vivo* studies have been conducted to assess the impacts of interferon administration on the spread of retrovirus infection and progress of the disease. Treatment of macaques with pegylated IFN-α2a during SIV challenge increased host resistance to systemic infection, despite the evidence suggesting that inflammation intensifies virus acquisition and disease progression.[97]Although once SIV infection is established in the host, continued pegylated IFN-α2a treatment resulted in a decrease of antiviral gene expression, which increases the susceptibility to the spread of infection and greater $CD4^+$ T-cell depletion compared to placebo,[97] and a similar conclusion was depicted by Asmuth *et al.*[98] when chronically SIV-infected macaques were injected with pegylated IFN-α2a. Sandler *et al.*[97] highlighted the importance of timing and duration of type-I IFN administration in shaping the course of the disease development. More specifically, early type-I IFN signaling was critical for early and long-term control of SIV replication and virus reservoir in macaques. At the same time, a delay of as few as 3 days in antiviral gene expression after SIV infection is established can result in accelerated disease progression.[97] Accordingly, type-I IFN treatment requires different therapeutic strategies at each stage of the course of HIV-1 disease.

IFN-ε ROLE IN FEMALE REPRODUCTIVE TRACT

IFN-ε, a novel type-I IFN, was identified in humans (chromosome 9p21) and mice (chromosome 4).[99] IFN-ε was found to be located within type-I IFN locus as a single copy. It comprises conserved progesterone receptor binding site in the proximal promoter but interestingly the response elements for pattern-recognition receptors are limited as opposed to other type-I IFN.[73,99] IFN-ε shares only 30% amino acid homology to consensus IFN-α and to IFN-β. Based on coding sequence, IFN-ε is most closely related to IFN-β. Human IFN-ε is 15 residues longer than mouse IFN-ε and they share 54% amino acid identity and 15% amino acid similarity.[99] The predicted structures of IFN-ε have overall similarity to the type-I IFN and it was confirmed later on by the ability to bind to the type-I IFN receptor complex.[73,99] Contrary to other classical type-I IFN, IFN-ε is not induced by pattern-recognition receptors pathways, IFN-ε is expressed constitutively by epithelial cells of the female reproductive tract and its expression level is regulated by sex hormones. The detectable levels of IFN-ε expression are highest in the proliferative stage (estrogen dominant) and lower in secretory phase (progesterone dominant) of the menstrual cycle.[73] The expression level of IFN-ε (with potential antimicrobial activity) concurs with the 'window of vulnerability for HIV-1' when the conditions are optimized for fertilization and pregnancy. Fung *et al.*[73] revealed the protective role of IFN-ε in the female reproductive tract by maintaining basal levels of ISGs (*2'5'oas, Irf-7, Isg15*) in the murine model. *IFN-ε*-deficient mice, challenged with genital HSV-2 or *Chlamydia muridarum* infection, showed increased susceptibility to infection compared to wild-type mice. There was no change in the expression of *Ifn-ε* in wild-type mice, as it is not pathogen induced, and protection was granted by activating recruitment of natural killer cells.[73] The quite distinct role and importance of IFN-ε in mucosal immunity was the focal point in different studies but still the mechanism is not fully understood. It is intriguing to evaluate IFN-ε in HIV-1 heterosexual transmission (Figure 1), which raises the question of how IFN-ε may protect the female reproductive tract from HIV-1 infection. Which HIV-1-related restriction factors are favorably induced by IFN-ε? What immune cell types are activated? Does IFN-ε support trans-infection mechanism from DCs to target cells? Could it be considered as a candidate microbicide?

CONSIDERATIONS FOR PROSPECTIVE CANDIDATE MICROBICIDE

Finding safe and effective non-ARV-based anti-HIV-1 microbicides, delivered efficiently and accepted by users, would provide new venues for the control of the HIV/AIDS epidemic. Candidate microbicide that progress in the production pipeline will be tested *in vitro, ex vivo* (vaginal mucosal explants, female reproductive tract immune cells, microflora) and *in vivo* on animal models (bone/liver/thymus mice, and non-human primates), despite the fact that impressive preclinical data are not always predictive of the success of such microbicides formulation to prevent HIV infection in humans. However, studying the effects of candidate microbicides throughout the menstrual cycle on vaginal cells and tissues (first cells to encounter HIV-1 in heterosexual transmission), epithelial integrity, inflammatory responses and vaginal microbiota is becoming a prerequisite. In addition, it is important to test the efficacy of candidate microbicides in the presence of semen as shown by Zirafi *et al.*[100] where there was an enhancement of HIV-1 infectivity in presence of semen resulting in the impaired antiviral activity of most candidate microbicides except maraviroc (CCR5 antagonist).[100] It is thought that the amyloid fibrils in semen enhance HIV infectivity by promoting viral attachment to the target cells.[101,102] A mutually non-exclusive explanation would be the induction of proinflammatory cytokines (IL-6 and IL-1β), chemokines (IL-8 and MCP-1), and IL-7 by seminal plasma that facilitate HIV infection *In vivo*.[103–105]

IFN-ε has a unique role at the mucosae immunity and the probability of considering it as future candidate microbicide is

dependent on many factors. IFN-ε (i) is not induced by PPR as seen with other type-I IFNs, (ii) endogenous IFN-ε is well tolerated as it is expressed constitutively by epithelial cells in the female reproductive tract where levels are hormonally regulated, (iii) is known to provide protection against common sexual transmitted infections in mice (HSV-2 and *Chlamydia*), (iv) is not suppressed by seminal fluids for its expression, (v) is induced by human ectocervical epithelial cells following exposure to seminal fluid, (vi) is required to maintain basal levels of ISGs in the female reproductive tract, (vii) is activated to facilitate recruitment of natural killer cells, (viii) is exclusively expressed by epithelial cells of mucosal tissues in *Indian* rhesus macaques (can be used for preclinical studies) and (ix) is important to enhance lymphocytes recruitment (cytotoxic $CD8^+CD4^+$ T-cells subset in lymph nodes but not memory $CD8^+$ T-cells responses), and to promote migration of antigen-specific $CD8^+$ T-cells to the gut mucosae, and reduced inflammation.[73,103,106,107] Together, these features highlight the antiviral potential of this naturally produced cytokine IFN-ε, which may offer unique protection against HIV-1 in the female reproductive tract.

The anti-HIV mechanisms of type-I IFNs have most impact at the acute stage of infection, but they are 'too late' and 'less efficient' to suppress HIV-1 replication once infection is firmly established within the host. IFN-ε provides a new modality of immune protection of the female reproductive tract in comparison with other type-I IFNs. These initial results of IFN-ε contribution to mucosal immunity fit with the principal mechanisms of HIV-1 protection. It would be very important to study the effect of exogenous application of IFN-ε throughout the menstrual cycle on the epithelial cells (tight junctions), the immune cells population in the female reproductive tract, fertilization, pregnancy and fetus.

The key question is whether IFN-ε is able to elicit protective immune responses without fueling immune activation to support HIV-1 replication *in vivo*; whereas detection of a proinflammatory signature will be a major criterion. Investigating the effect of IFN-ε on the residing immune cells will give an insight on its relevance in antiviral protection. Some of the focal points will be around its effect on DCs as they are the most potent antigen-presenting cells and the connection between the innate and adaptive immune systems. In addition, scrutinizing the induced ISGs and their antiviral activity will be central to understand IFN-ε protective mechanism. Despite the fact that IFN-ε is an endogenous protein, side effects may occur and this demands caution in timing and dosages. IFN-ε could be considered for different formulations for delivery as vaginal gel or intravaginal ring, plus the ability to combine it with other candidate microbicides may add a boost protection. The big challenge would be identifying the exact set of effector molecules induced by IFN-ε at the vaginal mucosa and their roles in protection against heterosexual transmission of HIV-1. The answers to these questions may hold the key for the development of the next generation of microbicide to offer greater protection against HIV-1 and/or other sexual transmitted diseases via the female reproductive tract.

CONCLUDING REMARKS

Although none of the candidate microbicides has progressed for clinical application currently, the innovation in microbicide's formulations and advancement in preclinical and clinical trials have already paved the way for more rapid development of future microbicides. Understanding the female reproductive tract immunobiology, and the transmission of HIV-1, is a prerequisite in the quest of finding a safe and effective candidate microbicide to prevent HIV-1 acquisition. Candidate microbicides offering novel and promising prevention modality should be investigated in depth. Better knowledge will lead to improved approaches to prevent HIV-1 transmission and address important issues in women's sexual and reproductive health needs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Pre-exposure Prophylaxis for Human Immunodeficiency Virus

The Past, Present, and Future

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KEYWORDS

- Pre-exposure prophylaxis • Implementation • Adherence • HIV prevention • Biomedical intervention

KEY POINTS

- Our knowledge of pre-exposure prophylaxis (PrEP) as a means to prevent human immunodeficiency virus (HIV) acquisition has increased greatly over the decade.
- Evidence from clinical trials conducted among multiple high-risk populations suggests that oral PrEP, if adhered to, can be effective in reducing the risk of HIV acquisition among HIV-uninfected individuals.
- The key issues with PrEP use that have arisen as a result of these trials warranting further research include adherence, risk of drug resistance, and behavioral disinhibition.
- Studies further informing PrEP utilization are ongoing to address issues such as alternate dosing strategies and delivery methods, long-term side effects, and effectiveness in real-world settings.
- PrEP implementation is underway; future studies and activities will need to focus on optimizing PrEP regimens and adherence, increasing education and uptake among high-risk populations and providers, and establishing systems to monitor and evaluate PrEP use.

INTRODUCTION

Human immunodeficiency virus (HIV) prevention research has rapidly advanced over the last decade, with several large-scale research studies demonstrating that antiretrovirals (ARVs) can be used not only for the prevention of mother-to-child transmission, post-exposure prophylaxis, and treatment as prevention (TasP) but also for

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pre-exposure prophylaxis (PrEP). PrEP for HIV prevention involves the use of ARV medications, optimally delivered in concert with risk-reduction counseling and behavioral interventions, such as condom provision and use, to prevent HIV infection among those who are HIV uninfected but at high risk for infection. PrEP entails having an HIV-uninfected individual take ARVs orally or topically (either vaginally or rectally) to prevent sexual or parenteral infection with HIV.[1,2]

The concept of PrEP is not new and has been used as a prevention method for a variety of illnesses, including prevention against rabies[3] and malaria[4] among those traveling to endemic areas. PrEP was considered as a strategy to reduce the number of new HIV infections given the continued high HIV incidence rates both nationally and globally. Globally, an estimated 2.5 million new infections occur every year[5]; in the United States, there are an estimated 50,000 new infections annually.[6] Without an effective HIV vaccine on the horizon, the need for high-impact HIV prevention tools is essential. These tools include interventions such as TasP for HIV-infected individuals; routine HIV testing and linkage to care; and biomedical interventions, such as male circumcision.[7]

Further research into PrEP will add to the toolbox of HIV prevention methods.

This article describes the prior research that informs our current understanding of PrEP, summarizes ongoing research in the area, and highlights key issues that must be addressed in order to optimize the use of this HIV prevention tool.

THE PAST: DEVELOPMENT OF PRE-EXPOSURE PROPHYLAXIS AS AN HIV PREVENTION INTERVENTION

Prior Evidence of Use of Antiretrovirals for Prevention

Proof of concept for the use of PrEP to prevent HIV infection stems from research conducted in both animals and humans. The use of PrEP and postexposure prophylaxis to prevent mother-to-child transmission of HIV has been proven to reduce the risk of transmission by as much as 99%.[8–10] Similarly, the use of postexposure prophylaxis in situations in which a person has had a high-risk sexual or parenteral exposure, or a high-risk occupational exposure, has been shown to be effective with an 81% reduction in transmission.[11] This proof of concept led to the first HIV PrEP trials using animal models.

Animal Trials

The biological plausibility of using ARVs as PrEP for HIV prevention was first examined using animal models as early as 1995. These animal studies also assisted in understanding issues regarding which drugs would be efficacious, drug delivery, and dosing. Among the many ARVs available, tenofovir disoproxil fumarate (TDF), a nucleoside reverse transcriptase inhibitor, has been widely studied for use as both oral and vaginal PrEP. This ARV is generally well-tolerated in HIV-infected persons and has minimal side effects.[12] The combination of tenofovir (TDF) and emtricitabine (FTC) (Truvada) has also been extensively studied for use with PrEP and has been used in many of the animal and clinical trials conducted to date.

The first few nonhuman primate studies assessed the efficacy of injectable TDF for PrEP. In 1995, Tsai and colleagues[13] published data that found that 4 weeks of daily injections of TDF starting 48 hours before, 4 hours after, or 24 hours after intravenous simian immunodeficiency virus (SIV) challenge resulted in 100% protection in macaques. In 1998, Van Rompay and colleagues[14] also concluded that 2 injectable doses of TDF 4 hours before and 20 hours after oral SIV challenge resulted in 100% protection among newborn macaques.

Although the initial injectable PrEP animal studies found 100% protection, early studies examining daily oral dosing with TDF followed by oral or rectal exposure did not. A series of studies found either no or partial efficacy after oral exposure[15,16] or delayed infections after rectal exposure.[17] Not until 2008 did studies looking at FTC in combination with oral TDF begin to show efficacy in reducing the risk of infection. In 2008 and 2010, studies were published that demonstrated that daily oral and intermittent oral PrEP with FTC and TDF/FTC was efficacious, providing either partial or complete protection.[18–20] These findings demonstrated not only that TDF/FTC was able to provide a high level of protection but also that TDF/FTC could be used in lieu of TDF for PrEP, thereby lowering the risk of potential drug resistance in the event of seroconversion.[19] Finally, studies of vaginal delivery of 1% TDF gel applied topically before exposure were also shown to be fully protective.[21]

Hence, these animal studies provided evidence that either TDF or and TDF/FTC could be used to prevent infection; given through injections; taken orally either daily or intermittently; and, most importantly, were efficacious in providing protection from oral, rectal, and vaginal exposure.

Randomized Clinical Trials Among Humans

These nonhuman primate study findings coupled with other confirmatory animal studies[22] supported the introduction of clinical trials of PrEP among humans. Since 2011, results from phase I, II, and III clinical trials have focused on different medications, delivery methods, and high-risk populations. Many of these studies have confirmed the efficacy of ARVs for HIV prevention among high-risk populations, including men who have sex with men (MSM), high-risk heterosexual men and women, serodiscordant couples, and most recently among injection drug users (IDUs) (**Table 1**).[23–28]

Following safety evidence provided by phase I and phase II studies of PrEP among HIV-uninfected persons using TDF and TDF/FTC,[22,29] several landmark studies demonstrated the efficacy of PrEP in reducing HIV transmission. Five key studies have paved the way for future use of PrEP: the Center for AIDS Program of Research in South Africa (CAPRISA) 004, Pre-exposure Prophylaxis Initiative (iPrEx) trial, TDF2, Partners PrEP, and the Bangkok Tenofovir study. The CAPRISA 004 trial sought to assess the safety and efficacy of using a 1% vaginal gel formulation of TDF among heterosexual women in Africa.[23] Participants used the gel both before and after coitus. After 30 months of follow-up, it was determined that the use of the TDF gel reduced the risk of infection by 39% when compared with the placebo arm. Subsequent as-treated analyses found that among women taking greater than 80% of doses, the efficacy increased to 54%, although these post hoc analyses should be interpreted cautiously. Thus, the CAPRISA study was able to confirm that a vaginal gel for PrEP could be used safely and effectively by women for HIV prevention.

The iPrEx trial was one of the first to examine the efficacy of daily oral TDF/FTC use for PrEP among MSM and among transgender women compared with placebo. Approximately 2500 participants were enrolled from multiple countries and followed for a median of 21 months. In as-treated analyses, daily oral TDF/FTC was found to reduce the risk of infection by 44% among participants in the TDF/FTC arm, with further as-treated analyses indicating that among those taking pills on 90% or more of days, the efficacy increased to 73% and was more than 90% in those with detectable drug levels.[26]

The TDF2 study, conducted in Botswana, sought to assess the efficacy of oral TDF/FTC as PrEP among heterosexual men and women. Participants were randomized to receive either daily oral TDF/FTC or placebo. Taking daily TDF/FTC reduced the risk of

Table 1
Summary of completed PrEP trials

Study Name	Study Start Date	Target Population and Sample Size	Study Locations	Study Regimen	Overall Efficacy (%) (95% CI)	Efficacy Among Adherent Population[a] (%)
Bangkok Tenofovir Study	2005	IDUs (N = 2413)	Thailand	Oral TDF	49 (10–72)	74
CAPRISA	2007	Heterosexual women (N = 889)	South Africa	Vaginal TFV	39 (6–60)	54
TDF2	2007	Heterosexual men and women (N = 1219)	Botswana	Oral TDF/FTC	62 (22–83)	78
iPrEx	2007	MSM and transgender women (N = 2499)	Brazil, Ecuador, Peru, South Africa, Thailand, United States	Oral TDF/FTC	44 (15–63)	73–90
Partners PrEP	2008	Serodiscordant heterosexual couples (N = 4758)	Kenya, Uganda	Oral TDF Oral TDF/FTC	TDF: 67 (44–81) TDF/FTC: 75 (55–87)	86–90
FEM-PrEP	2009	Heterosexual women (N = 2120)	Kenya, South Africa, Tanzania	Oral TDF/FTC	6 (−52 to 41)	18–25
VOICE	2009	Heterosexual women (N = 5029)	Uganda, South Africa, Zimbabwe	Oral TDF Oral TDF/FTC	TFV: 15 (−20 to 40) TDF: −49 (−129 to 3) TDF/FTC: −4 (−49 to 27)	Study stopped for futility

Abbreviations: CAPRISIA, Center for AIDS Program of Research in South Africa; CI, confidence interval; iPrEx, Pre-exposure Prophylaxis Initiative; TFV, 1% tenofovir vaginal gel; VOICE, Vaginal and Oral Interventions to Control the Epidemic.

[a] Adherence was determined using different outcomes according to each study protocol.

HIV infection by 62% and increased to 78% among those confirmed to have received drug in the prior 30 days.[24] When one previously undiagnosed HIV-infected participant receiving TDF/FTC developed drug resistance, the TDF2 study results highlighted the important potential for drug resistance among seroconverters and the need to identify these people as early in their infections as possible.

The Partners PrEP study was conducted in Kenya and Uganda among HIV-1 serodiscordant couples. Participants were randomized to one of three study arms: daily oral TDF, daily oral TDF/FTC, or matching placebo.[27] Final analysis showed a 67% reduction in the risk of HIV transmission among the TDF arm and a 75% reduction among the TDF/FTC arm with no issues regarding safety or tolerability.[27] When assessing efficacy among those with detectable TDF drug levels, efficacy was 86% and 90% among the TDF and TDF/FTC arms, respectively.[30]

Finally, the Bangkok Tenofovir study in Thailand was the only study to date to evaluate the use of PrEP among IDUs.[31] A unique feature of this study was that study participants received oral TDF or placebo via directly observed therapy, rather than independently. Primary analysis found a 49% reduction in HIV infection among the participants receiving TDF compared with a 74% reduction among those observed while taking their medications who had detectable TDF drug levels.[25]

Although these landmark studies support the efficacy of oral PrEP among MSM, heterosexuals, men, women, and IDUs, as well as vaginal PrEP among women, 2 other large PrEP studies have shown little to no efficacy among women. The Vaginal and Oral Interventions to Control the Epidemic (VOICE) study, which enrolled women living in Southern Africa, randomized women into one of 5 study arms. These arms included daily vaginal gel with and without TDF, daily oral TDF, daily oral TDF/FTC, and daily matching oral placebo. Initial analysis found that neither the daily oral nor the vaginal TDF arms reduced the risk of HIV infection, and final analyses found that the daily oral TDF/FTC arm was also not efficacious in reducing the participants' risk of HIV infection.[32] Since publication of these findings, additional analyses determined that adherence, as measured by product use, was very low among study participants and may have explained these disparate results.

Similar to the VOICE trial findings, the FEM-PrEP study sought to assess the efficacy of PrEP among African heterosexual women, randomizing participants to receive either daily oral TDF/FTC or placebo.[28] The study was terminated prematurely after interim analyses found similar rates of HIV infection among both study arms. Poor adherence, as in the VOICE trial, was thought to explain the lack of efficacy, as low levels of plasma TDF were measured among women who became infected during the study period.[33] These studies underscore the important role of adherence in both researching and implementing PrEP.

Implementation of Pre-exposure Prophylaxis

With the results from most of the phase III trials showing efficacy, in July 2012, the US Food and Drug Administration (FDA) approved licensure of TDF/FTC for daily oral use as PrEP among HIV-uninfected persons at high-risk for infection.[34] Although other ARVs have been evaluated for use as for PrEP, TDF/FTC is currently the only approved ARV with this indicated use. This instance was the first time that an ARV for HIV has been approved for both prevention in uninfected persons and for treatment of HIV. In response to the availability of this new biomedical prevention intervention, the US Centers for Disease Control and Prevention has issued guidance documents for clinicians considering prescribing PrEP to MSM, IDUs, and heterosexuals at high risk for HIV.[35–38] The World Health Organization has also issued guidance for the use of PrEP among serodiscordant couples, MSM, and transgender women who have sex with men.[39]

Multiple demonstration projects are underway to assist in the scale-up of PrEP as an HIV prevention method in the United States and in Africa; these include open access programs to support PrEP use provided by the pharmaceutical company that makes Truvada,[40] HPTN 073,[41] and various locally administered agency-based programs.[42]

Lessons Learned from the Past

Safety

All of the aforementioned studies have shown acceptable levels of safety and tolerability. Most common short-term side effects reported in these studies have included nausea, headaches, and weight loss[24,26–28]; however, monitoring for longer-term side effects, such as hepatic toxicity, kidney toxicity, and bone density loss, will need to be conducted.

Resistance

Poor adherence when using ARVs for PrEP can increase one's risk of infection with resistant strains if a person seroconverts and can subsequently limit one's future treatment options.[43] Reassuringly, when participants on the studies referenced seroconverted, drug resistance among incident cases was relatively rare. As few as none and as many as 3 participants were reported to develop resistance,[12,24,26,27,44] usually to FTC, although it is important to remember that these studies were not statistically powered to assess the development of resistance.

Adherence

Adherence has often been described as the Achilles heel of PrEP, with both the VOICE and FEM-PrEP studies emphasizing the essential role that adherence plays in ensuring the efficacy of using ARVs for PrEP.[45] As discussed, among five key PrEP clinical trials, efficacy was notably higher among the more adherent participants when compared to the overall study population.[24,26–28,46–49] For example, in iPrEX, the overall efficacy was 44%; but among those with detectable drug, the efficacy increased to 92%.[26] Similarly, the FEM-PrEP study showed that the overall efficacy was a mere 6%, possibly because of low adherence.[28]

For many of these studies, post hoc, as-treated analyses should be interpreted cautiously, as they violate assumptions of intention-to-treat analysis and, thus, may reflect underlying differences between persons who adhere to medications and those who do not, rather than the intrinsic benefits of the medication itself. Therefore, going forward, when prescribing PrEP, education, assessment of a patient's ability to adhere to treatment, as well as follow-up safety monitoring visits will be critical in reducing the risk of HIV acquisition and development of resistant infection. Studies, described hereafter, are underway to further study interventions to increase adherence among PrEP users.

Behavioral disinhibition

With the availability and use of PrEP, concerns have been raised regarding behavioral disinhibition/risk compensation (eg, having more high-risk sex partners or engaging in more unprotected and higher-risk sex acts because one is taking PrEP).[50,51] PrEP is a part of a combination prevention method; therefore, all persons receiving it through clinical trials also receive risk-reduction counseling and are encouraged to use condoms. None of the studies described earlier have observed any significant behavioral disinhibition, yet this may present a real concern among persons using PrEP in nonclinical trial settings. Adherence to PrEP regimens needs to be a focus area as PrEP is scaled up in community settings; ensuring adequate complementary behavioral education and services alongside PrEP delivery will also be critical.

THE PRESENT: ASSESSMENT OF NEW REGIMENS, DELIVERY MECHANISMS, AND FEASIBILITY

The issues presented in the previous section regarding PrEP safety, the development of viral resistance among seroconverters, adherence, and behavioral disinhibition inform many of the studies currently in progress. Even as PrEP is rapidly translated into practice, research continues to further our knowledge of this biomedical prevention strategy.[52–54] Current studies are designed to identify prevention regimens more effective than TDF/FTC with less viral resistance potential as well as to develop new modes of administration that overcome barriers to adherence.[55–65] Further, several open-label demonstration projects are being conducted that aim to characterize the correlates of PrEP uptake in key affected populations.[41,42] This section describes examples of ongoing research that leverage past successes of PrEP and strive to expand opportunities for PrEP use into the future.

New Pre-exposure Prophylaxis Regimens

Given the use of TDF/FTC in many first-line regimens for HIV-infected persons, one key concern is identification of a PrEP agent for which the development of resistance among seroconverters, however few there are, would have less impact. Maraviroc (MVC) was FDA approved in 2007 as the treatment of HIV following demonstration of virologic suppression among HIV-infected persons.[66] This CCR5 antagonist has been used safely among this population and found to have an acceptable safety profile, and it is currently not a first-line treatment regimen.[54,66–68] MVC was considered a potential agent for use as PrEP because of its biological mechanism of action. Unlike TDF and FTC, MVC surrounds the CCR5 coreceptor, allowing MVC to interrupt viral binding early in the HIV lifecycle. Early primate studies demonstrated that MVC prevented simian HIV (SHIV) infection against rectal HIV-1 challenges as well as when it was used as a microbicide agent against vaginal challenges, although more recent studies of MVC among macaques indicated no HIV prevention activity against rectal SHIV compared with controls, even in the presence of adequate blood levels.[19,67,69–71] Other studies have shown protection against vaginal introduction of SHIV in macaques using MVC,[72–74] though results using intravaginal rings (IVR) among primates have been mixed.[75,76] These characteristics in combination with its ability to concentrate high levels of drug in the cervicovaginal and rectal tissues relative to plasma levels make MVC a potentially powerful PrEP agent to explore in humans. MVC has been studied among 450 HIV-uninfected persons with rheumatoid arthritis,[77] but this was for short-term (12 weeks) use only and not among a population identified as at high risk for HIV. However, its favorable safety results contributed to another ongoing clinical trial in the United States. In 2011, the National Institutes of Health–funded HIV Prevention Trials Network (HPTN) launched HPTN 069, a phase II, randomized, placebo-controlled, double-blinded, multisite study of MVC as PrEP, comparing 3 arms: MVC versus MVC+TDF versus MVC+FTC versus TDF+FTC (**Table 2**). This study, initially open only to men and transgender women (male at birth), expanded eligibility in 2013 to include biologically born women. Data from HPTN 069, will characterize the safety and tolerability of MVC among an HIV-uninfected population at an elevated risk for HIV and determine whether this potentially promising PrEP agent will move forward to phase III efficacy trials.[68]

Novel Formulations

In addition to the search for additional PrEP agents that are effective, better tolerated, and with low potential to develop resistance, as well as not competing with first-line

Table 2
New PrEP agents under study in 2014

Agent	Description of Drug	Methods of Delivery Under Study
Maraviroc	CCR5 antagonist	Oral; vaginal ring
Dapivirine	Non-nucleoside reverse transcriptase inhibitor	Vaginal ring
GSK1265744/LA	Integrase strand-inhibitor; long-acting analogue to dolutegravir	Oral; injectable (long acting)
TMC-278/LA	Long-acting formulation of rilpivirine	Oral; injectable (long acting)

treatment regimens as does TDF/FTC, a key goal among current research is the development of formulations to overcome barriers to adherence. As described earlier, all of the studies evaluating PrEP as prevention against sexual HIV acquisition,[24,27,28,49] and even the directly observed therapy administration to IDU,[25,46] were undermined by poor adherence. As a result, new long-acting formulations that are either injectable or, for women, used as IVR hold promise to overcome barriers to PrEP adherence. Multiple products showing promise in this regard are currently in the pipeline (see **Table 2**). For example, the recently FDA-approved integrase strand-inhibitor dolutegravir (Tivicay) has been shown to be an effective treatment among both ARV-experienced and ARV–naïve HIV-infected patients.[55–57,59–64] With its favorable safety profile and characteristics amenable to formulation as a long-acting agent (eg, potency, solubility, and melting point), this long-acting analogue to dolutegravir, GSK1265744LA, is potentially seen as an ideal candidate for long-acting PrEP, either as a monthly or a quarterly injection.[55–57,59–64] In addition to its physical properties, GSK1265744LA has been shown to be highly effective as PrEP when tested among macaques in a recent trial of SHIV prevention following rectal challenge.[55,56] A phase IIb randomized trial of the safety and tolerability of this agent is expected to begin among low-risk men and women in 2014 in 2 separate complementary studies in the United States, South America, and sub-Saharan Africa.

Another long-acting agent, TMC-278LA, is the long-acting formulation of (marketed as Edurant alone and together with TDF/FTC in Complera) a non-nucleoside reverse transcriptase inhibitor approved for use in the United States by the FDA in 2011. This agent has similarly been shown to be safe, tolerable, and have a favorable profile for long-acting delivery.[57,58,62] This treatment has been used effectively among HIV-infected persons, including as the first-line treatment of treatment-naïve individuals.[62] Small-scale, phase I studies have shown TMC-278LA to be well tolerated when administered intramuscularly.[58,62,78,79] This agent, too, is slated to begin a phase IIb randomized trial of the safety and tolerability among low-risk men and women in 2014 in the United States and sub-Saharan Africa.[80] The availability of long-acting formulations, particularly those that need only be administered quarterly, will offer significant adherence benefits compared with daily use.

IVRs offer another formulation of PrEP that will be useful in overcoming adherence barriers for women. These flexible rings do not require insertion by a health care provider, as they can be inserted by women monthly.[53,54,79] Benefits of the ring when compared with a long-acting injectable include the potential for simultaneous administration of PrEP and hormonal contraceptive; the ability to remove the ring in the event of changing HIV risk profiles; and, in the event of allergic reaction or side effect, increased ability to halt the dosing.[81] Previous studies have indicated efficacy at preventing vaginal SHIV infection after challenge with dapivirine, though the IVR with MVC has been less effective in primate trials.[81]

Open-Label Investigations

Perhaps as critical to the PrEP conversation as are new formulations is the question of scale-up. PrEP is one of the first available biomedical HIV prevention interventions; as such, few data exist regarding optimal methods of implementation among those at highest risk of HIV. Simply the availability of PrEP does not ensure uptake, particularly among persons at the highest risk of HIV who may not have access to health care or costly prescriptions.[82–88] In addition to ongoing clinical trials of new and currently approved PrEP agents described earlier, investigations are being conducted to characterize the way PrEP is disseminated outside of the ideal clinical trials environment.

Several investigations of open-label PrEP are currently ongoing in the United States. These investigations include iPrEx open label extension (OLE), the optional, open-label follow-on study to the iPrEx study[89]; HPTN 073, offering open-label TDF/FTC to black MSM in concert with a client-centered case management intervention[41]; and local demonstration sites exploring provision of PrEP, largely in urban centers.[90–92] These studies generally explore correlates of PrEP uptake, adherence behavior, sexual and other HIV-related risk behavior while taking PrEP, and myriad characteristics that will eventually inform wider distribution of PrEP. Outside of research trials and demonstration sites, there are few ways to obtain PrEP free of charge, but there are pharmaceutical company-sponsored assistance programs[40] and copay programs for PrEP[93] available. In addition, private insurance as well as that through the Affordable Care Act[1] will pay for or subsidize PrEP prescriptions with varying degrees of preauthorization and documentation. Despite these offerings, the number of PrEP prescriptions in the United States since FDA approval of TDF/FTC remains quite low.[94,95]

THE FUTURE: PRE-EXPOSURE PROPHYLAXIS IMPLEMENTATION

In the previous section, the authors provided an overview of the current research efforts to assess new PrEP regimens, novel PrEP formulations, and the feasibility of scaling up PrEP in high-risk populations in nonclinical trials settings. In this section, the authors address 4 areas that will need to be considered when strategizing about the scale-up of PrEP on a population-wide basis: (1) optimization of PrEP regimens, (2) delivery of PrEP, (3) engagement of high-risk populations; and (4) guidelines, goals and monitoring, and evaluation.

Optimization of Pre-exposure Prophylaxis Regimens

The numerous clinical trials summarized earlier clearly indicate that even in the research setting, a substantial proportion of persons who might benefit from PrEP are unable or unwilling to adhere to a daily oral regimen. Similar to other biomedical prevention behaviors, such as oral contraceptives[96] or preventive vaccines,[4,97] adherence to daily regimens or to prevention guidelines involve the complex interplay between knowledge, attitudes, and behavior. Taking daily medication to prevent HIV acquisition requires numerous behavioral steps. Perception of self-risk must be sufficient to take a pill daily, even if behavioral risks are not present each day, because of the need to maintain adequate blood levels.[26] An individual's assessment that the medication is worth taking despite the side effects, as well as change in acceptance of medication-related risks over time because of the unknown long-term impacts of taking PrEP,[45,47,98] may result in varying adherence over the life cycle. Overcoming concerns about stigma if possession of the medications alone discloses high-risk behavior or the concern that having ARVs makes others think that patients are HIV infected are also barriers to adherence.[48,99] Finally, even with appropriate knowledge, self-risk assessment, and the means to obtain and take the medication, daily

adherence can be a struggle[45–48]; many people do not adhere, even with no frank reasons for nonadherence.

Additionally, the impact of PrEP regimens on the development of ARV resistance in persons who become HIV infected while taking PrEP will also need to be considered. Monitoring provided by a clinical trial environment differs substantially from a community-based care approach. For example, study participants in trials are generally tested for HIV monthly or every 8 weeks, whereas in real-world settings, testing is recommended every 3 months. Persons on PrEP who do seroconvert are at an increased risk of resistance the longer the duration of treatment on PrEP continues after infection because of the difference in treatment regimens and doses prescribed for infection versus prevention. Reconciling clinical trials' standards with care delivery in real-world settings with regard to appropriate monitoring will be an important area of focus as PrEP is made more widely available.

Concurrently, the safety of longer-term PrEP administration will need to be considered, as current research studies have followed those taking PrEP for only 2 to 3 years. The IPrEx OLE study is continuing to follow PrEP users taking TDF/FTC and will soon be able to provide information on longer-term safety and efficacy; similarly long-term follow-up studies both within and outside of clinical trial settings will need to be conducted as new, longer-acting PrEP regimens are developed.

Lastly, the targeting of PrEP use during periods of risk will continue to emerge as an important issue. PrEP scale-up has been compared with that of birth control,[96] in that women may elect to use oral or injectable birth control methods during periods of time when they do not desire to get pregnant. Similarly, persons at risk for HIV may elect to use PrEP for short periods of time during high-risk activity (eg, multiple sexual partners), for longer periods of time (eg, serodiscordant relationships), or may cycle on and off PrEP. This usage is also similar to models such as malaria prophylaxis, which can be used for a short duration (travel to malaria-endemic areas) or for longer durations (eg, living in malaria-endemic areas).[100]

Delivery of Pre-exposure Prophylaxis

Challenges to delivery and scale-up of PrEP have been identified, largely because of the issues around payment coverage for the drugs,[101] uncertainties of prescribing among many community-based noninfectious disease physicians (where most HIV-uninfected persons are seen for care),[102,103] minimal access to studies or free demonstration projects,[42] and challenges in engaging and treating high-risk populations who are likely most in need of the prevention intervention that PrEP represents.[82,104,105] First, there are issues regarding which clinical providers will prescribe PrEP and monitor its use by patients. Most HIV-uninfected persons who will be eligible for PrEP are seen by community-based noninfectious disease physicians who may be unaware of PrEP guidelines, feel uncomfortable discussing sexual or drug use behavior with patients, and do not have substantial experience prescribing ARVs.[102,106–108] Alternatively, PrEP could be delivered by infectious disease specialists with expertise in HIV and ARVs; but their capacity and willingness to provide PrEP for large numbers of HIV-uninfected persons may vary by provider. Another challenge presaged by the minimal availability of free or discounted PrEP is the transition into community-based access following the discontinuation of subsidized PrEP. Future programs will need to overcome this challenge and prospectively identify procedures for roll-off from clinical trials and open-label demonstration projects once these programs end.

Additionally, there are important cost, insurance coverage, and access considerations that will also need to be considered if PrEP is to be implemented on a wider basis given the high cost of ARVs. Although cost-effectiveness studies have indicated

that PrEP is cost-effective in areas with high HIV prevalence, generalized epidemics, and among high-risk populations,[101,109–114] the longer-term costs of HIV testing and monitoring will also need to be considered.[101] At present in the United States, financial coverage for PrEP depends on one's health insurance or willingness to self-pay; it will be important to monitor the impact of the Affordable Care Act on coverage for PrEP as well as changing private insurance paradigms for coverage of PrEP. Currently, there is limited access to PrEP through research studies and demonstration projects,[42] and Gilead Sciences, which manufactures Truvada, has developed a patient assistance program to assist patients who seek PrEP but do not have insurance coverage.[40]

Globally, there are country-specific issues related to PrEP access, including payment options and the development of national PrEP guidelines. In addition, issues related to resource allocation have been raised regarding supporting PrEP implementation both domestically and globally when there are still many untreated HIV-infected persons in need of ARV therapy.[115] The World Health Organization has issued guidance that, for serodiscordant couples when the HIV-infected partner may not yet be eligible for ARV treatment as per country guidelines, PrEP can be considered for the uninfected partner for 6 months as a bridge to treatment.[39] Moreover, the use of TasP is now established as an important component of HIV prevention programs both domestically and globally, which could potentially modulate enthusiasm for directing limited resources toward PrEP.

Engagement of High-Risk Populations

Despite the efficacy of PrEP in reducing the individual risk of HIV acquisition, many potential users in high-risk populations remain unaware of the potential benefits of PrEP and how to access this intervention.[116,117] Educational initiatives are being developed for persons at high HIV risk who may benefit from PrEP, including mobile phone applications, Web sites to assess one's risk for HIV, adherence interventions,[118] and the issuance of PrEP guidance documents for interested participants and providers by state health departments.[119,120]

Stigma remains a considerable barrier to PrEP provision, in the context of prescribing and using PrEP as well as adherence. Because ARV use for prevention and treatment can easily be confused, uninfected persons may be reluctant to use PrEP as friends and family members may think they are HIV infected.[45] In addition, the use of PrEP also signals high-risk sexual or drug use behavior and may suggest gay or bisexual orientation among men, drug use in men and women, and nonmonogamy in couples. This problem may be obviated by clinic-based injections when long-acting formulations become available, but daily regimens and IVRs will not eliminate these factors. For oral PrEP, changes in packaging that distinguish the use of PrEP for prevention from treatment may ultimately facilitate acceptance of and adherence to PrEP, much as oral contraceptives benefit from packaging that enables daily adherence. Risk compensation is a frequent issue that emerges in discussions of PrEP scale-up, as the prevention benefits of PrEP as a biomedical intervention can be mitigated by behavioral factors, such as an increase in numbers of sexual partners or a decrease in condom use.[121]

Consideration of issues related to gender will also be important as PrEP use becomes more widespread to ensure that this prevention modality meets the needs of women. In the United States, women have been underrepresented in PrEP studies and demonstration projects, although women are included in the Centers for Disease Control and Prevention's PrEP guidelines and the FDA approval of TDF/FTC for PrEP.[34,36] Globally, women have been well represented in PrEP trials; but caution will be required translating lessons learned outside of the United States to women

in the United States, just as the converse is true for MSM. The complex relationship for women regarding PrEP in the context of pregnancy prevention, pregnancy, and desired family building will also necessitate further exploration.[122,123]

Guidelines, Goals and Monitoring, and Evaluation

The use of oral PrEP for individuals at high risk of HIV infection has been shown to be a safe and effective HIV prevention intervention. Scaling the use of PrEP to a population-based level,[124] however, will be an ongoing public health challenge that will require awareness of numerous issues, several of which are described earlier. Moving forward, a higher degree of granularity and regular updating of PrEP guidelines will be needed as new ARVs are demonstrated to be safe and effective for PrEP use; longer-acting PrEP formulations become available; different patterns of intermittent PrEP use are evaluated; PrEP uptake increases in various high-risk populations; and the role of PrEP is further evaluated in discordant couples in which the HIV-infected partner is virologically suppressed on ARVs. For example, more data will be needed with regard to how to use PrEP over the life cycle: as behavioral risk profiles change, guidelines for how PrEP use should change will be necessary. Similarly, for discordant couples in which the HIV-infected partner is adherent to ARV and virologically suppressed, more data will be needed to discern whether the addition of PrEP is cost-effective and warrants added medication risks to the uninfected partner. Moreover, although it may be premature at present, the development of local, regional, and global quantifiable goals for PrEP use will be useful to promote the scale-up of PrEP use to impactful levels, especially if long-acting PrEP formulations become available. Lastly, as PrEP use becomes more widespread,[125] innovative monitoring and evaluation systems will need to be developed for PrEP uptake, adherence, ARV resistance, safety, and efficacy. As PrEP becomes more widely available and people become more aware about its benefits, research inquiries into these more nuanced facets of PrEP deserve exploration.

SUMMARY

In the past decade, enormous progress has been made in the development of PrEP as an HIV prevention intervention, with the safety and efficacy of PrEP now demonstrated in MSM, IDUs, and heterosexuals. Current research is ongoing to assess new PrEP ARV regimens, alternative and long-acting PrEP delivery mechanisms, and the feasibility of implementing PrEP in high-risk populations. In the years ahead, the HIV prevention field will continue to address several critical issues, including the optimization of PrEP regimens, how best to support the delivery of PrEP on a population-level scale, engagement of high-risk populations, the updating of PrEP guidelines, and the establishment of PrEP-related goals and monitoring and evaluation systems.

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Short communication

The blind leading the blind: Use and misuse of blinding in randomized controlled trials

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ABSTRACT

The use of blinding strengthens the credibility of randomized controlled trials (RCTs) by minimizing bias. However, there is confusion surrounding the definition of blinding as well as the terms single, double, and triple blind. It has been suggested that these terms should be discontinued due to their broad misinterpretation. We recommend that, instead of abandoning the use of these terms, explicit definitions of blinding should be adopted. We address herein the concept of blinding, propose standard definitions for the consistent use of these terms, and detail when different types of blinding should be utilized. Standardizing the definition of blinding and utilizing proper blinding methods will improve the quality and clarity of reporting in RCTs.

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1. What is blinding?

Clinical trial outcomes may be biased by human expectations, especially when treatment allocation, assessment methods, and outcome measures have a subjective component [1]. At a basic level, blinding (also called "masking," especially in ophthalmology studies) is utilized to eliminate or minimize these biases. For example, subjects entering a study might be differentially allocated to unblinded treatment groups such that those deemed most likely to benefit from the active treatment are assigned to receive it, while the remaining subjects are allocated to placebo (selection bias). Even when subjects are randomly allocated to receive active or placebo treatment, those who are knowingly randomized to a treatment that is perceived as superior to a placebo may offer overly optimistic responses that create an exaggerated treatment effect (self-report bias). These subjects may also differ in their tendency to drop out of a trial or in their desire to seek additional and potentially confounding treatments versus those allocated to placebo [2], which complicates the interpretation of clinical trial results.

Investigators and research staff are also prone to bias. A subjective investigator-graded outcome scale is a commonly reported end point in clinical trials and knowledge of a subject's treatment assignment may consciously or unconsciously influence the investigator to assign better evaluations to those who are taking the active test product (observer bias). Unblinded randomized controlled trials (RCTs) result in larger treatment effects compared to blinded studies [3,4] and diagnostic test performance is overestimated when evaluations are made with prior knowledge of the diagnosis [5].

2. Confusion in blinding terminology

The double-blind RCT is widely considered the gold standard study design that most convincingly demonstrates the effectiveness of a product or intervention [6]. However, there is great confusion surrounding the definition of blinding and, specifically, what conditions constitute a single-, double-, or triple-blind trial [7]. A review of 200 double-blind RCTs reported 8 unique combinations of two or more groups

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that were blinded, including subjects, investigators, outcome assessors, data managers, biostatisticians, and (rarely) manuscript writers [8]. Almost 1 in 2 double-blind RCTs do not specify which groups were actually blinded [8]. The definition of blinding is so misunderstood that the most recent CONSORT Statement (CONSORT 2010) recommends that the terms "single blind," "double blind," and "triple blind" be abandoned and, instead, blinded RCTs should only detail who was blinded after treatment allocation and how [9,10]. Given the broad misunderstanding of the concept of blinding, all individuals involved in clinical trial design and conduct should develop a basic understanding of blinding terminology and methods. We briefly address herein the concept of blinding and propose standard definitions for the consistent use of these terms in clinical trials.

3. Types of blinding and the proposed standard definitions

Although the definitions of single, double, and triple blind vary widely among physicians and textbooks [7], the proposed standardized definitions of these terms are offered as follows.

3.1. Single blind

A single-blind trial is traditionally defined as one where only subjects are blinded to treatment allocation, and almost 3 out of 4 clinical research textbooks concur with this definition [7]. Clinical trials that utilize blinding of any single group are sometimes labeled as single blind, but this definition is uncommon. In order to improve clarity in the reporting of clinical trials, we propose that the term "single blind" should be reserved only for studies where subjects, but not investigators or outcome assessors, are blinded. Furthermore, the blinding status of other groups (e.g., data managers and biostatisticians) should be identified but should not impact the designation of "single blind."

3.2. Double blind

The standard definition of double blind is that neither the subjects nor the investigators (including those who administer treatment) and outcome assessors are aware of the treatment assignment, regardless of the blinding status of other groups [7]. We concur with this definition and propose that the term "double blind" should be specifically reserved for trials where these groups are blinded and the blinding status of other groups should also be described.

3.3. Triple blind

There is great disagreement among physicians and textbooks regarding the definition of triple blind and, in fact, no single definition predominates [7]. However, the common thread among most proposed definitions and, as such, our proposed definition of triple blind is blinding of subjects, investigators (including those who administer treatment), outcome assessors, and those involved in data management (i.e., data managers and biostatisticians). Again, the blinding status of all groups should always be specified in accordance with the recommendations of CONSORT 2010 [9,10].

4. Who needs to be blinded?

A rarely discussed aspect of blinding involves *a priori* consideration of which groups involved in a clinical study need to be blinded. Open label, or unblinded, clinical trials are susceptible to self-report and observer bias, although the use of these designs is sometimes unavoidable. For example, a clinical trial comparing the effectiveness of a minimally invasive procedure versus open surgery cannot easily blind subjects or investigators to the assigned treatment. Consequently, studies incorporating sham procedures and inactive devices have become more widespread in order to minimize the confounding effects associated with subject knowledge of treatment assignment [11,12].

The single-blind trial, where only the subjects are blinded to the intervention, is conducted when the investigator requires knowledge of treatment allocation (e.g., sham surgery). This design is effective in preventing the subject knowledge of the treatment assignment from confounding study outcomes. Nonetheless, this design remains prone to bias from the investigator, especially in trials with subjective endpoints, e.g., investigator-graded degree of clinical improvement, since they may harbor a vested interest in a positive subject outcome.

As previously mentioned, the double-blind RCT represents a distinct improvement over open-label and single-blind trials and, when feasible, represents the strongest design for minimizing subject- and investigator-related bias. Double-blind RCTs are often required to attain regulatory approval for new drugs, dietary supplements, and, in some cases, medical devices. The potential bias reduction associated with double blinding is noteworthy as a meta-analysis indicated that some double-blind studies reported 15% lower treatment effects compared to unblinded studies [3].

The rarely utilized triple-blind trial arguably offers only minor advantages beyond that of a double-blind design. Blinding of data managers and biostatisticians is generally less critical than blinding of subjects and investigators, because data management personnel usually have no contact with subjects or site staff and because data analysis is typically conducted only after the database has been locked. Therefore, the risk of introducing significant bias is lower in these groups, especially when pre-defined data management methods and statistical analysis plans are utilized. However, bias may result when statistical analyses are performed in the absence of pre-specified guidelines. The implementation of triple-blind trials also incorporates logistic challenges that must be considered, which partly explains why they are rarely utilized [13]. For example, a requirement for the biostatistician to remain blinded adds a level of complexity to the study implementation since the person who prepares the randomization list would need to be someone other than the study biostatistician.

5. Assessment of blinding success

There has been considerable discussion regarding the value of evaluating blinding success after a study has been completed by asking subjects and/or investigators to guess which treatment each subject was administered [14]. Formal statistical methods for evaluating blinding success have even

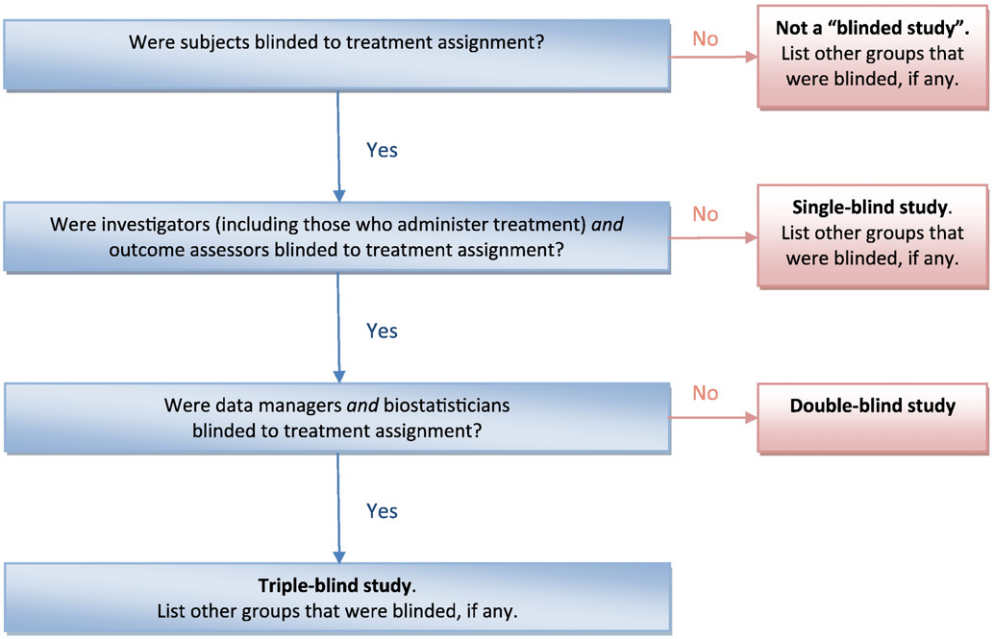

Fig. 1. Blinding definitional flowchart.

been proposed [15]. The evaluation of blinding success is controversial and has generated numerous publications for and against the practice [14,16–25]. Comprehensive coverage of this topic is, therefore, beyond the scope of this brief communication.

6. Conclusion

Although the latest CONSORT statement encourages authors and editors to discontinue use of the term "blinded trial" [9,10], these terms are heavily entrenched in clinical research jargon [26] and their use will likely continue despite this recommendation. Furthermore, specifying the type of blinding used is in agreement with the International Conference on Harmonisation (ICH) guidelines [27]. We therefore propose that, instead of abandoning the use of this term, explicit definitions for these terms should be proposed and advanced, ultimately resulting in the widespread adoption of standardized terminology. We have presented a standard definition of blinding and have created a flowchart to aid in decision-making that identifies the most common groups that are blinded in clinical studies (Fig. 1). Standardizing the definition of blinding and utilizing proper blinding methods will improve the quality and clarity of reporting in RCTs.

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Microbicides and PrEP Studies: What Have We Learned?

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Preexposure Chemoprophylaxis for HIV Prevention in Men Who Have Sex with Men

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ABSTRACT

BACKGROUND

Antiretroviral chemoprophylaxis before exposure is a promising approach for the prevention of human immunodeficiency virus (HIV) acquisition.

METHODS

We randomly assigned 2499 HIV-seronegative men or transgender women who have sex with men to receive a combination of two oral antiretroviral drugs, emtricitabine and tenofovir disoproxil fumarate (FTC–TDF), or placebo once daily. All subjects received HIV testing, risk-reduction counseling, condoms, and management of sexually transmitted infections.

RESULTS

The study subjects were followed for 3324 person-years (median, 1.2 years; maximum, 2.8 years). Of these subjects, 10 were found to have been infected with HIV at enrollment, and 100 became infected during follow-up (36 in the FTC–TDF group and 64 in the placebo group), indicating a 44% reduction in the incidence of HIV (95% confidence interval, 15 to 63; $P=0.005$). In the FTC–TDF group, the study drug was detected in 22 of 43 of seronegative subjects (51%) and in 3 of 34 HIV-infected subjects (9%) ($P<0.001$). Nausea was reported more frequently during the first 4 weeks in the FTC–TDF group than in the placebo group ($P<0.001$). The two groups had similar rates of serious adverse events ($P=0.57$).

CONCLUSIONS

Oral FTC–TDF provided protection against the acquisition of HIV infection among the subjects. Detectable blood levels strongly correlated with the prophylactic effect. (Funded by the National Institutes of Health and the Bill and Melinda Gates Foundation; ClinicalTrials.gov number, NCT00458393.)

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*Other members of the Preexposure Prophylaxis Initiative (iPrEx) study team are listed in the Supplementary Appendix, available at NEJM.org.

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A TOTAL OF 2.7 MILLION NEW INFECTIONS with the human immunodeficiency virus (HIV) were diagnosed worldwide in 2008, according to the Joint United Nations Program on HIV/AIDS (UNAIDS). Combination antiretroviral therapy for patients with HIV infection restores health and may decrease the transmission of the virus to uninfected partners.[1] Therapy also decreases mother-to-child transmission.[2]

Postexposure chemoprophylaxis is recommended after occupational or nonoccupational exposure to HIV-infected fluids.[3] The use of such chemoprophylaxis requires that people recognize when they might have been exposed to HIV and that they start therapy within 72 hours. Both challenges are substantial limitations to the use of postexposure chemoprophylaxis.[4,5]

We selected emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) combination therapy in a single tablet (FTC–TDF) for evaluation of preexposure prophylaxis because of several favorable characteristics.[6] (Details are provided in the introduction in the Supplementary Appendix, available with the full text of this article at NEJM.org.) The protective activity of FTC and TDF has been shown in mice transplanted with human immune cells[7] and in nonhuman primates.[8-10] In these studies, there were increased levels of efficacy when both agents were used together, as compared with the use of either agent alone. The administration of the drug both before and after exposure was important for maximizing the protective benefit.[11]

Daily preexposure prophylaxis with oral TDF had an acceptable side-effect profile in a trial involving West African women.[12] A tenofovir 1% vaginal gel reduced HIV infection rates by 39% among women.[13] Men and transgender women who have sex with men are disproportionately affected by the global epidemic.[14,15] Surveys of such persons in the United States indicate that the current use of preexposure prophylaxis is rare, although the majority would consider such use if evidence of safety and efficacy became available.[16,17]

In this multinational study, called the Preexposure Prophylaxis Initiative (iPrEx) trial, we aimed to evaluate the safety and efficacy of once-daily oral FTC–TDF as compared with placebo for the prevention of HIV acquisition among men and transgender women who have sex with men.

METHODS

PROTOCOL DEVELOPMENT

We developed the concept and protocol for this study using methods that came to be approved as "good participatory practices" by UNAIDS.[18] The development of the protocol was sponsored by the National Institute of Health's Division of Acquired Immunodeficiency Syndrome (DAIDS). The protocol was approved by national government public health authorities in Peru, Ecuador, South Africa, Brazil, Thailand, and the United States and by the ethics committee at each site. All subjects provided written informed consent. The study coordinator vouches for the fidelity of the report to the protocol. The study protocol is available at NEJM.org, and a detailed description of the methods is provided in the Supplementary Appendix.

STUDY POPULATION AND RANDOMIZATION

Inclusion criteria were male sex at birth, an age of 18 years or older, HIV-seronegative status, and evidence of high risk for acquisition of HIV infection. Subject codes were randomly assigned in blocks of 10, stratified according to site. The subject codes were assigned consecutively at the study sites to eligible subjects at the time of the first dispensation of a study drug. Serologic testing for hepatitis B was performed at screening.

STUDY VISITS

Study visits were scheduled every 4 weeks after enrollment. Each 4-week visit included drug dispensation, pill count, adherence counseling, rapid testing for HIV antibodies, and taking of a medical history. Chemical and hematologic analyses were performed at weeks 4, 8, 12, 16, and 24 and every 12 weeks thereafter. During screening, a computer-assisted structured interview collected information about education level, self-identified sex, and alcohol use, along with subjects' perceived study-group assignment at week 12. High-risk behavior was assessed by interview every 12 weeks, and physical examinations and evaluations for sexually transmitted infections were performed at least every 24 weeks. Visits through May 1, 2010, are included in this report of the primary analysis of safety and efficacy. The visit cutoff date was set by the study sponsor without any access to interim findings and was intended

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to ensure observation of the targeted number of seroconversion events (85). The use of study drugs was intensively monitored and promoted (for details, see Methods in the Supplementary Appendix).

STANDARD PREVENTION INTERVENTIONS

At every scheduled visit, subjects received a comprehensive package of prevention services, including HIV testing, risk-reduction counseling, condoms, and diagnosis and treatment of symptomatic sexually transmitted infections, including gonorrhea and chlamydia urethritis, syphilis, and herpes simplex virus type 2 (HSV-2). In addition, at 24-week intervals, subjects were screened for asymptomatic urethritis, syphilis, antibodies to HSV-2, and genital warts and ulcers; treatment was provided when indicated. Sexual partners were offered treatment of sexually transmitted infections that were diagnosed in the subject. Subjects were linked to local prevention and treatment services when required to receive standard-of-care services. All subjects were instructed to protect themselves from HIV with conventional methods, since they were unaware of their study-group assignment. Subjects who reported a recent unprotected exposure to an HIV-infected partner were referred for postexposure prophylaxis (at sites where such therapy was available), and the administration of a study drug was temporarily suspended. Vaccination against hepatitis B virus (HBV) was offered to all susceptible subjects.

LABORATORY TESTING

Testing for HIV antibody was performed on whole blood with the use of two different rapid tests at every scheduled visit, and reactive rapid tests were tested with the use of Western blot analysis of serum (Fig. S1 in the Supplementary Appendix). Subjects with failed rapid tests were retested during the visit. HIV plasma RNA testing with the use of an assay with a lower limit of quantitation of 40 copies per milliliter was performed if seroconversion was detected within 12 weeks after enrollment. RNA testing was also used to identify the first date of laboratory evidence of infection for the as-treated analysis. Testing for drug-resistance genotyping and phenotyping was performed with the use of clinically validated assays on the basis of the viral load at the seroconversion visit.

SUBGROUP ANALYSIS OF DRUG LEVELS

A prespecified subgroup analysis was performed to investigate whether drug levels correlated with protective effect. Subjects with HIV infection were matched with two control subjects, one from each study group who were selected from among seronegative subjects, according to study site (Fig. S5 in the Supplementary Appendix). Plasma was tested for the presence of FTC and tenofovir (TFV), and peripheral-blood mononuclear cells were tested for FTC triphosphate (FTC-TP) and TFV diphosphate (TFV-DP), which are the active intracellular metabolites of FTC and TFV, respectively, with the use of validated liquid chromatography and tandem mass spectrometry assays.

STUDY OVERSIGHT

The study was designed by four of the investigators in collaboration with all the site investigators and communities. DAIDS reviewers approved the protocol, which was developed by the study investigators, and monitored the conduct of the trial at study sites. The Bill and Melinda Gates Foundation also provided funding but did not have a role in protocol development or site monitoring. Gilead Sciences donated both FTC–TDF and placebo tablets and provided travel-related support for meetings conducted by non-Gilead investigators. The role of Gilead Sciences in the development of the protocol was limited to sections regarding the handling of the study drugs. Neither Gilead Sciences nor any of its employees had a role in the accrual or analysis of the data or in the preparation of the manuscript. DAIDS agreed to give Gilead 30 days to comment on the manuscript, but there was no agreement to accept suggestions. The first author wrote the first draft of the manuscript (except for the drug-level sections, which were drafted by another investigator) and decided to submit the manuscript for publication. The protocol statistician and data manager vouch for the accuracy of the data, and the protocol chair and site investigators vouch for the completeness of the reported data.

STATISTICAL ANALYSIS

Data were collected on case-report forms and faxed to a DataFax server at DF/Net Research. It was determined that the observation of 85 incident HIV infections would yield a power of at least 80% with a one-sided alpha level of 0.05 to

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reject a null hypothesis of efficacy of 30% or less if the true efficacy were 60% or more. The modified intention-to-treat analysis included available data for all subjects except those with HIV RNA detected in their enrollment sample. The as-treated analysis used a time-dependent covariate indication as to whether the subject was known to fall below the prespecified level of study-drug compliance (50%) on any of the following: records of study-drug dispensation alone, pill-use calculation on the basis of study-drug dispensation and returns, and subjects' self-report. For the as-treated analysis, pills from unreturned bottles were assumed to have been taken, and late visits were included in the analysis if the last dispensation allowed pill use on 50% or more of days. Safety analyses included all subjects.

RESULTS

STUDY SUBJECTS

Of 4905 subjects who were screened, 2499 were enrolled in the study from July 10, 2007, through December 17, 2009, at 11 sites in six countries (Fig. 1). The baseline characteristics of the two study groups were similar (Table 1). All subjects were born male, although 29 (1%) reported their current gender identity as female. The ages of the subjects ranged from 18 to 67 years; the FTC–TDF group was on average 9 months older than the placebo group (mean age, 27.5 vs. 26.8 years; P=0.04).

Among HBV-susceptible subjects at screening, 94% accepted HBV vaccination. We enrolled 13 subjects with chronic HBV infection that was detected at screening, and acute HBV infection was reported as an adverse event in 3 additional subjects (2 in the FTC–TDF group and 1 in the placebo group) after enrollment when elevated liver aminotransferase levels were observed. All the HBV infections resolved with detectable levels of immunity.

FOLLOW-UP AND ADHERENCE

The cohort was followed for 3324 person-years with a variable duration of observation (median, 1.2 years; maximum, 2.8 years) (Fig. 1). There were no significant trends in visit completion rates over time. Most subjects said they did not know their study-group assignment at week 12, and those who guessed their assignment were evenly distributed between the two groups (Table S3 in the Supplementary Appendix). No subjects were told their study-group assignment during the course of the trial. A study drug was temporarily discontinued in 21 subjects (8 in the FTC–TDF group and 13 in the placebo group) so that they could receive postexposure prophylaxis for HIV (P=0.28).

The rate of self-reported pill use was lower in the FTC–TDF group than in the placebo group at week 4 (mean, 89% vs. 92%; P<0.001) and at week 8 (mean, 93% vs. 94%; P=0.006) but was similar thereafter (mean, 95% in the two groups). At each visit, a portion of subjects (approximately 6%) did not report the number of pills missed. The percentage of pill bottles returned was 66% by 30 days and 86% by 60 days. The rate of pill use that was estimated according to pill count also increased during the first 8 weeks and then remained stable at a median ranging from 89 to 95%, depending on whether pills from unreturned bottles were counted as having been taken or not taken. On the basis of pill-dispensation dates and quantities, the rate of pill use decreased during the first year, from 99% to 91%, a trend that contrasted with pill counts and self-report, which indicated an increased rate of use.

Figure 1 (facing page). Enrollment and Outcomes.
The most common laboratory abnormalities that led to exclusion were elevations in hepatic aminotransferase levels, hyperbilirubinemia, and renal insufficiency. A total of 18 enrollees (0.7%) did not meet all eligibility criteria, including 2 subjects with preexisting diabetes mellitus, who were instructed to stop taking a study drug when the history was discovered. All enrolled subjects, including those who were subsequently found to be ineligible, were followed for HIV infection and safety. Quarterly-visit attendance is shown. Visits were considered to have been completed if they occurred before the subsequent visit window, with completion rates of 75 to 94% for all visits. The completion rate was more than 86% for all visits before week 132. Visits occurred within the protocol-defined window of ±5 days in 62 to 86% of visits.

SEXUAL PRACTICES

Sexual practices were similar in the two groups at all time points (P=0.97) (Fig. S2 in the Supplementary Appendix). The total numbers of sexual partners with whom the respondent had receptive anal intercourse decreased, and the percentage of those partners who used a condom increased after subjects enrolled in the study. There were no significant between-group differences in the numbers of subjects with syphilis (P=0.49), gon-

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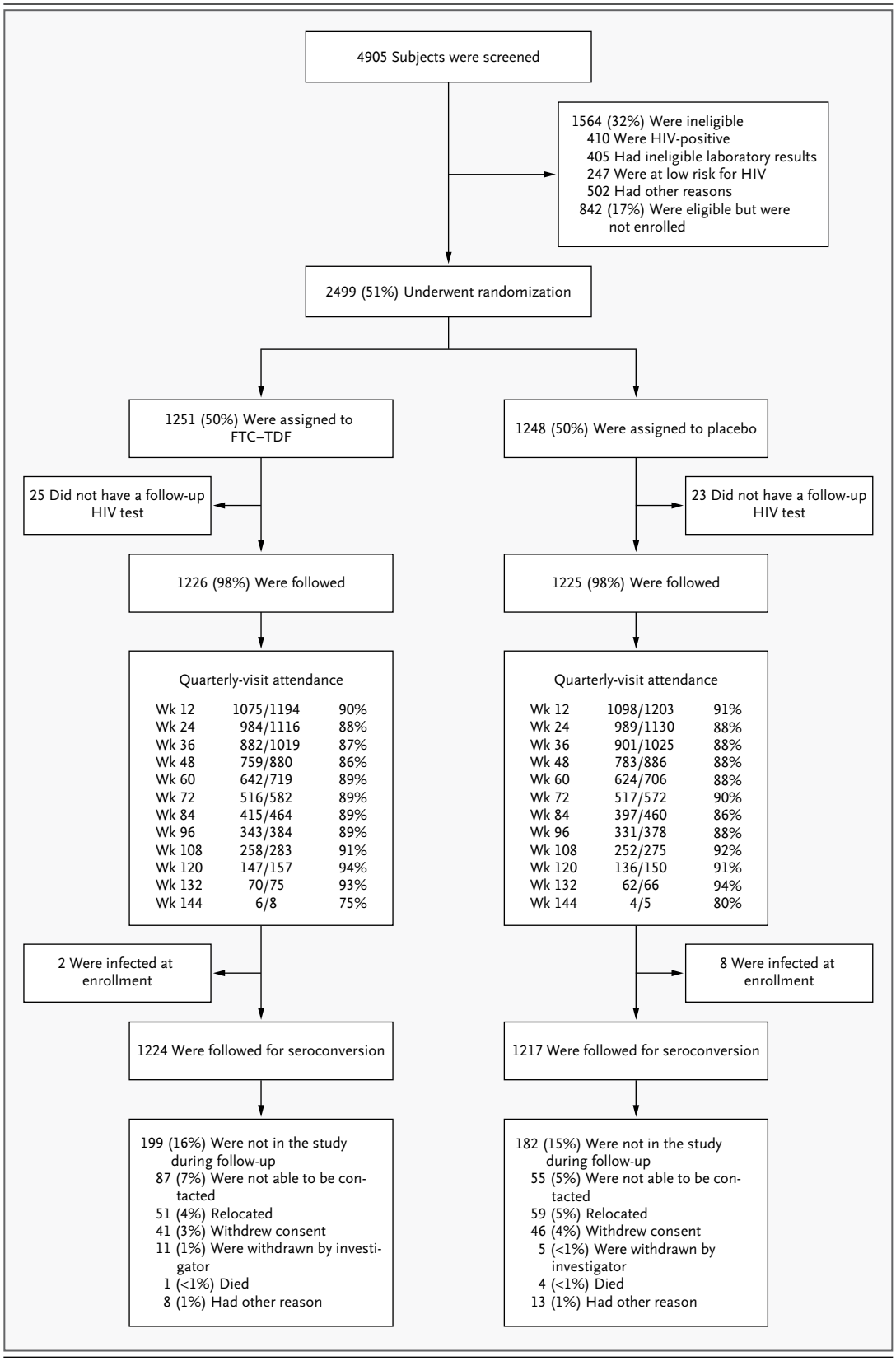

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Table 1. Baseline Characteristics of the Subjects.*

Characteristic	FTC–TDF (N=1251)	Placebo (N=1248)	P Value
Age group — no. (%)			0.04
18–24 yr	591 (47)	662 (53)	
25–29 yr	274 (22)	241 (19)	
30–39 yr	249 (20)	224 (18)	
≥40 yr	137 (11)	121 (10)	
Education level — no. (%)			0.26
Less than secondary	279 (22)	244 (20)	
Completed secondary	430 (34)	453 (36)	
Postsecondary	525 (42)	539 (43)	
No answer or missing data	17 (1)	12 (1)	
Race or ethnic group — no. (%)†			0.40
Black	117 (9)	97 (8)	
White	223 (18)	208 (17)	
Mixed race or other	849 (68)	878 (70)	
Asian	62 (5)	65 (5)	
Hispanic	900 (72)	906 (73)	0.72
No. of alcoholic drinks (on days when subject drank in past month) — no. (%)			0.66
0	206 (16)	184 (15)	
1–4 per day	348 (28)	345 (28)	
≥5 per day	666 (53)	687 (55)	
No answer or missing data	31 (2)	32 (3)	
City and country of residence — no. (%)			1.00
Lima, Peru	470 (38)	470 (38)	
Iquitos, Peru	230 (18)	230 (18)	
Guayaquil, Ecuador	150 (12)	150 (12)	
Rio de Janeiro	147 (12)	147 (12)	
São Paulo	39 (3)	37 (3)	
San Francisco	70 (6)	70 (6)	
Boston	43 (3)	44 (4)	
Chiang Mai, Thailand	57 (5)	57 (5)	
Cape Town, South Africa	45 (4)	43 (3)	
Sexual risk factors at screening			
No. of partners in past 12 wk	18±35	18±43	0.51
Unprotected receptive anal intercourse in past 12 wk — no. (%)	732 (59)	753 (60)	0.37
Unprotected anal intercourse with partner with positive or unknown HIV status in past 6 mo — no. (%)	992 (79)	1009 (81)	0.34
Transactional sex in past 6 mo — no. (%)	517 (41)	510 (41)	0.84
Known partner with HIV in past 6 mo — no. (%)	23 (2)	32 (3)	0.22
Sexually transmitted infections diagnosed at screening			
Syphilis seroreactivity — no./total no. (%)	164/1240 (13)	162/1239 (13)	0.95
Serum herpes simplex virus type 2 — no./total no. (%)	458/1241 (37)	430/1243 (35)	0.24
Urine leukocyte esterase positive — no. (%)	23 (2)	22 (2)	1.00
Hepatitis B virus status — no. (%)			0.11
Susceptible	827 (66)	803 (64)	
Immune because of natural infection	247 (20)	222 (18)	
Immune because of previous vaccination	149 (12)	190 (15)	
Current infection with hepatitis B virus	7 (1)	6 (<1)	
Indeterminate	21 (2)	27 (2)	

* Plus–minus values are means ±SD. Percentages may not total 100 because of rounding. FTC–TDF denotes emtricitabine and tenofovir disoproxil fumarate.
† Race or ethnic group was self-reported.

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Table 2. Adverse Events.*

Adverse Event	FTC–TDF (N=1251)		Placebo (N=1248)		P Value†
	no. of patients (%)	*no. of events*	*no. of patients (%)*	*no. of events*	
Any adverse event	867 (69)	2630	877 (70)	2611	0.50
Any serious adverse event	60 (5)	76	67 (5)	87	0.57
Any grade 3 or 4 event	151 (12)	248	164 (13)	285	0.51
Grade 3 event	110 (9)	197	117 (9)	225	0.65
Grade 4 event	41 (3)	51	47 (4)	60	0.57
Elevated creatinine level	25 (2)	28	14 (1)	15	0.08
Headache	56 (4)	66	41 (3)	55	0.10
Depression	43 (3)	46	62 (5)	63	0.07
Nausea	20 (2)	22	9 (<1)	10	0.04
Unintentional weight loss (≥5%)	27 (2)	34	14 (1)	19	0.04
Diarrhea	46 (4)	49	56 (4)	61	0.36
Bone fracture	15 (1)	16	11 (<1)	12	0.41
Death	1 (<1)‡	1	4 (<1)	4	0.18
Discontinuation of study drug					
Permanently	25 (2)	26	27 (2)	33	0.82
Permanently or temporarily	79 (6)	99	72 (6)	92	0.49

* A listing of all laboratory abnormalities and clinical adverse events of grade 2 or higher that were reported in 25 or more subjects (1%) is provided in Tables S9 and S10 in the Supplementary Appendix. FTC–TDF denotes emtricitabine and tenofovir disoproxil fumarate.
† P values were calculated by the log-rank test.
‡ This death was due to a motorcycle accident.

orrhea (P=0.74), chlamydia (P=0.43), genital warts (P=0.53), or genital ulcers (P=0.62) during follow-up (Table S4 in the Supplementary Appendix).

SAFETY

In testing for elevations in serum creatinine levels, there were 41 instances of elevations that were at least 1.1 times the upper limit of the normal range or more than 1.5 times the baseline level. Of these elevations, 26 (2%) were in the FTC–TDF group and 15 (1%) were in the placebo group (P=0.08). Two of these elevations increased in grade, accounting for a total of 43 creatinine adverse events (Table 2, and Table S9 in the Supplementary Appendix). Overall, 18 creatinine elevations (44%) remained in the normal range, and 36 (88%) were not confirmed on the next test. A total of 10 elevations led to discontinuation of a study drug (7 in the FTC–TDF group and 3 in the placebo group); study drugs were restarted in 9 subjects. Serum creatinine levels were elevated at more than one consecutive test in 5 subjects in the FTC–TDF group (<1%) and in none of the subjects in the placebo group. All elevations in the serum creatinine level resolved after the discontinuation of a study drug, within 4 weeks in 3 subjects, within 12 weeks in 1 subject, and within 20 weeks in 1 subject. Four of the subjects resumed taking FTC–TDF without recurrence of the elevation.

Moderate nausea (grade 2 and above) was reported more frequently in the FTC–TDF group than in the placebo group (22 vs. 10 events, P=0.04), as was unintentional weight loss of 5% or more (34 vs. 19 events, P=0.04) (for details, see Table S10 in the Supplementary Appendix).

EFFECT OF FTC–TDF ON HIV ACQUISITION

HIV rapid testing was performed at 39,613 visits, during which there were false reactive tests for 3 subjects at 7 visits; each subject had multiple negative tests afterward. HIV seroconversion was observed in 110 persons, of whom 10 had plasma HIV RNA subsequently detected in specimens obtained at the enrollment visit. A finding of fewer than 40 copies per milliliter of plasma HIV RNA was documented for the other 100 HIV-infected

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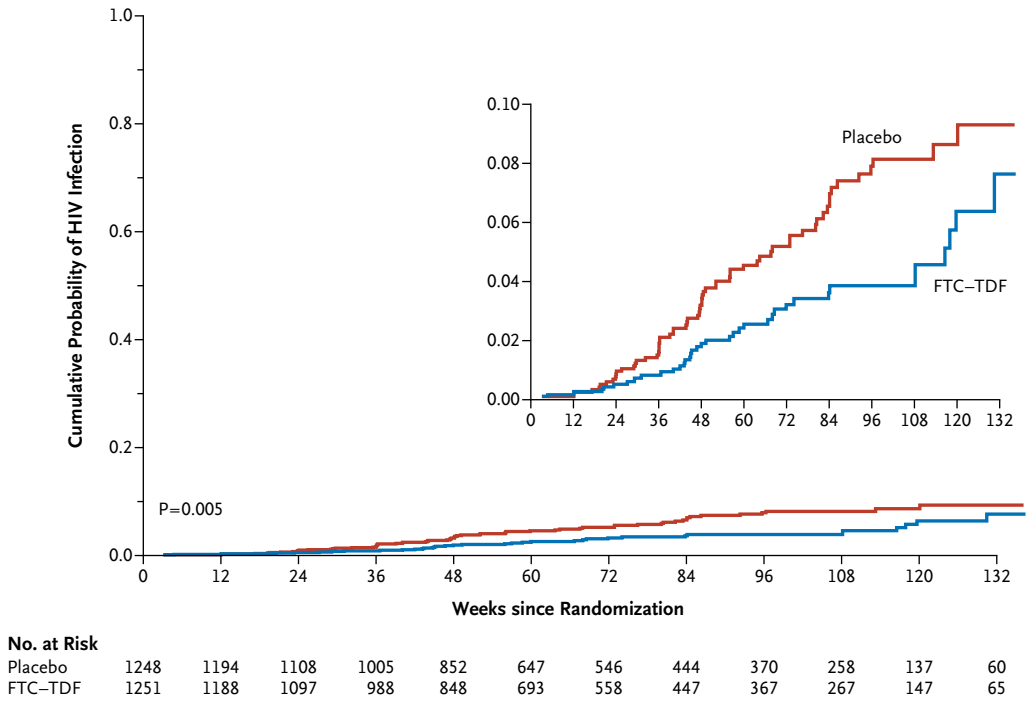

No. at Risk

Placebo	1248	1194	1108	1005	852	647	546	444	370	258	137	60
FTC–TDF	1251	1188	1097	988	848	693	558	447	367	267	147	65

Figure 2. Kaplan–Meier Estimates of Time to HIV Infection (Modified Intention-to-Treat Population).
The cumulative probability of HIV acquisition is shown for the two study groups. The efficacy of preexposure prophylaxis with emtricitabine and tenofovir disoproxil fumarate (FTC–TDF) was 44%, as compared with placebo (P=0.005). The inset graph shows a more detailed version of the overall graph up to a probability of 0.10.

subjects before seroconversion. Among the 100 subjects with emergent HIV infection, 36 occurred in the FTC–TDF group, and 64 occurred in the placebo group, representing a relative reduction of 44% in incidence in the modified intention-to-treat population (95% confidence interval [CI], 15 to 63; P=0.005) (Fig. 2). After adjustment for the difference in age between the two groups, the efficacy was 43% (95% CI, 14 to 62). The rate of pill use on 50% or more of days was recorded on the basis of pill counts, self-report, and dispensation records at 81% of visits on which efficacy was 50% (95% CI, 18 to 70; P=0.006). This rate did not differ significantly (P=0.48) from the efficacy at visits with less than 50% pill use of 32% (95% CI, −41 to 67%) (Fig. 3). Efficacy of less than 30% could not be ruled out in the modified intention-to-treat analysis (P=0.15) or in the prespecified as-treated analysis at 50% pill use (P=0.09). There was no evidence of a change in HIV efficacy with longer follow-up (P=0.44).

In prespecified analyses of efficacy according to subgroup, efficacy was higher among subjects who reported at screening that they had previously had unprotected receptive anal intercourse than among those who did not (efficacy, 58%; 95% CI, 32 to 74) (Fig. 3). There was no significant between-group difference in protection on the basis of region, race or ethnic group, male circumcision, level of education, alcohol use, or age. In post hoc analyses, pill use on 90% or more of days was recorded at 49% of visits on which efficacy was 73% (95% CI, 41 to 88; P<0.001). Among all subjects, without exclusion for HIV infection at enrollment or the degree of compliance to the drug regimen, the efficacy was 47% (95% CI, 22 to 64; P=0.001).

Among the 10 subjects in whom plasma HIV RNA was subsequently detected in specimens obtained at enrollment, 5 had symptoms of an acute viral syndrome at enrollment, 2 had symptoms 1 week later (prompting an interim study visit), 1 had an anal sore, and 2 had leukopenia at enrollment. In these subjects, the clinicians did not suspect acute HIV infection, because the symptoms were attributed to an upper respira-

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Subgroup	FTC–TDF	Placebo	FTC–TDF	Placebo	Hazard Ratio (95% CI)	P Value
	no. of patients		*no. of events*			
Analysis						
Intention-to-treat	1251	1248	38	72	0.53 (0.36–0.78)	0.001
Modified intention-to-treat	1251	1248	36	64	0.56 (0.37–0.85)	0.005
As treated						0.48
<50% Pill use	NA	NA	13	17	0.68 (0.33–1.41)	
≥50% Pill use	NA	NA	23	47	0.50 (0.30–0.82)	
Pill use						0.02
<90% Pill use	NA	NA	28	34	0.79 (0.48–1.31)	
≥90% Pill use	NA	NA	8	30	0.27 (0.12–0.59)	
Age						0.36
<25 yr	591	662	22	37	0.67 (0.40–1.14)	
≥25 yr	660	586	14	27	0.41 (0.24–0.87)	
Education						0.16
<Secondary education	279	244	12	12	0.89 (0.40–1.98)	
≥Secondary education	955	992	23	52	0.46 (0.28–0.74)	
Ethnic group						0.79
Non-Hispanic	351	342	4	8	0.48 (0.14–1.60)	
Hispanic	900	906	32	56	0.57 (0.37–0.89)	
Region						0.62
Andean	850	850	32	55	0.59 (0.38–0.91)	
Non-Andean	401	398	4	9	0.43 (0.13–1.39)	
Risk at screening						0.01
URAI	732	753	23	56	0.42 (0.26–0.68)	
No URAI	519	495	13	8	1.59 (0.66–3.84)	
Daily alcohol use						0.38
0–4 Drinks	554	529	15	32	0.43 (0.23–0.80)	
≥5 Drinks	666	687	19	32	0.63 (0.36–1.11)	
Circumcised						0.22
No	1085	1074	34	55	0.62 (0.40–0.95)	
Yes	162	170	2	9	0.23 (0.05–1.06)	
HSV-2 at screening						0.32
Negative or indeterminate	783	813	17	38	0.46 (0.26–0.82)	
Positive	458	430	19	26	0.70 (0.39–1.2)	

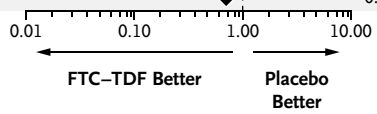

Figure 3. HIV Incidence among Subjects Receiving FTC–TDF, According to Subgroup.

The efficacy of emtricitabine and tenofovir disoproxil fumarate (FTC–TDF) is 1 minus the hazard ratio. Hazard ratios of less than 1 indicate efficacy, and 95% confidence intervals (shown by horizontal lines) that do not cross 1 indicate significant evidence of efficacy. All subgroup analyses were prespecified except for testing for herpes simplex virus type 2 (HSV-2) at screening and pill use at the rate of 90%. P values for the intention-to-treat analysis and the modified intention-to-treat analysis apply to the hypothesis of any evidence of efficacy; P values for other comparisons refer to the hypothesis that efficacy differed between the two strata. NA denotes not applicable, and URAI unprotected receptive anal intercourse.

tory tract infection, sinusitis, or other non-HIV cause.

Of the preexisting HIV infections at enrollment, two occurred in the FTC–TDF group and eight in the placebo group (P=0.06). Among subjects who were infected after enrollment, the numbers with detectable plasma HIV RNA before seroconversion were 5 of 36 (14%) in the FTC–TDF group and 7 of 64 (11%) in the placebo group (P=0.75). The time to seroconversion after RNA detection was similar in the two groups (P=0.55). After the discontinuation of a study drug, seroconversion rates were similar among 320 subjects (161 in the FTC–TDF group and 159 in the placebo group) (P=0.42). These subjects had a total of 1173 visits for HIV testing after the discontinuation of a study drug (642 in the FTC–TDF group and 531 in the placebo group).

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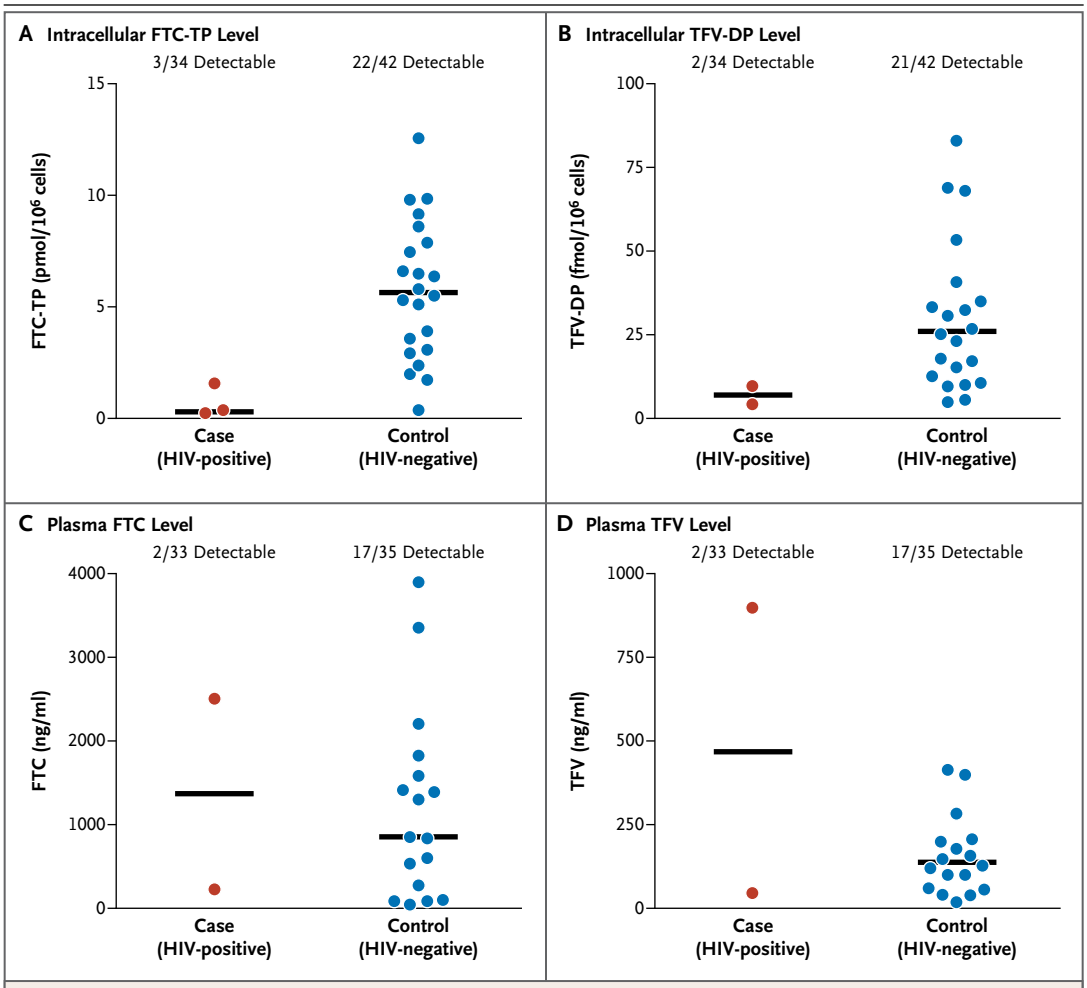

Figure 4. Levels of Study-Drug Components in Blood of Subjects Receiving FTC–TDF, According to HIV Status. Shown are intracellular levels (Panels A and B) and plasma levels (Panels C and D) of components of emtricitabine and tenofovir disoproxil fumarate (FTC–TDF), quantified in specimens obtained from subjects in the FTC–TDF group. FTC-TP denotes emtricitabine triphosphate, and TFV-DP tenofovir diphosphate. The horizontal lines in each panel indicate medians.

During these visits, 5 seroconversions were observed (2 in the FTC–TDF group and 3 in the placebo group).

DRUG-LEVEL DETECTION AND PROPHYLACTIC EFFECT

Among subjects who became infected with HIV, the median time between the tested specimen date and the last uninfected visit was 35 days (interquartile range, 28 to 56). No drug was detected in any plasma or cell specimens from subjects in the placebo group. Among subjects in the FTC–TDF group, at least one of the study-drug components was detected in 3 of 34 subjects with HIV infection (9%) and in 22 of 43 seronegative control subjects (51%) (Fig. 4). Of the 3 HIV-infected subjects with a detectable level of a study drug, none had cell-associated drug levels higher than the median for the 22 seronegative control subjects in whom a study-drug component was detected. Only 8% of subjects with HIV infection and 54% of control subjects who were considered "on treatment" on more than 50% of days had a detectable level of a study drug in plasma or peripheral-blood mononuclear cells (Table S8 in the Supplementary Appendix). Detection of the different drug components was more than 95% concordant (Table S6 in the Supplementary Appendix).

In the FTC–TDF group, among subjects with a detectable study-drug level, as compared with

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those without a detectable level, the odds of HIV infection were lower by a factor of 12.9 (95% CI, 1.7 to 99.3; P<0.001), corresponding to a relative reduction in HIV risk of 92% (95% CI, 40 to 99; P<0.001). After adjustment for reported unprotected receptive anal intercourse, the relative risk reduction was 95% (95% CI, 70 to 99; P<0.001).

EFFECT OF FTC–TDF ON HIV INFECTION

Plasma HIV RNA levels and CD4+ T-cell counts were similar among subjects with seroconversion in the two groups (Fig. S4 in the Supplementary Appendix). Among the 10 subjects who were infected at enrollment, 3 had FTC-resistant infections (2 of 2 in the FTC–TDF group and 1 of 8 in the placebo group) (Table S5 in the Supplementary Appendix). No TDF-resistant infections were observed. Among 36 subjects in the FTC–TDF group and 64 subjects in the placebo group who became infected with HIV during the trial, no FTC or TDF resistance was detected.

DISCUSSION

Once-daily oral FTC–TDF provided 44% additional protection from HIV among men or transgender women who have sex with men who also received a comprehensive package of prevention services. The protective effect of FTC–TDF was significant but not as high as originally hypothesized during the design of the study. Although reported pill use was high, drug exposure that was measured objectively was substantially lower. The intracellular assay that was used in this study is expected to detect TFV-DP for 14 days or more after the last dose of TDF is taken (see Methods in the Supplementary Appendix). Other evidence of low drug exposure included the lack of drug resistance observed among emergent infections and the absence of suppression of the HIV RNA level in plasma at the seroconversion visit. There was no evidence of delayed seroconversion among subjects who were infected in the FTC–TDF group. More information will be available after the entire cohort stops receiving the study drug.

The estimate of biologic activity of FTC–TDF persists after adjustment for high-risk sexual practice, suggesting that the correlation between drug detection and protection is primarily due to the drug and not to other characteristics of subjects that may link poor adherence with higher risk. The testing of a larger number of specimens, from more subjects at more times, is needed to better define the minimum protective drug concentration. Protective drug levels may differ according to the type of exposure (rectal vs. penile). Drug level may have a role in monitoring trials, programs, and individual users. Methods for inexpensively measuring long-term drug exposure, such as that afforded by analysis of hair,[19] would be helpful once such a method is fully validated.

Side effects may have contributed to low pill use among some subjects. As with treatment of HIV infection and the use of FTC–TDF in postexposure prophylaxis,[20] the initiation of FTC–TDF preexposure prophylaxis was associated with self-limited start-up symptoms in a few subjects. The trial design involving a placebo may also have contributed to lower-than-expected pill use. All subjects were counseled that the study pill might be a placebo or an active drug having no proven benefit. Open-label research and program development could provide users with clearer information about expected benefits and risks, which might increase the use and efficacy of preexposure prophylaxis. Engagement with communities and additional behavioral research are needed to develop methods of counseling that better support such use.

The initiation of chemoprophylaxis either before or after exposure should be deferred in patients with signs or symptoms of a viral syndrome, which are often present during acute HIV infection.[21,22] The initiation of postexposure prophylaxis in patients who are RNA-positive but antibody-negative has been linked with acquisition of resistance to FTC and lamivudine (3TC),[5] as occurred in subjects in the FTC–TDF group who were already infected at enrollment in our trial. Ways to increase recognition of acute HIV infection would include routine measurement of body temperature and testing for HIV antibodies to evaluate viral syndromes, regardless of whether the presentation suggests HIV infection or another cause. Testing for HIV RNA at the time of the initiation of preexposure prophylaxis should be considered where available.

TDF treatment is known to cause decreases in renal function,[23] and there were trends toward more creatinine elevations in the FTC–TDF group than in the placebo group. Most creatinine elevations were self-limited and were not confirmed on repeat testing of a new specimen, as might occur due to dehydration, creatine use, or exercise. The ability to detect safety outcomes, including drug resistance, may have been decreased by

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lower-than-expected drug exposure. In light of evidence of the efficacy of FTC–TDF, more information is needed about possible subclinical effects that may affect bone mineral density, low-level drug resistance, and proximal renal tubular function. Flares of hepatitis caused by HBV after stopping preexposure prophylaxis with TDF were not seen in West African women,[12] but more information is needed. These issues are being investigated in existing trials of preexposure prophylaxis.

Reported high-risk behavior decreased substantially after enrollment and remained lower than at baseline during the trial. Safer behavior was also observed in a trial of preexposure prophylaxis with TDF in West African women[12] and may reflect the services (e.g., counseling, testing, and dispensing of condoms) that are provided as part of such interventions. In addition, taking a pill a day may have served as a daily reminder of imminent risk and may have promoted planning for sex, which has been associated with lower HIV risk.[24] Behavioral changes during future open-label use of preexposure prophylaxis may differ because of an increased expectation of benefits, although such "risk compensation" was not observed during an open-label study of postexposure prophylaxis, during which benefits were expected.[25]

The optimal regimen for preexposure prophylaxis has not been established, and data from the subjects in our study cannot be applied to other populations. Alternative regimens in different populations are being studied. (Details are available in the Discussion in the Supplementary Appendix and at www.avac.org.)

In our study, preexposure prophylaxis with oral FTC–TDF among men and transgender women who have sex with men addressed an important unmet need in public health. HIV prevalence is higher in this population than in other groups in almost all countries.[14] In the United States, rates of HIV infection among such men and transgender women have climbed since the early 1990s, affecting in particular black and Hispanic subpopulations.[26] Intensive counseling in behavioral risk reduction for such subjects has not been shown to be better than standard counseling.[27] Although male circumcision partially protects heterosexual men,[28-30] penile circumcision is not expected to protect those who are exposed on the rectal mucosa.[31] Heterosexual women were partially protected by tenofovir 1% vaginal gel,[13] but the safety and utility of tenofovir topical gels for rectal use is not yet known. In the FTC–TDF group, there was increased efficacy among subjects who reported having unprotected receptive anal intercourse, which is the main mode of HIV transmission among the subjects in our study and increases the risk of heterosexual women who engage in the practice.[32] We showed that such subjects with a high risk of exposure to HIV can be mobilized to participate in prevention initiatives and that preexposure prophylaxis is effective for slowing the spread of HIV in this population.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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APPENDIX

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Antiretroviral Prophylaxis for HIV Prevention in Heterosexual Men and Women

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ABSTRACT

BACKGROUND

Antiretroviral preexposure prophylaxis is a promising approach for preventing human immunodeficiency virus type 1 (HIV-1) infection in heterosexual populations.

METHODS

We conducted a randomized trial of oral antiretroviral therapy for use as preexposure prophylaxis among HIV-1–serodiscordant heterosexual couples from Kenya and Uganda. The HIV-1–seronegative partner in each couple was randomly assigned to one of three study regimens — once-daily tenofovir (TDF), combination tenofovir–emtricitabine (TDF–FTC), or matching placebo — and followed monthly for up to 36 months. At enrollment, the HIV-1–seropositive partners were not eligible for antiretroviral therapy, according to national guidelines. All couples received standard HIV-1 treatment and prevention services.

RESULTS

We enrolled 4758 couples, of whom 4747 were followed: 1584 randomly assigned to TDF, 1579 to TDF–FTC, and 1584 to placebo. For 62% of the couples followed, the HIV-1–seronegative partner was male. Among HIV-1–seropositive participants, the median CD4 count was 495 cells per cubic millimeter (interquartile range, 375 to 662). A total of 82 HIV-1 infections occurred in seronegative participants during the study, 17 in the TDF group (incidence, 0.65 per 100 person-years), 13 in the TDF–FTC group (incidence, 0.50 per 100 person-years), and 52 in the placebo group (incidence, 1.99 per 100 person-years), indicating a relative reduction of 67% in the incidence of HIV-1 with TDF (95% confidence interval [CI], 44 to 81; $P<0.001$) and of 75% with TDF–FTC (95% CI, 55 to 87; $P<0.001$). Protective effects of TDF–FTC and TDF alone against HIV-1 were not significantly different ($P=0.23$), and both study medications significantly reduced the HIV-1 incidence among both men and women. The rate of serious adverse events was similar across the study groups. Eight participants receiving active treatment were found to have been infected with HIV-1 at baseline, and among these eight, antiretroviral resistance developed in two during the study.

CONCLUSIONS

Oral TDF and TDF–FTC both protect against HIV-1 infection in heterosexual men and women. (Funded by the Bill and Melinda Gates Foundation; Partners PrEP ClinicalTrials.gov number, NCT00557245.)

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The use of antiretroviral medications for the prevention of HIV type 1 (HIV-1) transmission is a promising strategy for reducing the spread of HIV-1.[1-4] Antiretroviral treatment for persons infected with HIV-1 provides important clinical benefits and substantially reduces infectiousness.[5-7] Antiretroviral prophylaxis is a potential HIV-1–prevention strategy for those not yet infected with HIV-1, administered either as postexposure prophylaxis after high-risk occupational or nonoccupational exposure or as preexposure prophylaxis in those with ongoing HIV-1 exposure.[8,9] The rationale for antiretroviral prophylaxis in persons with ongoing exposure is based on its efficacy in infants exposed to HIV-1 during birth and breast-feeding[10] and the partial or full protection it confers against mucosal simian HIV challenge in primates.[11] In perinatal-transmission studies and animal models, the protective benefits of antiretroviral prophylaxis were maximized when the antiretroviral medication was administered both before and after HIV exposure.[12]

The efficacy of preexposure prophylaxis for HIV-1 protection in humans has been evaluated for tenofovir, in the form of a vaginal gel or as oral tenofovir disoproxil fumarate (TDF) or oral TDF coformulated with emtricitabine (TDF–FTC). Studies in animal models suggest that TDF–FTC provides greater protection against HIV-1 than TDF alone.[11] The possibility of differential efficacy, safety, and cost suggests that TDF and TDF–FTC could be compared as potential preexposure prophylaxis agents. Persons at ongoing risk for HIV-1 acquisition in whom preexposure prophylaxis could be studied include persons who are HIV-1–seronegative but are in a partnership with a person already infected with HIV-1 (an HIV-1–serodiscordant partnership).[13,14] We conducted the Partners Preexposure Prophylaxis (PrEP) Study, a multisite, phase 3, randomized, double-blind, three-group, placebo-controlled trial of daily oral TDF or TDF–FTC given as preexposure prophylaxis against HIV-1 acquisition among East African heterosexual men and women in HIV-1–serodiscordant partnerships.

METHODS

STUDY OVERSIGHT

The Bill and Melinda Gates Foundation funded the study but did not oversee the protocol. Gilead Sciences donated the study medication but had no role in data collection, data analysis, or manuscript preparation. All authors vouch for the completeness and accuracy of the data presented.

STUDY POPULATION

From July 2008 through November 2010, we enrolled heterosexual couples in which one partner was infected with HIV-1 and the other partner was not infected (HIV-1–serodiscordant couples) from nine sites in Kenya and Uganda (see Tables S1 and S2 in the Supplementary Appendix, available with the full text of this article at NEJM.org).[15] The HIV-1–seronegative partners had normal renal function, were not infected with hepatitis B virus, and were not pregnant or breast-feeding. The HIV-1–seropositive partners were not receiving antiretroviral therapy and did not meet Kenyan or Ugandan guidelines for initiation of antiretroviral therapy.

The full study protocol and statistical analysis plan are available at NEJM.org. The study protocol was approved by the University of Washington Human Subjects Review Committee and ethics review committees at each of the study sites (see Table S3 in the Supplementary Appendix). All participants provided written informed consent in English or their local language.

RANDOMIZATION AND STUDY PROCEDURES

At enrollment, partners seronegative for HIV-1 were assigned, in a 1:1:1 ratio, to one of the three study groups: once-daily TDF, TDF–FTC, or placebo. Randomization was achieved by means of fixed-size block randomization, with stratification by site. TDF was given at a dose of 300 mg, and FTC was given at a dose of 200 mg; these doses are also the standard for treatment of HIV-1. The study regimens were indistinguishable in appearance, and investigators, except for statistical staff at the central coordinating center, were unaware of the study-group assignments.

All participants received a comprehensive package of HIV-1 prevention services: HIV-1 testing with counseling before and after testing, individual and couples risk-reduction counseling, screening and treatment for sexually transmitted infections, free condoms with training and counseling, and referral for male circumcision and postexposure prophylaxis according to national policies. Vaccination against hepatitis B virus was also offered.

Participants seronegative for HIV-1 had monthly visits that involved HIV-1 testing, dispensation

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of 30 days of study medication, collection of the prior month's unused medication, individualized adherence counseling, and standardized assessment of sexual behavior and side effects (see Table S4 in the Supplementary Appendix). Serum chemical and hematologic analyses were performed at 1 month and quarterly thereafter. Women were tested monthly for pregnancy; study medication was withheld in women who became pregnant, and they were referred for antenatal care and allowed to resume study medication when no longer pregnant or lactating.

Partners seropositive for HIV-1 were followed quarterly (see Table S5 in the Supplementary Appendix), with HIV-1 primary care services administered and with CD4 counts obtained every 6 months. Those who became eligible for the initiation of antiretroviral therapy according to national guidelines were actively counseled to initiate treatment and referred to local clinics.

END POINTS

The primary end point was seropositivity in partners previously seronegative for HIV-1. Monthly HIV-1 serologic testing involved two rapid HIV-1 antibody tests in parallel. Study medication was temporarily withheld if either test revealed seroreactivity and was permanently discontinued if enzyme-immunoassay testing confirmed HIV-1 acquisition (see Table S6 in the Supplementary Appendix). For persons with enzyme immunoassay–confirmed acquisition of HIV-1, samples were then tested by HIV-1 Western blotting and RNA polymerase-chain-reaction (PCR) assay at the University of Washington and were adjudicated by an HIV-1 end points committee. Because the study medication was taken by the seronegative partner, HIV-1 sequence analysis to assess transmission within the study partnership was not required for end-point determination and was not performed. For all participants in whom seroconversion occurred, archived plasma samples from visits before seroconversion were tested by means of the HIV-1 RNA PCR assay; those who had detectable HIV-1 RNA in samples from the time of enrollment were excluded with respect to analysis of the primary end point because HIV-1 infection had occurred before randomization.

STATISTICAL ANALYSIS

The study was end-point–driven. We calculated that 147 HIV-1 seroconversion events for each comparison (TDF vs. placebo and TDF–FTC vs. placebo) would provide 80% power, with a one-sided alpha level of 0.025, to detect a 60% relative decrease in the incidence of HIV-1 infection, with a lower bound of the 95% confidence interval excluding a 30% relative decrease in incidence (the null hypothesis).[15] We further calculated that a sample size of 4700 couples would achieve the target number of study end points, with 24 to 36 months of follow-up per couple and an expected incidence of HIV-1 infection of 2.75 per 100 person-years in the placebo group.[16]

The primary analysis was a modified intention-to-treat analysis, excluding only data from participants with HIV-1 RNA detected in plasma by means of PCR assay at enrollment. We used Cox regression, stratified according to site, to estimate the relative rates of time to first positive HIV-1 serologic test and the Kaplan–Meier method to estimate the cumulative probability of HIV-1 infection.

The study data were reviewed every 6 months by an independent data and safety monitoring board. For statistical monitoring, the Lan–DeMets spending approach was used to adjust the O'Brien–Fleming sequential-monitoring boundaries[17,18]; interim monitoring boundaries were computed by means of S+SeqTrial software (version 2.0, TIBCO). During its closed March 2011 session, the board noted a strong trend toward HIV-1 protection in the active preexposure prophylaxis groups and called an ad hoc meeting for July 10, 2011. At the July meeting, after reviewing data through May 31, 2011, the board recommended that the results of the study be publicly reported and the placebo treatment discontinued, because predetermined stopping rules were met with the demonstration of HIV-1 protection from preexposure prophylaxis. The present analysis includes updated data collected through July 10, 2011. Analyses were conducted using SAS software (version 9.2, SAS Institute).

RESULTS

STUDY PARTICIPANTS

We screened 7856 couples with discordant HIV-1 serostatus. We enrolled 4758 couples and followed 4747: 1584 randomly assigned to TDF, 1579 to TDF–FTC, and 1584 to placebo (Fig. 1). For 62% of the couples followed, the HIV-1–seronegative partner was male (Tables 1 and 2). Among participants seropositive for HIV-1, the median CD4 count was 495 cells per cubic millimeter (inter-

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quartile range, 375 to 662), 80% had a CD4 count of 350 cells or more per cubic millimeter, and the median plasma HIV-1 RNA level was 3.9 $\log_{10}$ copies per milliliter (interquartile range, 3.2 to 4.5). Overall, baseline characteristics were similar across the three study groups.

FOLLOW-UP AND ADHERENCE

Retention was 96% or greater during the study period (Fig. 1), with 4722 of the 4747 followed participants (99.5%) completing at least one post-randomization HIV-1 test, for a total of 7830 person-years of follow-up for the assessment of

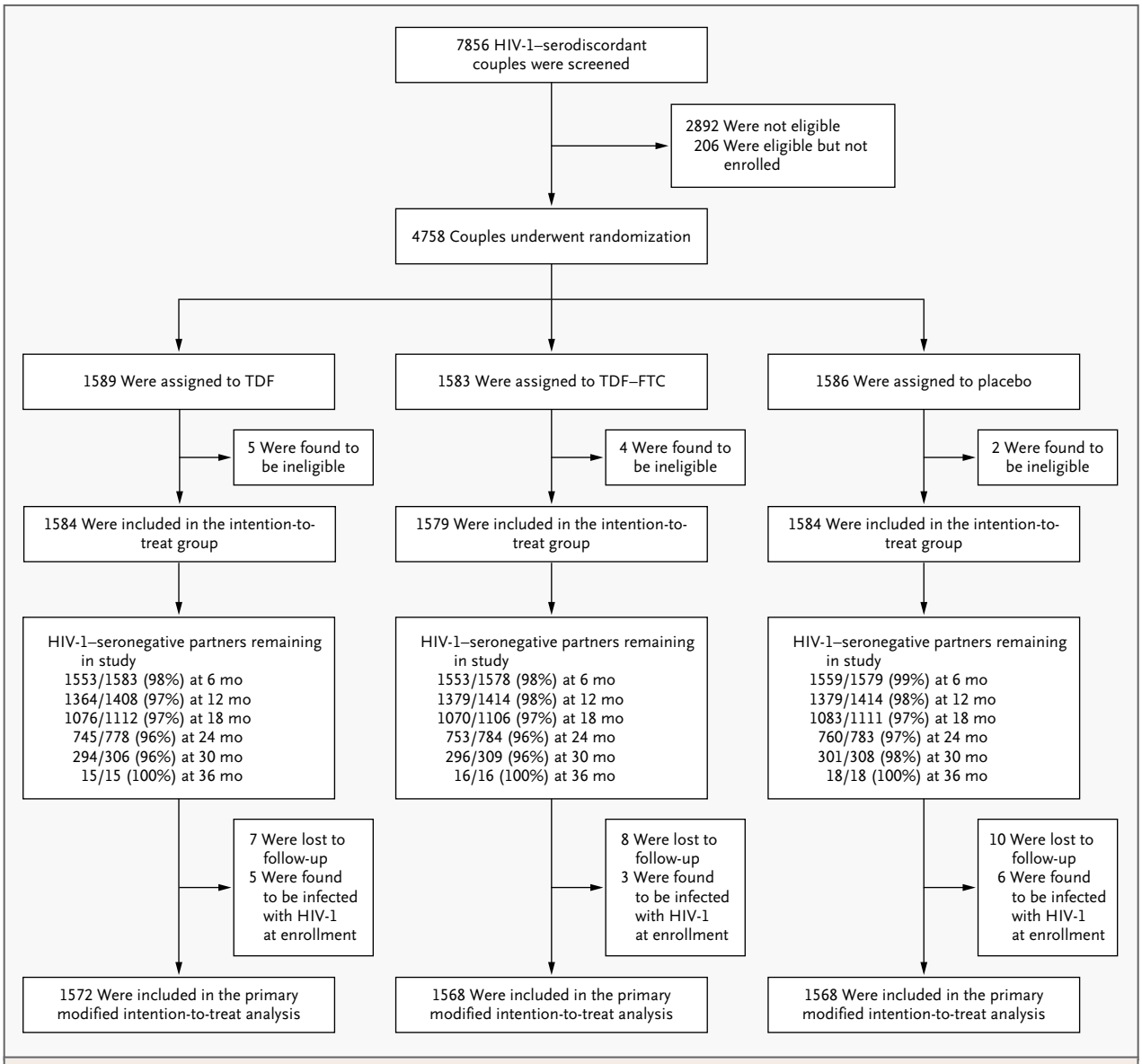

Figure 1. Enrollment and Follow-up of the Study Participants.

The most common reasons for ineligibility were HIV-1–seropositive partners' meeting national criteria for antiretroviral therapy initiation or already taking antiretroviral therapy (59%) and HIV-1–seronegative partners' being pregnant (2%), breast-feeding (0.4%), or having chronic active hepatitis B infection (10%). Less than 3% of ineligible couples met one of the exclusion criteria of creatinine elevation, glycosuria, or proteinuria in the HIV-1–seronegative partner, which were designed to minimize potential renal toxic effects from tenofovir (TDF) exposure. A total of 11 couples were enrolled and randomly assigned to one of the study groups but were later found not to meet all the eligibility criteria; they were discontinued from the study at the time their ineligibility was discovered, and their data were not included in analyses. At least 96% of HIV-1–seropositive partners remained in the study at any point during the follow-up period, and this percentage was similar across the three study groups. FTC denotes emtricitabine.

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Table 1. Baseline Characteristics of the Study Participants, According to Study Group and HIV-1 Serostatus.*

Characteristic	TDF (N=1584 couples)		TDF–FTC (N=1579 couples)		Placebo (N=1584 couples)	
	Seronegative Partner	Seropositive Partner	Seronegative Partner	Seropositive Partner	Seronegative Partner	Seropositive Partner
Male sex — no. (%)	986 (62)	598 (38)	1013 (64)	566 (36)	963 (61)	621 (39)
Age — no. (%)						
18–24 yr	184 (12)	268 (17)	177 (11)	287 (18)	172 (11)	273 (17)
25–34 yr	721 (46)	657 (41)	690 (44)	636 (40)	688 (43)	629 (40)
35–44 yr	480 (30)	474 (30)	498 (32)	460 (29)	513 (32)	509 (32)
≥45 yr	199 (13)	185 (12)	214 (14)	196 (12)	211 (13)	173 (11)
Education — yr						
Median	7	7	7	7	7	7
Range	4–10	4–9	4–10	4–9	4–10	4–9
Any monthly income — no. (%)	1275 (80)	1069 (67)	1236 (78)	1052 (67)	1259 (79)	1079 (68)
Any sex with outside partner in prior month — no. (%)	150 (9)	84 (5)	134 (8)	106 (7)	122 (8)	103 (7)
CD4 cell count — cells/mm^3						
Median	NA	491	NA	497	NA	499
Range	NA	370–661	NA	380–664	NA	375–663
HIV-1 plasma RNA — $\log_{10}$ copies/ml†						
Median	NA	3.9	NA	3.9	NA	3.9
Range	NA	3.2–4.5	NA	3.1–4.5	NA	3.2–4.5
Circumcised (men only) — no./total no. (%)	533/986 (54)	198/598 (33)	540/1013 (53)	177/566 (31)	509/963 (53)	202/621 (33)
Using contraception (women only) — no./total no. (%)‡	263/598 (44)	290/986 (29)	275/566 (49)	324/1013 (32)	299/621 (48)	321/963 (33)
Pregnant — no./total no. (%)	0/598	152/986 (15)§	0/566	135/1013 (13)	0/621	118/963 (12)
Infection with *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, or *Trichomonas vaginalis* — no./ total no. (%)¶‖	86/1551 (6)§	117/1520 (8)	93/1557 (6)§	122/1525 (8)	126/1550 (8)	137/1534 (9)
Syphilis seropositivity — no./total no. (%)¶**	59/1569 (4)	73/1576 (5)	60/1572 (4)	52/1573 (3)	62/1569 (4)	73/1571 (5)
HSV-2 seropositivity — no./total no. (%)¶††	835/1506 (55)	NA	814/1507 (54)§	NA	875/1512 (58)	NA

* FTC denotes emtricitabine, HIV-1 HIV type 1, NA not applicable, and TDF tenofovir.

† Plasma HIV-1 RNA concentrations were quantified in enrollment samples by means of batch testing at the University of Washington with the use of the Real-Time HIV-1 RNA assay (Abbott) at a limit of quantification of 80 copies per milliliter.

‡ Contraception includes hormonal oral, injectable, or implantable contraceptive agents; intrauterine devices; and hysterectomy or bilateral tubal ligation. A total of 83% of the HIV-1–seronegative women and 85% of the HIV-1–seropositive women using contraception used a hormonal agent.

§ Results of only four comparisons with the placebo group were significant: prevalence of *Neisseria gonorrhoeae, Chlamydia trachomatis,* or *Trichomonas vaginalis* infection among HIV-1–seronegative partners receiving TDF (6%, vs. 8% receiving placebo; P=0.04) and receiving TDF–FTC (6%, vs. 8% receiving placebo; P=0.02), prevalence of HSV-2 seropositivity among HIV-1–seronegative partners receiving TDF–FTC (54%, vs. 58% receiving placebo; P=0.03); and prevalence of pregnancy among HIV-1–seropositive women receiving TDF (15%, vs. 12% receiving placebo; P=0.04).

¶ Data on sexually transmitted infections were available for more than 95% of participants.

‖ Participants were treated for symptomatic sexually transmitted infections that were found. *T. vaginalis* was the most common infection, accounting for 75% of all the infections detected. *N. gonorrhoeae* and *C. trachomatis* were tested for by different means (APTIMA Combo 2 [Gen-Probe] or COBAS Amplicor [Roche Diagnostics]) than was *T. vaginalis* (APTIMA Trichomonas vaginalis [Gen-Probe] or InPouch TV [BioMed Diagnostics]).

** Syphilis serologic testing was by means of rapid plasma reagin, confirmed with the use of a treponema-specific assay.[15] Seropositivity could indicate current or past infection.

†† Herpes simplex virus type 2 (HSV-2) testing was done by means of the HerpeSelect 2 enzyme immunoassay (Focus Technologies) at enrollment only; an index value of 3.5 or greater was considered a positive result.[19]

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Table 2. Baseline Characteristics of the Study Couples, According to Study Group.*

Characteristic	TDF (N=1584)	TDF–FTC (N=1579)	Placebo (N=1584)
Married — no. (%)	1543 (97)	1540 (98)	1552 (98)
Years living together			
Median	7.0	7.1	7.3
Range	3.0–13.5	3.0–14.0	3.0–14.0
Number of children in partnership			
Median	2	2	2
Range	1–4	1–4	1–4
No children — no. (%)	343 (22)	368 (23)	342 (22)
Years aware of HIV-1–serodiscordant status			
Median	0.5	0.4	0.4
Range	0.1–2.0	0.1–2.0	0.1–2.0
Number of sex acts in prior month			
Median	4	4	4
Range	2–8	3–8	2–8
Any unprotected sex acts in prior month — no. (%)	442 (28)	416 (26)	409 (26)

* Characteristics of the couple were reported by the HIV-1–seronegative partner. No comparisons between either treatment group and the placebo group were significant.

HIV-1 incidence accrued (median, 23 months; interquartile range, 16 to 28; range, 1 to 36). Study medication was dispensed at 96% of the attended visits. The most common reason for not dispensing study medication was pregnancy (with an incidence of 11.9, 8.8, and 10.0 pregnancies per 100 woman-years in the TDF, TDF–FTC, and placebo groups, respectively; P>0.05). Time off the study medication due to pregnancy and breastfeeding accounted for 5.3% of the follow-up time among women (2.0% among all participants). Study-medication interruptions for safety-related reasons accounted for less than 1% of the overall follow-up time: 0.6% in the TDF group, 0.7% in the TDF–FTC group, and 0.6% in the placebo group.

The primary study measure of adherence was monthly counts of the returned study bottles and tablets: 98% of the dispensed study bottles were returned, and 97% of dispensed study tablets were taken (see Table S7 in the Supplementary Appendix). Factoring in missed visits, all reasons for nondispensation of study medication, and nonadherence to dispensed study pills, we calculated that study medication was in use during 92.1% of the total follow-up time.

EFFECT OF TDF AND TDF–FTC ON HIV-1 ACQUISITION

HIV-1 seroconversion was observed in 96 participants, of whom 14 had plasma HIV-1 RNA retrospectively detected in specimens obtained at enrollment (5 receiving TDF, 3 receiving TDF–FTC, and 6 receiving placebo) (Fig. 1, and Fig. S1 in the Supplementary Appendix). Of 82 HIV-1 infections developing after randomization, 17 were in the TDF group, 13 were in the TDF–FTC group, and 52 were in the placebo group, indicating relative reductions in the rates of HIV-1 acquisition of 67% due to TDF (95% confidence interval [CI], 44 to 81; P<0.001) and 75% due to TDF–FTC (95% CI, 55 to 87; P<0.001), each relative to placebo (Fig. 2). The HIV-1–protective effects of TDF–FTC and TDF were not significantly different (P=0.23). With both TDF (P=0.003) and TDF–FTC (P<0.001), efficacy of less than 30% was ruled out in the primary modified intention-to-treat analysis. The intention-to-treat analysis including data for participants who were infected with HIV-1 at randomization yielded similar results (Fig. 3).

As compared with placebo, among women, the efficacy of TDF was 71% (P=0.002) and of TDF–FTC 66% (P=0.005); among men, the efficacies were 63% (P=0.01) and 84% (P<0.001), respectively. The HIV-1–protective effects of TDF and TDF–FTC were not statistically different according to sex. Protection against HIV-1 was generally similar between subgroup categories for other prespecified subgroups analyses (Fig. 3). During the follow-up period, 21% of the partners seropositive for HIV-1 (22% in the TDF group, 20% in the TDF–FTC group, and 21% in the placebo group) started combination antiretroviral therapy; the HIV-1–protective effects of TDF and TDF–FTC were similar to those observed in the primary modified intention-to-treat analysis if follow-up time after the HIV-1–seropositive partner started antiretroviral therapy was excluded (see Table S8 in the Supplementary Appendix).

ANTIRETROVIRAL RESISTANCE

Of the 96 persons who had seroconversion to HIV-1 positivity, 92 (96%) had plasma samples available for amplification of the HIV-1 RNA to assess for resistance (Table S9 in the Supplementary Appendix). Among the 8 participants in the TDF and TDF–FTC groups who were found to have been infected at randomization, HIV-1 with resistance to the study medications developed in 2 participants: 1 in the TDF group had a TDF-

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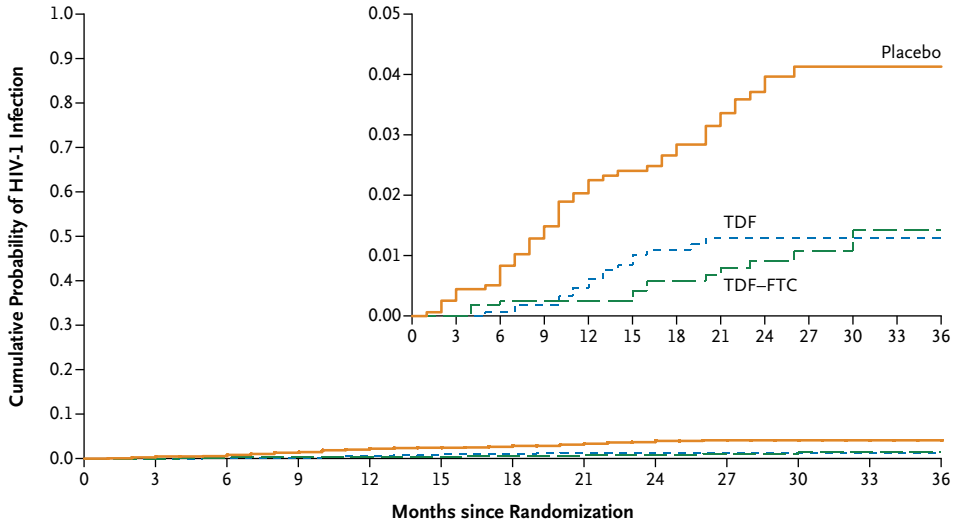

No. at Risk													
TDF	1572	1559	1547	1498	1350	1223	1062	902	735	510	287	108	15
TDF–FTC	1568	1557	1546	1493	1371	1248	1059	901	743	525	291	114	16
Placebo	1568	1557	1544	1487	1347	1224	1061	902	744	523	295	120	18

Figure 2. Kaplan–Meier Estimates of the Primary End Point in the Modified Intention-to-Treat Analysis, According to Study Treatment.

resistant virus (K65R mutation), and 1 in the TDF–FTC group had an FTC-resistant virus (M184V mutation). No participants who acquired HIV-1 after randomization were infected with an HIV-1 strain with the K65R or M184V mutation.

DETECTION OF TENOFOVIR AND PROPHYLACTIC EFFECT

Among 29 participants in the TDF and TDF–FTC groups who became infected with HIV-1, 31% had a detectable tenofovir level in a plasma sample obtained at the seroconversion visit, as compared with 82% of 902 samples from a random subgroup of 198 participants who did not acquire HIV-1 (Table S10 in the Supplementary Appendix). A detectable level of tenofovir, as compared with an undetectable level of the drug, was associated with estimated reductions in the relative risk of acquiring HIV-1 of 86% (with TDF) and 90% (with TDF–FTC).

SEXUAL BEHAVIOR

At enrollment, 27% of partners seronegative for HIV-1 reported having sex without a condom with their HIV-1–seropositive partner during the prior month. This percentage decreased during the follow-up period (to 13% and 9% at 12 and 24 months, respectively) and was similar across the study groups (see Fig. S2 in the Supplementary Appendix). The proportions of participants reporting outside partnerships and acquiring sexually transmitted infections during the follow-up period did not differ significantly across the study groups (see Table S11 in the Supplementary Appendix).

SAFETY AND ADVERSE EVENT PROFILES

There were no significant differences in the frequency of deaths, serious adverse events, or serum creatinine or phosphorus abnormalities across the study groups (Table 3, and Table S12 in the Supplementary Appendix). Neutropenia was seen more commonly in the TDF–FTC group (17% of participants with a grade 1 or 2 event and 1% with a grade 3 or 4 event) (see Table S13 in the Supplementary Appendix) than in the TDF group (15% of participants with a grade 1 or 2 event and 1% with a grade 3 or 4 event) or the placebo group (12% of participants with a grade 1 or 2 event and 1% with a grade 3 or 4 event). The active study medications were associated with modestly increased reports of gastrointestinal side effects

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Subgroup	TDF	TDF–FTC	Placebo	Hazard Ratio (95% CI), TDF vs. Placebo	P Value	Hazard Ratio (95% CI), TDF–FTC vs. Placebo	P Value
	no. of events/total no. (rate per 100 person-yr)						
Overall							
Modified intention-to-treat population	17/1579 (0.65)	13/1576 (0.50)	52/1578 (1.99)	0.33 (0.19–0.56)	<0.001	0.25 (0.13–0.45)	<0.001
Intention-to-treat population	22/1584 (0.84)	16/1579 (0.61)	58/1584 (2.22)	0.38 (0.23–0.62)	<0.001	0.27 (0.16–0.48)	<0.001
Sex of HIV-1–seronegative partner					0.65		0.24
Male	9/984 (0.56)	4/1010 (0.24)	24/959 (1.49)	0.37 (0.17–0.80)		0.16 (0.06–0.46)	
Female	8/595 (0.81)	9/566 (0.95)	28/619 (2.81)	0.29 (0.13–0.63)		0.34 (0.16–0.72)	
Age of HIV-1–seronegative partner					0.79		0.06
<25 yr	3/184 (1.07)	6/177 (2.34)	10/170 (4.04)	0.28 (0.08–1.01)		0.59 (0.21–1.61)	
≥25 yr	14/1395 (0.60)	7/1399 (0.30)	42/1408 (1.78)	0.34 (0.18–0.61)		0.17 (0.07–0.37)	
Unprotected sex with study partner during past mo					0.05		0.77
No	14/1138 (0.72)	8/1161 (0.40)	30/1170 (1.50)	0.47 (0.25–0.89)		0.27 (0.12–0.58)	
Yes	3/441 (0.46)	5/415 (0.78)	22/408 (3.60)	0.13 (0.04–0.44)		0.22 (0.08–0.58)	
Country					0.94		0.46
Kenya	7/699 (0.61)	7/697 (0.60)	22/694 (1.90)	0.32 (0.14–0.74)		0.31 (0.13–0.74)	
Uganda	10/880 (0.69)	6/879 (0.41)	30/884 (2.07)	0.33 (0.16–0.68)		0.20 (0.08–0.48)	
Circumcision status of HIV-1–seronegative men					0.54		0.42
Circumcised	6/542 (0.70)	3/543 (0.34)	13/512 (1.52)	0.46 (0.17–1.20)		0.22 (0.06–0.79)	
Uncircumcised	3/440 (0.40)	1/467 (0.12)	11/447 (1.45)	0.28 (0.08–1.00)		0.09 (0.01–0.68)	
Plasma HIV-1 RNA level of HIV-1–seropositive partner					0.39		0.79
<50,000 copies/ml	13/1277 (0.61)	9/1279 (0.42)	32/1263 (1.51)	0.40 (0.21–0.76)		0.28 (0.13–0.58)	
≥50,000 copies/ml	4/269 (0.90)	4/271 (0.90)	18/289 (3.93)	0.23 (0.08–0.69)		0.23 (0.08–0.68)	
CD4 count of HIV-1–seropositive partner					0.03		0.39
250–349 cells/mm^3	8/312 (1.56)	4/297 (0.78)	10/299 (1.95)	0.79 (0.31–2.01)		0.39 (0.12–1.26)	
≥350 cells/mm^3	9/1267 (0.43)	9/1279 (0.43)	42/1279 (2.01)	0.21 (0.10–0.44)		0.21 (0.10–0.44)	

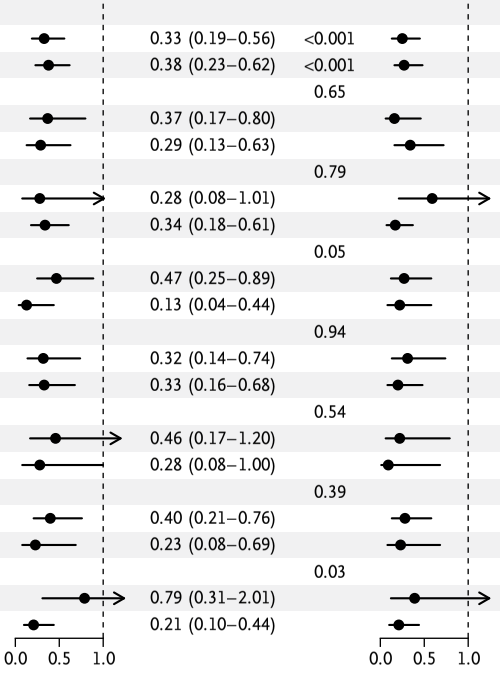

Figure 3. Hazard Ratios for HIV-1 Infection Overall and among Subgroups, According to Study Group.

P values for the modified intention-to-treat and the intention-to-treat analyses are for the hypothesis of any evidence of efficacy (i.e., testing against a null hypothesis of 0%); P values for the other comparisons correspond to a test for significant interaction in the site-stratified Cox proportional-hazards model. Of 45 HIV-1 infections in women, 5 occurred in women (2 in the TDF group and 3 in the placebo group) who had not received study medication for more than 3 months owing to pregnancy or breast-feeding.

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Table 3. Adverse Events, According to Study Group.*

Adverse Event	TDF (N=1584)	P Value vs. Placebo	TDF–FTC (N=1579)	P Value vs. Placebo	Placebo (N=1584)
	no. (%)		*no. (%)*		*no. (%)*
Any adverse event	1350 (85)	1.00	1362 (86)	0.42	1350 (85)
Any serious adverse event	118 (7)	1.00	115 (7)	0.89	118 (7)
Death†	8 (1)	0.80	8 (1)	0.80	9 (1)
Any grade 4 event	34 (2)	0.64	44 (3)	0.58	39 (3)
Any grade 3 event	289 (18)	0.35	293 (19)	0.24	268 (17)
Confirmed laboratory events‡					
Elevated creatinine§					
Grade 1	16 (1)	0.57	18 (1)	0.28	12 (1)
Grade 2 or 3	3 (<1)	0.62	2 (<1)	0.62	1 (<1)
Decreased phosphorus¶					
Grade 2	134 (8)	0.56	128 (8)	0.79	124 (8)
Grade 3	8 (1)	0.50	12 (1)	1.00	12 (1)

* All clinical adverse events of grade 2 or higher and all confirmed laboratory adverse events that were reported in 1% or more (≥47) of study participants are listed in Table S9 in the Supplementary Appendix. P values were calculated with the use of Fisher's exact test except for those for death, which were calculated by means of the Cox proportional-hazards model of time to death.

† In the TDF group, two participants died from trauma, two from alcohol poisoning, and one each from esophageal carcinoma, lung abscess, shigella gastroenteritis, and acute abdomen. In the TDF–FTC group, three participants died from trauma and one each from poisoning, pulmonary embolism, pulmonary tuberculosis, gastroenteritis, and acute febrile illness. In the placebo group, three participants died from trauma and one each from electrocution, suicide, hematemesis, complications of diabetes, febrile illness, and hypotension.

‡ Laboratory adverse events are reported here only if they were confirmed by means of repeat testing, ideally conducted within 7 days after the event.

§ One confirmed grade 3 case of elevated creatinine was observed in the study, in a 46-year-old male participant receiving TDF who had had seroconversion to HIV-1 seropositivity and had discontinued study medication 22 days previously. The creatinine level returned to normal after hydration. No confirmed grade 4 creatinine events were observed.

¶ According to the study protocol, there was no grade 1 decrease in phosphorus level, and no confirmed grade 4 phosphorus decreases were observed. Clinically significant proteinuria (≥grade 1) was observed in association with 27 confirmed grade 2 phosphorus decreases (nine participants in each of the three study groups) and 1 confirmed grade 3 event (in the placebo group). Glycosuria of grade 1 or greater was observed in association with 7 confirmed grade 2 phosphorus decreases (two participants each in the TDF and placebo groups and three in the TDF–FTC group) and 2 confirmed grade 3 events (one each in the TDF and placebo groups).

and fatigue as compared with placebo, primarily during the first month of administration (see Table S14 in the Supplementary Appendix).

DISCUSSION

In this study of heterosexual men and women with a partner known to have HIV-1 infection, once-daily oral TDF and TDF–FTC were associated with risk reductions of 67% and 75%, respectively, against HIV-1 infection when provided in conjunction with other HIV-1 prevention services. Both TDF and TDF–FTC showed significant, and a similar magnitude of, HIV-1 protection for both women and men.

Clinical trials of tenofovir-based preexposure prophylaxis have had conflicting results. Once-daily oral TDF–FTC reduced the risk of HIV-1 acquisition by 44% in a multicountry study among men who have sex with men and by 62% among young heterosexuals from Botswana,[20,21] and the use of 1% tenofovir vaginal gel decreased the incidence of HIV-1 among South African women by 39%.[22] Biologic and behavioral hypotheses have been proposed to explain the failure of two trials of preexposure prophylaxis among African women to show protection against HIV-1 infection,[23,24] including a lack of adherence to daily doses of preexposure prophylaxis, vaginal concentrations of tenofovir achieved with oral dosing that may be particularly sensitive to nonadherence,[25] sexually transmitted infections or other cofactors

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affecting infection with HIV-1 in young women, high HIV-1 concentrations in the seropositive partner during primary HIV-1 infection, and innate or acquired immunologic factors that may provide adjunctive protection in long-term couples with HIV-1 serodiscordance. Further study is needed to understand which, if any, of these factors influence the efficacy of preexposure prophylaxis.

Although we studied established couples known to be HIV-1–serodiscordant, all HIV-1 transmissions ultimately occur between serodiscordant partners. Our findings provide proof of concept that preexposure prophylaxis can reduce HIV-1 acquisition in heterosexual populations.

High adherence is essential to achieve clinical benefits from antiretroviral agents for HIV-1 treatment,[26] and emerging evidence suggests that adherence to preexposure prophylaxis is also important for HIV-1 prevention. In the Preexposure Prophylaxis Initiative trial involving men who have sex with men, the relative reduction in the risk of HIV-1 infection from TDF–FTC preexposure prophylaxis was 44% overall but was 73% among the participants with an adherence of 90% or more (as measured by means of pill counts) and 92% among the participants with detectable tenofovir levels in the blood — although only half the participants had detectable levels.[20]

In our study, retention and pill-count adherence were high, tenofovir was detected in 82% of samples from randomly selected participants, and detectable tenofovir levels were associated with a reduction in the relative risk of HIV-1 infection of more than 85%. The high proportion of samples with detectable tenofovir levels is consistent with the 92% study-drug coverage we calculated on the basis of missed visits, withholding of the study drug, and nonadherence, with the absolute difference of 10 percentage points most likely reflecting the fact that pill counts can overestimate adherence if pills are not returned.

Analyses of objective adherence measures across preexposure prophylaxis trials will be informative for understanding the relationship between adherence and protection against HIV-1 infection. In a subgroup of our study cohort, intensive monitoring of adherence by means of pill bottles with caps that electronically monitor bottle openings and monthly unannounced visits to the home for purposes of pill counting supported high adherence,[27] and in-depth interviews have emphasized that trust and the support of a partner reinforce high adherence.[28] Strategies to promote and achieve high adherence outside clinical-trial settings will be necessary to achieve maximum public health benefits of preexposure prophylaxis.

We found similar degrees of protection against HIV-1 with TDF and TDF–FTC, in contrast to findings in studies of animal models.[11] Dual-agent preexposure prophylaxis would most likely be more expensive than single-agent preexposure prophylaxis, and the potential for differential tolerability and antiretroviral resistance in persons with HIV-1 seroconversion despite the use of preexposure prophylaxis should be considered in decision making with regard to public health policies regarding preexposure prophylaxis. We are continuing the TDF and TDF–FTC groups of our study, including offering randomization to TDF or TDF–FTC to participants originally assigned to the placebo group, to gather additional information on the relative safety, efficacy, and HIV-1 resistance of TDF as compared with TDF–FTC.

In our study, 25% (two of eight) of participants who had acute HIV-1 infection at the time of study-drug initiation had viral resistance develop (through the M184V mutation in one and the K65R mutation in the other). The initiation of preexposure or postexposure prophylaxis in persons with acute HIV-1 infection can select for resistance; strategies to improve the recognition of acute infection are needed.[20,29,30] Resistance was rare in partners in whom seroconversion occurred after randomization, of whom a minority had detectable tenofovir levels.

Adherence to preexposure prophylaxis, protection against HIV-1 infection, and antiretroviral resistance appear to be tightly interwoven. Low adherence provides little HIV-1 protection but little risk of resistance if infection is acquired. High adherence potentially blocks most transmissions, and the few persons who acquire HIV-1 despite preexposure prophylaxis potentially have an increased risk of drug resistance. Four participants with HIV-1 seroconversion in our study became infected with HIV-1 that was resistant to nonnucleoside reverse-transcriptase inhibitors, which should not have been selected for by the study medication and instead probably reflects circulating resistance, which is increasingly being detected in Africa.[31]

When used for HIV-1 treatment, TDF is known to cause small decreases in glomerular filtration that are of uncertain clinical significance.[32] In

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our population of HIV-1–seronegative participants without preexisting renal impairment, we found no evidence of clinically significant elevations in serum creatinine. Additional studies are needed of proximal renal tubular function, bone mineral density, and other aspects of long-term safety of TDF-based preexposure prophylaxis, as well as safety in pregnant, breast-feeding, or adolescent women, among whom HIV-1 rates are high.[33,34]

For couples known to have HIV-1 serodiscordance, antiretroviral treatment of the partner infected with HIV-1 provides substantial, though incomplete, protection against HIV-1 transmission; 25 to 30% of HIV-1 infection in serodiscordant couples are from infected partners outside the couple.[5,7] Mathematical modeling may help guide policy decisions regarding optimal targeting and timing of treatment and preexposure prophylaxis for reducing HIV-1 incidence in couples.[35] Antiretroviral-based HIV-1–prevention strategies may be particularly important for couples seeking to have children.[36-38] In addition, preexposure prophylaxis offers an HIV-1 prevention strategy for uninfected persons with partners who do not know their HIV-1 status or who are infected with HIV-1 but have not begun antiretroviral therapy.

Successful prevention of HIV-1 infection on a population scale will need to incorporate multiple, evidence-based biomedical and behavioral strategies to achieve maximum benefits. The HIV-1 incidence in this study was lower than that seen in previous studies of HIV-1–serodiscordant African couples,[14,39] emphasizing the importance of and synergy among HIV-1 testing in individuals and couples, risk-reduction counseling, and other prevention services, in combination with antiretroviral preexposure prophylaxis, for reducing the risk of HIV-1 infection in heterosexual populations. Potential implementation of preexposure prophylaxis as a public health measure will require clinical monitoring, methods for encouraging adherence, and ensured access to antiretroviral therapy for HIV-1–infected persons. Nonetheless, to stem the global HIV-1 epidemic, effective primary HIV-1 prevention strategies are critical.

The views expressed are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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APPENDIX

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Review

Rectal pre-exposure prophylaxis (PrEP)

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ABSTRACT

Rectal pre-exposure prophylaxis (PrEP) will be a critical component of HIV prevention products due to the prevalence of unprotected receptive anal intercourse among men who have sex with men and heterosexual couples. Given the biological considerations of this compartment and the complexity of HIV infection, design of a successful rectal microbicide product faces a number of challenges. Important information is being compiled to begin to address deficits in knowledge toward design of rectal PrEP products for men and women. Aspects of formulation development and preclinical and clinical evaluation of rectal products studied to date are summarized in this review. This article is based on a presentation at the "Product Development Workshop 2013: HIV and Multipurpose Prevention Technologies," held in Arlington, Virginia on February 21–22, 2013. It forms part of a special supplement to Antiviral Research.
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Contents

1. Introduction . . . S17
   1.1. Are rectal microbicides needed? . . . S17
   1.2. Oral vs topical rectal PrEP. . . . S18
2. Preclinical evaluations for rectal PrEP. . . . S18
   2.1. Animal testing for rectal PrEP. . . . S18
      2.1.1. Small animal models . . . S18
      2.1.2. Non-human primate models . . . S19
3. Early rectal PrEP development . . . S19
4. Design of rectal specific products . . . S21
5. Questions to be addressed and future directions. . . . S22
   Acknowledgements . . . S22
   References . . . S22

1. Introduction

HIV remains a significant global health challenge. Although advances in antiretroviral therapy have extended the life expectancy of HIV infected individuals, much work is still needed in the area of prevention. This review aims to summarize the role of rectal pre-exposure prophylaxis (PrEP) in global HIV prevention. Specifically, it provides justification of need and an overview for the current status of rectal microbicide research. Emphasis has been placed on preclinical evaluation and product development issues specific to rectal microbicides. As this field is in its early stages, the current gaps in knowledge and future directions of the science in the field are also presented.

1.1. Are rectal microbicides needed?

The CDC has reported that male to male sexual contact continues to be the highest transmission category for HIV followed by heterosexual contact (CDC, 2012). This statistic accounts for gay and bisexual men representing the population most severely impacted by HIV. Anal intercourse is a common practice among men who have sex with men (MSM). A high prevalence rate of unprotected receptive anal intercourse (RAI) in MSM has been

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shown in a number of studies including the EXPLORE study which evaluated high risk behaviors among MSM in six US cities (Koblin et al., 2003). However a number of studies have shown that heterosexual couples also engage in anal intercourse (Civic, 2000; Erickson et al., 1995; Gross et al., 2000; Mosher et al., 2005) with lifetime anal intercourse estimates in heterosexual couples ranging from 6% to 40% (McBride and Fortenberry, 2010) with up to 10% of sexually active women in the US engaging regularly in RAI. In a CDC report which polled people ages 15–44, 44% of men and 36% of women admitted to having ever had anal sex (Chandra et al., 2011). The risk associated with HIV transmission through unprotected receptive anal intercourse (URAI) is 1.7% per act while the risk associated with unprotected vaginal intercourse is only 0.08% (Boily et al., 2009). This statistic more than likely contributes to URAI being the highest transmission category for HIV acquisition.

A number of anatomical and physiological factors contribute to greater risk of HIV transmission through rectal intercourse (McGowan and Dezzutti, 2013). The rectal epithelia consist of a single layer of cells as opposed to the multilayer squamous epithelium of the vagina and ectocervix. The pH of the rectum is closer to neutral and is an open tube orientation with potential of HIV reaching as far as the splenic flexure. There is a large surface area which requires protection. Finally the gastrointestinal tract is populated with a large number of HIV-1 infectable cells (Ullrich et al., 1998; van Marle et al., 2007). All of these factors make the rectal route a more susceptible route for HIV infection.

1.2. Oral vs topical rectal PrEP

Assessments of prevalence studies on anal intercourse as well as men and women's willingness to use microbicide products have indicated a need for the development of a rectal microbicide product. Products designed to protect men or women from HIV transmission through URAI could be either an oral PrEP product or topical PrEP product (also referred to as topical microbicide). With respect to oral PrEP, the U.S. Food and Drug Administration has approved Truvada, an oral tablet combining two antiretroviral drugs (emtricitabine (FTC)/tenofovir disoproxil fumarate(TDF)), for use in uninfected individuals who have a high risk of HIV infection. A key clinical study toward approval of this product was the iPreX study. This study, conducted in 2499 high risk MSM, showed significant reduction of HIV acquisition in men treated with daily oral FTC/TDF (Grant et al., 2010). In addition to oral PrEP, topical rectal PrEP products are being designed. Clinical efficacy studies of vaginal topical microbicides indicate that achieving significant tissue concentration of drug for some APIs may be critical to achieving efficacy (Karim et al., 2011). For oral PrEP studies it is observed that although high tenofovir (TFV) blood level was achieved, drug level at the mucosal site was much lower. However with topical PrEP one can achieve high drug concentration in the local tissue while limiting systemic exposure to the drug. From not only an efficacy standpoint but also considering resistance and toxicity there may be benefit to rectal topical PrEP products.

To date rectal microbicide clinical trials have only evaluated UC781 or TFV. Preclinical studies have been conducted for a number of additional microbicide drug candidates which were intended for vaginal administration. However they also have potential for administration by the rectal route. These include cellulose acetate, PRO2000, SPL7013, vena gel (Abner et al., 2005), dextrin sulfate (Fletcher et al., 2006), C34, T20, T1249, L'644 (Harman et al., 2012), TMC120 (dapivirine) (Herrera et al., 2011), saquinavir, MIV-150, carrageenan, zinc acetate (Kenney et al., 2013, 2012), BufferGel, C31G, octylglycerol (Patton et al., 2009), maraviroc, griffithsin (Wang et al., 2012). Vaginal administration of several of these drug candidates was tested in the clinic. Notably PRO2000 (McCormack et al., 2010) carrageenan (Skoler-Karpoff et al., 2008) were found to be safe but not efficacious against vaginal transmission of HIV. Early efforts in the area of rectal microbicides looked at the safety or efficacy of products designed for vaginal use in the rectal compartment. More recently rectal specific dosage forms are being designed for application as rectal PrEP products. Additionally dual compartment (vaginal and rectal) products are also being formulated.

2. Preclinical evaluations for rectal PrEP

Pharmaceutical product development requires a considerable amount of preclinical assessment prior to its entry into the clinic. Once a lead drug candidate has been identified preformulation evaluations, formulation development and assessment, and preclinical studies are required. For rectal microbicide products preclinical safety, stability, and efficacy have been evaluated in *in vitro*, *ex vivo*, and animal studies. An algorithm for preclinical evaluation for vaginal and rectal microbicide products was presented by Buckheit and Buckheit (2012). This algorithm incorporates assessment in the presence of biological relevant fluids and tissues. One of the key preclinical evaluations which has been explored is the utilization of the colorectal explant system for safety and efficacy testing of rectal microbicide drug candidates and products. This model developed by Dezzutti et al. Abner et al. (2005) has been utilized to screen a large number of microbicide candidates and commonly used lubricants for their impact on the excised target tissue of interest. The model also allows for product and drug candidate evaluation in the presence of biologically relevant fluids.

2.1. Animal testing for rectal PrEP

Both small and large animal models have been implemented in rectal PrEP product development. Specifically animal models have been used to provide preclinical safety data as well as efficacy or proof-of-concept for PrEP strategies administered both vaginally and rectally. Rodents, rabbits, non-human primates (NHP), and sheep have been applied for such evaluation of vaginal or rectal products. One of the earliest studies which evaluated rectal product application safety was conducted by Phillips and Zacharopoulos in the mouse model (Phillips and Zacharopoulos, 1998). In this study, the rapid exfoliation induced by rectal application of N-9 was demonstrated. This effect was also shown in the NHP model in a study conducted by Patton et al. (2002). Several early vaginal HIV prevention products were evaluated for safety in the rectal compartment in the macaque. Buffergel (Patton et al., 2004), Savvy (Patton et al., 2006b) and VivaGel (Patton et al., 2006a) are among those early products with nonspecific action against HIV which were tested rectally that demonstrated safety in the macaque model. Efficacy has also been evaluated in animal models in the field. The 1% TFV gel product was the first to demonstrate efficacy in blocking HIV transmission effectively by rectal application in a mouse model (Chateau et al., 2013). Additionally, NHP models have also been utilized to evaluate the efficacy of rectal PrEP products. The first study to demonstrate efficacy in this macaque model was conducted by Tsai et al. (2003). This study showed complete protection from rectal HIV challenge after rectal administration of 1% or 2% cyanoviran gel. Additional examples of specific applications of these animal models in preclinical development are provided in the following section of this review.

2.1.1. Small animal models

Rodent models are generally used in drug development to screen active compounds for efficacy and safety. Although rodents

provide a cost effective model, they are distantly related to humans biologically. The anatomical and physiological properties of their reproductive and gastrointestinal tracts are different from those of human and non-human primates. Despite model limitations, rodents are commonly used for preclinical safety evaluations of rectal PrEP drug candidates and products.

Native mice and rats cannot be infected with HIV. However researchers have engineered rodents to be more susceptible to HIV infection. One humanized mouse model used in microbicide evaluation is the BLT (Bone Marrow Liver Thymic) mouse model. BLT mice contain reconstituted human immune cells distributed throughout the body including the intestines and female reproductive tract (Denton et al., 2012; Hu and Yang, 2012). The presence of human CD4+ T-cells, macrophages, and dendritic cells render BLT mice susceptible to mucosal HIV-1 transmission via vaginal and rectal routes. This model has been applied to efficacy evaluation for vaginal products (Denton et al., 2008). It was recently utilized to evaluate the efficacy of 1% TFV rectally administered demonstrating that the humanized mouse model can be used in rectal microbicide assessment (Chateau et al., 2013).

In addition to mouse models, rabbits are also used to evaluate rectal product safety. The rabbit rectal irritation test has been used to evaluate both vaginal microbicide products and rectal specific dosage forms for rectal safety (Wang et al., 2012, 2011).

2.1.2. Non-human primate models

An ideal animal model for HIV infection should be able to mimic HIV transmission and pathogenesis as well as evaluate the safety and effectiveness of antiretroviral drug candidates and other antiviral strategies. NHPs are genetically closest to humans with similar immunology, anatomy, and physiology. NHPs are the primary animal models for investigating HIV transmission, immunology, and pathogenesis (Lackner and Veazey, 2007; Lederer et al., 2009). The most widely used NHP model is the macaque, including rhesus, pigtail, and cynomolgous. However, HIV-1 cannot replicate efficiently in macaques due to host restriction factors, notably TRIM5α protein, which blocks viral infection by inactivating incoming capsids (Stremlau et al., 2004). Accordingly, chimeras encoding the HIV-1 envelope or reverse transcriptase (SHIV, or RT-SHIV) are used in macaque models (Ambrose et al., 2007; Daniel et al., 1985; Uberla et al., 1995).

Macaque models have been used to evaluate vaginal (Patton et al., 2006a) and rectal (Patton et al., 2009; Tsai et al., 2003) microbicide products, vaccines (Palermo et al., 2011) and the combination of vaccines with microbicide products (Barouch et al., 2012). Specific examples of the use of non-human primates to evaluate the efficacy of oral and rectal PrEP products in inhibiting HIV transmission include Cyanovirin-N (Tsai et al., 2003), TFV (Cranage et al., 2008), MIV-150 (Kenney et al., 2012) and Maraviroc (Massud et al., 2013). Each of these drug candidates has been shown to be protective in this model. In the study evaluating Cyanovirin-N (CN), when 2 ml was given rectally to cynomolgus macaques at 1% or 2% the product was found to fully protect animals from SHIV89.6P infection without observed toxicity. The Cranage study which evaluated colorectal pretreatment with TFV gel (1%) using Indian rhesus macaques showed that tenofovir was able to protect from rectal challenge with $SIV_{mac251/32H}$. Interestingly, in this study the plasma concentration of TFV was monitored and shown to peak rapidly following rectal administration. A strong positive association between the concentration of TFV in the plasma 15 min after rectal application of gel and the degree of protection in macaques challenged with virus was seen. The results indicate that a plasma concentration of TFV above 120 ng/ml effectively protected the animals against infection. The NNRTI MIV-150 in carrageenan gel was investigated in rhesus macaques to prevent rectal transmission of SHIV (Singer et al., 2011). Results showed that MIV-150/carrageenan protected the animals from infection following rectal viral challenge. In the MIV-150 study, pre-challenge application was found to be more effective than a post-challenge application indicating a need to implement the appropriate regimen strategy for achieving efficacy with this rectal microbicide candidate. More recently the NHP model was used to evaluate the efficacy of oral administration of maraviroc (MVC) (Massud et al., 2013) following rectal HIV challenges. MVC is a potent CCR5 co-receptor antagonist. In this study, MVC was evaluated for its potential to prevent rectal SHIV transmission using a macaque model through weekly SHIV162p3 exposures. It is found that oral maraviroc lacked prophylactic efficacy despite high drug concentrations in rectal tissues (40 times higher than that required to block SHIV infection of peripheral blood mononuclear cells (PBMCs) *in vitro*). Conversely, a macaque study evaluating a rectal specific MVC gel (1%) showed that 4 of 6 macaques receiving MVC gel remained protected following 10 viral challenges (Heneine W and Dobard C, personal communication).

These animal models provide a tool for the evaluation of safety and efficacy of PrEP products by offering a platform to investigate parameters which can affect prophylactic efficacy of antiretrovirals (ARVs) including pharmacokinetics, drug dissolution/release, drug permeability and tissue penetration and pharmacodynamics. Importantly, both the macaque and humanized mouse models have been used to evaluate the efficacy of oral and topical PrEP products against rectal HIV exposure. Although animal testing is an important component of any pharmaceutical development plan, it should be kept in mind that none of the models presented exactly mimics human HIV infection. Each model presents its own limitations due to species-specific anatomy and physiology and biological aspects of HIV infection.

3. Early rectal PrEP development

Although, a number of preclinical studies have been conducted on rectal microbicides (Table 1), a limited number of clinical studies have been completed or are underway (Table 2) (McGowan, 2012; McGowan and Dezzutti, 2013). The initial focus of early clinical studies was on the rectal safety assessment of existing vaginal microbicide gels. The first Phase 1 clinical trials designed toward this effort were HIVNET-008 (nonxynol-9 gel) (Tabet et al., 1999), RMP-01 (UC781 gel) (Anton et al., 2011), RMP-002/MTN-006 (TFV gel) (Anton et al., 2012) and MTN-007 (TFV reduced glycerin gel) (McGowan et al., 2013). The vaginal products used in these studies possessed physicochemical attributes (such as low pH and hypertonicity) which are not optimal for rectally delivered products. Target specifications for vaginal products differ from those for rectal microbicide products due to physiological differences between the vaginal and colorectal compartments. Consequently, Phase 1 gastrointestinal adverse events were observed for some of the vaginal products evaluated when administered rectally. For the N-9 product and its matching placebo, high rates of histological abnormalities were observed (Tabet et al., 1999). No safety issues were observed for rectal use of the UC781 gel in a 36 participant Phase 1 study and *ex vivo* efficacy was demonstrated. However, the sponsor (CONRAD) of the vaginal UC781 gel product has terminated this gels development program due to other reasons. Rectal use of vaginal TFV gel in a Phase 1 study involving 18 participants (RMP-002/MTN-006) resulted in product related gastrointestinal adverse events including diarrhea, bloating, urgency, abdominal pain, and flatulence. However, rectal use of the reformulated reduced glycerin TFV gel formulation resulted in no product related gastrointestinal adverse events with no reported cases of urgency, bloating or nausea in the 65 participants in the MTN-007 Phase 1 study. The field has reached a consensus

Table 1
Agents that have been evaluated in preclinical studies.

Agents	Study	Status	References
Cellulose acetate, Pro2000,SPL 7013, Vena Gel, UC781	Explant model	Completed	Abner et al. (2005)
PRO2000, Dextrin sulfate, Tenofovir	Explant model	Completed	Fletcher et al. (2006)
Tenovofir	Explant model	Completed	Dezzutti et al. (2012a,b,c)
C34, T20, T1249, L'644	Explant model	Completed	Harman et al. (2012)
Tenofovir, emtricitabine, UC781, dapivirine (TMC 120)	Explant model	Completed	Herrera et al. (2011)
Saquinavir	Explant model	Completed	Stefanidou et al. (2012)
Tenofovir	Humanized mouse model	Completed	Chateau et al. (2013)
BufferGel, nonoxynol-9, C31G, octylglycerol, polystyrenen sulfate, cellulose sulfate, SPL7013, Carraguard, UC781	Non-human primate safety model	Completed	Summarized in Patton et al. (2009)
Tenofovir/griffithsin combo gel	Explant model, rabbit rectal irritation model	Completed	Wang et al. (2012)
Maraviroc gel tenofovir/maraviroc combo gel	Explant model, rabbit rectal irritation safety model, non-human primate efficacy model	Ongoing	
Maraviroc, griffithsin	Explant model	Ongoing	
MIV-150, carageenan, zinc acetate	Explant model	Ongoing	
Tenofovir/IQP 0528 gel	Explant model	Completed	Dezzutti et al. (2012c)
Cyanovirin gel	Non-human primate efficacy model	Completed	Tsai et al. (2003)
Tenofovir gel	Non-human primate efficacy model	Completed	Cranage et al. (2008)
MIV-150 gel	Non-human primate efficacy model	Completed	Singer et al. (2011)

Table adapted from McGowan 2013 with updates.

Table 2
Agents that have been evaluated in clinical trials.

Agents	Sponsor	Trial	Study stage	Status	ClinicalTrials.gov identifier	References
Nonoxynol-9 gel	NIAID	HIVNET-008	Phase 1	Completed	NCT00000929	Tabet et al. (1999)
UC781 gel	CONRAD	RMP-01	Phase 1	Completed	NCT00408538	Anton et al. (2011); Ventuneac et al. (2010)
VF[a] of TFV gel	CONRAD	RMP-02/MTN-006	Phase 1	Completed	NCT00984971	Anton et al. (2012)
RGVF[b] of TFV gel	CONRAD	MTN-007	Phase 1	Completed	NCT01232803	Mcgowan et al. (2013)
RGVF of TFV gel	CONRAD	Project Gel	Phase 1	Ongoing	NCT01283360	
VF, RGVF, RF[c] of TFV gel	Ian McGowan	CHARM-01	Phase 1	Ongoing	NCT1575405	
VF, RGVF, RF of TFV gel	Ian McGowan	CHARM-02	Phase 1	Ongoing	NCT01575418	
RGVF of TFV gel	CONRAD	MTN-017	Phase 2	Ongoing	NCT01687218	

Table adapted from McGowan 2013 with updates.
[a] VF, vaginal TFV gel (1%).
[b] RGVF, reduced glycerin TFV gel (1%).
[c] RF, rectal specific TFV gel (1%).

to avoid hyperosmolar products in the rectal compartment. This view is based on observations of induced mucosal damage due to dehydration of epithelial cells and consequential cell shrinkage caused by extremely hyperosmolar test and commercial products with osmolality over 3000 mOsm/kg (Begay et al., 2011; Dezzutti et al., 2012a; Fuchs et al., 2007). Furthermore, increased vulnerability to sexually transmitted infections has been associated with the consistent use of hyperosmolar lubricant products (Gorbach et al., 2012).

The development of dual compartment microbicide products is being explored. The pre-clinical safety and efficacy of a reduced glycerin (reduced osmolality) TFV gel developed by CONRAD was evaluated for its potential to be used in both compartments (Dezzutti et al., 2012b). The reduced glycerin gel with decreased osmolality resulted in improved epithelial integrity of polarized ectocervical and colorectal explants following exposure over that observed for the original TFV gel. This data suggests better safety profile for the reduced glycerin TFV product for rectal use. Another product evaluated for pre-clinical safety and efficacy as a dual compartment microbicide product was a combination gel containing TFV and IQP-0528 (Dezzutti et al., 2012c). Epithelial integrity of ectocervical and colorectal explants remained intact after a 24 h exposure of the gels despite the osmolality of the single entity product containing only IQP-0528 and the combination product (TFV and IQP-0528) still being somewhat hyperosmolar (below 900 mOsm/kg and similar to that of the reduced glycerin TFV gel) (Ham et al., 2012). More recently, Kenney et al. (2013) developed a non-ARV dual compartment gel (zinc acetate in carrageenan) with nearly iso-osmolality and close to neutral pH which was shown to be safe and effective against SHIV-RT infection in macaques given vaginally and HSV-2 infection in mice given both vaginally and rectally.

Studies are ongoing to develop critical data needed for the development of rectal specific microbicide formulations with properties which are better suited for use in the rectal compartment (Wang et al., 2012, 2011). The Combination HIV Antiretroviral Rectal Microbicide (CHARM) program is developing both single entity and combination antiretroviral rectal specific products. Through these efforts a rectal specific TFV 1% gel in a carbopol and sodium carboxymethylcellulose base was developed. This newly developed product entered clinical trials this year. The ongoing CHARM-01 and CHARM-02 Phase I clinical trials evaluate the safety, acceptability, pharmacokinetic/pharmacodynamic profile and distribution of three TFV containing gel formulations including the original vaginal TFV gel (1%), the reduced glycerin TFV gel (1%) and the rectal specific TFV gel (1%) products. In preclinical evaluations, this rectal specific TFV product was shown to be safe and effective in *in vitro* and *ex vivo* studies, and was safe when evaluated *in vivo* in a rabbit rectal irritation study. Additionally, this group has developed a rectal specific combination gel product which contains TFV and griffithsin, a lectin viral entry inhibitor drug candidate. The combination product was also shown to be

safe and effective *in vitro*, *ex vivo* and *in vivo* (Wang et al., 2012) in preclinical assessments. Currently this same group is developing a combination rectal specific product which contains TFV and maraviroc within the scope of CHARM. All rectal specific microbicide products developed to date share similar properties of iso- or near iso-osmolality and neutral pH, proper rheological profile, adequate drug *in vitro* release profile, sufficient bioactivity and lack of toxicity in preclinical assessments. Table 3 provides examples of vaginal, rectal specific and dual compartment products for comparison.

4. Design of rectal specific products

To date all formulations used in clinical studies have been aqueous based gels (hydrogels) because of the perceived acceptance of gels over other dosage forms (Carballo-Dieguez et al., 2008). However, hydrogel products may present greater challenges than suppositories or other dosage forms given the high water content required for hydrogel formulations. Such aqueous based formulations may be problematic for drug candidates with limited water solubility or with aqueous instability. It should be noted that the dosage form and formulation components will not only alter drug stability but also drug release and pharmacokinetics which may subsequently impact safety and the efficacy of rectal microbicide products. Considering that a number of rectal microbicide drug candidates are highly water insoluble, particularly several NNRTIs and a number of other ARVs being evaluated, alternative formulations or addition of cosolvents or other strategies to enhance active pharmaceutical ingredient solubility should be assessed. The addition of cosolvent in formulations may pose a potential factor for increased safety risk. Glycerin, a commonly used cosolvent excipient to enhance API solubility, can impart hyperosmolar conditions on the rectal or colon tissues. This hyper-osmolality may result in adverse effects in the gastrointestinal tract potentially leading to enhanced HIV-1 infection (Begay et al., 2011; Fuchs et al., 2007). In the clinical trial RMP-02/MTN-006 (Anton et al., 2012) gastrointestinal adverse events were significantly increased with multiple day dosing of the hyperosmolar TFV gel. Fewer adverse effects were observed in the succeeding clinical trial MTN-007 which used a formulation containing a reduced amount of glycerin (McGowan et al., 2013).

The safety profile of a drug or drug product may be different when given by the rectal route as opposed to other routes of administration (McGowan, 2012). Given differences in the fragility of colorectal tissue combined with rectal compartment tissue distribution variances, it is important to thoroughly evaluate rectal safety not only for products specifically designed for rectal administration but also for products designed for vaginal administration which may be applied to the rectal compartment. In addition to the safety of rectal microbicide products, the convenience and acceptability of the products as well as its applicator should also be considered. When N-9 was investigated as a potential rectal microbicide in the HIVNET-008 Phase I study, results showed that the rectal microbicide was not only impacted by adverse effects from N-9 (Tabet et al., 1999) but also by adverse effects associated with applicator use (Gross et al., 1999).

Clearly, it is imperative to develop a rectal compartment specific microbicide product with improved safety and possibly better efficacy. Many factors can impact the success of rectal microbicide product development such as physiological and anatomical factors of the rectum, formulation type, active pharmaceutical ingredient, excipient choice, physicochemical properties of the product, volume to be delivered and applicator type. It is important to obtain information on safety, efficacy, and acceptability at the early stages

Table 3
Comparison between rectal, vaginal and dual compartment products.

Properties	Rectal specific product	Vaginal product		Dual compartment products	
	RF[a] gel	VF[b] gel	UC781 gel	RGVF[c] gel	TFV/IQP-0528 gels[*]
Ingredients	Carbopol 974P, Na-CMC[d], glycerin (2.5%), EDTA[e], sodium hydroxide, purified water methlyparaben, propylparaben	HEC[f], citric acid, glycerin (20%), EDTA sodium hydroxide, purified water, methlyparaben, propylparaben	Caropol 974P, methylcellulose, glycerin, sodium hydroxide, purified water, methlyparaben, propylparaben	HEC, citric acid, glycerin (5%), EDTA sodium hydroxide, purified water, methlyparaben, propylparaben	Poloxamer, HPC[g], carbopol 974P, EDTA, glycerin (5%), sodium hydroxide, purified water, methlyparaben, propylparaben
Physical appearance	Semisolid	Translucent, colorless, a little hazy semisolid	Transparent, colorless, viscous semisolid	Off white to light yellow semisolid	Transparent, colorless viscous semisolid
Formulation type	Hydrogel	Hydrogel	Hydrogel	Hydrogel	Hydrogel
pH	7	4.5	5.2	4.6	4.8
Osmolality (mOsm/kg)	479 With a target of iso or near iso-290	3111 With an unspecified target	NA[h] With an unspecified target	836 With a target of less than 1000	882 With a target of less than 1000
Rheology profile	Shear thinning	Shear thinning	Shear thinning	Shear thinning	Shear thinning
Viscosity (cp) at 10 rpm	3049	9921	NA	9161	50,000[**]
Reference	McGowan (2012)	McGowan (2012); Dezzutti et al. (2012a,b,c)	Anton et al. (2011); Ventuneac et al. (2010)	McGowan (2012); Dezzutti et al. (2012a,b,c)	Ham et al. (2012)

[a] RF, rectal specific TFV formulation.
[b] VF, vaginal TFV formulation.
[c] RGVF, reduced glycerin TFV formulation.
[d] Na-CMC, sodium carboxymethylcellulose.
[e] EDTA, disodium edetate.
[f] HEC, hydroxyethyl cellulose.
[g] HPC, hydroxypropylcellulose.
[h] NA, not available.
[*] Only evaluated in preclinical studies.
[**] Data was taken at 1 s^{-1}, which equivalent to about 0.3 rpm.

of rectal microbicide product development. Attention to these factors will lead to a greater probability of success in design of a safe and effective rectal product which men and women are willing to use.

The single cell lining and large surface area of the rectal compartment create a poor barrier to pathogen entry. The rectal epithelium is composed of absorptive cells and goblet cells which secrete mucin. These factors contribute to the challenges associated with design of pharmaceutical products for the rectal compartment. Further there are a number of physiological factors which impact drug absorption from this compartment including colonic content, compartment localization, pH, presence of mucin, and lack of buffering capacity of rectal fluids. Isotonic and neutral (pH of 7) products would be optimal as rectal microbicides.

Several dosage forms could be applied as rectal microbicide product platforms including gels, creams, foams, suppositories, enemas, and douches (Carballo-Dieguez et al., 2008; Kinsler et al., 2013; Tsai et al., 2003). A significant contributor to product effectiveness is user compliance. In an acceptability study conducted by Newman et al., formulation, scent, applicator, color, and taste were identified as factors that will impact rectal microbicide acceptability (Newman et al., 2013). In a recent randomized crossover trial, Pines et al. (2013) compared the acceptability of three distinct anorectal delivery systems (enema bottle pre-filled with a clear isotonic liquid Normosol-R, a disposable vaginal applicator pre-filled with 4 ml of a clear water based, condom compatible isotonic gel Pre-Seed™, and a 1.4 g anorectal suppository Tucks™). Although the magnitude of differences between complete product acceptability scores differed by age, gender, and data collection mode, overall participants found the applicator with water based gel to be most acceptable. Acceptability studies in this area to date suggest that gels are the most acceptable dosage forms for rectal microbicide development (Carballo-Dieguez et al., 2008; Newman et al., 2013; Peinado et al., 2013; Pines et al., 2013). However, it should be noted that these studies evaluated only a limited number of dosage form options.

An additional parameter to consider for rectal microbicides is the volume of product to administer rectally, Carballo-Dieguez et al. (2008) demonstrated that up to 35 ml of a transparent and odorless placebo gel (Femiglide®) would be acceptable to men who engage in RAI and would be required to achieve effectiveness in a volume escalation trial. Studies to date on rectal administration of gel products have predominantly used a vaginal applicator for delivery of the product into the rectum. Carballo-Diéguez et al. initiated the work towards the development of rectal microbicide specific delivery device (funded by NICHD R01 046060). However the feasibility of the applicator remains to be answered.

In a study toward development of rectal specific microbicides (Wang et al., 2011) two fundamentally different approaches to establishing an effective rectal microbicide product were applied. One approach involved creating a formulation that spreads easily and coats all tissue surfaces of the rectum and distal colon rapidly prior to RAI, this would be achieved by a fluid product; the other approach involved creating a deformable, erodible barrier that could remain relatively localized at the administration site until distributed by rectal intercourse, and this strategy is achievable with a gel formulation. In this work, a series of rectal placebo formulations with a wide range of physicochemical properties was developed which was shown to be non-toxic in both *ex vivo* colorectal tissue and *in vivo* rabbit rectal irritation study. Namely, four product types: aqueous fluid, aqueous gel, lipid fluid and lipid gel, were developed to create a wide range of spreadability, erodibility, retention at site of administration, and effect on permeability. Several key parameters for rectal specific product development were defined in this work. Iso-osmolality, neutral pH, appropriate viscosity of less than 200 cps for liquid product and greater than 5000 cps for gel products, compatibility with latex condoms, demonstration of safety and efficacy in preclinical assessments were set as target specifications. This set of placebos was evaluated in clinical testing for their spreading characteristics and effect on rectal tissues. Hendrix et al. have established a technique to study distribution of rectal microbicide gel candidates which combines the use of single photon emission computed tomography (SPECT)/computed tomography (CT) (Cao et al., 2012), magnetic resonance imaging (MRI) and sigmoidoscopy (Fuchs et al., 2013; Hendrix et al., 2008). When assessed using this technique, these rectal microbicide products showed a wide range of clinical parameters regarding modification of mucosal permeability and product distribution within the rectal compartment. Mucosal barrier modifications are evaluated in this method by combining a radioisotope probe (technetium sulfur colloid) with the test product. Analysis of data collected from this trial is under way. Results from this study have served as a basis for the development of the rectal specific product which has recently entered clinical trials through the CHARM program.

5. Questions to be addressed and future directions

Although significant advances have been made in the field of rectal microbicides or rectal PrEP there are still a number of questions to be answered. The biology pertinent to HIV infection in this compartment and key aspects of interactions between compartment and dosage forms have yet to be fully understood. Contributions of product functionality, defining user preferences for specific dosage form options, volume of product which should optimally delivered, and coverage of target site requirements still remain to be defined. Although work has begun on the design of applicators for delivery of products into the rectal compartment, the feasibility of such applicators has not been established. Traditional gel products serve as the preponderance of products currently being evaluated but novel or alternative dosage forms should be explored which would provide greater user acceptability, convenience, and product targeting and efficacy. Proof of concept has been shown for ideas such as combined delivery of multiple active agents and dual compartment formulations but their utility must still be established. Given the prevalence of URAI and increased risk of HIV infection through this route, it is essential to rapidly fill these knowledge gaps so that a pharmaceutical product can be designed which can safely and effectively protect men and women from acquisition of HIV through the rectal route.

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ORIGINAL ARTICLE

Use of a Vaginal Ring Containing Dapivirine for HIV-1 Prevention in Women

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ABSTRACT

BACKGROUND

Antiretroviral medications that are used as prophylaxis can prevent acquisition of human immunodeficiency virus type 1 (HIV-1) infection. However, in clinical trials among African women, the incidence of HIV-1 infection was not reduced, probably because of low adherence. Longer-acting methods of drug delivery, such as vaginal rings, may simplify use of antiretroviral medications and provide HIV-1 protection.

METHODS

We conducted a phase 3, randomized, double-blind, placebo-controlled trial of a monthly vaginal ring containing dapivirine, a non-nucleoside HIV-1 reverse-transcriptase inhibitor, involving women between the ages of 18 and 45 years in Malawi, South Africa, Uganda, and Zimbabwe.

RESULTS

Among the 2629 women who were enrolled, 168 HIV-1 infections occurred: 71 in the dapivirine group and 97 in the placebo group (incidence, 3.3 and 4.5 per 100 person-years, respectively). The incidence of HIV-1 infection in the dapivirine group was lower by 27% (95% confidence interval [CI], 1 to 46; P=0.05) than that in the placebo group. In an analysis that excluded data from two sites that had reduced rates of retention and adherence, the incidence of HIV-1 infection in the dapivirine group was lower by 37% (95% CI, 12 to 56; P=0.007) than that in the placebo group. In a post hoc analysis, higher rates of HIV-1 protection were observed among women over the age of 21 years (56%; 95% CI, 31 to 71; P<0.001) but not among those 21 years of age or younger (–27%; 95% CI, –133 to 31; P=0.45), a difference that was correlated with reduced adherence. The rates of adverse medical events and antiretroviral resistance among women who acquired HIV-1 infection were similar in the two groups.

CONCLUSIONS

A monthly vaginal ring containing dapivirine reduced the risk of HIV-1 infection among African women, with increased efficacy in subgroups with evidence of increased adherence. (Funded by the National Institutes of Health; ClinicalTrials.gov number, NCT01617096.)

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More than half of the 35 million persons currently living with human immunodeficiency virus type 1 (HIV-1) infection are women. A majority of these women reside in sub-Saharan Africa,[1] a region that has some of the highest incidences of HIV-1 infection in any population worldwide.[2-4] The use of antiretroviral medications as pre-exposure prophylaxis is a promising approach to the prevention of HIV-1 acquisition.[5] Several clinical trials of the antiretroviral tenofovir showed such protection against HIV-1.[2,6-8] However, in three trials involving African women, adherence to tenofovir-containing pills and vaginal gels was low, and HIV-1 protection was not shown.[3,4,9] Across trials of tenofovir-based prophylaxis, a sizable proportion of participants were not adherent, a finding that emphasizes the need for additional options, particularly ones that women can control and longer-acting approaches that do not require daily or coitally dependent use.[5]

Vaginal rings can provide sustained and controlled release of medications. For example, rings containing exogenous hormones are licensed for contraception and estrogen replacement.[10] For HIV-1 prevention, an antiretroviral-containing vaginal ring could provide long-acting HIV-1 protection while reducing systemic exposure to the active pharmaceutical ingredient and delivering the anti–HIV-1 agent at the site of viral transmission. Dapivirine is a non-nucleoside HIV-1 reverse-transcriptase inhibitor that has activity against a broad range of HIV-1 subtypes. In two phase 1 trials,[11,12] genital biopsy tissue samples obtained from women using dapivirine vaginally in the form of a ring, films, and gels were substantially less susceptible to HIV-1 when challenged ex vivo than were tissue samples obtained from placebo-treated women. A monthly vaginal ring containing dapivirine was found to be safe and acceptable in phase 1 and 2 studies, with typical plasma levels of the drug that were lower by a factor of 1000 than levels in women receiving oral dapivirine.[11-15] We conducted a phase 3, randomized, double-blind, placebo-controlled trial of the dapivirine vaginal ring among African women.

METHODS

STUDY POPULATION

From August 2012 through June 2015, we enrolled and followed healthy, sexually active, nonpregnant, HIV-1–seronegative women between the ages of 18 and 45 years at 15 research sites in Malawi, South Africa, Uganda, and Zimbabwe (Tables S1 and S2 in the Supplementary Appendix, available with the full text of this article at NEJM.org).[16] The primary objectives were to determine the efficacy and safety of the dapivirine vaginal ring as compared with a placebo ring; after insertion, the ring is used for 4 weeks and then replaced with another. Community members from each site provided input into trial design and conduct. The trial protocol, which is available at NEJM.org, was approved by the ethics review committee at each site (Table S3 in the Supplementary Appendix). All participants provided written informed consent.

STUDY PROCEDURES

At enrollment, women were assigned in a 1:1 ratio, with the use of fixed-size block randomization, stratified according to site, to receive either a silicone elastomer vaginal matrix ring containing 25 mg of dapivirine or a placebo vaginal ring. Both the dapivirine and placebo rings were manufactured by QPharma under contract with the International Partnership for Microbicides. The dapivirine and placebo rings were indistinguishable, and with the exception of staff members at the central statistical and data management center, investigators and participants were unaware of the randomization assignments until completion of the trial. Women were taught how to insert and remove the vaginal ring and counseled to wear it for the entire month.

Women returned for monthly follow-up visits, which included HIV-1 serologic testing, safety monitoring, and individualized adherence counseling (Table S4 in the Supplementary Appendix). At each visit, a new ring was provided, and the ring that had been used during the previous month was collected. Women were tested monthly for pregnancy, and the study ring was withheld from women who became pregnant; they resumed use of the study ring when no longer pregnant or lactating. All participants received a package of HIV-1 prevention services, including counseling with respect to HIV-1 risk reduction, partner HIV-1 testing, treatment of sexually transmitted infections in participants and partners, and free condoms. (Details regarding the trial design are provided in the Supplementary Appendix.)

OBJECTIVE ASSESSMENT OF ADHERENCE

Plasma samples that were collected quarterly were tested for the presence of dapivirine with the use of a validated ultra-performance liquid chromatography–tandem mass spectrometry assay (Clinical Pharmacology Analytical Laboratory), with a lower limit of quantification of 20 pg per milliliter.[17] To aid in distinguishing cases in which the ring was removed during the month and then reinserted before a clinic visit, the detection of a plasma dapivirine level of more than 95 pg per milliliter (a level nearly always achieved within 8 hours of continuous use) was used to define adherence.[13,14] While the trial was ongoing, plasma samples were assayed and results were reviewed by the trial leaders. To preserve blinding, samples from both the dapivirine group and the placebo group were tested, and results were summarized only as the percentage of samples with dapivirine detected, overall and for each site. After the first year of the trial, testing for residual dapivirine in used rings was initiated with the use of acetone extraction and high-pressure liquid chromatography (Parexel). Women were defined as being adherent if the returned ring contained less than 23.5 mg of dapivirine (i.e., with >1.5 mg released).[13,14]

PRIMARY END POINTS

The primary efficacy end point was HIV-1 infection, identified with the use of a standard seroconversion algorithm (Fig. S1 in the Supplementary Appendix). The study ring was temporarily withheld while confirmatory testing was pending and was permanently discontinued if testing confirmed HIV-1 acquisition. Archived plasma samples from visits before seroconversion were tested for HIV-1 RNA on polymerase-chain-reaction (PCR) assay, and participants with detectable HIV-1 RNA at the time of enrollment were excluded from the primary analysis. Participants completed a final study visit 4 weeks after the last product-use visit to assess for delayed HIV-1 seroconversion, and women who tested positive for HIV-1 at that visit and who had detectable HIV-1 RNA at the last product-use visit were included in the primary analyses because HIV-1 infection had occurred during the product-use period.

The primary safety end point was a composite of any serious adverse event, any grade 3 or 4 adverse event, and any grade 2 adverse event that was assessed by the trial clinicians as being related to dapivirine.

STUDY OVERSIGHT

The National Institutes of Health funded the trial. The authors designed the trial, gathered and analyzed the data, prepared the manuscript, and were responsible for the decision to submit the manuscript for publication. The International Partnership for Microbicides supplied the vaginal rings, was the regulatory sponsor, and participated in the design of the trial, the interpretation of the results, and the preparation of the manuscript. The ring manufacturer, QPharma, had no role in the design or implementation of the trial. The authors vouch for the accuracy and completeness of the data and analyses.

STATISTICAL ANALYSIS

The trial was designed with power of 90% to detect a risk of HIV-1 infection that was 60% lower in the dapivirine group than in the placebo group, with a one-sided alpha level of 0.025. Like other trials of new HIV-1 prevention interventions,[6,7,18] this trial was powered so that the lower boundary of the 95% confidence interval would exclude a 25% reduction in risk, with the primary analysis comparison for the trial planned against a standard null of 0% (Table S5 in the Supplementary Appendix). Under these assumptions, a minimum of 120 HIV-1 acquisition events would be required to achieve the statistical power posited in the design of the trial. An endpoint–driven design was used, and the trial continued until the target number of HIV-1 end points had been accrued and all participants had been followed for a minimum of 12 months, in accordance with regulatory guidance regarding compilation of safety information for new HIV-1 prevention strategies.[19]

An annual incidence of HIV-1 infection of 3.9% in the placebo group was assumed, and a sample size of 3476 women was planned. After the trial started, another HIV-1 prevention trial that was conducted at several sites in our trial showed an HIV-1 incidence of more than 5% per year.[3] As a result, in October 2013, the sample size for this trial was recalculated to approximately 2600 women, and the statistical analysis plan was modified accordingly. At the same time, the analysis plan was further modified for a fully powered analysis that would exclude all

data from 2 of the 15 sites, since these 2 sites had shown lower-than-anticipated participant retention and lower product adherence (with adherence levels of <50%, according to measurement of plasma dapivirine levels) than at the other sites. Further enrollment at the 2 sites was discontinued, but enrolled participants were permitted to continue in follow-up. The sample-size recalculation and plan to exclude data from the 2 sites were approved by the independent data and safety monitoring board and reviewed by regulatory agencies. Interim statistical monitoring, which was performed on the basis of data from all 15 sites, used the Lan–DeMets spending approach to adjust the O'Brien–Fleming sequential monitoring boundaries.[20,21]

The primary analysis of HIV-1 protection was performed according to the intention-to-treat principle with the use of Cox regression, stratified according to site, to estimate the relative rates of time until HIV-1 acquisition. Two analyses were defined: one included data from all 15 sites and a second excluded data from the 2 sites at which enrollment had been discontinued early (i.e., 13 sites were included). Prespecified subgroup analyses were planned. When it was determined that age was significantly related to the efficacy of HIV-1 protection, a post hoc analysis was designed to characterize more fully that relationship by dividing the population into age-categorized thirds containing approximately equal numbers of participants with HIV-1 infection, thus balancing the statistical power for the exploratory subgroups. All analyses were conducted with the use of SAS software, version 9.4 (SAS Institute), and R software, version 2.15.1 (R Project for Statistical Computing). A P value of less than 0.05 was considered to indicate statistical significance, and all P values are two-sided.

RESULTS

STUDY PARTICIPANTS

Of 5516 women who underwent screening, 2629 were enrolled: 1313 in the dapivirine group and 1316 in the placebo group (Fig. 1). The median age was 26 years (interquartile range, 22 to 31). Less than half (41%) were married, and 85% had completed some secondary schooling. Nearly all (99.5%) reported having had a primary sex partner during the 3 months before trial enrollment, and 17% reported more than one partner; 57% reported the use of a condom with the most recent sex act. Transactional sex in the previous year was reported by 6%, and anal sex during the previous 3 months by 2%. Nearly two thirds (64%) reported that their primary partner was aware that they would be using a vaginal ring in a research trial. Characteristics were similar in the two groups (Table 1).

FOLLOW-UP AND ADHERENCE

The rate of retention of participants for assessment of incident HIV-1 infection was 85% or more during follow-up (Fig. 1), with 2614 participants (99.4%) completing at least one post-randomization HIV-1 test and 4280 total person-years of follow-up accrued for assessment of HIV-1 incidence. The median follow-up was 1.6 years (interquartile range, 1.1 to 2.3), and the maximum follow-up was 2.6 years; 1024 women contributed more than 2 years of follow-up. The most common reason for not dispensing the study ring was pregnancy, which occurred at an incidence of 3.9 per 100 person-years in the dapivirine group and 4.0 per 100 person-years in the placebo group (P=0.82).

In the dapivirine group, the drug was detected in 82% of plasma samples at levels of more than 95 pg per milliliter (Fig. S2 in the Supplementary Appendix). Detection increased during the first year of use and was relatively stable thereafter. In the subgroup of visits in which returned rings were available, 84% contained less than 23.5 mg of dapivirine, and dapivirine levels in plasma and in returned rings were correlated (Fig. S3 in the Supplementary Appendix). In general, for visits at which plasma dapivirine levels were less than 95 pg per milliliter, the residual dapivirine levels in used rings were similar to levels in unused dapivirine rings, whereas residual dapivirine levels in used rings were lower for visits at which plasma dapivirine levels were more than 95 pg per milliliter. However, a range of residual dapivirine levels was observed, with low levels observed for some visits with low plasma dapivirine levels and high levels observed for some visits with plasma dapivirine levels of more than 95 pg per milliliter.

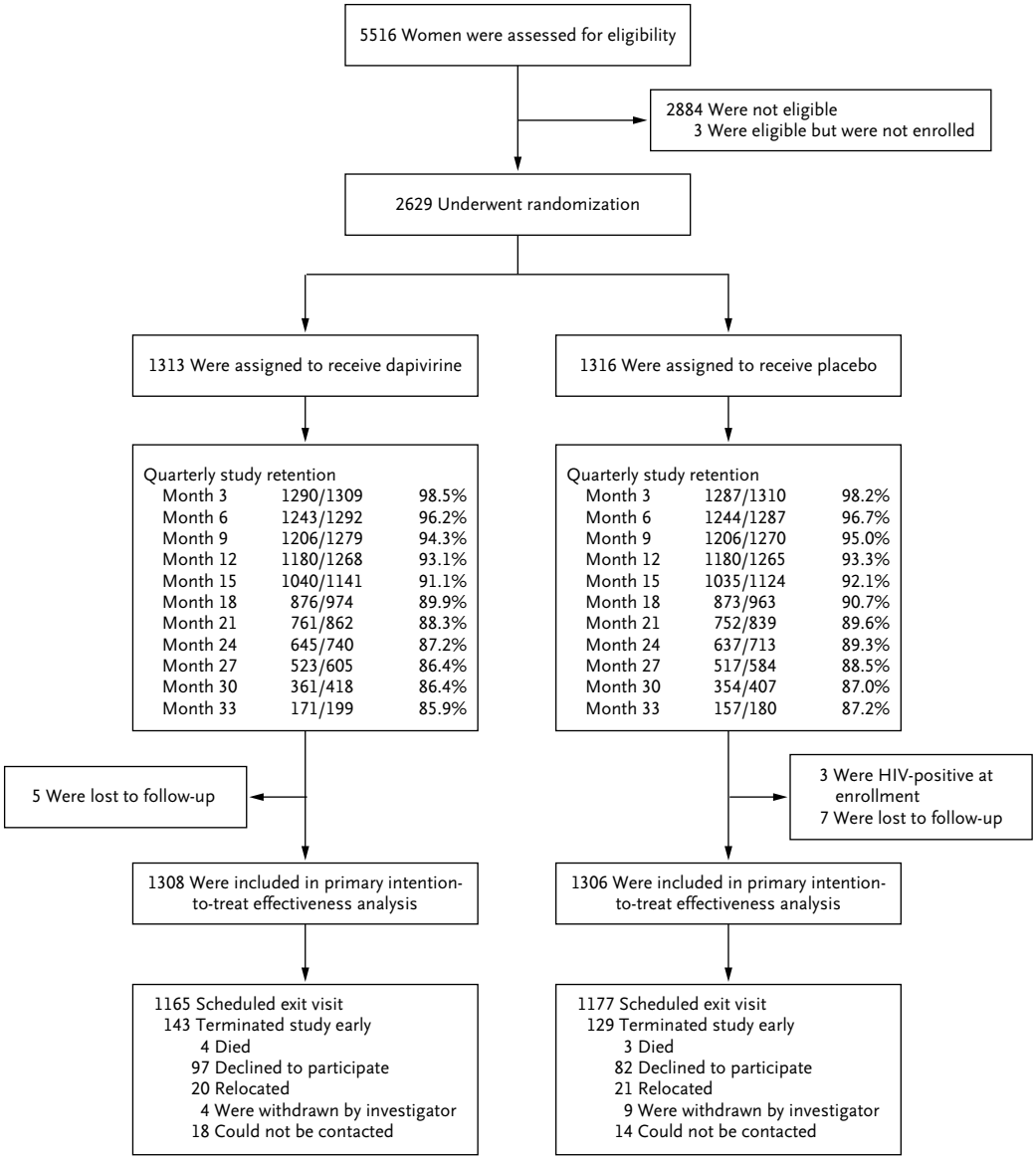

Figure 1. Enrollment and Outcomes.

Of the 5516 women who underwent screening, 2629 were enrolled, 2884 were not eligible, and 3 were eligible but did not enroll. The most common reason for ineligibility was seropositivity for HIV-1. Participants were enrolled at 15 study sites: 9 in South Africa, 3 in Zimbabwe, 2 in Malawi, and 1 in Uganda. Of the 2629 women who enrolled, 1426 (54%) were from South Africa, 678 (26%) from Zimbabwe, 272 (10%) from Malawi, and 253 (10%) from Uganda. The per-site enrollment numbers are provided in Table S1 in the Supplementary Appendix. Trial retention was defined as the provision of an HIV-1 test result, and women who withdrew early from the study were counted as having missed visits thereafter. Participants attended 91% of scheduled study visits (97% after accounting for early withdrawals from the study). Interruptions in the use of the study rings owing to pregnancy and breast-feeding accounted for 2% of study follow-up time, and protocol-required, safety-related temporary interruptions of product use accounted for less than 1%.

Table 1. Characteristics of the Participants at Baseline.*

Characteristic	Dapivirine Group (N=1313)	Placebo Group (N=1316)
Age — yr		
Mean	27.2±6.1	27.3±6.3
Median (range)	26 (18–44)	26 (18–45)
Secondary school education or higher — no. (%)	1101 (84)	1124 (85)
Earns own income — no. (%)	605 (46)	581 (44)
Currently married — no. (%)	527 (40)	547 (42)
Partner aware of ring use — no. (%)	835 (64)	845 (64)
Two or more male sex partners in the past 3 mo — no. (%)	212 (16)	227 (17)
No. of episodes of vaginal intercourse in the past 3 mo	26.4±24.3	26.5±24.9
Condom use during last vaginal sex — no. (%)	775 (59)	733 (56)
Anal sex in the previous 3 mo — no. (%)	25 (2)	29 (2)
Transactional sex in past yr — no. (%)†	73 (6)	89 (7)
Contraceptive method — no. (%)		
Intrauterine device	162 (12)	163 (12)
Oral contraceptive pills	143 (11)	144 (11)
Depot medroxyprogesterone acetate	515 (39)	555 (42)
Norethisterone enanthate	200 (15)	181 (14)
Hormonal implant	258 (20)	243 (18)
Sexually transmitted infection — no. (%)		
Chlamydia trachomatis	175 (13)	141 (11)
Neisseria gonorrhoeae	58 (4)	51 (4)
Trichomonas vaginalis	91 (7)	90 (7)

* Plus–minus values are means ±SD. There were no significant differences between the two groups at baseline.
† The incidence of transactional sex was measured by means of audio computer-assisted self-interview.

EFFECT OF DAPIVIRINE VAGINAL RING ON HIV-1 ACQUISITION

Across all 15 sites in the trial, a total of 168 incident HIV-1 infections occurred during the product-use period: 71 in the dapivirine group and 97 in the placebo group (incidence, 3.3 and 4.5 per 100 person-years, respectively). The incidence of HIV-1 infection in the dapivirine group was lower by 27% (95% confidence interval [CI], 1 to 46; P=0.05) than that in the placebo group (Fig. 2A). At the two sites that were excluded from the primary analysis because of lower-than-expected protocol and product adherence when the groups were still masked, 29 HIV-1 infections were reported: 17 in the dapivirine group and 12 in the placebo group. After the exclusion of the data from these sites, there were a total of 139 infections among 2395 participants: 54 in the dapivirine group and 85 in the placebo group. The incidence of HIV-1 infection in the dapivirine group was lower by 37% (95% CI, 12 to 56; P=0.007) than that in the placebo group (Fig. 2B). An efficacy of HIV-1 protection of less than 25% was not ruled out in either analysis (P=0.88 for 15 sites and P=0.30 for 13 sites).

In subgroup analyses, HIV-1 protection was generally similar to that seen overall (Fig. S4 in the Supplementary Appendix). However, the efficacy of HIV-1 protection differed significantly according to age, with an efficacy of 61% (95% CI, 32 to 77; P<0.001) among women 25 years of age or older and 10% (95% CI, −41 to 43; P=0.64) among those under the age of 25 years (P=0.02 for interaction).

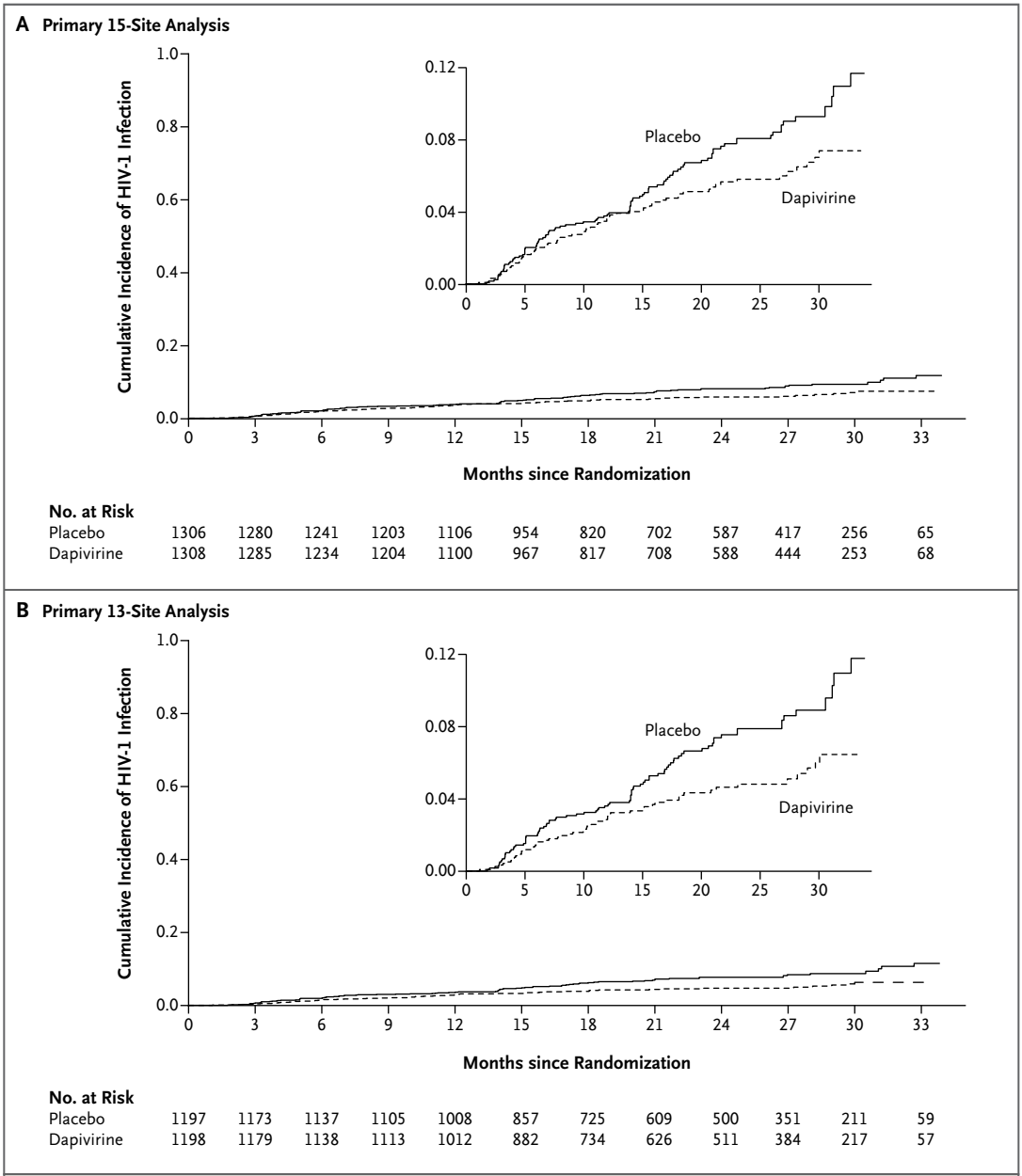

Figure 2. Cumulative Incidence of HIV-1 Infection in Two Analyses.

Shown is the cumulative probability of HIV-1 acquisition, as estimated by means of Kaplan–Meier methods, in analyses performed in two populations. Panel A shows the results for the overall 15-site analysis, in which the HIV-1 incidence was 3.3 per 100 person-years (95% confidence interval [CI], 2.6 to 4.2) in the dapivirine group and 4.5 per 100 person-years (95% CI, 3.7 to 5.5) in the placebo group. Panel B shows the results after the exclusion of data from 2 sites because of lower-than-expected protocol and product adherence, in which the HIV-1 incidence was 2.8 per 100 person-years (95% CI, 2.1 to 3.6) in the dapivirine group and 4.4 per 100 person-years (95% CI, 3.5 to 5.5) in the placebo group. Insets show the same data on an enlarged y axis. Excluded from the analyses were data for 3 participants in the placebo group who had HIV-1 infection before enrollment and for 3 participants (1 in the dapivirine group and 2 in the placebo group) who became infected after the product-use period.

To better characterize the relationship between age and HIV-1 protection seen in the prespecified subgroup analysis (age <25 vs. ≥25 years), an exploratory analysis was conducted post hoc. Age-categorized subgroups with balanced statistical power were created after dividing the 2395 participants into three groups with approximately equal numbers of those with HIV-1 infection as follows: ages 18 to 21 years, 451 participants with 44 HIV-1 infections (incidence in the placebo group, 5.4 per 100 person-years; 95% CI, 3.2 to 8.4); ages 22 to 26 years, 752 participants with 51 HIV-1 infections (incidence in the placebo group, 6.1 per 100 person-years; 95% CI, 4.3 to 8.3); and ages 27 to 45 years, 1192 participants with 44 HIV-1 infections (incidence in the placebo group, 3.0 per 100 person-years; 95% CI, 2.0 to 4.4) (Fig. 3A). Lack of HIV-1 protection, along with lower adherence, was seen in participants who were 18 to 21 years of age, with an efficacy of HIV-1 protection of –27% (95% CI, –133 to 31; P=0.45) (Fig. 3B). For women who were older than 21 years of age, the efficacy of HIV-1 protection was 56% (95% CI, 31 to 71; P<0.001), and the rate of adherence was more than 70% overall, as defined by dapivirine detection in plasma, in returned rings, and in the composite of those two measures.

SAFETY

There were no significant between-group differences in the frequency of the primary safety end points (Table 2, and Table S6 in the Supplementary Appendix) or in other adverse events commonly detected in the trial population (Fig. S5 in the Supplementary Appendix). Incident sexually transmitted infections occurred at a similar rate in the two groups (Table S7 in the Supplementary Appendix). Finally, among participants who acquired HIV-1 infection, there was no significant between-group difference in the numbers of participants with non-nucleoside reverse-transcriptase inhibitor mutations suggesting antiviral resistance (8 of 68 participants [12%] in the dapivirine group and 10 of 96 [10%] in the placebo group, P=0.80) (Table S8 in the Supplementary Appendix).

DISCUSSION

In this multicountry trial, a vaginal ring containing 25 mg of dapivirine that was renewed every month showed evidence of HIV-1 protection, demonstrating the efficacy of a sustained-release approach to delivering antiretroviral prophylaxis. As seen in other studies of HIV-1 prophylaxis,[5,7] the protective effect of the dapivirine vaginal ring, as compared with placebo, was significant but not as high as hypothesized in the design of the trial. Greater HIV-1 protection was observed among subgroups of women who had evidence of higher rates of adherence than among those with lower rates of adherence. HIV-1 protection was not observed for women between the ages of 18 and 21 years, and objective markers of adherence were lower in this subgroup than in those older than 21 years.

Strong relationships between adherence and HIV-1 protection have been seen in studies of HIV-1 prophylaxis, with several studies among African women showing low adherence.[3,4,9] In our trial, a majority of participants had objective evidence of adherence to the use of the dapivirine ring. We predefined dapivirine levels in plasma and in used rings to indicate adherence on the basis of phase 1 and 2 studies. However, our definitions could have led to an overestimation of adherence because participants were categorized as being adherent even though they may have used the ring for only a portion of the month (and possibly only for a few hours before a clinic visit). Further pharmacokinetic analysis may help define whether there is a threshold of use of dapivirine that provides protection against HIV-1, analogous to investigations that followed the initial clinical trials of tenofovir-based strategies.[22,23] Some studies of other treatment and prevention interventions have shown that use and resultant benefits often decline over time.[2,22] In contrast, we found that adherence appeared to increase after the first months of use, which may indicate that some time was needed for participants to become comfortable with the ring, and HIV-1 protection was sustained during follow-up of 24 months or more. Qualitative analyses showed that nonadherence to HIV-1 prophylaxis strategies in clinical trials may be related to characteristics of the participant (e.g., a younger age), the product (e.g., side effects), and the research process (e.g., concern about unproven safety and efficacy).[24,25] Notably, studies of the oral antiretroviral agent tenofovir have shown higher adherence in open-label studies after a demonstration of safety and efficacy than

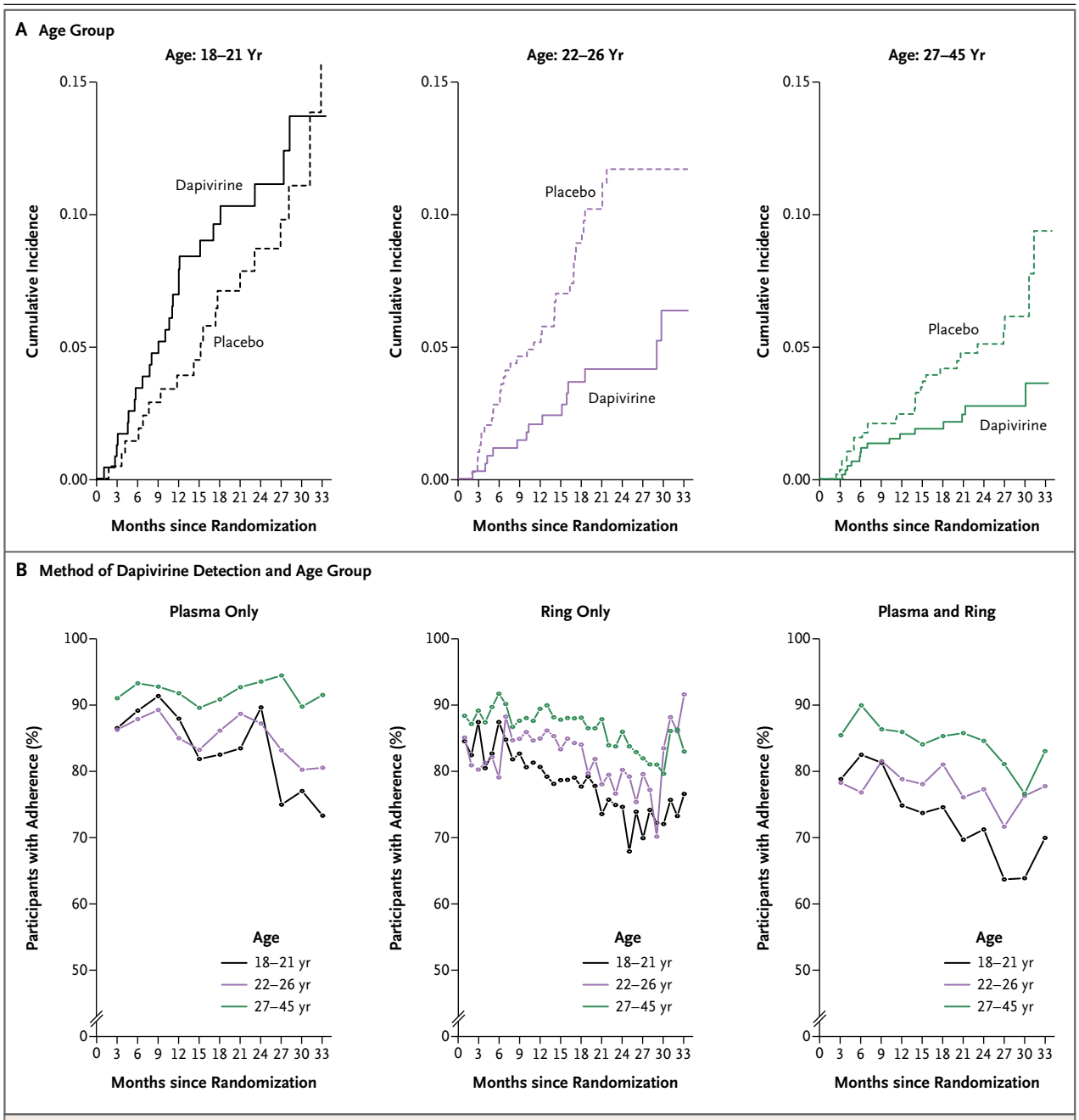

Figure 3. Cumulative Incidence of HIV-1 Infection, According to Age Group and Method of Dapivirine Detection.

Panel A shows that the efficacy of HIV-1 protection in the dapivirine group was –27% (95% CI, –133 to 31) for those 18 to 21 years of age, 56% (95% CI, 19 to 76) for those 22 to 26 years of age, and 51% (95% CI, 8 to 74) for those 27 to 45 years of age, as compared with placebo. The combined efficacy of HIV-1 prevention for participants over the age of 21 years was 56% (95% CI, 31 to 71; P<0.001). Panel B shows the percentage of plasma samples with a dapivirine value of 95 pg per milliliter (the adherence cutoff), the percentage of used rings with a residual dapivirine level of less than 23.5 mg (also the adherence cutoff), and a composite of dapivirine levels in plasma and residual rings. In the plasma-and-ring analysis, adherence levels were higher by a factor of 1.6 (P=0.07) among women 22 to 26 years of age and by a factor of 4.1 (P<0.001) among women 27 to 45 years of age than among women 18 to 21 years of age, as calculated by means of a generalized linear mixed-effects model with random intercepts and slopes and logit link after the exclusion of data from the first quarter because of small numbers. Returned rings were not available for the first calendar year of the trial, so the curves do not include all participants at all time points.

Table 2. Adverse Events.*

Adverse Event	Dapivirine Group (N=1313)	Placebo Group (N=1316)
	no. (%)	
Primary safety end point*	180 (14)	186 (14)
Any serious adverse event	52 (4)	48 (4)
Death	4 (<1)	3 (<1)
Any grade 4 event	22 (2)	23 (2)
Any grade 3 event	151 (12)	162 (12)
Any grade 2 event assessed as related	7 (1)	9 (1)

* The primary safety end point of the study was defined as any serious adverse event, any grade 3 or 4 adverse event, and any grade 2 adverse event assessed by the treating clinician as being related to the study product. P=0.80 for the overall comparison by the chi-square test.

had been seen in the initial blinded, placebo-controlled trials.[26-28]

Both behavioral and biologic effects may have contributed to a lack of HIV-1 protection in women between the ages of 18 and 21 years in this trial. Adherence to use of the ring appeared to be lower in this group than in women above 21 years of age; lower adherence to HIV-1 treatment and use of contraceptives has been reported among persons from 18 to 21 years of age.[29,30] The genital tract of women in this age group may be more susceptible to HIV-1 infection and potentially also more difficult to protect with antiretroviral prophylaxis strategies.[31,32] Further research is needed to understand the unique prevention needs of young women, including open-label evaluations that may allay concern about the efficacy of HIV-1 prevention, safety, or use of placebo. In addition, studies are needed to assess whether HIV-1 protection could be achieved in younger women with levels of adherence that were greater than those seen in this trial. The dapivirine ring significantly reduced HIV-1 incidence by more than half among women over the age of 21 years; women between the ages of 22 and 26 in the placebo group had an annualized incidence of HIV-1 of more than 6%, a finding that emphasizes the great need for prevention strategies for this population.

The concept of a topical microbicide for vaginal or rectal use for HIV-1 prevention was first proposed more than two decades ago.[33] Microbicide products — including gels, films, foams, and rings — have been evaluated in multiple studies among at-risk women and men. Microbicides can provide personal control over HIV-1 prevention and offer the ability to be used discreetly. Affordability is an important challenge to HIV-1 prophylaxis interventions, and topical delivery of antiretroviral drugs may allow minimal toxicity monitoring, which would improve cost-effectiveness. Only one previous trial, a phase 2 evaluation of a vaginal gel containing tenofovir, showed evidence of HIV-1 protection,[2] but this result was not confirmed in subsequent studies.[3,9] The results of an ongoing second phase 3 trial of the dapivirine vaginal ring (the Ring Study; ClinicalTrials.gov number, NCT01539226) will be important for improving our understanding of this intervention.

African women bear a disproportionate burden of the global HIV-1 epidemic. In the placebo group in our trial, the annualized HIV-1 incidence was more than 4%, despite monthly HIV-1 testing and risk-reduction counseling, testing of male partners, screening and treatment of sexually transmitted infections, and provision of free condoms. Our results show that a vaginal ring containing an antiretroviral drug can provide some protection against HIV-1 acquisition.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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APPENDIX

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How Good is Good Enough?
Efficacy vs. Effectiveness

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Comparative Effectiveness Research/Health Technology Assessment (HTA)

The "Efficacy-Effectiveness Gap": Historical Background and Current Conceptualization

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ABSTRACT

Background: The concept of the "efficacy-effectiveness gap" (EEG) has started to challenge confidence in decisions made for drugs when based on randomized controlled trials alone. Launched by the Innovative Medicines Initiative, the GetReal project aims to improve understanding of how to reconcile evidence to support efficacy and effectiveness and at proposing operational solutions. **Objectives:** The objectives of the present narrative review were 1) to understand the historical background in which the concept of the EEG has emerged and 2) to describe the conceptualization of EEG. **Methods:** A focused literature review was conducted across the gray literature and articles published in English reporting insights on the EEG concept. The identification of different "paradigms" was performed by simple inductive analysis of the documents' content. **Results:** The literature on the EEG falls into three major paradigms, in which EEG is related to 1) real-life characteristics of the health care system; 2) the method used to measure the drug's effect; and 3) a complex interaction between the drug's biological effect and contextual factors. **Conclusions:** The third paradigm provides an opportunity to look beyond any dichotomy between "standardized" *versus* "real-life" characteristics of the health care system and study designs. Namely, future research will determine whether the identification of these contextual factors can help to best design randomized controlled trials that provide better estimates of drugs' effectiveness.

Keywords: efficacy-effectiveness gap, pragmatic clinical trials, outcomes research, pharmaceuticals.

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Introduction

Regulatory approval of a new drug requires evidence of a positive efficacy-safety ratio: the extent to which the drug does more good than harm [1] is usually measured using randomized controlled trials (RCTs). Prelaunch drug development relies heavily on RCTs. When new drugs are launched in the market, little is known about their impact under routine prescribing practice and utilization of drugs, conventionally known as effectiveness [2]. The concept of the "efficacy-effectiveness gap" (EEG) describes possible discrepancies and complementary scientific evidence on efficacy and effectiveness. The awareness raised around this concept [3–5] results from how it may impact clinical and policy decisions on drugs. Research initiatives that aim to improve understanding of how evidence of efficacy and effectiveness can be reconciled and introduced at an earlier stage of drug development have been launched worldwide [6,7].

In the context of the GetReal project [7], the objectives of this narrative review on the EEG were 1) to understand the historical background in which the concept of EEG has emerged and 2) to describe the conceptualization of EEG.

Methods

A narrative-focused literature review of documents published in English to synthesize knowledge on EEG and address the objectives set out by the authors was carried out.

Identification of Documents

First, a broad search of the gray literature was performed across Internet Web sites from governmental authorities [8,9] and nongovernmental initiatives for EEG [6,9–12], as well as

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Table 1 – Search algorithm on the "efficacy-effectiveness gap".

Efficacy		
1	Efficacy	efficacy [Title/Abstract]
2	Clinical trials	"Clinical Trial, Phase II" [Publication Type] OR "Clinical Trial, Phase III" [Publication Type] OR "Clinical Trial, Phase IV" [Publication Type] OR "Controlled Clinical Trial" [Publication Type] OR "Multicenter Study" [Publication Type] OR "Randomized Controlled Trial" [Publication Type] OR "Clinical Trials, Phase II as Topic" [Mesh] OR "Clinical Trials, Phase III as Topic "[Mesh] OR "Clinical Trials, Phase IV as Topic" [Mesh] OR "Controlled Clinical Trials as Topic" [Mesh] OR "Multicenter Studies as Topic" [Mesh] OR clinical trial*[Title/Abstract] OR clinical trial*[Text Word] OR "randomized" [Title/Abstract] OR "randomized" [Title/Abstract] OR "randomized" [Text Word] OR "randomised" [Text Word] OR "randomization" [Title/Abstract] OR "randomization" [Title/Abstract] OR "randomization" [Text Word] OR "randomization" [Text Word] OR "random allocation" [Title/Abstract] OR "double blind" [Title/Abstract] OR "double blinded" [Title/Abstract] OR "double masked" [Title/Abstract] OR "single blind" [Title/Abstract] OR "single blinded" [Title/Abstract] OR "single masked" [Title/Abstract]
3	Explanatory trials	Explanatory [Title/Abstract] AND (trials [Title/Abstract] OR trial [Title/Abstract] OR studies [Title/Abstract] OR study [Title/Abstract])
4		1 AND (2 OR 3)
Effectiveness		
5	Effectiveness	Effectiveness [Title/Abstract] OR "translational research" [Title/Abstract]
6	Real life	("Real Life" [Title/Abstract]) AND (study [Title/Abstract] OR studies [Title/Abstract])) OR ("real word" AND study) [Title/Abstract] OR ("real word" AND data*) [Title/Abstract] OR (observational [Title/Abstract] AND (study [Title/Abstract] OR studies [Title/Abstract])) OR ("Real Life" [Title/Abstract] AND conditions [Title/Abstract]) OR naturalistic [Title/Abstract] OR "patient-oriented research" [Title/Abstract]
7	CER	"comparative effectiveness" [Title/Abstract] OR CER [Title/Abstract]
8	Pragmatic studies	(Pragmatic [Title/Abstract] OR Practical [Title/Abstract]) AND (trials [Title/Abstract] OR trial [Title/Abstract] OR studies [Title/Abstract] OR study [Title/Abstract])
9		5 AND (6 OR 7 OR 8)
Concept		
10	Gap	Gap*[Title/Abstract] OR issue*[Title/Abstract] OR complexit*[Title/Abstract] OR barrier*[Title/Abstract] OR facilitator*[Title/Abstract] OR problem*[Title/Abstract] OR bias [Title/Abstract]
11		4 AND 9 AND 10

CER, cost-effectiveness research.

Google and Google Scholar (for books and theses). This approach identified key authors and the main keywords to be used subsequently in a focused literature search (second step). This search was performed applying an algorithm (see Table 1) to the Embase, PubMed, and Cochrane Collaboration Web sites. Finally, the search was completed using a "snowball method" across the reference lists from the articles identified. The search ended when saturation was reached and no additional information relating to the identified paradigms could be extracted.

Selection and Appraisal of Documents

Documents were selected when 1) providing key elements about the historical background in which the EEG concept emerged and 2) when reporting insights (definitions and solutions) on the EEG concept. Quality appraisal of the document was performed ensuring "relevance" and "clarity" of the research questions, both regarding the qualitative research [13] and unprompted judgment, that is, our expertise [14].

Analysis and Data Synthesis

To identify different "paradigms," all the selected documents were used to extract and synthetize how the phenomenon of interest (EEG) was understood or discussed (the authors' perspective). A simple inductive analysis of the documents' content was performed because no paradigm classification had been defined before our study. The identification of different items took place as the data were being collected and analyzed. One researcher (C. N.) read the documents and annotated the main items in the margins. Subsequently, other researchers were involved (L.A. and M.R.), proceeding to a more analytical listing of items and the identification of paradigms.

Results

Search Results

The search algorithm allowed the identification of 672 academic manuscripts after removing duplicates. These were screened on title, and for a further 100 articles, the abstracts or the full manuscript was read.

Historical Background

The growing number of available medical technologies since the 1950s and the increasing amount of related information have generated a need for rigorous assessment of the potential benefit [15], information synthesis, and knowledge dissemination [16] of medical interventions.

Standardization of Trials and Information Synthesis

The standardization of clinical trials started in the 1950s with the implementation of founding principles for RCTs [17], but the importance of basing the assessment of therapies through clinical trials—rather than relying on prescribers' "opinions" [18]—reached the wider medical community nearly 20 years later. During this period, the concept of "attitude" [19] in a trial (explanatory *vs.* pragmatic) was introduced: the "explanatory attitude" related to the objective of acquiring information about the efficacy of drugs, whereas the "pragmatic attitude" related to the objective of gaining information about their effectiveness. These two attitudes were described as leading to two ways of interpreting results: understanding *versus* making decisions. The

"explanatory" attitude alone, however, has prevailed for more than 20 years.

In turn, methods for synthetizing the information from several clinical trials (meta-analyses) were standardized between 1980 and the early 1990s [20]. The Cochrane Collaboration [21] was created to facilitate the development of systematic reviews and meta-analyses of RCTs.

Knowledge dissemination

The creation of the American Consensus Development Program [22] in 1977 was an early attempt to improve the dissemination of biomedical research into clinical practice. Similarly, the paradigm of evidence-based medicine (EBM) emerged to promote good clinical practice [23] and was presented as "a practice integrating individual clinical expertise with the best available external clinical evidence from systematic research" [24]. (Fig. 1).

The EEG

Notwithstanding the standardization of clinical trials, methods to synthetize evidence, and platforms for knowledge dissemination, a chasm between research and clinical practice [25] has been noted and called the "knowledge gap" [22] or the "efficacy-effectiveness gap" [26]. Lehman et al. [26] explained: "Effectiveness refers to the impact of treatment under usual treatment conditions, in which patient factors […], provider factors […], and service system factors […] that can affect treatment outcomes, are not controlled."

Over the years, several ways of understanding the EEG have been suggested. Through our literature review, we have identified that the EEG concept falls into three major paradigms (see Table 2) relating to the role of 1) health care settings, 2) methods used to assess drugs' effect, and 3) the interaction between the drug and contextual factors.

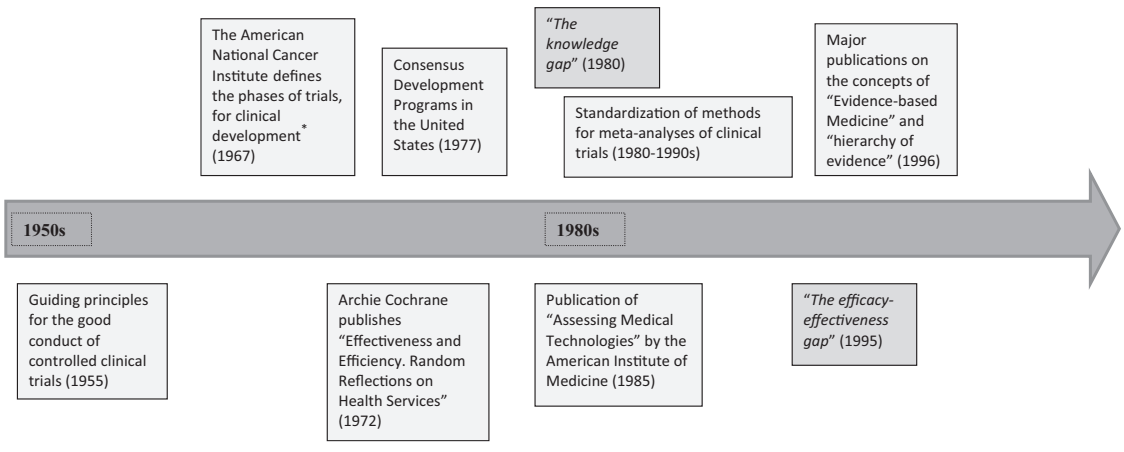
First Paradigm: The Issue of Behavior Toward Drugs' Prescription and Use

In a first paradigm, the EEG may be explained by characteristics of health care settings, including the "behavior" of caregivers and patients' adherence to treatment. In this paradigm, the optimal effect of the drug is thought to be distorted by real-life factors; namely, the EEG is understood as an issue of *behavior*.

First, the physicians' behavior has been thought to jeopardize the effectiveness of drugs and the dissemination of knowledge. One example can be found in a book published by the American Institute of Medicine, in which the authors stated that "patterns of medical practice often diverge from recommendations based on controlled clinical evaluations" [27, p. 176] and explained that one reason for this relates to physicians' behavior, defined as a combination of skepticism about innovation, lack of medical training, characteristics of the practice setting, and so forth. However, RCTs were thought to be the strongest method of assessing the efficacy of drugs. Similarly, it has been suggested that cognitive biases in interpreting new results (e.g., the tendency to embrace evidence supporting preconceived ideas) may compromise the generation and dissemination of evidence on efficacy into practice [28]. It is noteworthy that because clinical research and medical care involve human beings—physicians and patients—subjectivity cannot be set aside [29].

Second, the patients' adherence (i.e., the extent to which a patient's behavior corresponds to agreed recommendations from a health care provider [30]) has been suggested as a key factor to explain EEG, especially in chronic disorders [31–33]. The underlying rationale is that in clinical trials, attempts are made to maximize adherence, which would not be the case in daily practice. Numerous adherence-enhancement strategies have been developed (cognitive-educational interventions, self-reporting devices, or even telephone coaching [34]). Their impact on medication adherence, however, was found to be modest and not sustained over time [35]. In turn, the impact of enhancing medication adherence on effectiveness is yet to be established. Also, the relationship between the effect of a drug and drug adherence is complex and may apply in both directions: bad adherence by patients may lower the impact of treatments, and the poor efficacy (or tolerance) of a drug as perceived by the patient may lower the patient's adherence, in particular for medications that are expected to have a direct and identifiable effect on symptoms. In recognition of this intricate relationship, the investigators of a large pragmatic trial on antipsychotic drugs [36] have used the "discontinuation of treatment for any cause" as the primary outcome, explaining that "this measure integrates patients' and clinicians' judgments of efficacy, safety, and tolerability into a global measure of effectiveness […]."

Finally, the disparity in resource and access to care between settings has been considered as being responsible for the EEG [37,38]. For example, in elderly people with depression [39], the EEG has been described as the result of a combination of health care–related factors and intricate barriers, categorized into "patient barriers" (e.g., high costs of care, misdiagnosis of



* Zubrod, C.G. Multiclinic trials in cancer chemotherapy. *Can Med Assoc J* **97**, 101-3 (1967)

Fig. 1 – Historical background of the efficacy-effectiveness gap concept.

Table 2 – Summary of the three paradigms identified.

Paradigm	Description	Themes encompassed
1. The EEG is related to real-life characteristics of the health care system	The ideal effect of the drug is distorted by real-life characteristics of the health care system, related to the physician, the patient, and access to health care resources	• In routine practice, the *physicians'* "behavior" regarding medical guidelines and dissemination of knowledge is not optimal • In routine practice, the *patients' adherence* is not optimal • In routine practice, there are access barriers to health care resources
2. The EEG is related to an issue of the method used to measure the drug's effect	Efficacy and effectiveness studies use different study designs and design parameters, hence the EEG	• Concept of *evidence-based medicine* and *hierarchy of evidence*: the efficacy is the *real* effect of the drug; the RCTs are the criterion standard for measuring the drug's effect • Concept of *pragmatism*: RCTs' lack of generalizability; any direct dissemination of evidence coming from clinical trial into clinical practice is inadequate
3. The EEG is related to an issue of complex interaction	The drug's effect is the result of complex (and multiple) interactions between the biological effect of the drug and "real-life" contextual factors, hence the EEG	• Some contextual factors are (significantly) interacting with the drug's biological effect ("drivers of effectiveness") • An imbalance in the distribution of these factors between efficacy and effectiveness studies may cause an EEG

EEG, efficacy-effectiveness gap; RCT, randomized controlled trial.

depression in elderly people, and older people rarely seeking care), "provider barriers" (e.g., competing care of comorbidities), and "policy barriers" (e.g., geographic distance from care provider). It is believed that facilitating access to care could improve medical outcomes, as exemplified in a study on the "easy access to ambulatory service" program by patients with bipolar disorder [40].

Second Paradigm: The Issue of Method Used and Measure

A second paradigm holds that the EEG may be explained by the different study designs and methods being used to assess the impact of drugs (efficacy vs. effectiveness studies). In short, the EEG is understood here as an issue of *method/measure* in which the difference in effect size would stand in a difference relating to the study design.

There is indeed an association between the choice in study design parameters and the drug's effect size, as evidenced by Naudet et al. [41], who have investigated the impact of several design parameters of RCTs *versus* observational studies on the effect size of antidepressants. One of the reported results referred to the use of double blinding (*vs.* none), the comparison against placebo (*vs.* active comparator), or the use of intention-to-treat analyses (*vs.* per-protocol) as being associated with a lower effect size, regardless of patients and disease characteristics.

One finding of our literature review, however, is that within this paradigm, two concepts are opposed [42]: the concept of "hierarchy of evidence" supporting the superiority of RCT (due to a supposedly better internal validity) over nonrandomized studies, and the concept of "pragmatism" arguing that RCTs would lack generalizability (external validity) as compared with more pragmatic studies.

The Concept of Hierarchy of Evidence

The development of EBM and the concept of hierarchy of evidence might have introduced ambiguity regarding the role of RCTs within the frame of health technology assessment, with the underlying assumption that efficacy would be the *real* effect of the drug whereas effectiveness would be a *distorted* one, derived from "biased" real-life observational studies [43,44]. In a publication on EBM [24], its authors simultaneously stated that RCT "has become the gold standard for *judging* whether a treatment does more good than harm" and that EBM "is the conscientious, explicit, and judicious use of current best evidence in *making decisions* about the care of individual patients," which might have blurred the essential difference between assessing drugs' efficacy and choosing between drugs in clinical practice [19]. Furthermore, the concept of hierarchy of evidence [45] has consolidated RCTs and meta-analyses of RCTs as the leaders among type of studies aiming at assessing drugs' impact. This concept holds that the quality of study designs is set in stone, irrespective of the study objective, that is, whether it is intending to measure the biologic effect of the drug or not, or informing prescribers or policymakers on the best options to choose from in routine practice. Although a more balanced use of this hierarchy of evidence has been recently suggested [46,47], the concept of hierarchy of evidence may have contributed to the idea that "real-life" studies are not as legitimate as RCTs to provide evidence on the effect of drugs.

The Concept of "Pragmatism"

Conversely, it has been argued that RCTs lack generalizability. The "cautious generalization" of clinical trials was emphasized a long time ago [17], as well as the necessity to interpret results of clinical trials within the context of routine practice, using, for instance, the physicians' expertise [22]. The limited generalizability of RCTs, however, has been explained in more detail only recently. Many aspects of the traditional RCT design do not represent routine clinical practice: the selection of the population [48–50], its homogeneity [5], interventions not reflecting routine practice [51], and outcomes often not relevant enough for clinicians and patients [25]. The concept of pragmatism holds that this lack of generalizability has led to an EEG and that any direct dissemination of evidence arising from clinical trials into clinical practice might be inadequate [52,53]. In line with this concept, the generation of real-life evidence on the impact of drugs is becoming increasingly recommended, namely, for

pharmaceutical companies [10,51,54], in which such evidence is seen as complementary [55–57].

Third Paradigm: The Issue of Increased Variability and Interaction

More recently, a third paradigm has been developed in which any quantitative difference in drugs effect estimates, as measured in an experimental setting or in routine practice, may be understood as the result of interaction of multiple real-life characteristics on the purely biological effect of the drug. Eichler et al. [5] have explained that "to a large extent, the EEG may be considered a result of increasing variability of drug response owing to a combination of genetic, other biological and behavioral factors." The factors of potentially increasing variability in real life were categorized into 1) intrinsic biological characteristics of patients (genetics, physiology, comorbidities, etc.); 2) extrinsic environmental factors (diet, air pollution, health care system characteristics, etc.); and 3) behavioral factors (off-label prescriptions, patient adherence, etc.).

This paradigm encompasses the first two paradigms described earlier and also brings the EEG concept into a more operational level: if the EEG is related to the increasing variability (or the modification) of real-life factors, which statistically speaking corresponds to effect modification and/or interaction, then this gap may not only be explained but can also be anticipated and predicted.

To illustrate this paradigm, we report one study by Schneeweiss et al. [58], who have applied the patients' eligibility criteria commonly used in RCTs on statins to an observational study population, by sequentially excluding prevalent drug users, patients with contraindications, or patients with low adherence and so forth. They have examined the extent to which the 1-year mortality rate ratio estimates were changed and evidenced that using more restrictive eligibility criteria modified the mortality rate ratio estimates to the levels found in RCTs. These results suggest the existence of some interaction effect of patients' characteristics on the association between statins and mortality. On the same line, Ankarfeldt et al. [59] explored the impact of a high protein diet on weight change when RCTs and observational studies showed conflicting results. The authors suggested that being overweight and obese—which characterize the patients included in RCTs but not to the same extent, the patients included in observational studies—can act as an effect modifier on the association between a high protein diet and weight change. Another illustration is provided by Chassang et al. [60] who explored the impact of blinding (vs. open label) on the effect size of antidepressants. In line with Naudet et al. [41], the authors found that in double-blind RCTs, the treatment response tends to be smaller than in open-label studies because there is no "patients' beliefs effect" (expectancies over active treatment, leading to a patients' change in behavior) in double-blind RCTs. Although not new, the study results demonstrate—both through mathematical formalization and through empirical data analyses—that a difference in treatment effect size can happen only in the presence of interaction. This article provides a clear and formal demonstration of the role of interaction in the EEG.

Discussion

The present article is the result of extensive literature review on the EEG focusing on the current understanding of this concept.

Main Results

The present narrative study is thought to be the first to review the literature on the EEG, so as to reflect the whole spectrum of its conceptualization and to elicit the scientific paradigms that have influenced physicians and decision makers for decades.

Three Main Paradigms Render the EEG

A first paradigm holds that imperfections of the health care system in real life may explain why effectiveness outcomes are disappointing compared with efficacy outcomes. Thus, it is believed that all aspects of care in real life should be brought up to the standards of the experimental setting, with several levels of intervention: medical guidelines, knowledge dissemination, adherence-enhancement strategies, and so forth. These interventions are certainly commendable efforts to be made. The actual impact of such interventions, however, would be difficult to assess. Of note, this paradigm is not considering the possibility of the effectiveness being superior to the efficacy. One can imagine that the patient-physician relationship is a lever to patients' adherence, hence a better outcome in real life. Similarly, it is possible (and desirable) that in routine practice, physicians choose between therapeutic options so as to maximize the chance of success in one particular patient. A second paradigm advocates that the methodology used to measure a drug's effect (efficacy vs. effectiveness) has an impact on the drug's outcome, hence the EEG. Although this is probably often the case, one output of this review is that most of the debate has been focused on the pros and cons of each study design option. This debate is somewhat sterile because efficacy and effectiveness studies actually address different but complementary questions. In their pursuit of recognizing the complementary importance of explanatory and pragmatic designs, some authors have introduced the idea of "the explanatory-pragmatic continuum" [61]. Finally, a third paradigm has been identified, championing that a drug's therapeutic impact is the result of complex interactions between the drug's biologic effects and contextual factors (patient-related, provider-related, and health care–related). These interactions are at play whatever the study setting or design, but may differ in magnitude as a result of a drug being assessed in standardized (RCT) or in more flexible (real-life) conditions, hence the discrepancies in results. This third paradigm encompasses the first two and provides the opportunity to look beyond any dichotomy between standardized *versus* real-life behavior of physicians/patients, or between study designs/methods. Moreover, it may provide promising implications for future research.

Practical Implications for Researchers

The third paradigm provides a general research framework that allows the identification of those contextual factors interacting with the drug that have a particular impact on its effectiveness ("drivers of effectiveness").

All contextual factors may not equally impact a potential EEG. For instance, in the study by Schneeweiss et al. [58], several "levels of restriction" were applied to a cohort study population to replicate finding of RCTs, but did not share a similar impact on estimates for effects on statins: the restriction on "incident users" (by excluding prevalent users) had a greater impact than did the restriction on "adherent patients" (by excluding nonadherent patients). Through this example, we may hypothesize that the "incident/prevalent user" characteristic "drives" the effectiveness of statins, whereas "adherence" does not. This hypothesis, which of course would need to be further addressed, has practical implications in the design of an RCT to establish the effectiveness of statins among all potential users, including new users and possible switchers. A practical implication in designing an RCT on statins would be to include both incident and prevalent users, so as to be able to take this factor into account and minimize the risk of an EEG.

Another issue to consider is that drivers of effectiveness might depend on the drug and the disease under investigation. For instance, the choice between a double-blind or open-label design was found to influence antidepressants' effect estimates [41,60] because treatment expectancies on the treatment's effect are high in depression but this might not be the case for other drugs or diseases.

The implications for future research are numerous. The identification of the most impactful contextual factors may help to better design RCTs and if the latter are to be designed in a more "pragmatic" way [10], the emphasis in pragmatism should be put on these impactful contextual factors. Also, the contextual factors that are most likely to cause an EEG need to be identified "case by case," that is, for each disease area.

Study Limitations

First, the present review was not exhaustive but was not aimed to be so. The literature published on the EEG is extensive, and a selection was made using predefined criteria (publication in English, peer-reviewed articles, subjective appraisal of articles' quality). Also, the "snowball method" is thought to identify the most cited articles, usually published in journals in the higher impact factor scale range [62]. A selection bias preventing us from identifying all the existing paradigms on the EEG, however, cannot be excluded.

Second, our study focused on the gap in effectiveness outcomes and did not explore a gap in safety or benefit-risk ratio outcomes. Purposely made, this choice meets the objectives of the GetReal project, allowing us to better frame our research to that end. A gap in safety outcomes, however, may exist exactly in the same way as it does for effectiveness outcomes. The articles reviewed in the present study often discussed the two kinds of outcomes indifferently. For instance, Nallamothu et al. [50] have provided examples for a gap in knowledge in the risk of hyperkalemia in patients taking aldactone, which was underestimated in RCTs. Hernan et al. [63] have investigated the discrepancies of results between cohort studies and RCTs regarding the risk of coronary heart disease in women subject to hormone replacement therapy. The authors have evidenced that this discrepancy could be largely explained by differences in the distribution of time elapsed since menopause (within 10 years of menopause vs. more), with a significant interaction found for this factor. Overall, the results of the present study may certainly apply to safety or benefit-risk outcomes.

Finally, the present review did not explore to which extent the identification of an EEG may have impacted stakeholders decisions. The issue of the EEG was first raised by prescribers and health technology assessment (HTA) bodies, but has now reached the awareness of regulators [64]. The European Medicines Agency and European HTAs now offer parallel scientific advice to manufactures, where HTA needs for real-world data are being voiced early in development [65]. It would be interesting to investigate whether, and to which extent, regulatory decisions have been made in light of the issue of the EEG, that is, using real-life evidence. This is the objective and scope of another GetReal study in which we systematically reviewed phase 3 preauthorization trials that used more "pragmatic" designs (Karcher et al, 2015, submitted manuscript, unpublished data).

Conclusions

Identifying and targeting the contextual factors that have a meaningful impact on effect estimates for medications is a key priority for RCT design permitting better estimates of the effectiveness of drugs in addition to their efficacy. Within the GetReal Consortium [7], several studies are under way to identify the drivers of effectiveness in different therapeutic areas (schizophrenia, Hodgkin's lymphoma, diabetes, etc.). We hope that this review will help future research build on the identified paradigms.

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Review article

Apples and oranges? Interpreting success in HIV prevention trials

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Abstract

Background: In the last decade, several large-scale, clinical trials evaluating the efficacy of novel HIV prevention products have been completed, and eight are currently underway or about to be reported. Little attention has been given in the literature to the level of protection sufficient to warrant introduction, and there is concern that using the term "efficacy" to describe the effect of user-controlled methods such as microbicides may mislead policymakers.

Design: We review how the fields of family planning, vaccine science and mathematical modelling understand and use the terms efficacy and effectiveness, and explore with simple mathematical models how trial results of user-controlled products relate to common understandings of these terms.

Results: Each field brings different assumptions, a different evidence base and different expectations to interpretations of efficacy and effectiveness — a reality that could cloud informed assessment of emerging data.

Conclusion: When making judgments on the utility of new health technologies, it is important to use standards that yield appropriate comparisons for the innovation and that take into account the local epidemic and available alternatives.

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Keywords: Efficacy; Effectiveness; HIV prevention; Vaccine; Microbicide; Contraception; Family planning

1. Introduction

Over the last decade, several large-scale, clinical trials evaluating the efficacy of various novel HIV prevention products, including experimental vaccines, use of oral HIV drugs for pre-exposure prophylaxis (PrEP) and a variety of new microbicide candidates for preventing HIV transmission to women during vaginal sex, have been reported, and seven more are underway or about to be reported [1]. The trial designs include a wide range of effect sizes for the sample size calculation (33–60% reduction), but it is not clear what level of protection will be deemed sufficient to warrant introduction. This will depend in part on how policymakers interpret the numbers emerging from clinical trials and how these compare to their expectations regarding efficacy and effectiveness. Given that condoms are routinely cited as 95% efficacious, for example, some HIV stakeholders perceive anything less as unacceptable.

In assessing "how good is good enough", it is important to differentiate between user-controlled and provider-controlled methods, particularly in relation to the impact on estimates of biological efficacy that can be derived using trial data. There is also confusion in translating the results from trials into individual risks which are often easier for people to understand when deciding whether or not a product will work for them.

As a first step toward understanding these issues, we review the definitions and use of the terms efficacy and

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effectiveness across different fields, including vaccine science, HIV prevention, family planning and mathematical modelling. Then we use mathematical modelling to illustrate how these various measures relate in the particular case of HIV transmission and attempt to determine the appropriate standards for use with user-controlled methods.

2. Efficacy vs. effectiveness

With respect to health outcomes, *efficacy* is the improvement achieved with use of an intervention by participants under ideal conditions. It frequently refers to research settings or situations of perfect use. By contrast, *effectiveness* is reserved for the effect that can be achieved in practice, taking into account limited coverage, constrained resources and inconsistent or imperfect use.

Both efficacy and effectiveness are measures of *relative* rather than of *absolute* risk; they compare the incidence of a health outcome among individuals receiving an intervention vs. those who do not and are expressed as ratios. The *absolute* risk by contrast is expressed as a simple proportion.

2.1. The meaning of efficacy and effectiveness in trial results

For interventions like vaccines, where adherence by trial participants can be measured objectively, it is often possible to get an accurate estimate of biological efficacy from the results of a Phase 3 trial, provided loss to follow-up is minimal (see Appendix A for how vaccine efficacy is calculated). This is generally based on a per-protocol analysis, restricted to those who complete the full immunization schedule (perfect use).

When evaluating products that are user dependent, however, the trial results cannot be adjusted to derive efficacy unless there are reliable biological measures of product adherence. In trials of products such as microbicidal gels or PrEP, not all participants will use the product correctly or consistently, and measurement of use relies on self-reports. A Phase 3 trial therefore provides a combined measure of the product's biological efficacy and the pattern of use in the trial (adherence). Indeed, because of incorrect and inconsistent use, a trial that demonstrates a 40% reduction in incidence of HIV between its arms necessarily implies a product of higher efficacy.

2.2. Efficacy in the world of mathematical modelling

Modellers use the term "efficacy" differently to denote risk reduction for a *single act* of sexual intercourse with an infected partner rather than the protection achieved over time (as in trials). They use this *per-sex-act* efficacy in combination with many other factors — including the consistency with which it is used in different partnerships; the probability that the woman's sexual partner is HIV infected; whether he is in the high viremia phase; and whether either partner has other sexually transmitted infections — to project how use of a method may affect patterns of HIV transmission.

For outcomes that are relatively rare, like HIV, per-sex-act efficacy is a reasonable approximation of the efficacy of the method as understood by trialists or program planners. The same is not true, however, for more infectious pathogens, such as bacterial STIs, which have a higher transmission probability [2]. Thus, it is not always appropriate to equate the parameters used in modelling articles with the estimates of efficacy derived from clinical trials, although for HIV the values are comparable.

3. Current evidence of the efficacy of different HIV and contraceptive technologies

When the trial results for any new HIV prevention method become available, donors and policymakers will have to decide whether it merits further investment and ultimately introduction.[1] Their evaluation of the potential utility of any new method will depend in part on their assessment of the adequacy of existing options, their reading of available evidence and the assumptions they may bring from past experience.

Current evidence on the efficacy and effectiveness of different prevention technologies varies widely. Likewise, the fields of HIV prevention, family planning/reproductive health and vaccine science hold different working assumptions about what level of efficacy warrants introduction and use.

3.1. Efficacy of HIV prevention methods

In the world of HIV, the strongest evidence for an effective prevention strategy exists for male circumcision, which has been shown in three randomized controlled trials to reduce the risk of HIV acquisition among heterosexual men by roughly 50% to 60% [3–5]. The evidence for other HIV interventions is considerably less rigorous. For example, there have been no randomized controlled trials to assess the efficacy of the male and female condom against HIV and other STIs, although there have been several reviews of the available data [6,7]. Available estimates come from observational cohort studies of HIV discordant couples where a sero-negative person with a known exposure can be followed over time. When some individuals use condoms 100% of the time and some never use condoms, efficacy is calculated by taking one minus the ratio of the HIV incidence among "always users" of condoms vs. "never users", based on self-reports.

A Cochrane meta-analysis of these studies suggests a reduction in risk with consistent condom use of 80%, with the confidence limits for "consistent users" ranging between

[1] Because many new HIV prevention technologies are being developed with public sector financing, the decision to take a product forward will lie with donors rather than with a private company.

34% and 94% [8]. When inconsistent users are also considered, condoms reduce the annual HIV incidence by 69% [9].

Reviews of condom effectiveness have also sought to estimate the per-sex-act efficacy of the condom. In particular, Pinkerton and Abramson [10] used a Bernoulli model to "back calculate" the likely per-sex-act efficacy of male condoms associated with observed reductions in incidence among discordant couples. They concluded that condoms reduced the probability of HIV transmission per sex act by as much as 95%. It is this per-sex-act modelled figure that is often cited for both condom efficacy and effectiveness (see Table 1).

3.2. Efficacy of contraceptive methods

Since many women receive most of their sexual and reproductive health care from family planning and prenatal clinics, these sites are likely to be among the venues used to distribute any new HIV method aimed at women in the general population. Thus, family planning and reproductive health providers are likely to be among the various "gatekeepers" whose opinion will shape how women perceive any new HIV prevention method.

Family planning providers have been socialized in a world where contraceptive efficacies of 96% to 99% are not uncommon and anything less than 90% is largely dismissed as inadequate. After a decades-long effort to develop highly effective, long-acting contraceptive technologies (such as intrauterine contraceptives, injectables and implants), the benchmarks for contraceptive efficacy are now quite high (see Appendix A for equations of how contraceptive efficacy is calculated).

Estimates of contraceptive efficacy and effectiveness, however, vary in rigor. Evidence of efficacy for most methods comes from clinical investigations, but there have been no randomized, placebo-controlled trials of modern contraceptives. Sometimes clinical studies have randomized women to use different methods, but in many cases estimates derive from observational cohort studies, which track unwanted pregnancies among women using different birth control methods over the course of (usually) 1 year [11]. Like other observational studies, such research is subject to bias.

4. Explaining partial protection

Regrettably, data from trials do not translate easily into concepts accessible to individuals wishing to assess their own risk or to policymakers and donors seeking to make investment decisions among options. Research shows that individuals have a difficult time understanding notions of probability, especially when information is presented in relative, rather than in absolute, terms, as is the case with trials [12]. Relative measures are especially problematic because most people do not have an internalized notion of the underlying probabilities: for example, 42% of unmarried men and 40% of unmarried women aged 18–29 years in the United States believe that the chance of getting pregnant within a year while using the birth control pill is 50% or greater [13].

To compensate, the family planning field tends to rely instead on a measure known as the probability of contraceptive failure. Unlike efficacy and effectiveness, which represent a *relative* reduction in risk of unwanted pregnancy (a ratio), this provides women and providers with an estimate of the *absolute* probability that a woman will get pregnant if she uses a method over a period of a year (a proportion).

When discussing contraceptive options, providers commonly present two different pieces of information: probabilities of failure during a year of "perfect use" and during a year of "typical use" (including inconsistent *or* incorrect use) [14]. This metric has been shown to be far easier for women to interpret, because it helps them assess their personal likelihood of becoming pregnant within a time frame that is easy to grasp.

In general, there are large differences between probabilities of contraceptive failure during perfect use and during typical use for methods that require adherence, and smaller differences for less user-dependent products. For example, the annual risk of an unwanted pregnancy among women who use male condoms consistently and correctly is only 2% (perfect use), but it is 15% among typical users (see Table 1) [14].

Unfortunately, this approach is more difficult to take in the world of STIs and HIV, where the risk of HIV acquisition is largely defined by the infection status of one's sexual partner. Not only does the risk of infection per act of

Table 1
Estimates of condom efficacy and effectiveness

	HIV Prevention			Contraception	
	Always users vs. never users (efficacy)	Consistent and inconsistent users vs. never users (effectiveness)	Per-sex-act reduction in risk (efficacy based on modelling)	Perfect use (efficacy)	Typical use (effectiveness)
Efficacy or effectiveness	80%	69%	95%	98.9%	91.4%
Annual probability of failure	–	–	–	2%	15%

Sources: Refs. [8,14].

intercourse vary dramatically by pathogen but the risk of being "exposed" also varies dramatically by partner. Thus, it is much harder to construct a metric for women to understand their absolute risk of HIV infection over a year because it is defined as much by who they have sex with as it is by what prevention method they may use.

Likewise, with user-dependent methods like microbicides or condoms, focusing on the method's efficacy alone misses half the story. The protection that a prevention method confers is a function of both the inherent efficacy of the method and how consistently it is used. Indeed, given the particular transmission dynamics of HIV, consistency directly compensates for efficacy [15]. In other words, using a low-efficacy method consistently for HIV protection can confer as much protection as using a high-efficacy method inconsistently.

To illustrate this, Table 2 shows model projections of how a woman's absolute risk of acquiring HIV varies, depending upon the likelihood that her partner is HIV infected, and the extent to which she can use different methods of protection. Using simple model projections of the probability of HIV acquisition over a year, this model projects that, for the scenarios presented, and using HIV transmission probabilities appropriate for developing country settings [16], women in discordant partnerships have a one-in-five risk of acquiring HIV over a year if they do not use condoms, which drops to a risk of 0.2% for women in lower risk partnerships (two in 1000 risk).

Assuming that microbicide use reduces the per-sex-act risk of HIV acquisition by 60%, and condoms reduce the per-sex-act probability of HIV acquisition by 95%, we can see that different combinations of product use can achieve similar levels of protection. For example, if there is a 50/50 chance that a woman's next sexual partner is likely to be infected, based on the underlying HIV prevalence in her sexual network, she can achieve the same level of protection through 50% condom use or 75% microbicide use (6.4% vs. 6.2%). The analysis also illustrates that women could reduce their risk of HIV infection by more than a third if they were able to add relatively consistent microbicide use (75% use) on to their existing levels of condom use (0%, 20% or 50% condom use).

Table 2
Model estimates of the annual probability of a woman acquiring HIV in 1 year, assuming that a microbicide reduces the per-sex-act risk of HIV acquisition by 60%, for different patterns of product use and assumptions about the likelihood that her sexual partner is HIV infected

Percent of women becoming HIV infected in 1 year					
	Probability partner HIV infected				
	100	75	50	25	1
No product use	21.0	15.7	10.5	5.2	0.2
20% condom use, no microbicide use	17.7	13.3	8.9	4.4	0.2
0% condom use, 75% microbicide use	12.9	9.6	6.4	3.2	0.1
50% condom use, no microbicide use	12.4	9.3	6.2	3.1	0.1
20% condom use, 75% microbicide use	10.8	8.1	5.4	2.7	0.1
50% condom use, 75% microbicide use	7.5	5.7	3.8	1.9	0.1

Assumes one sexual partner, 100 sex acts per year; 5% of HIV-infected partners have increased risk of transmission due to high viremia; condoms reduce per sex act transmission risk by 95%; male-to-female HIV transmission probability 0.193%.
More information on the modelling behind this table is provided in Appendix A.

5. What do trial results tell us about product efficacy?

So, what do the relative risk numbers emerging from clinical trials tell us about product efficacy? That depends on the nature of the product and on how the results are reported. Because vaccines are administered by providers, Phase 3 clinical vaccine trials can yield true estimates of efficacy. Among participants who receive all doses, breakthrough infections more clearly reflect product failures rather than failure to use the product.

With interventions that require user adherence, Phase 3 trials do not yield true estimates of efficacy; rather they provide an unbiased measure of effect in the trial setting, but one that represents an average across all users, including those who did not use the product or did so inconsistently.

Nonetheless, it is possible to estimate product efficacy from the results of an effectiveness trial of a user-dependent product. Algebraic analysis suggests that a product's efficacy can be approximated by dividing the measured trial effectiveness by the consistency of gel use in the non–condom-protected sex acts (see Appendix A for supporting math).

Table 3 provides examples of the imputed efficacy that would be implied by relatively modest trial results of 30% to 40% reduction in acquisition of HIV among comparison groups. Depending on the level of product adherence achieved in the trial, the imputed product efficacy could range from 33% to 57% (or higher if reported adherence is lower than 70% among women not using condoms). It is this efficacy value that is most comparable with the 95% efficacy value cited for condoms.

While adherence is admittedly subject to over-reporting, any overestimation of use will lead to a conservative, rather

Table 3
Imputed product HIV efficacy, for different assumptions about the measured trial effectiveness, and levels of gel adherence in non–condom-protected sex acts

Measured effectiveness in trial	Gel adherence in non–condom–protected sex acts	Estimated product efficacy
30%	70%	43%
30%	80%	38%
30%	90%	33%
40%	70%	57%
40%	80%	50%
40%	90%	44%

Supporting math available in Appendix A online.

than to an inflated, estimate of product efficacy. (The higher the reported adherence, the lower the estimate of product efficacy.) By way of comparison, the reported gel adherence in non–condom-protected acts in the recent HPTN 035 trial of the Pro2000 microbicide gel was 68.2% (Barbara Richardson, Fred Hutchinson Cancer Research Center, personal communication). Among women in the MDP301 study who attended all 13 study visits, 56% reported using gel at every last sex act.

6. Interpreting trials: how good is good enough?

Given the different understandings of the terms efficacy and effectiveness, different stakeholders are likely to answer this question differently. Those coming from the world of preventive vaccines routinely accept vaccines that are 70% efficacious, including vaccines against pertussis, hepatitis B for newborns, and polio [17-19]. In family planning, the "gold standard" is considerably higher because there are many forms of contraception that achieve 95% to 99% efficacy in clinical studies (although in no study were women randomized to receive a placebo). But does a similarly high bar make sense for HIV where there are still groups of users who have no method of HIV protection they can reliably implement?

One such group is women in regular partnerships. Studies from around the world have shown that couples, especially women, find it extremely difficult to negotiate consistent condom use within on-going relationships. Yet, it is precisely within regular partnerships that many women are becoming infected [20,21]. While the female condom is theoretically available, in reality it remains scarce, expensive and ill-suited for relationships where women want to have children. By contrast, both women and men have responded enthusiastically to the use of microbicide gels within regular partnerships, with a significant proportion reporting increased sexual pleasure [22]. Moreover, mathematical modelling has shown that even a moderately effective microbicide could have a significant impact on HIV transmission in some settings [23]. Similar impacts have been demonstrated for partially effective HIV vaccines [24,25].

Nonetheless, many users and policymakers have internalized the belief that condoms reduce the risk of HIV acquisition by 95%, leaving the impression that this is the level of effectiveness against which any future HIV prevention methods should be compared. But this 95% figure is the estimated protection condoms provide in a single act of intercourse (not over time), derived from mathematical modelling. It is more appropriate to compare the results of trials of user-controlled methods such as microbicides, to the 69% reduction observed in the meta-analysis of consistent and inconsistent condom users over time.

Assessing what is "good enough" also depends on the underlying risk of disease. In settings where one in three sexually active adults are infected with HIV, as is the case in some parts of sub-Saharan Africa, a method that halves risk will be received differently than in settings where fewer than one in 1000 people are infected.

Likewise, perceptions will be shaped by how data are presented to women and to policymakers. Spermicides are 90% efficacious at preventing pregnancy, but among 100 women using spermicides for birth control for 12 months, 29% will have an unwanted pregnancy. These data describe the same product, but the first is a relative risk estimate comparing consistent users to nonusers, while the second communicates the absolute risk of becoming pregnant in a year among typical users [14].

Inconsistency between different types of trial analyses [intent-to-treat (ITT) vs. per protocol] can be a further cause of confusion, as demonstrated by the recent release of the results from Thai RV144 HIV vaccine study. Investigators from this trial first released to the press results from the trial's primary end point — a modified ITT analysis that included everyone who was randomized (even if they did not receive all of the trial's required injections), but excluded seven individuals found to be HIV positive at baseline. They later released a per-protocol analysis that included only participants who were HIV negative at baseline and who received all six injections. The modified ITT analysis found statistically significant protection (31.2% reduction; $p=.035$), whereas the per-protocol analysis did not (26.2% reduction; $p=.16$). The results were not numerically inconsistent with each other, but the ITT result reached significance because of the larger number of end points, and this created a sense of inconsistency [26].

At times, ITT and per-protocol results can diverge substantially. The Merck HPV vaccine, for example, is widely promoted as 99% efficacious (as revealed in the per-protocol analysis), but this efficacy estimate refers only to prevention of dysplasia and/or cancers caused specifically by HPV strains 16 and 18 among women who received all three doses of the vaccine. In an ITT analysis (which includes women who received less than three doses), the vaccine was 44% efficacious against HPV 16- and 18-related negative outcomes and only 18% efficacious against all HPV-related dysplasia or cancer [27]. Our point is not to question the public health importance of the HPV vaccine, but rather to highlight how confusing different analyses can be to the untutored eye.

Thus, when making judgments on the utility of new health technologies, it is important to use appropriate benchmarks and to compare innovations against available alternatives. Policymakers must be careful not to mix apples and oranges: they must separate trial results from extrapolated modelling estimates; consider per-protocol analysis and ITT analysis within the context of user-controlled and provider-controlled methods; and avoid setting inappropriate standards for HIV risk reduction based on metrics created for

family planning. People's future access to an expanded array of prevention options will depend in part on our collective ability to draw appropriate and informed comparisons.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.contraception.2010.06.009.

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Statistical issues in trials of preexposure prophylaxis

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Purpose of review
We discuss selected statistical issues in the design and analysis of preexposure prophylaxis (PrEP) trials. The general principles may inform thinking for other interventions in HIV prevention.

Recent findings
To date, four different designs have been used to determine the effectiveness of PrEP: randomized, double-blind, placebo-controlled; randomized, open-label, immediate or delayed access; nonrandomized comparison of HIV incidence according to the level of drug detected; comparison of the observed HIV incidence to the expected rate using historical control data. Open-label trials of PrEP, which assess public health effectiveness, complement the placebo-controlled trials which established the biological efficacy of TDF/ FTC. Future trials of PrEP will be highly challenging to design since a no PrEP group is difficult to justify and the natural control regimen, TDF/FTC, is highly efficacious.

Summary
Standard statistical paradigms for noninferiority trials should be reconsidered for evaluating alternative PrEP regimens.

Keywords
intention-to-treat, noninferiority, open-label, placebo-controlled, risk compensation

INTRODUCTION

Randomized controlled trials of preexposure prophylaxis (PrEP) have given rise to specific statistical challenges both in design and analysis. In this article we focus in depth on three issues: assessing the influence of risk compensation, dealing with patients with acute HIV infection at study enrolment, and the design of future studies in the context of a highly efficacious preexisting regimen.

Risk compensation and the limitation of placebo-controlled trials

'Risk compensation' is the adjustment of behaviour in response to a perceived reduction in risk, a critical issue in the public health implementation of PrEP because of the potential for increased risky sexual behaviour which could counteract biological efficacy [1]. Placebo-controlled randomized trials are regarded as the gold standard for establishing the biological efficacy of an experimental drug. A key rationale for using placebo in trials of PrEP agents has been to avoid bias because of differential exposure to HIV caused by different sexual behaviour in the randomized groups; this contrasts with the real-life situation, where individuals know if they are taking an active drug. A frequently unappreciated point is that risk compensation cannot be assessed by standard within or between group comparisons in a placebo-controlled trial [2]. The European Medicines Agency stated that 'The behavioural impact of PrEP on risk compensation and condom replacement cannot be assessed in prelicensure placebo-controlled trials' and that 'it is mandatory that the marketing authorisation application contains a risk management plan that adequately covers the public health impact of the PrEP intervention' [3].

In an imaginative analysis to gain insights into risk compensation in the Preexposure Prophylaxis Initiative (iPrEx) trial, Marcus *et al.* [4] compared patients who believed they were taking active drug

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KEY POINTS

- Open-label randomized trials are the only design that can reliably assess risk compensation.
- The proportion of prevalent acute infections can provide insights into underlying HIV incidence in the trial population.
- New paradigms are required for noninferiority trials of experimental PrEP regimens.

($n = 553$) with patients who believed they were taking placebo ($n = 223$). Patients who believed they were receiving active drug had higher number of receptive partners at baseline, but the difference between the two groups did not increase during follow-up after study drug was initiated. There was also no difference at any time point in the percentage of receptive anal intercourse partners using condoms. These results were interpreted as no evidence of risk compensation. However, this study has several limitations: confidence intervals were relatively wide (the analysis excludes 1429 patients who did not predict their treatment assignment); the accuracy of self-reported data on sexual practices; and the fact that groups were based on perceived assignment rather than certain knowledge as pertains in real-life. A further limitation is that risk compensation is a function of how effective an individual considers the intervention to be, and the very high biological efficacy of tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) was not known at the time the study was conducted.

Grant *et al.* [5▪] assessed and presented a detailed analysis of a cohort study of MSM enrolled from three previous randomized controlled trials of PrEP (including iPrEx) that were offered open-label PrEP. The authors assessed risk compensation by looking at longitudinal changes in behaviour, comparing patterns among men who accepted the offer of PrEP and those who declined it. Self-reported total number of sexual partners, noncondom receptive/insertive anal intercourse decreased during follow-up in both groups and to a similar extent. Syphilis incidence was also similar in the two groups. However, the fact that the control group was not randomized limits the interpretability of these data.

The most robust data on risk compensation to date were obtained in PROUD, a pragmatic, open-label trial which attempted to mimic how PrEP would be administered in routine clinical practice [6▪▪]. Eligible patients were randomized to receive daily TDF/FTC either immediately ($n = 275$) or after a deferred period of 1 year ($n = 269$). Data from the first year of follow-up allowed direct assessment of risk compensation. Patients were asked to complete monthly questionnaires and daily diaries about sexual behaviour but the completion rates of these were low, particularly in the deferred group. Accordingly the investigators reported cross-sectional analyses of sexual behaviour based on baseline and 1 year questionnaires only. No differences were found in terms of the total number of different anal sex partners but there was marginal evidence of a larger proportion of PrEP recipients at 1 year who reported receptive anal sex with 10 or more partners without a condom. An indirect, but more objective measure of risky sexual behaviour, is the diagnosis of other sexually transmitted infections (STIs) [7]. PROUD reported a slightly higher rate of diagnosis with any bacterial STI in the immediate PrEP group (57%) than in the deferred group (50%). However, after adjustment for the number of screens, there was no evidence of a difference between the groups in the frequency of bacterial STIs, either individually or overall.

A potentially important effect which could impact negatively on the cost–effectiveness of PrEP is that some men who have been using condoms consistently may stop doing so because they are able to access PrEP. Such men have not been eligible for PrEP trials to date and are unlikely to be formally eligible in PrEP implementation programmes. However, setting rigid criteria for PrEP access is not realistic, and if this phenomenon is real it will be difficult to detect it.

Acute HIV infections at study enrolment in the analysis

A clinical challenge with PrEP is the window period between exposure to HIV and the (assay-dependent) detection of infection, meaning that PrEP is inevitably initiated in some individuals who are already infected. The procedure used in most trials has been to perform a point-of-care serological test for HIV on the day of enrolment and to store an additional plasma sample that is retrospectively tested for HIV RNA, the earliest marker for HIV infection, if the patient had a reactive HIV antibody test at their first (or early) follow-up visit [8–12]. In real-life clinical practice, procedures are usually less stringent than in trials. United States guidelines recommend 'At a minimum, clinicians should document a negative antibody test result within the week before initiating (or reinitiating) PrEP medications' [13]. Also, samples may not be routinely stored, precluding the possibility of retrospective testing.

The primary efficacy analyses of trials have generally excluded patients with detectable HIV

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Table 1. Impact on effect measures of including or excluding patients with acute HIV infection at enrolment in the PROUD trial

	ITT (all patients)		Modified ITT (excluding acute cases)	
	Immediate	Deferred	Immediate	Deferred
No. of infections	5	21	3	20
Follow-up (person-years)	243.5	222.1	243.5	222.1
Incidence rate*	2.1	9.5	1.2	9.0
Rate difference*	7.4		7.8	
Number needed to treat	13.5		12.9	
Efficacy (%)	78		86	

Three patients (two Immediate, one Deferred) tested nonreactive by a third-generation rapid test on the day of enrolment but reactive with a joint antigen/antibody assay. ITT, intention to treat.

RNA at enrolment [modified intention-to-treat, (mITT)] on the grounds that PrEP cannot possibly avert infection in these individuals. (PrEP may have a postexposure prophylaxis effect but only if initiated within 48–72 h of exposure.) From an effectiveness rather than an efficacy perspective a full ITT analysis including all patients is arguably the more relevant [14]. In particular, analyses of safety outcomes should be intention-to-treat (ITT), particularly those relating to drug resistance, as viral mutations are particularly likely to emerge during acute infection under selective drug pressure.

In practice, ITT and mITT analyses in most studies produce very similar results as the number of prevalent acute infections is generally much smaller than the number of incident infections. However, it can make a material difference in studies where adherence to PrEP is high. For example, of the five infections in the immediate PrEP arm in PROUD, two occurred at enrolment. Here, the estimated efficacy is 78% under an ITT analysis compared with 86% under an mITT analysis (Table 1). Note that there is little effect on the rate difference (or number-needed-to-treat), the most relevant measure for public health.

Finally, in the following section we raise the possibility of using the number of prevalent acute infections (antibody negative/HIV RNA-positive result on enrolment sample) to measure the underlying 'force of infection' in the trial population. The method is described in the footnote to Fig. 1, which shows the inferred baseline incidence plotted against the observed incidence of infection among patients who were allocated to placebo in trials that tested enrolment samples for HIV RNA. With one exception, it over-estimates incidence is overestimated by a factor of between 2 and 3. There are two main possible explanations for this. First, the calculation is highly sensitive to the assumption about the mean interval between detectable circulating viral RNA and detectable circulating antibody, which may be incorrect. Second, patients may have been motivated to join the trial because they had recently been at especially high risk of exposure to HIV. Nonetheless, in large trials this approach can provide a rough estimate of the underlying rate of infection.

Future studies and the challenge of a highly-efficacious control regimen

Although TDF/FTC is the only drug currently approved by Food and Drug Administration for prevention, there is a pipeline of other agents,

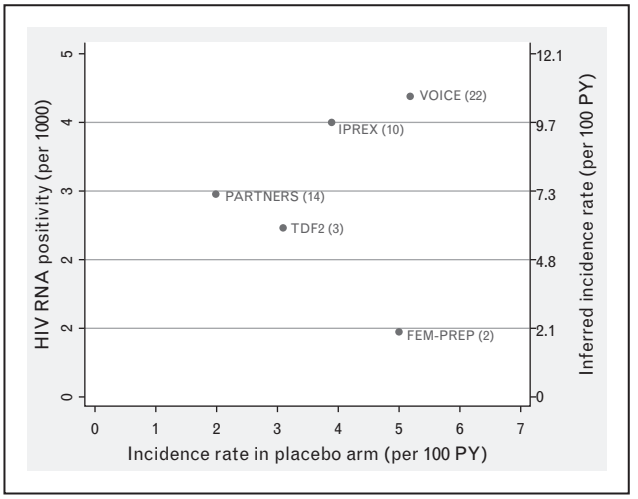

FIGURE 1. Proportion of patients with acute infection at enrolment plotted against incidence of infection observed in placebo recipients. Limited to studies that tested enrolment plasma samples retrospectively for HIV RNA. Value in brackets is number of acute infections observed in each study. The inferred incidence of infection at enrolment (plotted on right-hand vertical axis) was estimated by dividing the proportion of patients who were HIV RNA-positive/antibody negative by the mean interval between the detection of HIV RNA and antibody [20]. We assumed a value of 15.1 days using data from Eller *et al.* [21].

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Table 2. Outcomes in hypothetical study – low-incidence population

Group	Total follow-up	HIV infections	Rate (per 1000 PY)	Effectiveness compared to N (%) (95% CI)
Control (C)	5000	8	1.6	60 (5, 85)
Experimental (E)	5000	15	3.0	25 (−54, 64)
No treatment (N)	5000	20	4.0	–

CI, confidence interval.

particularly long-acting agents [15]. Given the proven biological efficacy of TDF/FTC, there are ethical barriers to conducting future clinical trials that include a no PrEP comparison group. Possible exceptions to this are populations where PrEP is not policy or where adherence to daily TDF/FTC is uncertain.

Donnell *et al.* comprehensively reviewed study designs for PrEP interventions, assuming daily TDF/FTC to be the control regimen [16▪▪]. They considered three different experimental regimens: a new daily drug, a long-acting drug, and a different TDF/FTC dosing strategy. For the first of these scenarios, a noninferiority design would be the natural choice. The study explored noninferiority margins of 1.10, 1.20, and 1.25 on a hazard ratio scale. For the highest noninferiority margin of 1.25, and assuming the experimental intervention to be equally effective to TDF/FTC, the authors show that a trial would have to accumulate a total of 844 HIV events to be sufficiently powered; this translates to a sample size of approximately 19 000 subjects for HIV incidence of 2.25/100 person-years and 2 years follow-up on average – an infeasible undertaking.

Further calculations were made under the assumption that the experimental agent is more effective than TDF/FTC, to enable smaller, more realistic sample sizes. However, in the face of strong evidence that TDF/FTC confers very high protection if adequate drug concentrations are achieved [17], this assumption is plausible only in comparisons with long-acting drugs in a population likely to experience barriers to adherence to a daily oral medication.

The large number of required events for noninferiority studies is driven mainly by the use of the hazard ratio (which is based on the multiplicative scale) for assessing noninferiority. From a public health perspective, the rate difference is the more important metric as it translates directly to the number needed to treat [18], and this concept can be utilized in the comparison of drugs as well as to a comparison of drug versus no treatment.

Suppose we did a clinical trial to compare an experimental preventive intervention (E) to daily TDF/FTC (control, C) in a group of 5000 volunteers. The trial randomizes 2500/arm and follows them for a total of 2 years, yielding the results in Table 2. The HIV rate ratio (relative to C) is 1.88 [95% confidence interval (CI) 0.74, 5.1]. Thus, the rate of HIV could be as much as five times higher for E and would clearly exceed any noninferiority margin. The rate difference is much narrower: 1.4 (95% CI −0.4 to 3.3)/1000 person-years. For every thousand people getting E rather than C for 1 year, the best estimate is that there would be 1.4 more infections (or 3.3 at most).

We now argue that information on the number of infections under the condition of no-treatment (N) is essential context, noting this group is not actually observed. Suppose, HIV incidence under N is 4.0/1000 person-years. The effectiveness of E compared to N is 25% (95% CI −54% to 64%) and the effectiveness of C compared to N is 60% (5–85%). It is helpful to compare the effectiveness estimate for E and C on the additive scale: 60 − 25% = 35% (95% CI −14 to 84%), which represents that proportional increase in the number of infections using E rather than C relative to the number of infections in the absence of PrEP. Thus given 5000 person-years follow-up we would expect 20 infections with no PrEP and 7 (15–8) more infections with the use of E rather than C (7/20 = 35%); this would seem to represent an appreciable loss of efficacy.

Consider an alternative scenario where the trial population is at 10 times higher risk of HIV and is highly adherent to both E and C (Table 3). Under this scenario, the effectiveness of E compared to N is 93% (95% CI 87–96%) and the effectiveness of C compared to N is 96% (92–98%). The HIV rate ratio is unchanged (1.88 = (1–93%)/(1–96%)), but the difference in effectiveness on the additive scale is much smaller: 96 − 93% = 3% (95% CI −1 to +8%). Given 5000 person-years follow-up, we still expect seven more infections with the use of E rather than C but this time against a background of 200 infections in the absence of PrEP. In this scenario, E would seem to be an acceptable alternative to C.

The fact that underlying HIV incidence as well as adherence to PrEP can vary greatly between populations implies the need to anchor any comparison to the number of HIV infection we would have

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Table 3. Outcomes in hypothetical study – high-incidence population

Group	Total follow-up	HIV infections	Rate (per 1000 PY)	Effectiveness compared to N (%) (95% CI)
Control (C)	5000	8	1.6	96 (92, 98)
Experimental (E)	5000	15	3.0	93 (87, 96)
No treatment (N)	5000	200	40.0	–

PY, person-years.

observed in the absence of PrEP. We propose, for wider discussion, the use of a two-part noninferiority definition:

$$\text{(i) } \lambda_E - \lambda_C < \Delta \text{ and (ii) } (\lambda_E - \lambda_C)/\lambda_N < \rho,$$

where λ_E, λ_C, and λ_N are estimates of HIV incidence in the E, C, and N groups respectively and the noninferiority margins (Δ, ρ) are appropriately chosen. (To simplify exposition, we have avoided attaching probabilistic statements to the lower confidence limits.)

For instance, in the low-incidence scenario the upper CI for $\lambda_E - \lambda_C$ is 3.3/1000 and the upper bound on $(\lambda_E - \lambda_C)/\lambda_N$ is 0.84 (or 84% more of total infections). In the high-incidence scenario, the upper CI for $\lambda_E - \lambda_C$ remains 3.3/1000 whereas the upper bound on $(\lambda_E - \lambda_C)/\lambda_N$ is now 0.08 (or 8% more of total infections). The first part of the definition is fully rigorous is the sense that it is intention-to-treat and does not rely on an external estimate of λ_N, but this is required for the second part of the definition. The Partners Demonstration project estimated this based on the placebo rate of HIV in the cohort prior to the treatment period [19]. An alternative approach could be to use the proportion of patients with HIV RNA detected in their enrolment sample, as described earlier. A final possibility is to use external data in the population from which the study patients are recruited, although this can be misleading. The PROUD study observed an HIV incidence of 9.0/100 person-years in the deferred group, which was approximately seven-fold higher than a national estimate of 1.34/100 person-years for MSM attending sexual health clinics [6▪▪]; this underscores that it may be difficult to assemble control groups that accurately reflect the HIV risk of individuals who seek participation in a trial.

CONCLUSION

Placebo-controlled and open-label trials of PrEP have addressed fundamentally different questions. The former evaluates the biological efficacy of the PrEP agent studied; the latter attempts to evaluate real-life effectiveness, reflecting the impact of risk compensation and actual adherence. Future trials of PrEP are highly challenging to design since daily TDF/FTC, the natural control regimen, is highly efficacious. New statistical paradigms for noninferiority trials are required, with statisticians and expert clinicians working closely together to develop these.

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Conflicts of interest

There are no conflicts of interest.

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Commentary

Beyond ''getting drugs into bodies'': social science perspectives on pre-exposure prophylaxis for HIV

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Abstract

Social scientists have much to contribute to the analysis of the real and potential contribution of pre-exposure prophylaxis (PrEP) to HIV prevention around the world. Beyond just a matter of clinical efficacy and getting pills into people's mouths, PrEP raises a number of important social-psychological questions that must be attended to in order to translate biomedical and clinical findings into uptake of PrEP among enough people at risk of HIV infection to produce population-level effectiveness. PrEP is a dynamic phenomenon with ''dialectical'' attributes that invite both optimism and cynicism as a desirable and effective HIV prevention strategy. PrEP disrupts traditional notions of ''safe'' and ''unsafe'' sex; it confers on its users a level of agency and control not generally achieved with condoms; and it affects sexual practices and sexual cultures in meaningful ways. As these dynamics play out in different contexts, and as new modes of PrEP administration emerge, it will be important for social scientists to be engaged in assessing their impact on PrEP implementation and effectiveness.

Keywords: PrEP; social science; HIV prevention; sexuality; risk compensation.

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Introduction

Among the HIV biomedical, clinical and advocacy communities, discussions of pre-exposure prophylaxis (PrEP) have largely focused on two questions: Is it clinically effective? and What are the structural and policy factors that impact its effectiveness when implemented? But, PrEP raises a number of other important social-psychological questions that also must be attended to in order to translate biomedical and clinical findings into uptake of PrEP among enough people at risk of HIV infection to produce population-level effectiveness. Social science has much to contribute in this regard.

Social scientists take as their starting point that PrEP is a dynamic phenomenon that is more than just a pharmacologic intervention – that is, getting PrEP to ''work'' is more complicated than simply ''getting drugs into bodies.'' Rather, PrEP embodies a range of interacting physiological, psychological and social realities that together affect not only an individual's risk or avoidance of HIV infection but also relationship dynamics, sexual cultures and social arrangements that have influence beyond HIV. We explore some of this dynamism and the issues it raises for further understanding of the role of PrEP in HIV prevention and to make the case that social science perspectives are essential as further implementation of PrEP ensues.

The dynamic nature of PrEP

PrEP emerges in, and itself effects, a dynamic situation. In the context of combination HIV prevention, PrEP provides another method in the ever-evolving constellation (or ''toolbox'') of evidence-based prevention strategies. It enhances the repertoire of choices individuals can make about how best to protect themselves from acquiring HIV, taking into account the realities of one's life, the nature of one's sexual and drug-using practices and relationships, and personal preferences about behavioural and technological ''interventions.''

Perhaps most importantly, PrEP's demonstrated efficacy among gay and other men who have sex with men (MSM) and transgender women [1], heterosexual men and women [2,3], and men and women who inject drugs [4] disrupt traditional notions of ''protected'' and ''unprotected'' sex, and of ''risky'' and ''safe'' sex and drug use [5–7] – notions that have been institutionalized in public health and community (especially gay community) discourse and practice for the past three decades. (As a case in point, PrEP's disruptive effect, alongside other advances in using antiretroviral (ARV) drugs for HIV prevention, recently helped to spur the U.S. Centers for Disease Control and Prevention (CDC) to stop using the term ''unprotected sex'' to refer to sexual intercourse without a condom [8].)

The promotion of ARV-based prevention approaches – whether ''treatment as prevention,'' ''pre-exposure prophylaxis'' or ''post-exposure prophylaxis'' – imbues the person taking ARV with a responsibility to care for his/her health as well as that of others [9]. Throughout the course of the HIV epidemic, the collective responsibility for preventing HIV has shifted from the promotion of condom use by HIV-negative persons to recommending that HIV-positive individuals begin

taking ARV early in the course of their infection. Efforts to promote PrEP could again shift the responsibility for prevention back towards HIV-negative individuals [10], underscoring the dynamic ways in which individuals (and couples) interact with drugs and the drugs change their realities.

The dialectics of PrEP

When PrEP first emerged on the HIV prevention landscape, much of the popular discourse surrounding it was framed in a binary and oppositional fashion, that is, either PrEP holds the promise to ending the HIV pandemic or PrEP is an insidious strategy that will exacerbate HIV epidemics and attendant social ills [11]. Many social scientists argue instead that PrEP as a technology is not inherently ''good'' or ''bad'' – it has both positive and negative potentialities simultaneously and produces something new entirely as a result of the dynamic tension between them [6]. This dialectic can be seen in a number of areas explored below.

Efficacy

Evidence from key clinical trials has shown that, if taken daily as prescribed, oral Truvada for PrEP is a highly efficacious HIV prevention strategy. It may reduce HIV acquisition by more than 90%, placing it right next to male latex condoms and access to sterile syringes as the most efficacious HIV prevention methods available today. But, data from the same clinical trials and others [12] indicate that most participants did not take oral PrEP as prescribed. Although adherence observed in clinical trials is likely to vary considerably from levels in the ''real world,'' trial results suggest that implementation programmes may need to greatly increase adherence levels in order to maximize the likelihood that PrEP will have a population-level impact.

Furthermore, although treatment-resistant mutations have not been witnessed in clinical trials as of yet, there is some concern that low adherence levels create the potential in individuals with partial adherence to develop resistance to certain classes of ARV, should they become HIV infected (and to transmit those strains onward). However, especially in light of recent data suggesting that even intermittent use of PrEP can be highly effective at preventing infections [13], evidence to date does not support this argument.

Agency and control

PrEP also has the potential to confer agency and control on HIV-uninfected persons who heretofore have had to depend on willingness of partners to use condoms or ARV as their primary prevention strategies [14,15]. The possibility of using PrEP without the knowledge of the other partner is a very important development for anyone who needs an HIV prevention method that can be used surreptitiously, as has been argued for microbicides, particularly for women [16,17].

Relatedly, PrEP in its current form as a once-daily pill is not coitally dependent, so individuals can take it at any time during the day they wish and not have it interrupt or interfere with any particular sexual episode (i.e., before, during or after intercourse). This unobtrusiveness, in combination with the relief of knowing one is protected from HIV, confers some level of control on the PrEP user. It also imbues PrEP with the potential to enhance sexual pleasure and fulfilment [18]. This should continue to be the case when other methods of PrEP administration (such as injectables and vaginal rings) become available, as these are also not coitally dependent and, to a great extent, can be used without others necessarily knowing [19].

However, although PrEP has this potential to confer agency and control in the user, it is not that simple. The only currently available PrEP method – Truvada – is the same pill that is used to treat HIV-positive persons. In many settings where HIV-associated stigma is high, being seen with ''the little blue pill'' (Truvada) implies being HIV infected, regardless of how a person on PrEP attempts to explain its use for prevention [18] The associated stigma may be a big disincentive for HIV-uninfected persons to take up PrEP [18,20,21]. Moreover, broad cultural and institutional stigma associated with sexuality, substance use and HIV may militate against access to PrEP services and engagement in related care in many settings.

PrEP and sexuality

One of the most controversial aspects of PrEP is that of ''risk compensation'' [5,15]. The fear is that PrEP users will decrease condom use or substitute PrEP for it, thereby enhancing the potential for increased sexually transmitted infections (STI), if not HIV transmission. But, arguing against PrEP based on the fact that it does not protect against other STIs is problematic in at least two ways. First, PrEP taken correctly confers as much, if not more, protection from HIV than do male latex condoms. Thus, if HIV prevention is the primary goal of PrEP, aversion of new HIV infections ought to be the outcome of relevance, and PrEP should be acknowledged as highly successful in this regard. Second, many people most at risk of acquiring HIV are the very ones who simply are not using condoms and who are, therefore, at risk of acquiring both HIV and other STIs [22]. PrEP may be an important way for these individuals to at least prevent the more dangerous disease – HIV – and, therefore, it ought not to be rejected because of what else it does not avert. Moreover, evidence to date from long-term follow-up from PrEP clinical trials and from open-label studies indicates that ''risk compensation'' has not occurred among either gay and other men who have sex with men (MSM) or heterosexuals using PrEP [23–25]. Although there has been incidence of hepatitis C and other STIs among those who use PrEP, no evidence yet exists to suggest that PrEP users experience increased rates of STI as compared to their at-risk counterparts.

Among gay men, fear about abandoning condoms goes beyond public health concerns and touches core issues in sexual culture. On the one hand, PrEP is creating a new form of ''safe sex'' that does not rely on barrier prevention methods (such as latex condoms), allowing its users to experience barrier-free intimacy without fear of contracting HIV. On the other hand, the potential for PrEP to confer a new level of agency, control and pleasure in sexual relations, in combination with the fears of ''risk compensation,'' has fuelled a new sexual moralism, particularly within gay communities. Early public debates in the gay community were framed around a controversial online essay that labelled PrEP users as ''Truvada whores'' [26–28]. Intended as a stigmatizing label, activists reappropriated the term as a message of pride

and launched a PrEP campaign with T-shirts starkly emblazoned with the phrase [29]. But, the cultural association of PrEP with a kind of ''unbridled'' sex may have contributed to its slow uptake [7].

Scholars have noted that the so-called ''PrEP wars'' resemble debates over birth control for women [30]. Many of the issues raised in argument against PrEP are identical to those invoked against female contraception, namely, cost, safety, the potential impact on sexual behaviour and the potential for unforeseen health risks associated with long-term use. These issues are not new or specific to HIV, and concern about them is largely driven by a version of sexual morality – that sex is taboo, that it is self-destructive and that sexual pleasure is sinful and disgraceful [7,31].

Despite its sociocultural baggage, PrEP already has begun to reshape the sexual landscape in many communities. For example, online sexual hook-up websites for gay men now offer an expanding variety of options for characterizing one's HIV status, with at least five HIV status options apparently now in circulation: *HIV-negative; HIV-negative and on PrEP; HIV-positive and not on treatment; HIV-positive with an undetectable viral load* and *I don't know* [32]. These provide users of the website with information they use to guide sexual practice and to imagine and define their sexual communities. The sociocultural implications of this shift are significant, as there are signs that the HIV-positive/negative binary that has persisted since the advent of the HIV test in the early 1980s may be eroding [33–35]. This trend has implications for serodiscordant relationships, as PrEP offers a way to safeguard health while preserving the relationship and promoting intimacy [36]. For heterosexual couples, PrEP provides a relatively easy way (as compared to previously favoured techniques, such as sperm washing or intrauterine insemination) to facilitate pregnancy without risking HIV transmission [37]. In short, in the context of PrEP, the risk of seroconversion is no longer the significant obstacle it has been to serodiscordant intimacy and partnership.

The ''substitutive'' nature of PrEP

Beyond ''risk compensation,'' there are other concerns about the ways in which PrEP may become a substitute for extant HIV prevention and treatment strategies, with resultant ill effects. At a policy level, there is some concern that governments and other payers will shift resources from behavioural counselling, HIV testing, condom promotion, social support and harm reduction services to PrEP programmes, with negative consequences for certain populations [38]. Some argue that PrEP likely will not benefit those most in need – including people who use drugs and/or have mental health problems, or who experience instability in their housing situations – because of cost and adherence issues, and, as such, PrEP use might enhance existing HIV-associated disparities [39]. Still others have argued that implementing PrEP in low-resource environments would be unethical because it would threaten to shift resources away from treatment [40,41]. These critics raise important issues of equality and justice. Their arguments point to the current institutional (financial and policy) context for funding HIV prevention in general (which in many settings is dismal) that result in the counterpoising of ART use for primary prevention and ART use for treatment.

Taking all these considerations together, it is clearly the case, as Peter Aggleton has noted, that ''PrEP is an HIV prevention strategy that may be useful to some people in some contexts some of the time'' [42]. If and how it is used, and with what potential effect, will vary across individuals, social groups, populations and social, political and economic systems. It is important that social scientists investigate how all this occurs and plays out globally over time, and with what consequences for individuals and societies.

Conclusions

The promise of PrEP is not yet being fully realized, in part because not enough is being done to understand the social dynamics of the prevention strategy. From a clinical standpoint, adherence may appear to be the problem that stands between PrEP and its potential impact. But from a sociological perspective, there is a much richer set of issues that shape PrEP and its social and clinical significance. PrEP's efficacy and effectiveness – alone and in combination with other HIV prevention methods – are not simply a function of ''getting drugs into bodies.''

Beyond adherence, implementing PrEP will require understanding how individuals and communities comprehend it. Do they believe it is effective? Do they trust the agencies and individuals promoting it? Do they think that they have access to it and can afford it? And, perhaps the most significant question is whether potential PrEP users understand themselves to be at risk of acquiring HIV, and, if so, whether that risk is sufficient for them to proactively engage in HIV prevention.

Beyond simple use or non-use of PrEP, social research can help us understand what meanings people assign to it. Is PrEP another ''little blue pill'' that they associate with ''recreational'' sex? Is it a symbol of love or intimacy with their partner? Is it a marker of the rich or elite who have the ''privilege'' to use it? PrEP's symbolic life will become just as important as its clinical efficacy in shaping how communities engage with it. Social science methods can help evaluate what impact PrEP has on sexual (and drug using) practices and cultures – beyond merely ''risk compensation.'' For example, ''neg + PrEP'' is fast becoming a new identity for gay men using online hook-up applications. How does this self-proclaimed status shape one's interactions with other men? Does taking PrEP encourage some users to explore sexual practices (such as receptive anal intercourse) that they once avoided for fear of infection? These possibilities are not just merely a matter of ''risk''; they shape sexual cultures and thus have important sociological implications.

Perhaps most importantly, social science can help us reveal what PrEP tells us about the state of our public health infrastructure and the organized AIDS response community. Nearly three years after PrEP's FDA approval, the drug remains relatively underutilized. What does the slow-paced embrace of PrEP by health departments, medical providers, HIV/AIDS advocates and AIDS service organizations tell us about institutions of public health and medicine and AIDS advocacy? These socio-structural questions provide important

pathways for understanding PrEP as an object circulating in social space more generally.

As interest intensifies in implementing and scaling-up PrEP in both clinical and community settings, as it now appears to be doing, the potential for social science research on PrEP – and the need to incorporate its findings – has never been greater.

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The authors declare they have no conflicts of interest.

Authors' contributions
Judith Auerbach led the writing process and wrote the first draft of the paper. Trevor Hoppe contributed significantly to the concepts and ideas in the text and to its further iterations. Both authors have read and approved the final version.

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Combination HIV Prevention Interventions: The Potential of Integrated Behavioral and Biomedical Approaches

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Abstract Combination HIV prevention interventions that integrate efficacious behavioral and biomedical strategies offer the potential to reduce new HIV infections. We overview the efficacy data for three biomedical HIV prevention approaches, namely microbicides, pre-exposure prophylaxis (PrEP), and HIV vaccination; review factors associated with differential acceptability and uptake of these methods; and suggest strategies to optimize the effectiveness and dissemination of combination HIV prevention approaches. A narrative review was conducted highlighting key efficacy data for microbicides, PrEP, and an HIV vaccination and summarizing acceptability data for each of the three biomedical HIV prevention approaches. Recommendations for the integration and dissemination of combined behavioral and biomedical HIV prevention approaches are provided. To date, microbicides and an HIV vaccination have demonstrated limited efficacy for the prevention of HIV. However, PrEP has demonstrated efficacy in reducing HIV incident infections. A diverse array of factors influences both hypothetical willingness and actual usage of each biomedical prevention method. Strategies to effectively integrate and evaluate combination HIV prevention interventions are urgently needed.

Keywords Combination HIV prevention · Microbicide · PrEP · HIV vaccine · Biomedical HIV prevention · Behavioral HIV prevention

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Introduction

Rates of new HIV infections in the USA remain relatively stable with approximately 50,000 incident cases diagnosed annually [1]. Globally, there were 2.3 million incident HIV cases in 2012 [2]. HIV prevalence continues to rise with an estimated 35.3 million people now living with HIV worldwide [2]. To curb the tide of new HIV infections, the HIV prevention "toolkit" has increased its arsenal of available prevention methods through the use of both behavioral and biomedical approaches. Combination HIV prevention interventions that integrate efficacious behavioral and biomedical strategies offer the potential to reduce new HIV infections and, as a consequence HIV prevalence worldwide [3•, 4, 5], particularly when implemented in conjunction with broader structural interventions [6•]. However, to enhance widespread dissemination and uptake of these methods, strategies to facilitate integration and acceptability of combined behavioral and biomedical HIV prevention strategies are essential.

Behavioral HIV prevention strategies focus on reducing high-risk practices including non-condom-protected sexual encounters and sharing of contaminated needles, among others. A multitude of efficacious behavioral HIV prevention approaches have been developed. For example, the US Centers for Disease Control and Prevention (CDC) have reviewed, evaluated, and identified efficacious behavioral HIV prevention interventions [7, 8]. These approaches employ a variety of intervention modalities (e.g., one-on-one, small group) and typically target at-risk populations (e.g., men who have sex with men, African American adolescents) [7]. When behavioral interventions are used alone, they may reduce high-risk sexual practices, use of contaminated needs, and other behaviors that increase HIV risk.

In addition to behavioral strategies, the HIV prevention "toolkit" has expanded to include biomedical prevention approaches. Biomedical HIV prevention approaches encompass

a diverse array of strategies at different stages of development, of varying efficacy, and at various states of approval including the following: (a) microbicides applied either to the vagina or to the rectum; (b) pre-exposure prophylaxis (PrEP) antiretroviral (ARV) medication use and use of ARV medications as a preventive strategy among high-risk seronegative persons; (c) post-exposure prophylaxis (PEP) and use of ARV medications following HIV exposure; (d) HIV vaccination; (e) medical male circumcision; (f) HIV testing, linkage, and retention in HIV care ("test-and-treat"); and (g) enhanced ARV adherence among HIV seropositive individuals (treatment as prevention). While not all of these biomedical HIV prevention strategies have demonstrated efficacy or are approved for widespread use, they offer the possibility to further curtail the HIV epidemic. Combination HIV prevention approaches that integrate both behavioral and biomedical strategies may offer an even greater potential to reduce HIV transmission among high-risk populations.

To illustrate the potential of combination prevention approaches, we first briefly overview the efficacy data for three biomedical HIV prevention approaches: microbicides, PrEP, and HIV vaccination. We then review factors associated with differential acceptability and uptake of each of these three methods. Lastly, we provide possible strategies to optimize the effectiveness and dissemination of combination HIV prevention approaches that integrate both behavioral and biomedical intervention strategies.

Overview of Literature Search Method

Database searches of PsycINFO, PsycARTICLES, Medline, and PubMed were conducted to identify published articles in peer-reviewed journals that either (a) provided efficacy data or evaluated the acceptability of microbicides, PrEP, or an HIV vaccine or (b) discussed strategies to optimize the implementation and dissemination of combination HIV prevention interventions. Combinations of the following search terms were used to identify relevant articles: *combination HIV prevention, microbicide, PrEP, pre-exposure prophylaxis, HIV vaccine, HIV vaccination, HIV, AIDS*. A search of references cited in relevant studies was conducted to identify additional articles. English language articles published in peer-reviewed journals were screened for inclusion in the review.

Microbicides

Unlike either male or female condoms that may require negotiation between sexual partners for their use, vaginal microbicides allow for greater control by women as an HIV prevention strategy [9]. Similarly, efficacious microbicides applied rectally may increase both men and women's protection from HIV via a method that allows for greater individual control [10]. Currently, there are a number of microbicides in various stages of development and testing. However, in the USA, there are no FDA-approved microbicides available.

Overview of Microbicide Efficacy A 2008 study reviewed 73 pre-clinical and 45 clinical trials of microbicides at various phases of development [10]. Generally, as of this 2008 review, completed phase I and phase II microbicide trials demonstrated microbicide safety and the products were well tolerated [10]. However, microbicide phase III trials had not demonstrated HIV prevention efficacy [10].

A subsequent 2012 meta-analysis examined the effectiveness of vaginal microbicides relative to placebo or no intervention conditions across 13 randomized controlled trials [11]. Across trials, tenofovir as a microbicide demonstrated initial efficacy in reducing HIV acquisition, but a subsequent large-scale trial was discontinued due to a decreased protective effect against HIV [11]. Further, there was no efficacy for reduced HIV acquisition from nonoxynol-9, cellulose sulfate, SAVVY, Carraguard, PRO 2000, or BufferGel [11]. Additionally, nonoxynol-9 demonstrated the potential to *increase* the probability of HIV acquisition via an increased risk of genital lesions [11].

Should a safe and efficacious microbicide be developed, its acceptability by potential users will be important for its successful marketing, uptake, and widespread dissemination [12]. Further, factors associated with differential acceptability can be used to tailor intervention messaging related to microbicide use and to address potential adherence challenges [12]. Dimensions of microbicide acceptability have been assessed for both hypothetical future microbicide products and also for individual microbicides during pre-clinical and clinical trials.

Acceptability of Future Potential Microbicides Two studies examined factors associated with the acceptability of future potential microbicides among men who have sex with men (MSM) [13] and women in the USA [14]. A qualitative study conducted with MSM examined the acceptability of microbicides, HIV vaccine, PrEP, and PEP [13]. Participants endorsed enthusiasm for a future potential microbicide if the product acted like a lubricant and was also efficacious in preventing HIV [13]. However, participants noted some concerns about product characteristics (e.g., consistency, taste, form of use) [13]. In a second study, US women provided feedback on the acceptability of future vaginal microbicides using a mixed methods study design [14]. Women noted concerns about the physical characteristics of potential microbicide gels (e.g., messiness, chemical makeup) and fears about possible discomfort and embarrassment using the product [14]. However, women highlighted the importance of having a self-controlled HIV prevention method, particularly if the microbicide was effective, discreet, long-lasting, comfortable, and affordable [14]. Quantitative analyses also examined the role of personal, relational, and attitudinal dimensions of

microbicide acceptability; microbicide acceptability was negatively associated with past experiences of physical or sexual violence or experiencing decreased power in their sexual relationships and positively associated with past vaginal contraceptive product use [14].

Acceptability of Microbicides During Pre-clinical or Clinical Trials During pre-clinical or clinical trials, acceptability of the candidate microbicides of nonoxynol-9 [15, 16], cellulose sulfate [17], BufferGel [9, 18, 19], PRO 2000 Gel [18–20], vaginal ring microbicide delivery modality [21], and tenofovir [22–24] was examined. In what follows, we briefly review acceptability data for each of the candidate microbicides.

In an early trial of nonoxynol-9 among sex workers in South Africa, there were no overall differences between the nonoxynol-9 and placebo groups for side effects; however, a minority of women noted adverse side effects including vaginal burning and pain during sex [16]. Participants noted no difficulties using the product and reported that the product was not detectable to their clients [16]. Subsequent to trial participation, a subset participated in qualitative focus groups regarding nonoxynol-9 acceptability [15]. Of note, this microbicide was found to be ineffective for sexually transmitted infection (STI)/HIV prevention [15]. Despite being told of the microbicide's lack of efficacy, qualitative findings indicated that women believed the gel afforded STI/HIV prevention benefits and alleviated STI symptoms and reproductive tract pain [15]. For example, women stated that the gel reduced vaginal wetness and discharge, rashes, and uterine, bladder, abdominal, and menstrual pain, despite the lack of trial evidence to support these beliefs [15].

A phase I trial of a cellulose sulfate (CS) gel assessed its acceptability relative to a placebo gel among HIV-infected women and their male partners [17]. Women reported less likelihood to use the CS gel relative to the placebo gel; 64 % of the sexually active women reported leaking of either the CS or placebo gels during sex [17]. Male partners indicated high support for the product if it became available and was efficacious, with women indicating a preference for a product that would not be detectable by their male partners [17].

In a trial of the vaginal microbicide BufferGel, HIV-seronegative women at low HIV risk in Malawi, Zimbabwe, India, and Thailand reported on the overall acceptability of the microbicide by themselves and their male partners [9]. Overall, 73 % of respondents indicated that if the microbicide was approved, they would use the product [9]. With regard to preferred features, 93 % liked the product's color, 92 % liked the ease of insertion, 81 % liked the product's smell, and 66 % appreciated the reusable applicator [9]. However, 49 % of participants indicated the microbicide "felt too wet or drippy," with 31 % indicating the microbicide soiled their clothing [9]. Qualitative findings suggested that women with heightened concerns of HIV risk and/or partner fidelity found BufferGel to be more acceptable; however, male partners expressed concerns that microbicide use might encourage greater promiscuity [9]. Given product characteristics, both men and women did not believe it would be feasible to use the microbicide without partners' awareness of use [9].

A mixed methods study examined the acceptability of PRO 2000 Gel, a topical microbicide in a phase I trial among both sexually active HIV-uninfected and sexually abstinent HIV-infected women in the USA and South Africa [20]. Global acceptability ratings indicated participants would purchase the microbicide and recommend the product to other women if it were to be made available [20]. With regard to desired features, women indicated that the ability to effectively prevent STI/HIV was critical, with a majority also endorsing a desire to have the gel also prevent pregnancy [20]. Physical characteristics (i.e., color, smell) of the product were also favorably reviewed, with some indicating a preference for a colorless product [20]. Qualitative findings highlighted some dissatisfaction associated with the gel leaking, both throughout the day and during sex [20].

A third study examined the acceptability of both BufferGel and PRO 2000 Gel relative to a placebo K-Y jelly condition for sexually abstinent HIV-uninfected and HIV-infected men used by direct application to the penis [19]. Men rated both BufferGel and PRO 2000 gel to be acceptable, with few noting that they would object to a potential partner using the product [19]. Further, men using both gels indicated high likelihood to use the product in the future [19]. A second trial of BufferGel and PRO 2000 Gel microbicides was conducted with women and their male partners from Malawi, South Africa, the USA, Zambia, and Zimbabwe [18]. Overall acceptability was high among both women and their male partners [18]. The ability for women to use a microbicide without her male partner's knowledge was perceived to be an important characteristic by women [18]. However, some men expressed less favorable attitudes regarding microbicides being a woman-initiated product [18].

An additional study examined the acceptability of a vaginal ring to continuously deliver microbicides via a mixed methods design among HIV-negative, sexually active women and their male partners [21]. The majority of women (69 %) reported high levels of interest in trying the vaginal ring and liked the possibility that the ring could prevent HIV and not interfere with sexual activity [21]. However, some (20 %) noted concerns that the ring would get lost inside the body, and 10 % endorsed concerns that the ring would come out [21].

Lastly, three papers reported on the acceptability of a tenofovir gel microbicide [22–24]. In a study comparing tenofovir gel versus a placebo microbicide, 97.4 % of the female South African participants found tenofovir gel to be acceptable, with 97.9 % indicating that they would use the gel if it prevented HIV [22]. Similarly, in a phase I trial of tenofovir gel with HIV-negative US women, 94 % reported

they would either "definitely" or "probably" use the gel if found to be effective [23]. Additionally, 81 % of participants' male partners indicated they would use the gel if found to be efficacious [23]. The majority of women (79 %) and men (76 %) also endorsed liking the gel [23]. Lastly, in a phase I trial of tenofovir gel, a mixed methods study examined the microbicide's acceptability among both HIV-uninfected and HIV-infected women [24]. Overall, quantitative acceptability ratings were high, with 94 % of participants indicating they would either definitely or probably use the gel [24]. During qualitative interviews, the physical characteristics of the product were found to be acceptable (e.g., color, odor), but participants noted dissatisfaction with gel leakage [24]. Most participants (90 %) reported that the gel increased her sexual pleasure, with 86 % noting that the gel increased her male partner's sexual pleasure; a majority of participants reported a desire to use the gel in combination with condoms [24]. Additionally, most participants preferred a gel that would not be noticeable to their male sexual partners [24].

Microbicide Acceptability: Measurement and Study Design Issues As noted in our review and reviews conducted by Mantell and colleagues [12] and Morrow and Ruiz [25], the bulk of studies investigating microbicide acceptability have focused on product formulation features or physical characteristics (e.g., texture, viscosity). Across studies, there is a lack of consensus regarding the definition and operationalization of microbicide acceptability [12]. Acceptability has often been conceptualized as either hypothetical intention to use a future product or actual product usage [18, 25]. However, studies investigating broader, theoretically driven constructs of relevance to microbicide acceptability (e.g., social and cultural norms relevant to microbicide use, partner acceptability) have been less common throughout all phases of microbicide development and testing [12, 25]. Further, as suggested by Morrow and Ruiz, there is a need for well-designed mixed methods' studies of microbicide acceptability [25]. Such studies should also account for study conditions when assessing the acceptability of microbicides; for example, in trials where condoms in conjunction with microbicide use is the recommended practice, acceptability should be assessed in light of these relevant study design features [25].

PrEP

Pre-exposure prophylaxis (PrEP) is the use of ARV medications by HIV-uninfected individuals before HIV exposure [26]. The US Food and Drug Administration approved Truvada® (emtrictiabine/tenofovir disoproxyl fumarate) in 2012 as an HIV prevention method to be used in combination with safer sexual practices [27]. PrEP treatments using tenofovir disoproxyl fumarate and emtrictiabine/tenofovir disoproxyl fumarate have demonstrated efficacy to prevent HIV among high-risk groups including serodiscordant heterosexual couples [28], heterosexual men and women [29], and MSM and transgender women [30]. However, in the FEM-PrEP trial of oral PrEP (emtrictiabine/tenofovir disoproxyl fumarate) with HIV-uninfected at-risk women in Kenya, South Africa, and Tanzania, the trial was halted before completion due to equivalent HIV incidence between study arms [31]. Furthermore, in the FEM-PrEP trial, adherence was poor with less than 40 % of the sample demonstrating recent PrEP use [31]. Despite the promise of PrEP, poor adherence underscores the need to better understand factors affecting the acceptability, uptake, and adherence to PrEP. Thus, in the following sections, we highlight studies exploring factors associated with oral PrEP acceptability.

PrEP Acceptability: Review of Measurement Issues A recent review examined the way in which acceptability was measured in both PrEP and treatment-as-prevention studies [26]. Within PrEP trials, acceptability was typically measured via individual adherence rates [26]. Additionally, four studies operationalized acceptability as willingness to take PrEP [26]. Eight studies focused on a multi-dimensional assessment of PrEP acceptability including factors such as access, perceptions of HIV risk, stigma, and willingness to use PrEP, among others [26]. Qualitative acceptability studies assessed an even broader array of constructs including perceived HIV transmission risk, safer sex beliefs, cost concerns, access concerns, potential side effects, desired PrEP information, and the role of regular HIV testing [26]. This review highlights the diversity by which PrEP acceptability has been measured. In what follows, we briefly review studies examining PrEP acceptability for both future potential products and also during randomized controlled PrEP trials.

Acceptability of Future Potential PrEP Use The acceptability of oral PrEP for future potential use has been assessed predominantly among MSM [13, 32–36] or gay or bisexual men in serodiscordant couples [37, 38]. Additional studies have explored the acceptability of potential future PrEP use among other at-risk populations [39–41] or among African American adults [42] and adolescents [43]. Two studies assessed PrEP acceptability among HIV healthcare providers [44, 45].

Among MSM in Seattle, PrEP acceptability was assessed among men attending either a Gay Pride event or an STI clinic [32]. Only 2 % endorsed current PrEP use, with 22 % stating that they knew of other MSM taking PrEP [32]. Forty four reported that they would take PrEP daily if it would prevent HIV, 31 % stated they would not take PrEP, and 20 % were undecided [32]. Correlates of willingness to take PrEP included an income below $15,000, younger than 40 years of age, and being recruited via the STI clinic [32]. A second study of US MSM examined PrEP use intentions [33]. Almost three quarters (74 %) of participants endorsed PrEP use intentions;

factors associated with PrEP use intentions included less education, moderate income, no perceived side effects, and PrEP being made available for free [33].

A sample of HIV-uninfected US MSM assessed their likelihood for using PrEP and potential risk compensation behaviors [34]. Overall, 70 % of participants reported willingness to use PrEP if it was at least 80 % effective [34]. Correlates of increased likelihood of use included being Black, increased engagement in HIV risk behaviors, and substance-related sexual risk behavior [34]. Among those indicating a desire to use PrEP, 35 % reported that they would decrease their condom use [34].

A qualitative study of MSM who engage in unprotected anal sex was conducted to examine knowledge and acceptability of PrEP and other biomedical HIV prevention approaches [13]. Overall, participants had little PrEP knowledge and acceptability of PrEP was mixed [13]. Participants identified potential PrEP benefits as providing HIV protection in serodiscordant relationships; however, participants identified a number of possible negative attitudes including concerns about side effects and efficacy [13].

Among a sample of MSM and male sex workers, a qualitative study explored the acceptability of PrEP in light of FDA approval [35]. While participants endorsed having little prior knowledge of PrEP, a majority endorsed willingness to use PrEP, citing potential barriers to use as cost and access to prescribing clinicians [35]. FDA approval was perceived as an important endorsement for PrEP's use and enhanced PrEP's acceptability [35]. However, other participants noted that other sources of information (e.g., physician recommendation, medical publications) were more critical than FDA approval to enhance PrEP acceptability [35].

Another study with MSM in France examined factors associated with willingness to participate in a future trial of PrEP usage "on-demand" (i.e., taking PrEP a few hours before potential HIV sexual exposure and a second dose a few hours later) [36]. Overall, 40 % of participants were interested in participating in a potential future trial [36]. Factors associated with willingness to participate included lower educational level, self-reporting a greater number of casual sexual partners, endorsing greater frequency of unprotected sexual encounters, and providing PrEP in a convenient geographic location [36].

Two studies examined PrEP acceptability among gay and bisexual men in serodiscordant relationships [37, 38]. Motivating factors for PrEP usage included a desire to prevent HIV, lower concerns and fears related to HIV transmission, the ability to engage in unprotected sex, and belief in PrEP's effectiveness [37]. Barriers to PrEP use included cost, short- and long-term side effects, potential adverse effects for incomplete adherence, and limited accessibility [37]. In a second study, participants noted high overall acceptability and willingness to use PrEP [38]. Factors associated with PrEP acceptability included the ability to engage in unprotected sex with an HIV-infected partner, protection from HIV, and decreased anxiety when engaging in sex with an HIV-infected partner [38]. Additionally, 64 % indicated that they would increase their HIV risk behaviors and 60 % reported they would decrease their condom use if using PrEP [38].

Another study examined willingness to use PrEP and PrEP acceptability among individuals at elevated HIV risk in seven countries (Peru, Ukraine, India, Kenya, Botswana, Uganda, South Africa) [39]. Overall willingness to use PrEP was high with 61 % indicating they "definitely" would use PrEP and 31 % indicating they "probably" would use PrEP; a slight majority (52 %) indicated they would disclose PrEP use to a partner [39]. Participants reported willingness to use PrEP even if there were associated side effects and costs and would require combined condom use or being regularly tested for HIV [39]. Correlates of increased likelihood to use PrEP included past medication adherence, being female, being younger, having fewer children, condom use in the past month, past HIV testing experience, and no current injection drug use [39].

In a sample of MSM, female sex workers, and male-to-female transgendered individuals in Peru, focus groups examined factors that would influence PrEP acceptability [40]. While there was general support for PrEP use, potential barriers to PrEP use included costs, PrEP efficacy, possible side effects, sexual risk disinhibition, potential experiences of stigma and discrimination, and mistrust of healthcare professionals [40].

A study of young truck-driving males in India assessed the acceptability of three biomedical HIV prevention approaches: HIV testing, circumcision, and PrEP [41]. PrEP was found to be acceptable to 85.9 % of the sample [41]. In multivariate analyses, correlates of PrEP acceptability included having a social network supportive of PrEP and having a history of prior sex with female sex workers [41].

One study examined the association between HIV risk behavior engagement, perceived HIV risk, and PrEP interest among a sample of African Americans attending an STI clinic [42]. Taking a single dose before sex was the most acceptable form of PrEP (77 %), followed by a weekly PrEP dosage (76 %) and taking a single dose 1 day before sex (75 %); a daily PrEP dosage was least acceptable (63 %) [42]. Further, participants indicated decreased PrEP interest due to current safer sex practices, concerns about side effects, and likelihood for low adherence [42]. Twenty percent also indicated they would use condoms less frequently if taking PrEP, and 7 % reported they would not use condoms at all if taking PrEP [42].

PrEP attitudes were examined among a sample of African American, urban dwelling adolescents [43]. Overall acceptability of PrEP was high, particularly if it was made available at no or little cost and was highly effective [43]. Participants also found the need for routine HIV testing on a regular basis to be an acceptable practice [43]. Factors that would enhance

uptake and use of PrEP included convenient access through health department clinics, pharmacies, or other venues that were easily accessible via public transportation [43]. Barriers to PrEP usage included potential side effects, high medication cost, partial effectiveness, low perceived HIV risk, potential negative peer reactions, burden associated with a daily medication, beliefs that PrEP would be for MSM only, and concerns about potential increases in HIV risk behaviors as a form of risk compensation [43].

Two studies examined PrEP acceptability among HIV healthcare providers [44, 45]. Only 19 % of those surveyed had prescribed PrEP, with the majority prescribing PrEP to MSM and individuals in serodiscordant relationships [44]. Providers noted concerns associated with ARV resistance and poor adherence along with the potential for increased engagement in HIV risk behaviors [44]. In a second mixed methods study of Italian HIV healthcare specialists, providers noted a number of concerns including duration of PrEP use, potential side effects and toxicity, ARV resistance, difficulties monitoring adherence, and the possibility of increased prevalence of other STIs [45]. In addition, providers noted concerns that funding for PrEP could adversely affect the availability of funds for HIV-infected individuals using ARVs [45]. Providers disagreed about the optimal population for PrEP, but suggested that HIV-uninfected individuals in serodiscordant couples would be a key target population rather than individuals reporting high rates of sexual risk behavior because of fears of increased risk compensation [45]. Quantitative findings indicated that 30 % would not prescribe PrEP; among those expressing a willingness to consider prescribing PrEP, 81 % would provide PrEP to at-risk individuals under some circumstances and 93 % would prescribe to serodiscordant partners [45]. Providers also endorsed greater acceptability for HIV prevention methods other than PrEP including greater HIV testing access, HIV care, and behavioral HIV prevention interventions [45].

PrEP Acceptability in Randomized Clinical Trials As part of actual PrEP trials, several studies report on PrEP acceptability. In the PrEP trial of tenofovir disoproxil fumarate among women in Ghana, adherence and PrEP acceptability were examined using a mixed methods design [46]. Across the 12-month trial, the average self-reported adherence level was approximately 82 % or greater [46]. In qualitative interviews with a subset of participants, acceptability was adversely affected by the experience of physical symptoms and challenges associated with consistent PrEP adherence [46]. For some of the women, a desire for privacy when taking PrEP was also identified as being important [46].

PrEP acceptability was qualitatively assessed in a phase I trial among MSM and female sex workers in Kenya [47, 48]. Overall, PrEP acceptability was high, particularly if shown to be highly efficacious, safe, and available at little or no cost [47, 48]. Further, participants identified the self-controlled nature of PrEP as appealing, particularly for individuals whose partners did not want to use condoms [47]. Additionally, some noted that PrEP may be more acceptable among certain cultures or religious faiths relative to other HIV prevention methods [47]. In contrast, participants reported dissatisfaction with physical characteristics of the PrEP pill including its large size, irregular shape, and blue color which at times discolored one's mouth [47]. Participants also noted experiencing a variety of side effects, especially during the early phases of use, that dampened their overall enthusiasm for PrEP and in some instances decreased their adherence [47].

Among serodiscordant heterosexual couples enrolled in a PrEP clinical trial, the study investigated couples' preferences for either early ARV initiation by the HIV-infected partner relative to PrEP usage by the HIV-uninfected partner [49]. A majority of uninfected men and women indicated willingness to use PrEP [49]. Among the subset of participants unwilling to use PrEP, concerns included the need for 5 years of medication, potential side effects, desire to use medication only when sick, and drug fatigue [49].

A qualitative study during a PrEP phase III trial with African serodiscordant couples examined factors associated with PrEP adherence and acceptability [50]. Participants identified PrEP as a valuable resource providing a source of hope to prevent HIV, particularly given the heightened transmission risks posed by HIV-infected partners [50]. Participants cited their motivation to adhere to the PrEP regimen, despite often experiencing logistical challenges (e.g., traveling long distances for PrEP) [50]. Participants also cited social support provided by family, partners, and research study staff to be particularly important to enhance PrEP adherence [50].

Among a sample of Ugandan, HIV-uninfected individuals in serodiscordant relationships, 99 % of participants stated that they would use PrEP if shown to be safe, effective, and available at little or no cost [51]. There were no differences in acceptability levels between the study conditions of daily versus intermittent PrEP usage [51].

In a PrEP trial with sexually active, HIV-infected individuals in South Africa, Uganda, and the USA, the acceptability of oral PrEP tablets, vaginal PrEP gels, or both were examined [52]. Across sites, oral PrEP, if found to be effective, was the most acceptable mode of administration [52]. Differences by study site emerged in the acceptability of PrEP formulations with greater acceptability of multiple PrEP formulations in South Africa and Uganda and greatest oral PrEP acceptability among the US participants [52].

HIV Vaccination

Since the early years of the HIV epidemic, research devoted to the development of a safe and efficacious vaccination to

prevent HIV has been underway. Unfortunately, there has been limited evidence for an efficacious HIV vaccine [53]. Further, the development of a safe and efficacious HIV vaccine does not guarantee that it will be acceptable by individuals at elevated HIV risk and also by the broader population. As observed with other available vaccines, availability of an efficacious vaccine does not guarantee high uptake. Thus, an examination of factors associated with HIV vaccine acceptability is essential for future intervention efforts should a safe and efficacious vaccine becomes available. Assessment of HIV vaccine acceptability has typically focused on factors related to either participation in a future potential HIV vaccine trial or actual trial participation as illustrated by the following review.

Summary of Previous Reviews of HIV Vaccination Acceptability A review conducted by Dhalla and colleagues examined motivating factors associated with participation in a HIV vaccination preparedness study in both developed and underdeveloped nations [54]. Social benefits of participation, particularly altruism, a desire to help one's community or others, or assist with efforts to find an efficacious vaccine were cited as key factors motivating study participation [54]. In addition, personal benefits including potential HIV protection, a desire to reduce personal HIV risk behaviors, and a desire for the study's monetary incentives were also cited as factors associated with participation in HIV vaccine preparedness studies [54].

A second review examined core themes of willingness to participate in an HIV vaccine trial [55]. The authors identified six key themes across the reviewed studies: (a) safety concerns (e.g., vaccine-induced seropositivity, insufficient efficacy); (b) fear or mistrust of the government, researchers, and/or pharmaceutical companies; (c) concerns or misunderstandings about the study's methodology (e.g., not fully understanding randomization and/or placebo conditions); (d) concerns about discrimination or stigmatization; (e) practical obstacles (e.g., insurance concerns, busy daily schedules); and (f) other concerns (e.g., dislike of needles) [55]. Additionally, in the quantitative studies reviewed, key barriers included concerns about vaccine safety and discrimination and stigmatization [55].

Lastly, a meta-analysis of HIV vaccine acceptability was conducted across 20 studies [56]. On an acceptability scale from 0 to 100 %, the average acceptability rating was 65.6 % [56]. The meta-analysis also compared the acceptability of potential vaccines varying possible vaccine efficacy, high versus moderate efficacy; high vaccine efficacy and belonging to a group at low HIV risk were associated with medium acceptability effect sizes [56]. Small effect sizes for HIV vaccine acceptability were identified for cost, perceived HIV susceptibility, side effects and safety concerns, vaccine fears, perceived vaccine benefits, duration of vaccine protection, and individuals' ethnicity, with African American ethnicity associated with lower vaccine acceptability [56].

HIV Vaccine Acceptability Assessed via Willingness to Participate in Future Potential HIV Vaccine Trials HIV vaccine acceptability has been assessed via willingness to participate in future potential HIV vaccine trials among samples of South African [57, 58] and US [59] adolescents, US undergraduate students [60–62], Canadian Aboriginal individuals [63], Thai immigrants to the USA [64], MSM in China [65], and MSM and transgendered individuals in Thailand [66]. Additional studies have examined HIV vaccine acceptability among other at-risk, ethnically diverse samples of US men and women [67–71].

A study of South African adolescents assessed motivating factors and barriers to participation in a potential HIV vaccine trial [57]. Motivating factors included a desire to protect oneself and their loved ones, community, and the broader global community from HIV [57]. Barriers included concerns about potential vaccine side effects and worries that vaccination might increase HIV acquisition [57]. Overall, 42 % of adolescents reported they would accept an HIV vaccine if one was available and efficacious, while 8 % said they would not accept a vaccine, and 50 % were unsure [57].

A second qualitative study of South African adolescents assessed barriers and motivators to future HIV vaccine uptake [58]. Barriers included cost, possible adverse side effects, lack of perceived HIV vulnerability, HIV testing fears and associated stigma, negative community HIV attitudes, mistrust of government and science, potential HIV vaccination stigma, and a preference for traditional African healing practices rather than vaccination [58]. In contrast, a core motivating factor was a desire for HIV protection, particularly when transmission risk was outside an individual's personal control (e.g., partner infidelity) [58]. Motivating factors noted by women included a desire to raise a family, while young men wanted to engage in unprotected sexual encounters with multiple partners [58]. Additionally, youth identified that support from the broader community, media, and government would enhance motivation and uptake of a future HIV vaccine [58].

A third study examined the acceptability of a future HIV vaccine among a sample of US adolescents [59]. Adolescents were asked to compare two hypothetical vaccines, one with 50 % efficacy and another with 90 % efficacy [59]. The lower efficacy vaccine was deemed to be unacceptable; however, adolescents reported that the higher efficacy vaccine could result in increased HIV risk behavior engagement [59].

Among a sample of US undergraduate students, the association between health belief model constructs and HIV vaccine acceptability was examined [61]. Approximately 65 % endorsed high levels of overall HIV vaccine acceptability [61]. Correlates of HIV vaccine acceptability included younger age, greater perceived HIV vulnerability, greater perceived HIV vaccination benefits, fewer perceived barriers to HIV vaccination, lower HIV vaccine fears, lower perceived HIV vaccine stigma, and non-membership in an HIV high-risk

group [61]. Additionally, this study examined vaccination characteristics of efficacy, cost, and the percentage of the population vaccinated as potential factors that would affect acceptability [60]. Overall, a vaccine with 80 % efficacy, that was free, and had been received by 90 % of the population was deemed to be most acceptable, while more expensive, less efficacious vaccines that had been received by a small minority of the population were least acceptable [60].

A second study of US undergraduates examined factors associated with acceptability for a hypothetical HIV vaccine [62]. On a scale from 0 to 100 %, the mean acceptability for a hypothetical vaccine was 70.2 % [62]. In a multivariate analysis, younger age, lower cost, greater HIV-related concerns, and a higher number of lifetime sexual partners were associated with greater acceptability [62]. However, perceived HIV invulnerability was associated with decreased vaccine acceptability [62].

Another qualitative study examined motivating factors and barriers to HIV vaccination among a sample of Canadian Aboriginal individuals [63]. A motivating factor was the desire to reduce HIV incidence and morbidity among the community [63]. However, HIV vaccine stigma, sexual orientation stigma, cost, lack of HIV vaccine information, pessimism regarding successful HIV vaccine development, mistrust of pharmaceutical companies, government, and the healthcare establishment and concerns about HIV acquisition via the vaccine were core factors associated with decreased acceptability [63]. Additionally, a general mistrust of western medical practices versus traditional medical practices was identified as a potential barrier to HIV vaccine acceptability [63]. Moreover, a vaccine that did not have 100 % efficacy was perceived as less desirable and the overall efficacy level would affect acceptability [63].

A mixed methods study examined HIV vaccine acceptability among a sample of immigrant Thai residents in the USA for eight different possible vaccine profiles [64]. Participants identified that efficacy, physical side effects, and costs were the key factors that influenced vaccine acceptability [64]. Specifically, participants reported the need for a vaccine with high level of effectiveness (>70 %), general optimism about a potential vaccination, and trust in the government and research community as key motivating factors to enhance acceptability [64]. However, significant physical side effects, high vaccine cost, and general fears about biomedical products and vaccinations were prominent barriers to acceptability [64]. Participants also noted some potential negative consequences should a vaccine become available; for example, some participants noted concerns that individuals would increase their engagement in HIV risk behaviors [64].

Another mixed methods study with Chinese MSM investigated factors associated with willingness to participate in a potential future HIV vaccine trial [65]. The majority (71 %) reported willingness to participate in a future trial and identified several potential benefits including receipt of HIV-related information, study-related incentives, free HIV testing and counseling, and enhanced motivation to reduce HIV risk behaviors [65]. However, participants voiced potential concerns including fears of contracting HIV and worries about how others would perceive them [65]. Multivariate analyses indicated that enhanced family support, HIV protection, and decreased stigma concerns increased willingness to participate in a potential future HIV vaccine trial [65].

A mixed methods study examined HIV vaccine acceptability among a sample of Thai MSM and transgendered individuals [66]. Overall mean acceptability (rated from 0 to 100) was 73.8 [66]. Factors associated with lower acceptability included vaccine-induced HIV seropositivity, efficacy concerns, potential side effects, limited duration of vaccine protection, cost, and limited social and community support for the vaccine [66]. Participants indicated that they would engage in risk compensation behaviors (i.e., increasing HIV risk behaviors as a result of receiving the vaccine) if a highly efficacious vaccine were to become available [66].

Another study examined potential gender differences and vaccine characteristics that may affect HIV vaccine acceptability among an ethnically diverse, at-risk US sample [67]. Overall mean acceptability (rated from 0 to 100) was 61 for men and 56 for women, with no difference by gender [67]. Efficacy of the proposed vaccine, protection against multiple HIV strains, and experiencing few side effects had the greatest impact on acceptability [68]. Gender-stratified analyses examined potential barriers to HIV vaccine acceptability [68]. Women noted concerns about their ability to secure health insurance due to possible vaccine-induced seropositivity, feared male partner reactions, and previous discrimination by health care workers as core barriers [67]. For men, core barriers to HIV vaccine acceptability included concerns that a vaccine would adversely impact their immune system or affect HIV test results [67]. In contrast, motivating factors for both men and women included family support of the vaccine and feeling safe with their sexual partners [67]. Men also endorsed that having friends supportive of the vaccine would increase HIV vaccine acceptability [67].

A qualitative study with women at increased HIV risk in Los Angeles examined barriers and motivating factors for a future HIV vaccination [69]. For barriers, women expressed concerns that a vaccine could result in HIV acquisition, would adversely affect future reproductive health, and that the use of syringes or injections could be a potential trigger for former injection drug users [69]. In addition, women noted that gender roles (e.g., caretaking for children) and concerns about partner reactions could also serve as barriers to HIV vaccine acceptability [69]. Furthermore, vaccination cost, mistrust of the government and scientific community, HIV stigma, and the potential for experiencing discrimination based on vaccination were also barriers [69]. In contrast, a desire for HIV

protection and the ability to normalize HIV vaccination as part of routine medical care were seen as factors that would increase vaccine acceptability [69].

One innovative qualitative study examined the association between willingness to participate in an HIV vaccine trial and HIV vaccine acceptability among an ethnically diverse US urban sample [70]. Barriers that were common to both willingness to participate in an HIV vaccine trial and HIV vaccine acceptability included fear of vaccine-induced HIV acquisition, false-positive HIV tests, potential side effects, partial vaccine efficacy, general mistrust of the government and pharmaceutical companies, low perceived HIV risk, HIV stigma, and relationship concerns [70]. A common motivating factor was a desire for HIV protection [70]. Unique barriers for willingness to participate included uncertain vaccine properties and overall study demands; motivators included altruistic attitudes, free medical care/insurance, and monetary incentives [70]. For vaccine acceptability, barriers included short duration of vaccine effectiveness, cost, access, and inability to protect against multiple HIV strains; motivators for HIV vaccine acceptability included a desire to engage in unprotected sex, endorsement of the vaccine by reputable sources, and the potential to improve overall health [70].

A qualitative study examined willingness to participate in an HIV vaccine trial by MSM in San Francisco, injection drug users in Philadelphia, and African Americans in Durham, NC [71]. Approximately 46 % of participants indicated they would volunteer for such a trial, 41 % would not volunteer, and 13 % were undecided [71]. Participants identified a number of potential risks associated with participating in such a study including adverse side effects, concerns about vaccine safety, vaccine-induced seropositivity, and experiencing social stigma [71]. However, participants also identified a number of potential benefits from participation including potential health improvements, financial compensation, a desire to curb the HIV epidemic, and altruistic beliefs regarding the potential impact on the broader community or society at large [71].

In a sample of US ethnic minority individuals in Los Angeles, focus groups were conducted to assess barriers and motivating factors of willingness to participate in an HIV vaccine trial [72]. Barriers to participation included (a) concerns of vaccine-induced HIV infection, (b) physical side effects, (c) uncertain vaccine efficacy, (d) unknown vaccine characteristics, (e) mistrust, (f) low perceived HIV risk, (g) study demands, (h) stigma, and (i) vaccine-induced HIV seropositivity [72]. Factors motivating participation included (a) desire for protection against HIV, (b) potential free health insurance or medical care, (c) altruistic beliefs related to participation, and (d) provision of monetary incentives [72].

HIV Vaccine Acceptability via Willingness to Participate in a Phase 2 or Phase 3 Vaccine Trials Two studies examined HIV vaccine acceptability among participants in either a phase 2 [73] or phase 3 [74] vaccine trial. In one study, individuals previously enrolled in an HIV preparedness study were contacted regarding their willingness to participate in a phase 2 vaccine trial [73]. Thus, the study examined the relationship between hypothetical and actual willingness to enroll in an HIV vaccination trial [73]. Of those who stated their hypothetical willingness to participate in a subsequent HIV vaccination trial, only a small minority actually enrolled in the phase 2 trial [73]. Correlates of phase 2 study participation refusal included being African American, being less than 40 years of age, not attending college, and having fewer than five sexual partners in the past 6 months [73]. Additionally, concerns about vaccine-induced seropositivity were noted along with specific concerns voiced by African American individuals related to government mistrust and vaccine safety [73].

A second study assessed HIV vaccine acceptability via the reasons provided for enrolling in a phase 3 HIV vaccine trial [74]. Participants cited altruistic reasons as their primary motivator for joining the study [74]. Additionally, approximately half noted desires for HIV protection and reductions in their HIV risk behaviors as motivating factors for joining the study [74].

Optimization of Combination HIV Prevention Approaches

With the advent of efficacious biomedical prevention strategies such as PrEP, there is now a growing array of HIV prevention approaches available. However, as described in the preceding review, not all biomedical HIV prevention approaches have demonstrated efficacy and myriad factors may affect their acceptability, uptake, and dissemination among at-risk populations. Additionally, strategies to effectively integrate biomedical modalities with behavioral interventions are lacking. Thus, future HIV prevention approaches need to address both behavioral HIV risk behaviors and incorporate efficacious biomedical modalities.

A concern raised through the development of biomedical HIV prevention methods is the potential negative impact on behavioral HIV prevention methods including condom use [75]. Thus, risk compensation is a concern for the future of combination behavioral and biomedical HIV prevention approaches. For example, in a study with gay and bisexual men in serodiscordant couples, the majority indicated they would increase their sexual risk practices and decrease condom use if taking PrEP [38]. Further, in the case of microbicide trials, there has been debate regarding the inclusion of a trial arm where participants only use condoms [10, 75]. However, since consistent condom use among high HIV prevalence regions is often low (e.g., 29.1 % consistent condom use at baseline among South African women in the CAPRISA-004 trial [76]), combining biomedical and behavioral HIV prevention strategies may be the optimal strategy to reduce incident HIV

infections. Thus, future intervention efforts should consider strategies to aid individuals' selection from the menu of available HIV prevention options [12] with consideration of a variety of factors including the overall efficacy of the method to prevent HIV, acceptability of the method, relevant relationship characteristics, and sexual practices, among others. Biomedical HIV prevention approaches should ideally be part of a comprehensive HIV prevention approach including HIV testing, monitoring of biomedical product safety, behavioral strategies to reduce HIV risk behaviors and potential risk compensation behaviors, monitoring and treatment of side effects, and structural interventions to increase widespread dissemination and scale-up of interventions [77]. However, the implementation science for combination HIV prevention approaches is lacking [77].

Use of CDC identified efficacious behavioral HIV prevention interventions [7], particularly those designed for use with clinic populations may be optimal behavioral strategies that can be integrated with the provision of biomedical HIV prevention approaches [78]. Such behavioral approaches will likely be essential to reduce risk compensation practices that may increase HIV risk [78]. Further, as suggested by Curran and Crosby, the use of booster behavioral sessions or intervention approaches to bolster HIV preventive behaviors will be important to incorporate and evaluate in combination HIV prevention intervention approaches [78]. Further, application of strategies for the implementation, dissemination, and widespread uptake of efficacious combination prevention approaches will be critical to increase public health impact [77, 79]. As identified in the above-reviewed literature, there are a number of potential challenges associated with the widespread dissemination of biomedical HIV prevention approaches including cost, optimal HIV/STI testing frequency, monitoring strategies, and the potential for behavioral risk compensation [79]. Moreover, there is a need to optimize adherence to biomedical HIV prevention options as they become available. For example, inconsistent adherence to an ARV-based vaginal microbicide product could potentially result in ARV resistance on the part of the user and increased HIV transmission risk to one's partner [80, 81]. Furthermore, in the PrEP efficacy trials, adherence was correlated with PrEP efficacy [78, 82].

In addition, targeting populations at highest risk for HIV will be important for combination HIV prevention approaches. However, some biomedical HIV prevention approaches have encountered challenges enrolling populations experiencing increased HIV prevalence. For example, Mills and colleagues [83] identified potential barriers that could adversely impact enrolling women into HIV vaccination trials including concerns about subsequent pregnancy, negative consequences within one's relationship, and other forms of discrimination. Thus, there is a need for the use of optimal behavioral strategies in the design and implementation of biomedical trials to ensure individuals at particular risk are included in the evaluation of biomedical prevention approaches that can subsequently be integrated with efficacious behavioral prevention strategies.

Despite the promise of combination prevention approaches, a limited number of randomized controlled trials have been conducted. In one such trial, a small cohort of young MSM (N=68) were randomized to one of three conditions: (a) a behavioral HIV prevention alone condition (Many Men, Many Voices: 3 MV); (b) 3 MV + tenofovir/emtricitabine; or (c) 3 MV + placebo medication [84]. Across the 24 weeks of follow-up assessment, there was no difference between the three groups in the frequency of unprotected anal sex with all three groups decreasing their frequency of unprotected anal sex [84]. Additionally, plasma detectable tenofovir levels steadily declined across the course of the trial suggesting concomitant declining adherence over time [84]. To evaluate the efficacy of combination prevention approaches, additional randomized controlled trials are urgently needed. Additionally, for combination approaches with demonstrated efficacy, strategies to broadly implement and disseminate such interventions offer the potential to further reduce the prevalence of new HIV infections. Further, there is a need to consider factors that would affect widespread dissemination when a particular biomedical HIV prevention approach is included in a combination prevention program [70, 85]. As identified by others, there are a number of challenges that may adversely impact widespread dissemination of biomedical HIV prevention methods including access, cost, provider and patient comfort discussing these methods, and need for ongoing maintenance and monitoring [85].

There is also a paucity of research investigating medical providers' and other stakeholders' acceptability of biomedical HIV prevention approaches. Since providers will be instrumental in the provision and recommendation of these products when they are available for use [12, 86], a greater understanding of providers' knowledge and acceptability for biomedical HIV prevention methods is essential in future research efforts. Further, there is a need for providers to have clear recommendations for best practices in the management of biomedical HIV prevention technologies that also incorporate behavioral HIV prevention strategies [81]. Such guidelines should also consider routine screening for HIV and other STIs along with monitoring of relevant side effects (e.g., toxicity) [78]. Additionally, assessing the acceptability of combination prevention programs by key community stakeholders may influence their acceptability and uptake in the communities they serve.

Conclusions

There is a need to consider how acceptability data from biomedical HIV prevention approaches at various stages of

development may affect uptake and adherence to those methods as they become available. For example, Buchbinder and colleagues [73] identified younger, ethnic minority individuals and those reporting lower HIV risk behaviors as less likely to enroll in an HIV vaccine trial. Thus, behavioral intervention strategies to increase the use of specific biomedical HIV prevention approaches may need to be tailored to specific populations. The use of theory-informed behavioral interventions could be particularly effective to address health-related beliefs that may adversely affect the acceptability and uptake of these methods. Furthermore, hypothetical willingness to use a biomedical product or engage in a study trial may not translate to actual uptake and use of the product and/or willingness to participate in a trial for a given biomedical prevention approach.

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Compliance with Ethics Guidelines

Conflict of Interest Jennifer L. Brown, Jessica M. Sales, Ralph J. DiClemente declare that they have no conflict of interest.

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ORIGINAL RESEARCH ARTICLE

Patient Preferences for Injectable Treatments for Multiple Sclerosis in the United States: A Discrete-Choice Experiment

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Abstract

Background and Objective Patients' perceptions and experiences of medication efficacy, medication adverse events, dosing frequency, and dosing complexity have been found to influence adherence to injectable disease-modifying treatments (DMTs) in patients with multiple sclerosis (MS). The aim of this study was to quantify patient preferences for features of injectable DMTs for MS.

Methods Adult patients in the United States (US) with a self-reported diagnosis of MS completed an online discrete-choice experiment survey to assess preference for a number of features of a hypothetical injectable DMT. Patients chose hypothetical treatments in paired comparisons, where each treatment was described by features or attributes, including the number of years until disability progression, the number of relapses in the next 4 years, injection time, the frequency of injections, the occurrence of flu-like symptoms (FLS), and severity of injection-site reactions. Random-parameters logit regression parameters were used to calculate preference weights of attribute levels and the relative importance of changes in treatment features.

Results Of the 205 patients who completed the survey, 192 provided sufficient data for analysis. The results indicated a broad range of tradeoffs that patients would be willing to make. With regard to this, the relative importance of an improvement in the number of years until disability progression from 1 to 2 (i.e., vertical distance between preference weights for these attribute levels) was 0.9 [95 % confidence interval (CI) 0.5–1.2], the relative importance of this change was approximately equivalent to that of an improvement from 12 injections per month to two (mean 0.8, 95 % CI 0.4–1.2), or approximately equivalent to a decrease from four to one relapses in the next 4 years (mean 0.8, 95 % CI 0.5–1.2), or FLS 3 days after every injection to 3 days after some injections (mean 1.0, 95 % CI 0.6–1.4).

Conclusions These results suggest that an improvement in treatment efficacy may be as important as a reduction in injection frequency or a reduction in some adverse events for patients who self-administer injectable DMTs for MS. Understanding the preferences of patients who use injectable treatments will inform the development of such treatments, which may in turn improve patient medication adherence and well-being.

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Key Points for Decision Makers

This study, conducted with a sample of individuals in the US with self-reported multiple sclerosis (MS), employed a discrete-choice experiment to quantify patient preferences for disease-modifying treatment (DMT) features associated with medication adherence.

Patients reported that certain changes in injection frequency were as important as changes in DMT efficacy and adverse events; as such, injection frequency may be an important factor for patients with MS in determining their adherence to DMTs.

The importance that patients place on MS treatment attributes may be of interest to healthcare providers, as well as to those who influence the selection of MS treatments.

1 Introduction

Recently, there have been a number of approvals of new multiple sclerosis (MS) therapies. Yet despite this, current first-line treatments still include injectable disease-modifying treatments (DMTs) that were introduced in the early- to mid-1990s [1]. These treatments are likely to remain the mainstay of MS therapy, given their well-established efficacy and safety record [2]. However, numerous studies have concluded that adherence (defined as the extent to which a patient acts in accordance with the prescribed interval and dose of a dosing regimen [3]) to injectable DMTs for patients with MS is suboptimal [4, 5]. Studies in patients with MS [4–10] have reported rates of medication adherence ranging from 27 to 83 %, depending on the methodology and whether the study used claims data to calculate indirect measures of adherence [6, 7, 11, 12] or surveys to collect self-reported measures of adherence [4, 5, 8, 13].

Patients' perceptions and experiences of medication efficacy, medication adverse events, dosing frequency, and dosing complexity have been found to influence adherence to injectable DMTs in patients with MS [4, 5, 8–16]. Adherence to injectable DMTs is typically higher when dosing frequency is lower [4, 5, 11–13, 17]; injection fatigue and injection fear, as well as injection-related adverse events [e.g., flu-like symptoms (FLS) and injection-site reactions (ISR)] are also common reasons cited for non-adherence [4, 9]. These results imply that experiences and/or perceptions of discomfort and inconvenience affect the choice of and the adherence to self-administered injectable DMTs. Several authors have suggested that improvements in administration of these treatments, such as a reduced dosing frequency, may reduce patient burden and improve adherence, as well as having economic benefits through fewer hospitalizations and emergency room visits [4, 6, 7, 12, 18]. Improvements in the dosing frequency and tolerability of DMTs may therefore improve adherence and directly reduce the number of injection-related adverse events.

This study was designed to assess the relative importance of a number of features of a (hypothetical) injectable DMT for patients with MS in the United States (US), using a discrete-choice experiment (DCE). The study investigators employed a broad set of features related to treatment with an injectable DMT such as efficacy and adverse events, as well as features describing administration, including dosing frequency and injection time. Previous studies have used similar methods to quantify patient preference for MS treatments, although with fewer/different parameters than this study [19, 20]. Patient preference studies for features of injectable DMTs provide information on the relative importance of these treatment features with respect to patient treatment choice and adherence. Collectively, data from these studies inform physicians on what features of treatment regimens and modalities are important to their patients and the associated burden of treatment [5, 8, 14], as well as informing healthcare providers and payors of any potential (direct and indirect) economic benefits that may arise from increased treatment efficacy and adherence [7]. Therefore, the objective of this study was to quantify the relative importance of the features of injectable DMTs that are associated with treatment choice and medication adherence in patients with MS.

2 Methods

2.1 Study Design

DCE studies [21–25] are based on the premise that medical treatments can be described as combinations of different features, or attributes. Attributes are defined in more detail in terms of levels; for instance, dosing frequency is an attribute with levels that could include daily, weekly, or monthly. The treatment attributes and attribute levels used to describe hypothetical MS treatments in this study were informed by (1) the characteristics of currently available injectable DMTs; (2) selected clinical study findings; and (3) consultation with clinical experts. The selection of attributes and levels is described in more detail in an appendix in the Electronic Supplementary Material. Based on this information, the set of six treatment attributes chosen for this study were the number of years until MS

symptoms get worse (i.e., disability progression with a focus on ambulation), the number of relapses in the next 4 years, injection time, frequency of injections, FLS, and ISR (Table 1). A broad range of attribute levels was included to simulate the real tradeoffs that patients with MS would have to make when deciding among injectable DMTs and to avoid a narrow focus on a limited range of treatment attributes, which might bias study results.

The survey design, administration, and analysis followed current good research guidelines [21]. The draft survey instrument was pretested using in-depth, in-person interviews with 15 adult patients with a self-reported physician diagnosis of MS. Treatment cost was not considered as an attribute; patients were asked to assume that all the medications would have the same cost. The final survey instrument is available as an appendix in the Electronic Supplementary Material.

In a series of treatment-choice questions, patients were asked to choose one of two hypothetical injectable treatment pairs for MS, defined by their attributes and attribute levels (Table 1). Figure 1 presents a sample treatment-choice question from the online survey instrument.

The experimental design followed good practice guidelines [26] and was generated using SAS® 9.3 analytics software (SAS Institute, Cary, NC, USA), which allowed for the optimization of design efficiency, level balance, correlations between level differences, and number of choice tasks [27–31]. The experimental design comprised 48 different treatment pairs. In order to maximize the quality of response and minimize fatigue or cognitive burden [32–34], the 48 paired comparisons in the experimental design were divided into six survey versions, each version containing eight treatment-choice questions. Each patient was randomly assigned to one of the six versions, and the order of the treatment-choice questions was randomized for each patient. In addition, one of the treatment-choice questions was repeated later in the sequence. To evaluate the stability of patients' choices, we calculated the percentage of patients who provided the same answer to both the original and the repeated treatment-choice questions. In total, patients answered nine treatment-choice questions. In addition, the final survey collected data on patient demographic and socioeconomic characteristics and experiences with MS and MS treatments.

Table 1 Attributes and levels for the treatment-choice questions

Attribute	Levels
Number of years until MS symptoms get worse	4 years
	2 years
	1 year
Number of relapses in the next 4 years	1 relapse
	3 relapses
	4 relapses
Injection time	3 s
	10 s
Frequency of injections	1 time each month (monthly)
	2 times each month (biweekly)
	4 times each month (weekly)
	12 times each month (3 times each week)
	30 times each month (daily)
Flu-like symptoms	No flu-like symptoms after any injections
	Symptoms for 1 day after some injections
	Symptoms for 3 days after some injections
	Symptoms for 3 days after every injection
Injection-site reactions	No reaction
	Mild

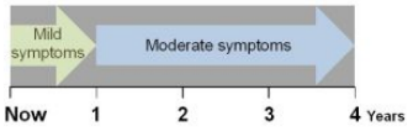
MS multiple sclerosis

To ensure that all patients were thinking about the same baseline, or initial MS symptoms, patients read a hypothetical reference scenario. Using a reference scenario ensured that each patient was considering the same initial disability level and the same change in disability. This, in turn, ensured that disability progression as a result of treatment was exogenous and that the changes in disability progression due to treatment were always improvements. Specifically, patients were asked to suppose that they had mild symptoms, as described using the Hohol, or Disease Steps, scale [35]. Patients were also asked to suppose that when their MS symptoms progressed, they would have moderate symptoms, again as described by the Hohol scale. The Hohol scale was selected because of the simplicity of the scale compared with the Expanded Disability Status Scale.

2.2 Study Sample

The target sample size for this study was 200 individuals. While the minimum required sample size for a DCE depends on a number of criteria [26], this sample size was within the range of sample sizes observed in most published studies [44].

Patients were recruited by All Global, a healthcare research firm, from an online panel of consumers in the US engaged for the purpose of survey research. All patients were aged 18 years or older and had a self-reported physician diagnosis of MS. The 25-min online survey was administered in May 2013. Patients were given a US$49 cash honorarium if they completed at least one treatment-choice question in the survey. The study was approved by

Medicine Feature	Medicine A	Medicine B
Number of years until MS symptoms get worse	1 Year 	2 Years
Number of relapses in the next 4 years	4 relapses	1 relapse
Injection Time	10 seconds	3 seconds
Frequency of injections	1 time each month (monthly)	4 times each month (weekly)
Flu-like symptoms	For 1 day after some injections	For 3 days after some injections
Injection-site reactions	None	Mild

	Medicine A	Medicine B
Which would you choose?	○	○

	Much more likely to miss doses with **Medicine A**	A little more likely to miss doses with **Medicine A**	Equally likely to miss doses with **Medicine A** and **Medicine B**	A little more likely to miss doses with **Medicine B**	Much more likely to miss doses with **Medicine B**
How likely is it that you would miss doses with these medicines?	○	○	○	○	○

Fig. 1 Example treatment-choice question. This is a screenshot from the final online US survey. The *underlined* entries in the "Medicine Feature" column were hyperlinks to the detailed descriptions of each medicine feature. *MS* multiple sclerosis

institutional review boards of the Research Triangle Institute (RTI). All patients provided informed consent prior to their inclusion in the study.

2.3 Analysis

To analyze the treatment-choice data and estimate preferences for the attribute levels, a random-parameters logit (RPL) model was approximated using NLOGIT Software version 5.0 (Econometric Software, Inc., Plainview, NY, USA). The RPL model accounted for the panel nature of the dataset (multiple observations obtained from the same respondent over time) and allowed for preference heterogeneity from patients by estimating a distribution of preferences for each parameter as well as a mean preference parameter [36, 37]. The dependent variable was the treatment choice, Medicine A or Medicine B. The independent variables were the levels for each of the attributes included in the study. All independent variables were effects-coded categorical variables, which allowed for the estimation of a unique parameter for each attribute level in the model [38]. All parameters were specified to be normally distributed random parameters.

The marginal log-odds resulting from the RPL model could be interpreted as 'preference weights', which indicate the relative importance of each attribute level. All mean attribute effects were normalized to be zero; the preference weight for each attribute level was then estimated relative to the mean effect for that attribute. To be able to compare the levels within an attribute, the 95 % confidence interval (CI) was calculated for each preference weight using the RPL estimate of the mean preference weight corresponding to the attribute level and the estimated standard error of that mean. If the 95 % CIs around

any two attribute levels did not overlap, the estimates were statistically different from each other at the 5 % level of significance or better.

The mean preference weights indicate the average relative importance of each attribute level in the sample and can be interpreted in two ways [36–38]. First, the vertical distance between any two preference weights for attribute levels, within a given attribute, indicate the importance of moving from one level of the attribute to the second level of the same attribute: the greater the difference, the more important the change from one level to the second level. Second, the relative importance of changes within an attribute could be compared with the relative importance of changes within a different attribute. This interpretation would be used to examine the tradeoffs that patients would be willing to make across treatment features.

In addition, to examine whether preferences for any treatment attributes depended on the levels of other treatment attributes, we included interactions between effects-coded (independent) variables. To examine whether preferences for any treatment attributes varied within subgroups, we included interactions between effects-coded (independent) variables and patient characteristics.

3 Results

To recruit respondents, All Global made the survey link available to web panelists who logged onto their account. From their account, interested panelists could learn about the survey opportunity and click through to the survey. We do not have information on the number of respondents who were provided the opportunity to be screened. A total of 498 patients opted to respond to the open survey, of whom 205 (41 %) met the inclusion criteria and completed at least one choice question in the survey. Thirteen patients (6 %) always chose either Medicine A or Medicine B in each of the treatment-choice questions and were excluded because this may indicate that these patients did not pay close attention to the choice questions. The final sample size for analysis was 192 patients. On average, patients had a 12-year diagnosis of MS [standard deviation (SD), 7]. The majority were female (78 %). More than two-thirds (68 %) reported having relapsing-remitting MS (RRMS). To manage MS symptoms, 45 % received regular injections. Sixty-five percent of patients not receiving regular injections at the time of the survey reported having received regular injections in the past. Thus, 81 % of patients had current or prior experience with injectable DMTs. Using the Hohol scale [35], 16 % of patients reported having mild symptoms of MS on most days, whereas 29 % reported having moderate symptoms. On average, patients reported experiencing four relapses (SD, five) within the last 2 years. A majority of patients (55 %) reported having experienced FLS resulting from MS treatments. Among patients with injection experience, 71 % reported having experienced a mild ISR (Table 2).

Table 2 Characteristics of study sample ($N = 192$)

Characteristic	Mean (SD) or %
Age (years)	50 (12)
Female	78 %
Employed full-time	22 %
Type of MS	
Relapsing-remitting	68 %
Secondary-progressive	16 %
Primary-progressive	7 %
Progressive-relapsing	5 %
Number of years since MS diagnosis	12 (7)
Number of relapses in last 2 years	4 (5)
Currently manages MS symptoms through	
Exercise or have physical therapy regularly	46 %
Over-the-counter medicines	26 %
Dietary supplements, such as vitamins and herbs	44 %
Alternative medicine, such as acupuncture	7 %
Oral medicine (pills) prescribed by doctor	46 %
Receive regular injections of medicine	45 %
None of the above	5 %
Receive regular intravenous infusions of medicine	11 %
Has never been prescribed a medicine to take on a regular basis to manage MS	6 %
Has never received injections on a regular basis to treat MS	29 %
MS symptoms, on most days	
No limitations	9 %
Mild symptoms	16 %
Moderate symptoms	29 %
Need cane for long distances	9 %
Need cane for short and long distances	21 %
Need bilateral support	7 %
Seconds it takes for medicine to come out of injection device	
Among those currently receiving regular injections	7 (5)
Among those who have received regular injections in the past	12 (18)
Ever had flu-like symptoms caused by your MS medicines	55 %
Has had a mild injection-site reaction	57 %

MS multiple sclerosis, *SD* standard deviation

The test of the stability of patients' choices indicated that most patients (158 of 192, or 82 %) provided the same response to both the original and repeated treatment-choice questions. This stability test result was similar to what has been observed in other studies [19, 39]. There are several reasons that respondents may have unstable responses to

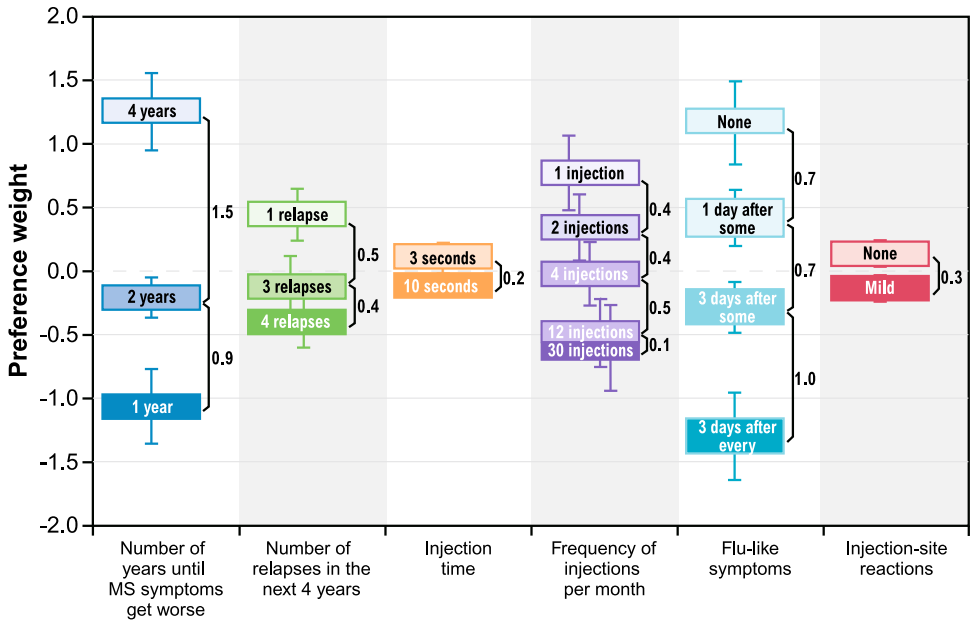


Fig. 2 Preference weights ($N = 192$). The *vertical bars* surrounding each mean preference weight denote the 95 % confidence interval about the point estimate. *MS* multiple sclerosis

choice questions, including learning and fatigue while answering the series of choice questions. Also, respondents' choice in any particular DCE question may depend on the content of previously answered choice tasks. While the stability test cannot distinguish the reason for response instability, we use this crude assessment as an indicator of the extent to which instability may be present in the study. Using an RPL model, no statistically significant differences were shown between RPL model results with and without the repeated question (data not shown). Therefore, repeated questions were included in the overall analysis, and no patients were removed due to the stability of their response.

Figure 2 presents the results of the RPL estimation, including preference weights and 95 % CIs for the attribute levels. Patient preferences were consistent with the expectation that better outcomes (e.g., better efficacy, fewer adverse events, more convenient administration) would be preferred to worse outcomes, providing evidence for internal validity of the study. For example, delaying MS disability progression by 4 years was preferred to delaying progression by 2 years, and delaying MS disability progression by 2 years was preferred to delaying progression by 1 year. The differences between the preference weights for these attribute levels were statistically significant ($P < 0.05$).

The mean relative importance of an improvement in the number of years until disability progression from 1 to 2 years was 0.9 (the vertical distance between preference weights for these attribute levels) (95 % CI 0.5–1.2; Fig. 2; Table 3). The relative importance of this change in disability progression was approximately equivalent to the relative importance of the number of relapses in the next 4 years from four to one (mean relative importance = 0.8; 95 % CI 0.5–1.2), the frequency of injections per month from 12 to two times (mean relative importance = 0.8; 95 % CI 0.4–1.2), and FLS from 3 days after every injection to 3 days after some injections (defined as "about one-half" of injections) (mean relative importance = 1.0; 95 % CI 0.6–1.4).

The mean relative importance of an improvement in the number of years until disability progression from 1 to 2 years was approximately twice (0.9 ÷ 0.5) as important as the mean relative importance of reducing the number of relapses in the next 4 years from three to one, three times (0.9 ÷ 0.3) as important as the mean relative importance of improving ISR from mild to none, and approximately four times (0.9 ÷ 0.2) as important as the mean relative importance of decreasing injection time in seconds from 10 to 3.

To assess whether preferences for selected attribute levels (i.e., levels of injection time, FLS, and ISR) varied systematically with treatment frequency, three additional RPL models with interaction terms were generated. The first of these models examined interactions between injection frequency and injection time, the second examined interactions between injection frequency and FLS, and the third examined interactions between injection frequency and ISR. None of these were statistically significant ($P > 0.05$), suggesting that there was no observed dependence of these three parameters on the level of injection frequency. In addition, interaction models examined the preference differences between four pairs of the following subgroups in the sample: 131 patients with RRMS and 61 patients with all other types of MS, 11 treatment-naïve

Table 3 Relative importance of selected changes in treatment attributes

Changes in treatment attribute levels	Mean relative importance	95 % confidence interval
Improvement in delay in disability progression from 2 to 4 years	1.5	1.1–1.8
Improvement in delay in disability progression from 1 to 2 years	0.9	0.5–1.2
Improvement in delay in disability progression from 1 to 4 years	2.3	1.7–2.9
Improvement in number of relapses from 3 to 1 in 4 years	0.5	0.2–0.8
Improvement in number of relapses from 4 to 3 in 4 years	0.4	0.1–0.7
Improvement in number of relapses from 4 to 1 in 4 years	0.8	0.5–1.2
Improvement in injection time from 10 s to 3 s	0.2	0.0–0.4
Improvement in number of doses per month from 2 to 1	0.4	0.0–0.9
Improvement in number of doses per month from 4 to 2	0.4	–0.0 to 0.8
Improvement in number of doses per month from 12 to 4	0.5	0.1–0.9
Improvement in number of doses per month from 12 to 2	0.8	0.4–1.2
Improvement in number of doses per month from 30 to 12	0.1	–0.4 to 0.6
Improvement in number of doses per month from 30 to 1	1.4	0.8–1.9
Improvement in flu-like symptoms from 1 day after some injections to none	0.7	0.4–1.1
Improvement in flu-like symptoms from 3 days after some injections to 1 day after some injections	0.7	0.4–1.0
Improvement in flu-like symptoms from 3 days after every injection to 3 days after some injections	1.0	0.6–1.4
Improvement in flu-like symptoms from 3 days after some injections to none	1.4	1.0–1.9
Improvement in injection-site reaction from mild to none	0.3	0.1–0.5

patients and 181 patients who were not treatment-naïve, 37 injection-naïve patients and 155 patients who were not injection-naïve, and 144 patients who reported that their current MS-related symptoms were moderate or worse and 48 patients who reported that their current MS-related symptoms were mild or better. All tests of the joint significance of the interaction terms indicated that there were no differences in joint preferences for treatment attributes between any of the two groups ($P > 0.05$). Tests of differences in individual preference weights showed only one notable difference in all four comparisons: patients with mild or better symptoms placed greater weight on decreasing the number of relapses from four relapses over the next 4 years to one relapse ($P < 0.01$) than patients with moderate or worse symptoms.

4 Discussion

Studies in MS have assessed adherence to injectable DMTs and associated adherence with features of injectable treatments, including injection frequency and injection-related adverse events (e.g., FLS and ISR) [4, 5, 9, 11–13, 17]. Such findings suggest that improvements in the dosing frequency of DMTs will improve adherence and, therefore, treatment outcomes. This study quantifies preferences of patients with MS for features of injectable DMTs, ranging from efficacy and levels of adverse events to features describing administration, such as dosing frequency.

This study found that increasing the time until disability progression and reducing FLS associated with injections were key drivers of patient preference. These features have previously been identified as among the most important treatment features affecting adherence to injectable DMTs [4, 5, 8–16]. Some improvements in DMT injection frequency were as important to patients as improvements in efficacy or injection-related adverse events. In particular, injections administered weekly or less often (i.e., two times per month or once per month) were preferred to injections administered daily or three times weekly. Reducing dosing frequency from three injections per week to two injections per month was as important to patients as lengthening the time until disability progression from 1 to 2 years or improving FLS from 3 days after each injection to 3 days after some injections. In some patients, frequent FLS are correlated with injection frequency; therefore, reducing dose frequency could reduce experiencing injection-related adverse events. However, a long time between injections also increases the incidence and severity of FLS, so the 'real-world experience' may be more complex [40]. Reducing dosing frequency from daily to monthly injections was similarly important to patients as lengthening the time until disability progression from 2 to 4 years or improving FLS from 3 days after some injections to none.

Given the evidence that injection fatigue, injection fear, and injection-related adverse events are associated with non-adherence [4, 9], these results indicate that changes in injection frequency may be as important as changes in efficacy or experiencing adverse events in determining a patient's choice of and adherence to injectable DMTs in MS.

Reducing the number of relapses was less important than delaying disability progression but still desirable to patients. Lengthening the time until disability from 1 to 4 years was 2.6 times more important than reducing the number of relapses over 4 years from four to one. This finding is consistent with the findings reported in Johnson et al. [19], in which delaying disease progression was more than twice as important as relapses. Because DMTs reduce relapses [41], physicians emphasize early initiation of treatment and reducing relapses (and, therefore, disability due to relapses) when prescribing treatment. The results of our study suggest that patients' opinions may differ from physicians' in that they appear to be more concerned about disability progression than relapses per se. Perhaps these results reflect recognition by patients that relapses often resolve over time, whereas disability progression is permanent. However, it is important to note that our study defined relapses such that they did not cause residual disability, which is not always the case. This was because this definition allowed us to independently identify the importance that patients place on relapse and disability progression, although it does not accurately represent the process by which disability can progress in patients with RRMS.

The preference weights for the different levels of injection time that were used in this study were not statistically different, indicating that patients did not distinguish between the levels of these attributes or had no pronounced preference for one attribute level to another. Furthermore, interactions between injection frequency and injection time, FLS, and ISR were not statistically significant. We therefore did not find any evidence that preferences for these three attributes were dependent on the level of injection frequency. Finally, the results of the study indicated that preferences for the treatment attributes did not differ among subgroups defined by whether the patients had treatment experience, whether the patients had injection experience, and whether the patients had RRMS or other types of MS. This could be a consequence of providing the participants with a reference scenario prior to taking part in the study. It could also be the result of insufficient sample sizes to detect differences in preferences between the subgroups. The only statistically significant difference that the study identified was that patients in this study with mild or better symptoms placed greater weight on reducing relapses than patients with moderate or worse symptoms. Although this suggests that MS-related symptoms may influence preference for a reduction in the number of relapses, we cannot determine whether these differences are due to patient perceptions that vary with disability levels or differences in information provided to patients with different disability levels.

There are a number of limitations to the study which are important when interpreting these results. One limitation is that 19 % of patients were injection-naïve; however, we found no evidence that preferences among patients with and without injection experience were statistically different from one another (data not shown). These results suggest that injection-naïve patients have well-formed preferences regarding features of injectable DMTs, although these patients do not have actual experience with injectable DMTs. Similarly, our analyses indicated that treatment-experienced and treatment-naïve patients had statistically similar preferences. Approximately 13 % of patients had not experienced a relapse in the previous 2 years and 12 patients reported never having experienced a relapse. Furthermore, stability testing suggested that only 82 % of patients were consistent with their choices.

While the use of DCEs in health applications to elicit preferences has been increasing [42–44], there are several potential limitations of this type of assessment. One inherent limitation is that the patients evaluated hypothetical injectable DMT profiles, and their choices among these injectable DMT profiles do not have the same significance as choices involving actual treatments. In addition, patients' actual treatment choices may reveal different implicit preference weights because actual treatment choices depend on a number of contextual factors (e.g., institutional and financial factors such as formulary status or treatment cost, respectively) that were beyond the scope of this study.

As in any survey research study, sample representativeness may be a potential study limitation. One of the inclusion criteria for survey respondents was that the patient had a self-reported physician diagnosis of MS. Although it is possible that some survey respondents did not actually have MS and simply participated in the survey to earn the modest compensation, the study was designed to minimize this possibility. When invited to take the survey, patients were not told about the subject of the survey. To screen respondents for self-reported physician diagnosis of MS, there was a question asking respondents to select from a list of health conditions that had been diagnosed by a healthcare provider. Only those selecting "multiple sclerosis" from that list were included in the study. The majority of the sample was female, which is consistent with the fact that MS is two to three times more common in women than in men [45]. Another limitation was that the sample was small relative to the population. We cannot

fully judge how representative our sample of patients with MS was or whether our results are generalizable to all individuals with MS in the US.

One must use caution in the interpretation of the study results. They should not be used to indicate the overall importance of treatment attributes, but rather the importance of changes in attribute levels that lie within the ranges of attribute levels include in this study. Also, while several of the treatment attributes in this study have been found to influence adherence in other studies, this study does not assess actual adherence, or intended adherence. These choices reflect respondents' assessments of how variations in attribute levels might affect adherence to hypothetical injectable MS treatments.

The study has a number of strengths derived from the use of best practices [21]. In particular, the survey was carefully designed in collaboration with clinical experts, was pretested using in-depth interviews with patients with MS in the US, and employed a state-of-the-art experimental design. The treatment-choice data were analyzed using advanced RPL methods that avoid estimation bias from unobserved variation in preferences across the sample and within-sample correlation in the choice sequence for each patient. We also tested for the effect of systematic preference differences by treatment experience and disability level.

5 Conclusion

This study provides evidence that patients with MS place approximately equal importance on changes in dosing frequency as they place on delays in disability progression and improvements in FLS caused by some injectable DMTs. The importance that patients place on dosing frequency and other MS treatment attributes may be of interest to healthcare providers recommending treatments to patients, as well as those who influence the selection of MS treatments when writing treatment guidelines, managing formularies, and/or developing reimbursement regimes.

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Compliance with Ethical Standards

Conflict of interest C. Poulos and J. Posner are employees of RTI Health Solutions, an independent scientific research organization; J. C. Yang was an employee of RTI Health Solutions at the time of the study. The study that is the subject of this manuscript was conducted by RTI Health Solutions and funded by Biogen. E. Kinter is an employee of Biogen. J. F. P. Bridges and A. Reder were expert consultants who were paid by Biogen for reviewing the protocol and the survey and participating in this research study, but not for the contributions on authoring this paper. Biogen has two injectable treatments that are approved by the US Food and Drug Administration (FDA)/European Medicines Agency (EMA): Avonex® and Plegridy™. The authors have full control of all primary data and agree to allow the journal to review the data if requested.

Contribution of each author Dr Poulos contributed to the study design, data analysis, interpretation of results, and writing and reporting. Dr Kinter contributed to the study design, interpretation of results, and reporting. Ms Yang contributed to the study design, data analysis, interpretation of results, and reporting. Dr Bridges contributed to the data analysis, interpretation of results, and writing and reporting. Mr Posner contributed to the study design, data analysis, and writing and reporting. Dr Reder contributed to the study design, interpretation of results, and reporting to ensure the clinical applicability and relevance of the survey instrument, data, and study results. All authors approved the manuscript.

Ethical standards The study was approved by an institutional review board (IRB) in the USA. The IRB operates under a Federal Wide Assurance (#3331) and is registered with both the Office for Human Research Protections and the US FDA. The study was reviewed for compliance with relevant US human subjects regulations (45 CFR 46). All patients provided informed consent prior to their inclusion in the study.

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Pharmacokinetic-Pharmacodynamic Relationships in HIV Prevention: Where Does The Drug Need To Be?

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EXPERT OPINION

1. Introduction
2. Methods
3. Mucosal tissue PK considerations
4. Mucosal tissue PK
5. Expert opinion

Pharmacokinetics of antiretrovirals in mucosal tissue

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Introduction: In the absence of an HIV vaccine or cure, antiretroviral (ARV)-based prevention strategies are being investigated to reduce HIV incidence. These prevention strategies depend on achieving effective drug concentrations at the site of HIV exposure, which is most commonly the mucosal tissue of the lower gastrointestinal tract and the female genital tract.
Areas covered: This article collates all known data regarding drug exposure in these vulnerable mucosal tissues and reviews important mechanisms of ARV drug distribution. Research papers and abstracts describing ARV pharmacokinetics (PK) in the female genital tract and lower gastrointestinal mucosal tissues available in MEDLINE® or presented at scientific conferences prior to December 2014 are reviewed in detail. Important influences on ARV mucosal tissue distribution, including protein binding, active drug transport and endogenous hormones are also reviewed.
Expert opinion: ARVs exhibit highly variable PK in mucosal tissues. In general, ARV exposure is higher in the lower gastrointestinal tract compared with the female genital tract, but concentrations required for protective efficacy are largely unknown. The expected site of HIV exposure represents an important consideration when designing and optimizing ARV-based prevention strategies.

Keywords: antiretroviral, HIV prevention, mucosal tissue, pharmacokinetics

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1. Introduction

As of the end of 2013, 35 million people worldwide were living with HIV/AIDS [1]. Highly active antiretroviral treatment (HAART) strategies effectively control HIV's progression into AIDS, restoring life expectancy and quality of life to that of an uninfected person. Nonetheless, of the 32.6 million HIV-infected persons living in lower- and middle-income countries, ~ 64% (20.9 million) do not have access to HAART [1]. Therefore, prevention strategies are needed to contain the epidemic.

In the absence of a licensed vaccine, the use of antiretrovirals (ARVs) for HIV prevention has been investigated. The utility of one of these approaches, treatment as prevention (TasP), was recently established in the landmark HPTN052 study where it was found that consistent suppression of blood plasma HIV RNA in HIV-infected subjects using early HAART reduced transmission to their uninfected partners by > 95% [2]. This study demonstrated the highest degree of protection of any HIV prevention trial. In the US, < 50% of HIV-infected patients on HAART exhibits a suppressed viral load [3]. This is an important limitation of the TasP approach and warrants further exploration into alternative HIV prevention strategies in uninfected individuals.

In 2012, Truvada®, a fixed-dose combination tablet of two nucleoside reverse transcriptase inhibitors (NRTIs) tenofovir disoproxil fumarate and emtricitabine, received FDA approval to be used as part of an HIV prevention package in high-risk individuals. Truvada received this licensing on the basis of a 44 – 75% decrease

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Article highlights.

- Antiretroviral (ARV) distribution to mucosal tissues is governed by a number of complex factors including lipophilicity, protein binding and active transport.
- The degree of ARV mucosal tissue distribution is highly variable between and within ARV therapeutic drug classes (**Figure 1**).
- As a drug class, nucleoside reverse transcriptase inhibitors (NRTIs) achieve higher exposure in the mucosal tissues compared with non-NRTI and protease inhibitors (**Figure 1**).
- In general, ARVs exhibit higher exposure in the lower gastrointestinal mucosal tissue compared with the female genital mucosal tissue.
- This article elucidates some important knowledge gaps in the field of mucosal tissue pharmacokinetics which warrant further study to better characterize, including: i) drug transporters and their regulation in mucosal tissues; ii) the effect of hormonal regulation and inflammation on mucosal tissue distribution; and iii) mucosal pharmacodynamic targets for HIV prevention.

This box summarizes key points contained in the article.

in HIV incidence in three clinical trials studying its use in diverse study populations including: serodiscordant heterosexual couples (Partner's PrEP) and other high-risk heterosexual individuals (TDF2) and men who have sex with men (MSM; iPrEX) [4-6]. Subsequently, a study investigating Truvada pre-exposure prophylaxis (PrEP) in intravenous drug users demonstrated 49% protection [7]. Truvada is the first relatively discreet, user-controlled HIV prevention option available to women. Yet, clinical trials in women have exhibited mixed results: two large Phase III trials, FEM-PrEP and VOICE, failed to show HIV prevention efficacy for tenofovir taken with or without emtricitabine [8,9]. These results were explained by the low degree of adherence to the daily regimens by study subjects. Subsequently, observational analyses have directly linked the effectiveness of Truvada in PrEP approach with adherence [10,11]. However, the degree of adherence required for protection may be contingent on the site of HIV exposure as evidenced by recent data demonstrating that only two doses per week of Truvada reduced HIV incidence by up to 90% in the MSM population of iPrEX [10]: a level of adherence that was also seen in FEM-PrEP and VOICE [8,9].

The most common mode of HIV transmission is through sexual activity, whereby mucosal tissues are the primary sites of HIV exposure. For TasP approaches to be successful, drug concentrations in infected individuals must be sufficient to suppress viral replication and shedding in the local anatomical sites associated with transmission, and may be necessary at high concentrations in the mucosal fluid serving as the viral source of transmission [12]. Conversely, PrEP approaches rely on drug concentrations in an uninfected individual being sufficient to prevent viral entry, integration or replication in HIV target cells in mucosal tissue and/or regional lymph nodes at the time of exposure [13]. Consequently, when considering the utility of TasP and PrEP for global reduction in HIV incidence, a thorough understanding of ARV pharmacology in mucosal tissues becomes exceedingly important to ensure that optimal drugs, doses and dosing schedules have been selected. For this purpose, the present review will examine ARV pharmacokinetics (PK) in the mucosal tissue types most commonly involved in HIV transmission – the lower gastrointestinal tract and the female genital tract.

2. Methods

Research papers and abstracts describing ARV PK in the female genital tract and lower gastrointestinal mucosal tissues published in the MEDLINE® database from January 2000 to December 2014 were identified using the following search terms: HIV, antiretroviral, pharmacokinetics, cervicovaginal fluid, cervical tissue, vaginal tissue, cervix, vagina, rectal fluid, rectal tissue and rectum. Individual drug names were also included in the search terms. All original articles and relevant reviews discussing ARV concentrations in the mucosal fluid or tissue of the gastrointestinal and female genital tract for any commercially available ARV s or ARV currently under investigation were reviewed in detail. Abstracts presented at scientific conferences prior to December 2014 were also included in this review.

3. Mucosal tissue PK considerations

Mucosal tissues represent a particularly dynamic microenvironment which is subject to a number of regulatory pathways including paracrine, autocrine and endocrine signaling. Understanding the pharmacological factors guiding drug distribution and how these are regulated within the tissue compartment is important for interpreting mucosal tissue PK data. In the subsections below, we describe three of these factors: protein binding, membrane drug transporter activity and endogenous hormones.

3.1 Protein binding

Protein binding is an important consideration in the PK profile of ARVs, because only the unbound fraction (i.e., free drug) is available for pharmacological activity. The extent of protein binding may affect a compound's ability to exit the vasculature and penetrate into the interstitial tissue space [14]. The primary proteins responsible for binding ARVs are albumin and α1 acid glycoprotein (AAG) [15]. The extent of binding to these proteins is highly variable among the ARV classes [16]. For instance, most protease inhibitors (PIs) are > 90% bound to AAG, whereas the non-NRTI (NNRTI), efavirenz is > 99% bound to albumin [16]. Importantly, the concentration of albumin and AAG are much lower in mucosal fluid relative to blood plasma. In one investigation of

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cervical mucus, the mean (SD) concentration of albumin and AAG was 73 (45) and 6.6 (3.6) mg/l, respectively. Both of these values are < 1% of the concentration found in blood plasma [17]. For one ARV, maraviroc, protein binding is reduced 10-fold in the female genital tract: from ~ 76% in blood plasma to ~ 7.6% in cervicovaginal secretions [18,19]. These findings imply that a higher degree of pharmacological activity might be achieved in mucosal fluid relative to plasma given the same total drug concentration in the two matrices.

Alteration in protein binding can affect pharmacodynamic (PD) markers such as the IC_{50}, and confound PK/PD analyses that extrapolate inhibitory quotients (the ratio between drug concentration at the end of the dosing interval and level of drug resistance of the viral isolate) to clinical trial data [16]. For example, the PI SC-52151 failed to show a clinical effect despite achieving an adequate concentration in the plasma [20]. This was later shown to be due to AAG binding, which reduced the activity of the drug by 17-fold in MT-2 cells [21]. Kempf *et al.* found that by including just 10% fetal calf serum in cell culture media, lopinavir was 96% protein-bound, thus providing proof of concept for *in vitro* manipulation to produce better PD estimates [22]. A recent *in vitro* investigation in $CD4^+$ T cells isolated from peripheral blood mononuclear cells (PBMCs) found that increasing the extracellular concentration of human serum albumin from 1 to 80 mg/ml resulted in reduced intracellular concentrations of approximately 8-fold for efavirenz (at a concentration of 5 ng/ml), 1.7-fold for raltegravir (at a concentration of 5 ng/ml) and 2.3-fold for etravirine (at a concentration of 2 ng/ml) [23]. Thus, careful consideration of the extent to which a drug is protein-bound is critical for translating *in vitro* data into the *in vivo* system.

3.2 Drug transporters

Transporter proteins are located on cell membranes of tissues such as the colorectal and genital tract mucosa and are highly involved in the uptake and efflux of drugs. Several ARVs are substrates of drug transporters within the ATP-binding cassette (ABC) and solute carrier (SLC) families [24]. Recent efforts have been directed at elucidating the role of mucosal tissue transporters in the distribution of drugs to the female genital and lower gastrointestinal tracts. Zhou *et al.* investigated the mRNA expression of several ABC efflux transporters as well as SLC and SLCO uptake transporters in human vaginal and ectocervical tissue [25]. Liver tissue was used as a reference standard due to abundant expression of the drug transporters investigated [26]. The efflux transporters MRP1 and MRP4 were highly expressed (50 – 100% of reference tissue) in cervicovaginal tissue, whereas MRP2 and the uptake transporters, OAT1 and OAT3, were < 2% of the reference tissue. This analysis did not include information regarding location of these transporters within the epithelium, which is important to interpret the data correctly. For instance, abundant expression of efflux transporters in the squamous epithelium adjacent to the lumen would increase elimination of drug substrates from the tissue. However, efflux transporters located within the basal lamina (the most interior layer of the epithelium) would increase transport into the squamous epithelial layer and lead to accumulation of drug substrates.

Nicol *et al.* addressed this by quantifying the mRNA and protein expression of three efflux (MDR1, MPR2, MRP4) and three uptake (OAT1, OAT3 and OATP1B1) transporters in cervical, vaginal and colorectal tissue using real-time polymerase chain reaction and immunohistochemistry techniques [27]. Zhou *et al.* findings agree with Nicol *et al.* analysis which revealed consistent expression of the ABC transporters at different levels in each tissue type. MDR1 and MRP2 mRNA expression in female genital mucosal tissue was found to be three- to four-fold higher than colorectal tissue ($p < 0.001$). Conversely, MRP4 mRNA in colorectal tissue was twofold higher than female genital mucosal tissue ($p < 0.05$). Whereas diffuse MDR1 protein expression was seen throughout the vaginal and ectocervical tissue, expression appeared to be localized to the luminal surface in endocervical and colorectal tissue. Diffuse MRP2 protein expression was observed in the epithelia of all tissue types. Finally, MRP4 protein expression appeared diffuse throughout the vaginal squamous epithelium and colorectal columnar epithelium. However, positively stained lymphocytes and monocytes appeared to be localized to the luminal surface of the colorectal epithelium. The influx transporters OAT1, OAT3 and OAT1B1 exhibited a lower degree of expression in all tissues compared with efflux transporters: < 25% of all mucosal tissue samples exhibited quantifiable expression and all detectable tissue samples were < 0.1% of expression compared to kidney tissue (used as a reference due to its abundant expression of uptake transporters) [26,27]. These data suggest that efflux transporters (MDR1, MRP2 and MRP4) play a role in drug distribution in female genital and colorectal tissues.

Table 1 summarizes the drug transporter affinity for available ARVs. Recent modeling work has determined that being a substrate of MRP1 positively correlates with female genital tract tissue concentrations, whereas being a substrate for MRP4 negatively correlates with tissue concentration [28]. The negative correlation between tissue penetration and MRP4 substrate status is in support of the findings that MRP4 expression appears diffuse throughout the female genital tract thus decreasing accumulation in the tissue.

Finally, the expression of efflux transporters appears to be regulated by inflammatory cytokines released in response to infection or injury [29,30]. Proinflammatory mediators such as IL-6, IL-1β and INF-γ have been shown to decrease the expression of BCRP and MDR1 [30-32]. Since mucosal inflammation may be the consequence of HIV infection [33], it is possible that stage of HIV infection may influence ARV drug distribution. Investigation with the antibiotic linezolid (an MDR1 substrate) has shown altered exposure in soft tissues correlated with degree of inflammation [34]. However,

Table 1. Antiretroviral drugs are substrates for drug transporters [24,68].

Class	Antiretroviral	Drug transporter
Nucleoside reverse transcriptase inhibitors	Abacavir	P-gp BCRP MRP4
	Emtricitabine	MRP1
	Lamivudine	BCRP OCT1 OCT2
	Tenofovir DF	P-gp
	Zidovudine	MRP4 BCRP OAT1 OAT2 OAT3 OAT4 CNT1 CNT3 ENT2
Integrase strand transfer inhibitors	Dolutegravir	P-gp, BCRP
	Raltegravir	P-gp
Protease inhibitors	Atazanavir	P-gp MRP1 MRP2
	Darunavir	P-gp OATP1A2 OATP1B1
	Lopinavir	P-gp MRP1 MRP2 OATP1A2 OATP1B1
Entry inhibitors	Maraviroc	P-gp

BCRP: Breast cancer resistance protein; DF: Disoproxil fumarate; P-gp: Permeability glycoprotein.

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the effect of local inflammation on ARV drug distribution to mucosal tissues is yet to be determined.

3.3 Endogenous hormones

Another important consideration is the effect of endogenous hormones on drug distribution to mucosal tissues. Hormonal and physiological variations occurring during the follicular, ovulatory and luteal phases of the menstrual cycle may alter certain PK parameters such as protein binding and oxidative hepatic metabolism [35]. In cultured epithelial cells of the female genital tract, estradiol and progesterone were found to regulate the intracellular concentration of tenofovir diphosphate (TFVdp) [36]. Additionally, MDR1 expression in the female genital mucosa appears to be subject to hormonal regulation with highest expression correlating with the luteal phase of the menstrual cycle [37] and lowest expression in postmenopausal women [27]. However, the literature describing the clinical implications of these physiological alterations for ARVs is extremely limited. Sheth *et al.* investigated trough cervicovaginal concentrations of tenofovir, emtricitabine and atazanavir in 20 HIV-infected women and found no correlation between cervicovaginal fluid concentration and menstrual cycle [38]. Likewise, cervical raltegravir trough concentrations in nine healthy volunteers demonstrated no correlation with phase of menstrual cycle [39]. However, the extensive between-subject variability in raltegravir cervical tissue concentrations (coefficient of variation = 195.7%) makes it difficult to conclude that menstrual cycle does not impact drug distribution to the female genital tract. No investigation to date has explored the impact of hormonal changes on drug distribution within the colorectal tissue.

4. Mucosal tissue PK

The distribution of ARV agents has been widely investigated in the female genital tract by measuring drug concentrations in cervicovaginal fluid. Cervicovaginal fluid allows for more intensive PK sampling within individuals and overcomes some of the challenges posed by tissue biopsies. Distribution of ARVs in the mucosal fluid of the lower gastrointestinal tract has not been widely studied. One study that measured drug concentration of dolutegravir in rectal fluid found that self-collected rectal fluid swabs were an unreliable surrogate for drug exposure in the rectal tissue because of large within- and between-subject variability [40].

When evaluating mucosal tissue PK, a ratio between drug exposure in the compartment relative to drug exposure in the blood plasma is commonly calculated to correct for plasma drug exposure with different drug combinations and dosing strategies. The ratio can be calculated with either single sampling times or by AUC. Utilizing the AUC approach minimizes spurious results due to lag times in distribution to peripheral compartments. **Figure 1** reports the penetration ratios for each ARV in the female genital or lower gastrointestinal tissue and includes ratios derived with AUCs (when available) or concentrations at the end of the dosing interval. A summary table of the methods and results of ARV PK studies in the female genital mucosal tissues up to 2011 can be found in a review by Else *et al.* [41].

4.1 Nucleoside reverse transcriptase inhibitors

Combination NRTIs form the backbone of most HIV treatment regimens. These agents require intracellular phosphorylation to their active metabolites, which compete with the host's endogenous nucleotides for incorporation into the elongating proviral DNA strand. In general, agents within this class exhibit a low degree of protein binding (< 50%) and hepatic biotransformation [42]. On phosphorylation, the intracellular active moieties are trapped within the cell membrane, and only parent compounds are readily detectable in extracellular fluid spaces such as blood plasma, cervicovaginal fluid and seminal plasma. Currently, one of the most commonly used NRTIs, tenofovir, is being investigated for PrEP in a 1% gel formulation. This section will review the mucosal tissue PK of orally and topically administered NRTIs.

Dumond *et al.* first described the median (interquartile range [IQR]) cervicovaginal fluid to blood plasma AUC_{0-tau} ratios following single and multiple doses of seven NRTIs in

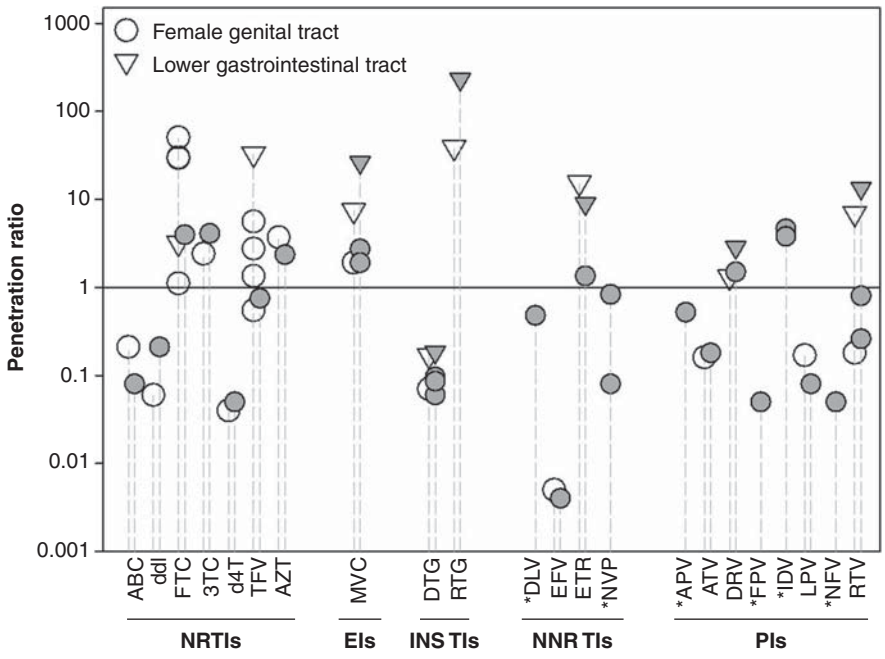

Figure 1. Illustration showing the antiretroviral female genital and lower gastrointestinal tract penetration ratios. This figure reports the mucosal tissue or fluid:blood plasma AUC ratios for 22 antiretrovirals stratified by class. Both single dose (open symbols) and steady state (closed symbols) are reported when available. The line of unity (mucosal tissue/fluid AUC = blood plasma AUC) is denoted by the solid reference line. In general penetration ratios tend to be higher in the lower gastrointestinal tract for most antiretrovirals. As a class NRTIs and the (EI), MVC, tend to demonstrate a high degree of penetration (> 1) into the female genital and lower gastrointestinal tracts whereas NNRTIs and PIs tend to exhibit low penetration (< 1).

*Reported ratio calculated from matched through concentration rather than AUC.

3TC: Lamivudine; ABC: Abacavir; APV: Amprenavir; ATV: Atazanavir; AZT: Zidovudine; d4T: Stavudine; ddI: Didanosine; DLV: Delavirdine; DRV: Darunavir; DTG: Dolutegravir; EFV: Efavirenz; EI: Entry inhibitor; ETR: Etravirine; FPV: Fosamprenavir; FTC: Emtricitabine; IDV: Indinavir; INSTIs: Integrase strand transfer inhibitors; LPV: Lopinavir; MCV: Maraviroc; NFV: Nelfinavir; NNRTI: Non-nucleoside reverse transcriptase inhibitor; NRTI: Nucleoside reverse transcriptase inhibitor; NVP: Nevirapine; PI: Protease inhibitors; RTV: Ritonavir; RTG: Raltegravir; TFV: Tenofovir.

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HIV-infected patients initiating or changing ARVs for HIV treatment. These are listed by rank order for the single dose in **Table 2**. Following the single dose, all agents were detected in cervicovaginal fluid within 4 h and reached peak concentrations within 6 h following a single dose [43]. Of the NRTIs, only the older agents, didanosine and stavudine, exhibited cervicovaginal fluid to blood plasma ratios of < 20%. Subsequently, tenofovir and emtricitabine were shown to exhibit 2 – 26 times higher cervicovaginal fluid exposure compared to blood plasma exposure when measured after a single dose over 14 days. The terminal half-life in plasma was estimated to be 47 h for tenofovir and 49 h for emtricitabine, and in cervicovaginal fluid, it was estimated to be 71 h for tenofovir and 40 h for emtricitabine [44]. Since these half-lives are similar to previously published emtricitabine triphosphate (FTCtp) and TFVdp data [45], this extended elimination phase was thought to reflect clearance and dephosphorylation of intracellular FTCtp and TFVdp.

The formation of TFVdp and FTCtp have been well characterized in the female genital tract following a single dose [44,46,47]. The most recent of these studies measured parent and intracellular concentrations at multiple time points over 48 h across a fourfold dosing range (150 – 600 mg for tenofovir disoproxil fumarate and 100 – 400 mg for emtricitabine) [46]. Whereas the time to peak concentrations (T_{max}) of tenofovir and emtricitabine is achieved within 6 h in cervical and vaginal tissues, the formation of the active metabolites are delayed, with a median T_{max} of 24 h for TFVdp and 12 h for FTCtp [46]. Although TFVdp was detected in vaginal tissue for up to 14 days following a single fixed-dose combination tablet of tenofovir disoproxil fumarate 300 mg and emtricitabine 200 mg, FTCtp was only detected for up to 2 days [44]. Louissaint *et al.* confirmed the persistence of TFVdp in vaginal tissue after a single dose, and calculated a terminal half-life of 53 h [47]. This tissue half-life is consistent with TFVdp's 49.7 h *in vitro* half-life in resting PBMC cultures [45].

Multiple PK investigations suggest differential distribution of tenofovir and emtricitabine between mucosal tissues types, which has important implications on ARV-based prevention strategies. PK analysis investigating exposure at 6 – 48 h following a single dose found 10-fold higher TFVdp exposure

Table 2. Distribution in cervicovaginal fluid after single and multiple doses.

Class	Antiretroviral	Number	CVF:BP AUC_{0-tau} Median (interquartile range)		Ref.
			Single dose	Multiple dose	
Nucleoside reverse transcriptase inhibitor	Zidovudine	3	3.71 (1.13, 6.04)	2.35 (1.21 – 21.2)	[43]
	Lamivudine	13	2.41 (1.09, 15.48)	4.11 (2.3 – 5.94)	[43]
	Tenofovir	10	1.35 (0.0023, 4.47)	0.75 (0.37 – 6.45)	[43]
	Emtricitabine	3	1.11 (0.91, 11.00)	3.95 (1.87 – 6.71)	[43]
	Abacavir	5	0.21 (0.08, 0.70)	0.08 (0.08 – 0.13)	[43]
	Didanosine	2	0.06 (0.06, 1.31)	0.21 (0.01, 0.4)	[43]
	Stavudine	3	0.04 (0.007, 0.35)	0.05 (0 – 0.12)	[43]
Non-nucleoside reverse transcriptase inhibitor	Efavirenz	6	0.005 (0.001, 0.008)	0.004 (0.001, 0.006)	[43]
	Nevirapine	7	Not available	0.83 (0.43, 1.58)	[52]
	Etravirine	12	Not available	1.2 (0.5, 4.8)	[56]
Integrase inhibitor	Raltegravir	6	Not available	4.4 (1.5, 1.7)	[64]
	Dolutegravir	8	0.07 (0.05, 0.18)	0.06 (0.04, 0.11)	[67]*
Protease inhibitors	Darunavir	8	Not Available	1.5 (1.0 – 1.6)	[79]
	Ritonavir	12	0.18 (0.005, 0.42)	0.26 (0.11, 1.69)	[43]
	Lopinavir	4	0.17 (0.003, 0.3)	0.08 (0.03, 1.28)	[43]
	Atazanavir	8	0.16 (0.1, 0.79)	0.18 (0.05, 0.66)	[43]

*Concentration data from HIV-negative subjects.
BP: Blood plasma; CVF: Cervicovaginal fluid.

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in colorectal tissue compared to female genital mucosal tissue [46]. However, FTCtp was 170 times higher in the female genital mucosal tissue compared to the rectal tissue. When these compounds were measured over 14 days following a single dose, tenofovir and TFVdp rectal tissue $AUC_{0-14days}$ remained were 60 – 100 times higher than female genital tract tissue [44,47]. The difference in the concentration gradient between these two studies is likely due to the delayed accumulation of TFVdp in rectal tissue (median $T_{max} \geq 48$ h) compared to female genital tract tissue (median $T_{max} = 24$ h) [46]. Emtricitabine and FTCtp $AUC_{0-14days}$ were ~ 10 times higher in female genital tract tissue than colorectal tissue [44].

Topical tenofovir 1% gel has been investigated for application in both the female genital tract and the colorectum. Once instilled, TFVdp can be detected in tissues within 0.5 h [48,49]. A randomized crossover PK study found that a 40 mg dose of topical tenofovir in the vagina achieved more than a 100-fold higher tenofovir and TFVdp concentrations in vaginal and cervical tissue compared to the standard 300 mg oral dose [50]. Maximum blood plasma concentrations following daily vaginal application of the gel was ~ 1% of the maximum concentration (C_{max}) following oral tenofovir 300 mg/day (median C_{max} = 3.9 vs 332 ng/ml, respectively). About 30 min after application, a separate study found that the gel yielded TFVdp concentrations in colorectal tissue 6 times higher than concentrations achieved after an oral dose [49] Maximum blood plasma concentrations following seven daily rectal administrations of the gel were ~ 3% of the exposure following a single dose of oral tenofovir 300 mg (median C_{max} = 8.61 vs 252 ng/ml). Rectal administration also led to detectable tenofovir concentrations in vaginal fluid: vaginal fluid C_{max} was ~ 2.5 logs lower than colorectal fluid but approximately twofold higher compared to vaginal fluid C_{max} following oral dosing [51].

4.2 Non-nucleoside reverse transcriptase inhibitors

The NNRTIs constitute several first-line ARV therapies in combination with NRTIs. By binding non-competitively to the reverse transcriptase enzyme, they inhibit transcription of viral RNA. Delavirdine, efavirenz and nevirapine are first-generation NNRTIs, whereas rilpivirine and etravirine are second generation. The penetration of most NNRTIs into the female genital tract is limited [14]. In a study by Min *et al.* cervicovaginal fluid:blood plasma ratios were 0.83 for nevirapine, 0.48 for delavirdine and < 0.08 for efavirenz [52]. These penetration ratios demonstrate an inverse relationship with the degree of protein binding exhibited by each agent (nevirapine = 60% bound, delavirdine = 98% bound and efavirenz ≥ 99.5%) [53-55]. The penetration of these agents into the colorectal tissue has not been studied.

In the DIVA02 study of 12 HIV-1-infected women, the etravirine median (IQR) cervicovaginal fluid:blood plasma ratio was 1.2 (0.5 – 4.8) [56]. Despite high (99%) protein binding, etravirine's low molecular mass and lipophilic structure may be responsible for the increased mucosal tissue penetration. A separate study in 12 HIV-negative men determined that after a single dose of etravirine, rectal tissue exposure was ~ 15-fold higher than blood plasma: with repeated dosing, rectal tissue exposure was approximately ninefold higher [57].

The PK of oral rilpivirine has not been investigated in mucosal tissues. However, interest has grown for the long-acting parenteral formulation of the drug, both for PrEP and to increase adherence in HIV treatment. Mucosal

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distribution with this formulation has been investigated in HIV-negative men and women at doses of 150, 300, 600 and 1200 mg given intramuscularly (i.m.) [58]. In men, at the 600 mg dose, the geometric mean (90% CI) rectal tissue:plasma ratio was 0.98 (0.85 – 1.11) on day 7, whereas in women, the geometric mean (90% CI) vaginal tissue: plasma ratio was 0.72 (0.4 – 1.05) on day 7. The authors concluded that the compartmental rilpivirine concentrations were proportional to the dose administered. A planned Phase II study of the long-acting injectable formulation of rilpivirine TMC278LA will evaluate the 1200 mg dose for chemoprophylaxis [59].

Dapivirine is also being developed as a topical microbicide formulated in intravaginal rings (IVRs), gels and films. In 24 HIV-negative women, concentrations attained in the lower female genital tract 28 days after placement of an IVR were 5 log units higher than maximum concentrations achieved in the plasma over the 28-day period [60]. Further, there was a linear relationship ($p = 0.0157$) between tissue concentration and protective effect [61]. The FAME-02 study in 60 HIV-negative women that compared the PK/PD and safety of gel (0.05%) and film (1.25 mg) formulations of dapivirine found that both formulations achieved low and comparable plasma concentrations (302.47 pg/ml for gel compared to 227.13 pg/ml for film). However, tissue concentrations of dapivirine from the film were four times lower than concentrations from the gel, and this correlated with greater infectivity in the *ex-vivo* challenge experiments with vaginal and cervical biopsies [62]. These novel formulations may be used to help achieve protective mucosal drug exposure with decreased systemic exposure and reduced dosing frequency.

4.3 Integrase strand transfer inhibitors

Integrase strand transfer inhibitors (INSTIs) represent the newest class of ARVs with the first agent in class raltegravir approved in 2007. This was followed by elvitegravir in 2012 and dolutegravir in 2013. As a class, these agents are potent, well tolerated and exhibit lower drug interaction potential than PIs and NNRTIs. Glucuronidation is the primary route of metabolism for raltegravir and dolutegravir, whereas elvitegravir is primarily metabolized by the CYP450 enzyme system. For this reason, some have been proposed as second-generation PrEP strategies. To date, raltegravir and dolutegravir have data available on drug penetration into mucosal tissues.

Raltegravir exhibits the lowest degree of protein binding (83%) within this class [63]. Data in HIV-negative and HIV-infected premenopausal women have shown median (IQR) raltegravir cervicovaginal fluid:blood plasma steady-state AUC_{0-12h} ratios of 0.93 (0.4, 1.7) and 4.4 (1.5, 7.2), respectively [64]. The DIVA trial investigated raltegravir trough concentrations in the blood plasma and cervicovaginal fluid of 14 HIV-infected women [65]. The median (IQR) cervicovaginal fluid:blood plasma ratio was 2.3 (1.4 – 4.1). Of the trial's three study subjects with bacterial vaginosis, two exhibited cervicovaginal fluid:blood plasma ratios that were 4- to 16-fold greater than the median, suggesting that inflammation may increase raltegravir exposure in the female genital tract. Raltegravir is a substrate for the MDR1 efflux transporter [66], which is highly expressed in the female genital tract and generally attenuated in the presence of inflammatory mediators; therefore, accumulation of raltegravir in inflamed female genital mucosal tissue is biologically plausible.

Conversely, dolutegravir exposure in female genital tract tissues and cervicovaginal fluid was found to be < 10% of blood plasma [67]. Dolutegravir is much more extensively protein-bound (≥ 98%) than raltegravir, which could potentially explain this low degree of distribution [68]. Despite this, 94% of samples collected from the female genital tract were above dolutegravir's protein adjusted IC_{90} of 64 ng/ml [67]. Since cervicovaginal fluid concentrations of AAG (the major serum protein responsible for binding dolutegravir) are ~ 1% of blood plasma [17], dolutegravir exposure in the female genital tract may be sufficient despite the compound's poor penetrative capacity.

In the lower gastrointestinal tract, raltegravir was found to rapidly distribute after a single dose, reaching concentrations in the terminal ileum which exceeded plasma concentrations within 2 h and peak concentrations within 4 h [69]. After a single 400 mg dose, the highest concentrations were achieved in the terminal ileum followed by the splenic flexure, and the rectal tissue ($AUC_{composite}$ = 594, 258, and 143 $ng*hr*ml^{-1}$, respectively). Under steady-state conditions with 400 mg dosed twice daily, the splenic flexure exhibited the highest exposure with an $AUC_{composite}$ of 2240 $ng*hr*ml^{-1}$ where the $AUC_{composite}$ was 788 $ng*hr*ml^{-1}$ in both the terminal ileum and the rectum. A single dose, dose-ranging PK study investigating 200 – 800 mg of raltegravir in healthy women found exposure in the lower gastrointestinal tract is ~ 25-fold greater than in the female genital tract, and colorectal exposure is similar between women and men [70]. Dolutegravir was found to be poorly distributed in the rectal tissue of healthy males, with a median $AUC_{composite}$ penetration ratio of 0.17 [40]. Despite low rectal exposure relative to blood plasma, 100% of concentrations from the lower gastrointestinal mucosa was above the protein-adjusted IC_{90} [40].

Cabotegravir is an investigational INSTI, which has a similar chemical structure as dolutegravir [71]. It is formulated as a long-acting injectable nanoparticle suspension for i.m. administration in HIV treatment and prevention [71]. Cabotegravir exhibits plasma PK which supports 4 and 8 weeks dosing intervals. Both dosing intervals are currently under investigation in Phase IIb studies for HIV treatment [72]. Similar to dolutegravir, a 400 mg i.m. dose of cabotegravir was found to exhibit cervical/vaginal and colorectal penetration ratios of 0.16 – 0.19 and ≤ 0.08, respectively, 2 – 8 weeks after a single injection [73]. Whereas median rectal tissue concentrations at 4 and 12 weeks following a 400 mg split i.m. injection were below the protein-adjusted IC_{90} (16.6 ng/ml), the median

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concentration in cervical and vaginal tissue was above this value at the weeks 4 and 12 visit, respectively [73]. Recently, Radzio *et al.* demonstrated 100% protection in macaques vaginally challenged with biweekly SHIV for up to 20 weeks after the last of 3 monthly 50 mg/kg injections [74]. This study found low penetration to the mucosal compartment similar to humans with cervicovaginal and rectal fluid:plasma AUC ratios of 0.16 and 0.38, respectively. Andrews *et al.* also demonstrated 100% protection in macaques that were rectally challenged with weekly SHIV for up to 20 weeks following two monthly 50 mg/kg injections and 5 weeks following a single injection [75]. In the single injection study, plasma concentration $\geq 1 \times PAIC_{90}$ correlated with 97% protection, whereas plasma concentrations $< PAIC_{90}$ resulted in 75% protection. Following plasma PK modeling and simulation, the group determined that an 800 mg split i.m. dose administered every 3 months would be carried forward into clinical PrEP trials [76].

4.4 Protease inhibitors

PIs inhibit the protease enzyme responsible for post-translational cleavage of viral proteins. These compounds generally have large molecular weights and exhibit extensive protein binding and hepatic biotransformation via the CYP450 system [77]. These pharmacological complexities, coupled with their post-integration mechanism of action, have resulted in this class being generally overlooked for HIV prevention strategies. Therefore, the literature regarding PIs' mucosal tissue PK is limited. Investigation of the cervicovaginal secretions of HIV-infected women receiving stable HAART revealed that indinavir and darunavir exhibit the highest degree of female genital tract penetration among the PIs. Median (range) trough cervicovaginal fluid:blood plasma ratios of 3.8 (0.99 – 10) and 4.64 (0.8 – 26.93) have been reported for indinavir [52,78]. For darunavir, the median (IQR) AUC cervicovaginal:blood plasma ratio is 1.5 (1.0 – 1.6) [79]. Amprenavir, ritonavir, atazanavir, lopinavir and saquinavir (listed in order of rank from highest to lowest) all exhibit ratios of < 1 [43,52,78]. Of all the PIs, indinavir exhibits the lowest degree of protein binding (54 – 70%), which possibly accounts for its high penetrative capacity [80].

Only darunavir and ritonavir have been studied in the lower gastrointestinal mucosal tissues of HIV-negative men. Following a single dose, darunavir and ritonavir were rapidly detected (within 1 h), reaching peak concentrations by 5 h [57]. Under steady-state conditions, the rectal tissue:blood plasma $AUC_{0-12\,h}$ ratio was ~ 2.7 for darunavir and 27 for ritonavir [57].

The potency and high genetic barrier to resistance exhibited by PIs make them favorable HIV treatment options. It has been previously shown that viral replication can persist in lymphoid tissue despite effective HAART therapy and that this persistence may correlate with decreased ARV exposure [81,82]. Since gut-associated lymphoid tissue may serve as a potential reservoir of persistent viral replication in patients, tissue penetration could be a theoretical consideration in designing ARV regimens, similar to the CNS Penetration-Effectiveness (CPE) score proposed by the CHARTER Group [83]. However, unlike the CPE score, increased mucosal tissue penetration has not yet been linked to more favorable outcomes.

4.5 Entry inhibitors

Maraviroc is a chemokine receptor antagonist that prevents the virus from binding to the CCR-5 co-receptor on $CD4^+$ T cells. Currently, it is the only clinically approved agent in its class. Maraviroc concentrates in the female genital tract [19]. In a study of 12 healthy volunteer women, $AUC_{0-12\,h}$ in cervicovaginal fluid was 3645 (1785 – 7505) $ng*h*ml^{-1}$ after a single 300 mg dose. After twice-daily dosing for 7 days, cervicovaginal fluid and vaginal tissue $AUC_{0-12\,h}$ was 7500 (3078 – 9090) and 4857 $ng*h*ml^{-1}$, respectively [19]. High concentrations of maraviroc were found in the rectal tissue of HIV-negative men both after single and multiple dosing at 300 mg doses [84]. Composite AUC ratios in the rectal tissue were 7.5- (single dose) to 26-fold (multiple dose) higher than blood plasma. The maraviroc accumulation ratio from single to multiple dosing in plasma and rectal tissue was 1.4 and 4.9, respectively, which suggests an additional mechanism of increased drug exposure in colorectal tissue. Since maraviroc's bioavailability is 23% [85], it is likely that local, luminal concentrations of maraviroc passing through the gastrointestinal tract contribute to increased rectal tissue exposure. A recent Phase I, single-dose PK study also found a 10-fold higher exposure of maraviroc in rectal tissue as compared to cervicovaginal tissue [70]. Since maraviroc is a substrate for the efflux transporter MDR1, and this transporter demonstrates 5.5-fold higher mRNA expression in vaginal tissue [27], it may be responsible for the differential penetration between female genital tract tissue and colorectal tissue.

Maraviroc has also been incorporated into silicone elastomer vaginal rings for use as a topical microbicide in combination with dapivirine. This system that contains 100 mg maraviroc and 25 mg dapivirine was recently studied as part of a Phase I clinical trial in 24 HIV-negative women who wore the ring for 28 days. Whereas dapivirine was detected in all 24 cervical tissue samples (mean concentration of 1.6 µg/ml), only 4 of 24 (16.67%) samples exhibited detectable concentrations of maraviroc [61]. This low release of maraviroc has resulted in reformulation efforts, which are ongoing [86].

5. Expert opinion

ARV-based prevention has previously demonstrated a high level of effectiveness in the context of mother-to-child transmission with nevirapine and zidovudine [87]. These prevention approaches have been explored for their potential to offer efficacious, relatively discreet, user-controlled HIV prevention options to adults at high risk of HIV acquisition. Because HIV is primarily transmitted through mucosal fluids, and early infection occurs within mucosal tissues, characterizing

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PK behavior within these peripheral compartments is important for optimizing PrEP and TasP initiatives.

Given that the PrEP end-user population is healthy adults, the ideal PrEP agent would have minimal side effects, a low drug interaction potential and allow for infrequent or as-needed dosing. Oral dosing formulations for two NRTIs (tenofovir and emtricitabine), raltegravir and maraviroc all exhibit excellent capacity to penetrate into mucosal tissue with concentrations that either match or exceed blood plasma concentrations with single and multiple doses. These agents' penetrative ability coupled with relatively uncomplicated drug interaction and side-effect profiles make them good candidates for PrEP. Also, alternative ARV formulations, such as 1% tenofovir gel and the dapivirine ring, are designed to achieve high local tissue concentrations and low plasma exposure. These formulations offer intermittent or reduced frequency dosing options.

In general, NNRTIs and PIs exhibit a low degree of penetration into tissue. Although complicated drug interaction and adverse events profiles impede their use in PrEP, NNRTIs and PIs are widely used in HIV treatment. Drugs within these classes that demonstrate extensive penetration, such as darunavir and etravirine, may provide some theoretical benefit to TasP approaches.

Last, the PK data demonstrating preferential distribution of certain ARVs, such as tenofovir, into the colorectal mucosa relative to the female genital mucosa strongly support the notion that the site of HIV exposure determines the degree of protection achieved by a PrEP regimen. This knowledge can be used to tailor PrEP strategies to the target end user. For instance, future PrEP trials or marketing initiatives directed at women are more likely to benefit from rigorous adherence counseling and intervention given that the female genital tract exhibits a smaller PK buffer for missed doses. In contrast, investigations of intermittent Truvada® PrEP regimens for MSM are strongly supported by PK data. This concept was recently confirmed with the success of the IPERGAY trial, which investigated an intermittent dosing regimen before and after sex in MSM [88].

Understanding the PK of ARVs in the mucosal tissue represents only one aspect of optimizing ARV use for HIV prevention. A thorough characterization of target concentrations required for protection and subsequently well-defined PK/PD relationships represents another important aspect of defining dosing strategies [89]. However, this has been inconsistently defined for ARV prevention. One reason is the lack of consensus on appropriate preclinical models of infection for defining concentration–response relationships. The limitations of *in vitro* and *ex vivo* models have been reviewed in detail elsewhere [90,91], but include a lack of tissue architecture and relevant microenvironment, and the use of clinically appropriate virus and viral inoculum.

Using clinical trial data from the CAPRISA004 study, Abdool Karim *et al.* determined that women with cervicovaginal fluid tenofovir concentrations of > 1000 ng/ml exhibited a higher degree of protection (74%) than those with < 1000 ng/ml (14%) [92]. Whether this target tenofovir concentration is also required for oral dosing strategies remains to be determined. Using data generated from the iPrEx study, Anderson *et al.* have proposed an EC_{90} concentration of 16 fmol TFVdp/million PBMCs with oral Truvada® dosing in MSM [93]. However, the concentrations used in this approach were derived from cryopreserved PBMCs which can lose up to 60% of TFVdp compared to traditional methods of cell isolation [93,94]. Therefore, this estimate likely underestimates the true effective concentration of TFVdp in PBMCs that results in colorectal tissue protection. Given the PK differences between the female genital and lower gastrointestinal tracts, it is also unlikely that these results can be extrapolated to a female population.

One recent preclinical and clinical approach to determine the concentration versus response relationship for tenofovir and emtricitabine measured intracellular metabolites (TFVdp and FTCtp) relative to competitive natural substrates of reverse transcriptase (deoxyadenosine triphosphate and deoxycytidine triphosphate) [95,96]. When combining these data into a mathematical model to simulate mucosal tissue exposure achieved by various doing strategies, 80% protection from HIV transmission was predicted for the female genital tract with daily Truvada dosing and 90% protection was predicted for the lower gastrointestinal tract with two doses per week. These predictions are in close agreement with observational data from clinical trials [10,11].

In the absence of well-defined efficacy target concentrations, important inferences can still be made from the available mucosal tissue PK data. Although it is not clear which anatomical locations should be preferentially targeted to reduce HIV transmission (e.g., mucosal tissues, regional lymph nodes), ARVs with mucosal tissue concentrations above the viral IC_{90} may be considered preferable for both PrEP and TasP. Since the extent of ARV mucosal tissue penetration is unpredictable, tissue PK data are essential for optimizing dosing strategies for HIV prevention.

Declaration of interest

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Review article

Drug transporters in tissues and cells relevant to sexual transmission of HIV: Implications for drug delivery

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ABSTRACT

Efflux and uptake transporters of drugs are key regulators of the pharmacokinetics of many antiretroviral drugs. A growing body of literature has revealed the expression and functionality of multiple transporters in female genital tract (FGT), colorectal tissue, and immune cells. Drug transporters could play a significant role in the efficacy of preventative strategies for HIV-1 acquisition. Pre-exposure prophylaxis (PrEP) is a promising strategy, which utilizes topically (vaginally or rectally), orally or other systemically administered antiretroviral drugs to prevent the sexual transmission of HIV to receptive partners. The drug concentration in the receptive mucosal tissues and target immune cells for HIV is critical for PrEP effectiveness. Hence, there is an emerging interest in utilizing transporter information to explain tissue disposition patterns of PrEP drugs, to interpret inter-individual variability in PrEP drug pharmacokinetics and effectiveness, and to improve tissue drug exposure through modulation of the cervicovaginal, colorectal, or immune cell transporters. In this review, the existing literature on transporter expression, functionality and regulation in the transmission-related tissues and cells is summarized. In addition, the relevance of transporter function for drug delivery and strategies that could exploit transporters for increased drug concentration at target locales is discussed. The overall goal is to facilitate an understanding of drug transporters for PrEP optimization.

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Contents

1. Introduction . . . 682
2. Critical barriers to achieving sufficient tissue drug concentration in pre-exposure prophylaxis (PrEP) . . . 683
   2.1. Barriers to achieving high ARV concentrations in sexually transmitted tissues and cells . . . 683
   2.2. The necessity of better understanding critical determinants of tissue drug exposure . . . 683
3. Drug transporters in sexual transmission-related tissues and cells . . . 684
   3.1. Expression and function of drug transporters in sexual transmission-related tissues and cells . . . 684
      3.1.1. Transporters in FGT (uterus, endocervix, ectocervix, vagina) . . . 684
      3.1.2. Transporters in colorectal tissues (Colon, colorectum) . . . 685
      3.1.3. Transporters in HIV target immune cells . . . 686
   3.2. Summary . . . 687
4. Drug transporters in the pharmacokinetics of ARVs . . . 687
   4.1. ABC transporters in the pharmacokinetics of ARVs . . . 687
      4.1.1. ABCB1 (P-gp) . . . 687
      4.1.2. ABCCs (MRPs) . . . 688
      4.1.3. ABCG2 (BCRP) . . . 688
   4.2. SLC transporters in the pharmacokinetics of ARVs . . . 688
      4.2.1. SLC22A family members (OATs and OCTs) . . . 688
      4.2.2. SLC28 and SLC29 family members (CNTs and ENTs) . . . 688

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4.2.3. SLCO family members (OATPs) . . . 689
4.3. Summary . . . 689
5. Drug delivery strategies to increase PrEP efficacy . . . 689
5.1. Physiochemical properties of antiretroviral drugs . . . 689
5.2. Formulation . . . 690
5.3. Nanomedicine and targeting approaches . . . 690
6. Future directions . . . 691
7. Concluding remarks . . . 691
Acknowledgments . . . 691
References . . . 691

1. Introduction

The efficacy of drug delivery to the target tissue and cell depends on several anatomical and physiological barriers such as mucous and cell membranes, protein binding, metabolizing enzymes, and drug transporters. Drug transporters are critical determinants of drug pharmacokinetics (PK), pharmacodynamics (PD), drug–drug interactions and safety. Significant progress has been made in understanding the uptake and efflux mechanisms of transporters, structural requirements of substrates, and exploiting drug transporters by inhibition and targeting to increase drug efficacy and reduce side effects. Kruijtzer et al. found that the co-administration of the breast cancer resistant protein (BCRP) and P-glycoprotein (P-gp) inhibitor, GF120918, resulted in a significant increase in the systemic exposure of oral topotecan. The apparent oral bioavailability increased from 40.0% without to 97.1% with GF120918 [1]. Studies have shown to use P-gp inhibitors to improve oral bioavailability and brain penetration of P-gp substrate drugs [2,3]. Although preclinical studies proved efficacy of transporter inhibitors [3], limited clinical success has been achieved mostly due to their non-specific inhibition leading to increased side effects. Newer strategies such as targeted nanoparticles are being developed to restrict transporter inhibition to the target of interest. It is well established that transporters are involved in antiretroviral (ARV) drug absorption and tissue exposure [4,5]. However, transporters relevant to sexual-transmission related tissues and cells, namely, female genital tract (FGT), colorectum, and immune cells are less studied. Because these tissues and cells are first exposed to human immunodeficiency virus (HIV-1) through sexual transmission, the transporters expressed in these targets could be highly relevant for prevention of HIV-1 acquisition through prophylactic strategies.

HIV pandemic continues to be a worldwide public health problem [6]. There has been a steady increase in the number of people living with HIV worldwide [6]. In 2012 alone, 1.6 million people died of acquired immunodeficiency syndrome (AIDS) globally, and the number of people living with HIV was estimated to be 35.3 million in 2012 [6]. Although current therapies can suppress the AIDS symptoms, they are not able to eradicate the virus in HIV-positive patients [7]. As a result, more than 2 million people are newly infected every year. Therefore efforts must be made to prevent the acquisition of HIV in healthy populations.

Sexual transmission is the cause of a vast majority of new HIV infections in sub-Saharan Africa, where 70% of global new infections occur. HIV sexual transmission can occur via vaginal as well as anal intercourse. Although approaches such as use of condoms, male circumcision, and reduction in the number of sex partners have been effective in reducing the acquisition rate of sexually transmitted HIV [6,8,9], effective implementation of these approaches is challenging. Currently, pre-exposure prophylaxis (PrEP) has been considered a self-controlled, promising strategy in the prevention of HIV sexual transmission. PrEP

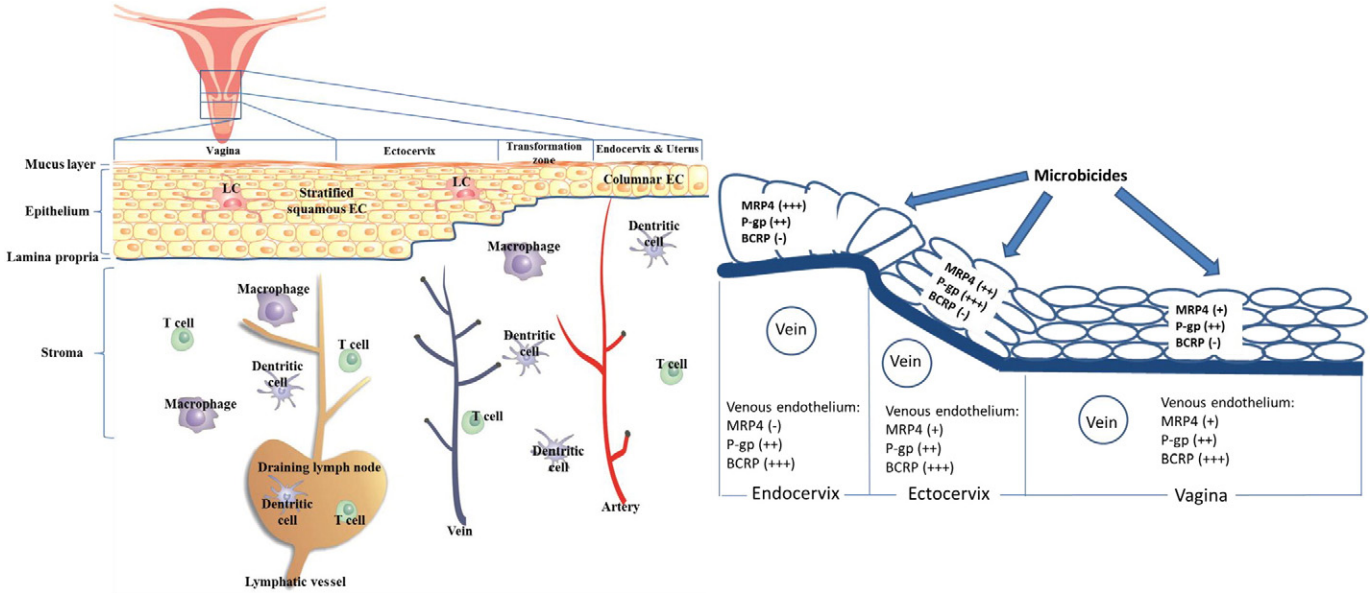

Fig. 1. Anatomy and transporter expression in the female genital tract. The FGT that is a potential site of sexual HIV transmission includes uterus, endocervix, ectocervix, and vagina. A mucus layer covers the epithelia of all these tissue segments, serving as a physical barrier to vaginally administered drugs. Stratified, squamous epithelial layers line the vagina and ectocervix, and a single-layer of columnar epithelial cells lines the endocervix and uterus. The epithelial layers and stroma are separated by the collagen-rich laminar propria. The CD4+ T cells, dendritic cells and macrophages are HIV target cells, and they are distributed in epithelial layers, stroma, and draining lymph nodes. The invading HIV particles can infect these immune cells and establish local tissue infection, expansion, and progress to systemic dissemination. Blood vessels (veins, arteries) and lymphatic vessels mediate the distribution of drug between the tissues and systemic compartments (circulating blood and lymph). LCs, Langerhan's cells, which are the dendritic cells residing in peripheral tissues. EC, epithelial cells. The figure is based on Zhou et al. [62].

includes the use of ARVs such as entry inhibitors (EIs), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and integrase inhibitors (IIs), which target the early steps of HIV life cycle before the integration of viral DNA into host genome [10–13] to prevent HIV infection in uninfected populations. The immune cells in female genital mucosal tissues such as T cells, macrophages, dendritic cells (DCs) with positive expression of CD4 receptor on cell surface are initially infected (Fig. 1) [14,15]. Consequently, PrEP drug candidates must reach submucosal immune cells to prevent the formation and local expansion of the "founder population" of the virus at the initial stage of viral exposure. These target immune cells are distributed at different depths throughout the female genital and colorectal tracts, therefore, achieving high immune cell concentration requires rapid and deep penetration of a sufficient amount of ARV drug into the tissue. Currently, most PrEP products are at different stages of development, and they can be administered via vaginal, rectal, oral, subcutaneous, or intramuscular routes. The rationale for using topically administered products is that vaginal and rectal administration could result in better accumulation of administered drugs in the cervicovaginal tissues, colorectal tissue, and immune cells associated with these tissues, compared to the oral and systemic routes [16]. In addition, topical administration results in lower systemic drug levels compared to oral and other administration routes, and is less likely to cause systemic side effects [17,18].

It has become a consensus in the field that cervicovaginal or colorectal tissue drug exposure is the key to the effectiveness of PrEP [19,20]. One of the major barriers for successful prevention of HIV acquisition using PrEP is the inability to achieve high concentrations of ARVs in tissues and cells relevant to sexual transmission of the virus. Besides physicochemical properties of drugs, anatomical, and physiological barriers, drug uptake and efflux transporters play a key role in the anti-HIV drug delivery to target tissues and cells [21]. Drug transporters are one of the major determinants of suboptimal tissue concentrations of ARVs [22]. Transporters can contribute to drug disposition, drug–drug interactions, emergence of resistant viral strains, toxicity, and inter-individual variability in ARVs efficacy [21,23]. Transporters relevant to ARVs are expressed in different body compartments such as gastrointestinal tract, cells in systemic circulation, tissues and cells involved in sexual HIV transmission, and organs involved in drug metabolism and elimination [21,24]. Specifically, drug transporter expression and functionality in human genital tract and colorectal compartments can significantly influence drug delivery to target tissues and cells that are involved in sexual HIV transmission. However, limited literature is available for the expression profile and functionality of transporters in specific anatomical compartments such as colorectum and vagina. This information is crucial in understanding the effect of transporters on drug delivery and ultimately safety and efficacy. An enhanced understanding of transporter-dependent tissue exposure could help delineate the causes of intra-individual and inter-individual variability in PK/PD, which are often observed with ARVs [25–27]. This new knowledge will facilitate the development of novel drug delivery strategies that enhance tissue drug exposure as well as reduce side effects. This review provides a comprehensive overview of transporters expressed in colorectal and female genital tissues and immune cells, their endogenous functionality, and their relevance to anti-HIV drug delivery, PK, and efficacy.

2. Critical barriers to achieving sufficient tissue drug concentration in pre-exposure prophylaxis (PrEP)

2.1. Barriers to achieving high ARV concentrations in sexually transmitted tissues and cells

It is challenging to achieve high ARV exposure in the tissues related to HIV sexual transmission, due to the physicochemical, physiological, and behavioral reasons. A number of PrEP drug candidates are either very hydrophobic (e.g., dapivirine) or quite hydrophilic (e.g., tenofovir, maraviroc). When administered topically, the absorption of these drugs can be limited due to the permeation barriers posed by the mucus layer and the epithelium (tight junctional proteins) of the cervicovaginal and colorectal tracts [28], as well as the blood and lymphatic drainage systems which serve to extract the drug from the tissue (Fig. 1). This is especially true for hydrophilic drugs that have low binding affinity to tissue proteins and cannot efficiently penetrate the plasma membrane. A Phase I trial (MTN-013/IPM-026) evaluating vaginal ring products containing dapivirine (hydrophobic) and maraviroc (MVC) (hydrophilic) revealed that MVC was not absorbed well based on the blood and cervical tissue concentrations and it did not exert protective effects compared to dapivirine [29]. Another extensively studied hydrophilic microbicide candidate, tenofovir (TFV), exhibited low penetration efficiency given the millimolar level drug concentration in the vaginal fluid but micromolar level concentration in the vaginal tissue [16].

Although hydrophobic drugs can be absorbed more efficiently compared to the hydrophilic drugs, their distribution within the tissues can be potentially problematic [30,31]. A study using excised human ectocervical tissue showed that film-released dapivirine mostly accumulated around the basal layers of cervicovaginal epithelium infiltrating the upper part of the stroma, while a small amount of drug reached the deep stroma [32]. This may be true for other hydrophobic drugs, which extensively bind to tissue proteins and consequently lead to poor penetration into deep stroma.

The low adherence often encountered with the use of once-daily products is another factor that can reduce ARV drug efficacy. Inconsistent efficacy results have been reported from different trials of 1% tenofovir gel [33–36], and the suboptimal results in CAPRISA 004, VOICE, and FEM-PrEP clinical trials have been attributed to poor patient adherence [13]. While long-acting dosage forms such as intravaginal rings are being actively pursued to resolve the adherence issue [13, 37–41], it is crucial to understand physiologic determinants of tissue drug exposure for the optimal exposure and efficacy outcomes.

As for the PrEP utilizing orally administered ARVs, the tissue distributions of some ARVs differ markedly in their ability to penetrate into mucosal tissues and fluids [22,42–44]. It has been observed that highly protein-bound drugs have lower tissue concentration i.e., lower tissue-to-plasma ratios, because only the free drug portion not associated with plasma albumin or α_1-acid glycoprotein can distribute into tissues. However, the tissue distribution cannot be solely explained by protein binding affinity of the ARVs; other mechanisms may play a role in the distribution of ARVs to cervicovaginal tissues [22,43]. Additionally, large inter-individual variability has been observed in the FGT drug concentration, for drugs within the same class and across different classes [22]. The variability in drug exposure in FGT is generally greater than that observed in plasma [22,43]. Therefore, a better understanding of the causes underlying the low tissue penetration and large inter-individual variability will facilitate the achievement of sufficient drug exposure after oral dosing.

2.2. The necessity of better understanding critical determinants of tissue drug exposure

Currently, the effective in vivo drug concentration remains unknown for many microbicide drug candidates being evaluated [19]. The consensus on PrEP drug delivery is to achieve the maximally tolerated drug concentration in the tissues and cells relevant to HIV sexual transmission [19]. For the ARVs with poor ability to penetrate tissues, an easy way of increasing the cervicovaginal tissue drug exposure is to increase the dose. However, high topical dose of some drugs is associated with altered vaginal microbiome and/or genital tract irritation [45], and poses challenges to the manufacturability and applicability of microbicide products given limited drug loading capacity of topical products. For the oral PrEP, high doses of ARVs may cause a variety of toxicities to the liver, kidney, and cardiovascular systems [46]. The necessity and challenges of achieving high drug exposure in a safe and effective

manner call for a need to better understand critical determinants of ARV exposure in the tissues and cells relevant to HIV sexual transmission. Notably, preclinical studies have demonstrated that the topically administered products could result in biologically relevant levels of dapivirine [47,48], MVC [49], and TFV [50–52] in the tissues related to HIV sexual transmission. The models used in these studies include human cervical and vaginal tissue explants, rabbits, and non-human primates. The wide use of these models in microbicide testing warrants the parallel investigation of the critical determinants of tissue drug exposure in these models. This is especially important given the apparent inconsistency between preclinical and clinical testing results for some drugs (e.g., MVC). This information can lead to better design of PrEP products and significant improvement of drug delivery to target tissues and cells.

In addition, multiple physiological barriers exist and limit drug penetration into the tissues. The FGT and colorectal tissue are covered with a mucus layer that can entrap topically administered drugs and reduce their contact with epithelial cells. In FGT, the vagina and ectocervix are lined with multi-layer squamous epithelial cells (up to 40 layers) [53,54], which serves as a permeation barrier to topical drug administration (Fig. 1) [28]. The endocervix, uterus, and colorectum are lined with single-layer columnar epithelial cells (Fig. 1), but the tight junction expression is more intense in these epithelial layers compared to the ectocervical and vaginal epithelia [55]. Since the target immune cells are distributed throughout the female genital and colorectal tracts [56], the administered drugs must penetrate deeply and accumulate in sufficient concentration in the tissue-associated immune cells.

3. Drug transporters in sexual transmission-related tissues and cells

A growing body of evidence has demonstrated that some efflux and uptake transporters are expressed in FGT, colorectal tissue, and immune cells (Table 1). Studies have demonstrated that efflux transporters of blood-derived immune cells limit the intracellular accumulation and/or efficacy of ARVs [57,58]. Although there is little direct evidence showing the functional role of these transporters in drug PK in the tissue, clinical PK studies comparing drug exposure in different physiologic compartments have implicated the presence of active drug transport mechanisms in the FGT [44,59]. Some transporter substrates, including MVC, lamivudine, and emtricitabine, are reported to be preferentially distributed in cervicovaginal tissues and/or fluids at higher levels than in plasma, after oral administration [44,59]. These differential drug distribution profiles cannot be solely explained by the differences in plasma protein binding and passive permeation, as drugs with similar plasma protein binding and permeability displayed different capabilities of distributing into cervicovaginal tissues [43,44,59,60].

Thompson et al. summarized published literature of 58 drugs and developed a mathematical model to predict a compound's cervicovaginal tissue penetration ratio (TPR) based on physicochemical properties and protein binding. The analysis showed that the TPR of orally administered drugs was significantly associated with its probability of being the substrate of two efflux transporters, multidrug resistance-associated protein 1 and 4 (MRP1 and MRP4) [61]. This analysis highlighted the possibility of in vivo functional role of these transporters, in the blood-to-tissue distribution of ARVs in lower FGT. Several efflux and uptake transporters are also expressed in human FGT, colorectal tissues, and immune cells (Table 1). In this section, expression, function and regulation of drug efflux and uptake transporters are reviewed and discussed.

3.1. Expression and function of drug transporters in sexual transmission-related tissues and cells

Drug transporters are transmembrane proteins that control the movement of substrates in and out of the cells. They are localized on plasma membrane or the membrane of intracellular organelles such as mitochondria [63]. Numerous studies have established the role of transporters in controlling drug accumulation, and in maintaining the homeostasis of endogenous substances in multiple tissues/organs including brain, liver and kidney [58,63–71], and in various types of cells including epithelial cells [62,72], endothelial cells [73], and immune cells [57,74]. ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies are transporters that are most relevant to efflux and uptake of ARVs respectively. The subfamilies of ABC and SLC transporters are reviewed elsewhere [75].

3.1.1. Transporters in FGT (uterus, endocervix, ectocervix, vagina)

Transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multi-drug resistance proteins (MRPs) from ABC

Table 1
Expression and localization of transporters in human tissues and cells relevant to HIV sexual transmission.

Compartments	Tissue/cell type	mRNA/protein expression	Cellular localization	Ref.
Female genital tract	Uterus	(+): P-gp, BCRP, MRP1, MRP4, MRP5, MRP7, OCT3, CNT1, ENT1,2 OATP3A1	Glandular cells of endometrium (P-gp, MRP4, CNT1, ENT1,2); endometrial capillaries (P-gp)	[76,81,83,88–90]
	Endocervix	(+): P-gp, BCRP, MRP2, MRP4 (−):OAT1,3, OATP1B1	Glandular epithelium and submucosal immune cells (P-gp, MRP2); vascular endothelium of stroma (BCRP)	[80,90–92]
	Ectocervix	(+): P-gp, BCRP, MRP1, MRP4, MRP5, MRP7, OAT2, OCT1,2,3, ENT1,2 (−): MRP2, MRP6, OAT1, 3, OATP1B1	Squamous epithelium and stromal tissue (P-gp); vascular endothelium of stroma (BCRP)	[24,89,90,92,93]
	Vagina	(+): P-gp, BCRP, MRP1, MRP2, MRP3, MRP4, MRP5, MRP6, MRP7, OAT2, OCT1,2,3, ENT1,2 (−):MRP3, 6, OAT1, 3, OATP1B1	Squamous epithelium and submucosal immune cells (P-gp and MRP); deeper layers of epithelial cells and vascular endothelial cells (MRP3,4).	[24,77,80,91,94]
Colorectal tissues	Colon	(+): P-gp, BCRP, MRP1, MRP2, MRP3, MRP4, MRP5, MRP7 (−): MRP2, OAT1, 2, 3,	Columnar epithelium (P-gp, BCRP, MRP3,4,5, OATP2B1,4A1,4C1); secretory epithelial cells (goblet cells) (MRP3,4, OATP1C1,2B1,4C1)	[76,89,93,95–109]
	Colorectum	(+): P-gp, MRP2, MRP4 (−):OAT1,3, OATP1B1	Columnar epithelium and submucosal immune cells (P-gp and MRP2)	[91,94,106]
Immune cells	Within cervicovaginal and colorectal tissues	(+): P-gp, MRP2, MRP4 (−): OAT1, 3, OATP1B1	A subset of submucosal lymphocytes and monocytes in cervicovaginal and colorectal tissues (P-gp and MRP2)	[94,95]
	Blood-derived	PBMCs (+): P-gp, BCRP, MRP1,2,3,4,6,7, OCT1, OAT2, ENT1,2, OATP2B1, 3A1		[89,110–127]
		Lymphocytes or CD4+ T cells (+): P-gp, BCRP, MRP1,2, OATP1A2, OATP3A1		[58,89,91,94,122,128–146]
		Macrophages (+): P-gp, MRP1,4,5,7 CNT1,2,3, ENT1,2, OCT1,3, OCTN1,2		[122,147–149]
		Dendritic cells (+): P-gp, CNT1,2,3, ENT1,2, OCT1,3, OCTN1,2		[122,150]

superfamily, and organic anion and organic cation transporters (OATs and OCTs), equilibrative transporters (ENTs), and organic anion-transporting polypeptides (OATPs) from SLC superfamily (Table 1) have been experimentally demonstrated to transport ARVs and are most frequently studied transporters in ARV PK. As listed in Table 1, P-gp, BCRP, MRP4 are consistently expressed along the entire FGT. BCRP appeared to be the most highly expressed transporter among ABC transporters in FGT. The uterine BCRP mRNA level was found to be the highest among all types of female reproductive tract tissues [76]. The BCRP mRNA level in endocervix, ectocervix, and vagina was comparable to or higher than that in liver [24,92]. Several other MRP isoforms are also expressed at different segments of FGT (Table 1). There are contradictory reports regarding the expression of MRP3. This is mainly due to the difference in detection methods and criterion of "positive expression." For example, Zhou et al. reported MRP3 mRNA level undetectable in human ectocervix and vagina, as the RT-PCR products for this transporter did not show visible bands in the agarose gel after electrophoresis [24]. However, Gunawardana et al. detected MRP3 protein in sporadic basal epithelial layers of human vagina using immunofluorescence staining, thus considering MRP3 as positively expressed [77]. Our group has also recently studied the mRNA expression and protein localization of three efflux transporters, P-gp, MRP4, and BCRP, in the lower genital tract of premenopausal women and pigtailed macaques [62]. The immunohistochemical (IHC) staining showed that P-gp and MRP4 were localized in multiple cell types including epithelial cells and vascular endothelial cells in the female lower genital tract while BCRP was predominantly localized in the vascular endothelial cells. Compared to human tissues, the macaque cervicovaginal tissues displayed comparable expression and localization patterns of the three transporters, although subtle differences were observed between the two species. Transporter expression was also studied in cervicovaginal cell lines such as End1/E6E7, Ect1/E6E7, and VK2/E6E7 [78].

Compared to the ABC transporters, SLC transporters have been generally less studied in the FGT. The most relevant SLC transporters OAT1, OAT3 and OATP1B1 were found to be not expressed in endocervix, ectocervix, and vagina [24], which are major sites of PrEP drug action. The type of cells that harbor transporter proteins varies among different segments of FGT (Table 1).

Transporter functionality has been explored in few studies. Schinkel et al. found that Mdr1a/1b double knockout resulted in 2.2-fold increase in digoxin accumulation in mouse uterus, after intravenous administration [79]. Using a rabbit model, Grammen et al. demonstrated that the systemic uptake of P-gp substrate talinolol after vaginal administration could be significantly increased with the co-administration (vaginal route) of the P-gp inhibitor verapamil [80]. These studies indicate that ARV drug delivery to FGT and systemic circulation can be increased with the concomitant use of transporter inhibitors.

The regulation of transporter expression and localization in FGT has also been reported. In premenopausal women, the cellular localization, staining intensity, and percentage of P-gp in uterine tissue varied with the phase of the menstrual cycle. The staining intensity increased from early proliferative to late proliferative endometrial stages while it diminished from early secretory to late secretory phase.[81] P-gp expression parallels that of nuclear progesterone receptor expression in the normal human endometrial cycle and early gestational endometrium. In addition, P-gp expression corresponds to rising plasma and tissue levels of progesterone as well as to morphologic changes in the endometrial glandular epithelium associated with the marked development of the secretory apparatus [81]. In addition to P-gp, the MRP4 protein expression was found to undergo cyclic change during the estrous cycle in intact bovine endometrial tissues [82]. In the uteri from peritoneal endometriosis patients, Gori et al. reported that MRP4 was expressed in eutopic and ectopic endometrium, where it was overexpressed in peritoneal lesions and localized in the cytoplasm of glandular epithelial cells. Attenuation of MRP4 mRNA and protein levels in endometriotic epithelial cells by Lipoxin A4 was found to be mediated through estrogen receptor α, as examined using receptor antagonists and small interfering RNA [83]. Our preliminary data indicate that P-gp and Mrp4 expression at mRNA and protein levels is regulated by menstrual cycle in a mouse model (data not shown). The cyclic change of transporter expression during the menstrual cycle is presumably a result of the cyclic change of the reproductive hormones (estrogens and progesterone). Hormones have been shown to affect transporter expression and functionality through a variety of mechanisms, and the transcriptional regulation through hormone-responding nuclear receptors is most well studied [84]. In addition to endogenous hormones, synthetic hormonal contraceptives such as Depo-Provera also have the potential to modulate transporter expression, due to their ability to bind nuclear receptors [85]. However, the effect of hormonal contraceptives on transporter expression in cervicovaginal tissues, colorectal tissue, and immune cells has not been reported. This information is important given that hormonal contraceptives are widely used in participants of PrEP clinical trials [86]. For example, in the CAPRISA 004 trial which provided critical evidence of the effectiveness of tenofovir vaginal gel, 80% of participants were on the progestin contraceptives [87]. The effect of the commonly used contraceptives warrant further investigation for better delineation of the mechanisms underlying suboptimal exposure and efficacy of ARVs in PrEP trials.

These studies demonstrate that efflux transporters expressed in FGT could play a functional role on drug absorption. Additionally, the low expression of uptake transporters in FGT concomitant with their expression in tissues/cells involved in drug removal from vaginal lumen suggests that effective drug delivery strategies should exploit efflux transporters to increase the tissue drug concentrations for PrEP-based HIV prevention. Factors relating to transporter expression changes with menstruation, and the anatomical location within the genital tract should be noted. The effect of hormones and hormonal receptor signaling on transporter expression could guide the design of combination drug delivery strategies.

3.1.2. Transporters in colorectal tissues (Colon, colorectum)

As shown in Table 1, P-gp, BCRP, MRPs1-7 are positively expressed in human colorectal tissues at moderate to high levels, compared to other genes expressed in colorectum [58,89,95,100,103,106,108], or compared to the expression level of the same transporter in liver [76]. The detection methods include qRT-PCR, Western blot, and IHC staining. De Rosa et al. compared the expression levels of multiple ABC transporters in the sigmoid colon of HIV-uninfected men [58]. In this evaluation, the ranking of transporter mRNA level was: MRP2 > MRP4 > MRP1 > P-gp > BCRP, and the ranking of transporter protein level was: MRP1 > MRP4 = BCRP > MRP2 > P-gp [58]. However, Zimmermann et al. reported that the rank order of the transporter mRNA level was: MRP3 ≫ MDR1 > MRP4 almost equal to MRP5 > MRP1 > > MRP2 [108]. One possible cause for this difference is the source of tissue samples. De Rosa et al. used tissues from young men with a median age of 39, while Zimmermann et al. used the tissues from both men and women with the average age of 62 [58,108]. Colorectal tissue P-gp, BCRP, MRP2, MRP3, MRP4 were localized on the plasma membrane of multiple cell types, including specialized epithelial cells with secretory/excretory functions, and endothelial cells of capillary blood vessels [93,94,99,104,151]. Furthermore, MRP4 was localized in endoplasmic-Golgi complex and basolateral location in goblet cells [104], MRP3 and MRP5 were localized on the basolateral membrane of glandular epithelial cells [104,152]. The regional distribution of transporters along the intestinal tract has also been studied. Blokzijl et al. reported that P-gp mRNA level in colon was 5-fold lower than the level in terminal ileum [95]. Prime-Chapman et al. examined the mRNA expression of MRP1-6 in human colon and rectum. All the 6 MRPs were detected in the colon, but MRP2 and MRP6 were not detectable in the rectum [106]. The differential expression of the same transporter between colorectum and female genital tract was also reported by Nicol et al. In this study P-gp and MRP2 were expressed at lower levels compared to vagina, while the colorectal MRP4 level was higher than the vaginal level [94]. As to the SLC transporters,

OAT2, CNT1, ENT1 were found to be highly expressed [89], while OAT1, OAT3, OCT2, OCT3 and OATP1B1 were negligible in human colorectum [94,103].

The functionalities of colorectal P-gp and BCRP have been studied using human tissues and preclinical models. Collett et al. studied the permeability of the P-gp substrate UK-338,003 through mouse ileum and colon. The colonic permeability of UK-338,003 was at least 40 times lower than that of ileum, with large asymmetry (basolateral-to-apical permeability was much larger than the apical-to-basolateral permeability). The application of P-gp inhibitor markedly increased the colonic permeability of UK-338,003 to the level comparable to that of ileum. Low apical-to-basolateral permeability of UK-338,003 was also observed in the ex vivo human distal intestine, however the increase in UK-338,003 permeability after the co-administration of P-gp inhibitor was significantly lower than the increase in mouse colon [98]. Enokizono et al. used 4-methylum-belliferone (4MU) as substrate to examine BCRP functionality on the everted mouse colon sacs. A marked reduction in the mucosal secretion clearance of the 4MU sulfate was observed in the colon of BCRP knockout mice compared to that of the wild-type mice [101].

The studies examining the regulation of colorectal transporters have focused on several ABC transporters. The effect of gender on human colonic transporter expression remains unknown, but there is no difference in colonic P-gp mRNA level between male and female mice [153]. The impact of inflammation on transporter expression is different for different transporters. Ulcerative colitis (UC), Crohn's disease (CD), collagenous colitis and diverticulitis cause tissue inflammation and strongly decreased P-gp mRNA and protein expression in colonic epithelial cells and submucosal mononuclear cells. The inflammation also down-regulates BCRP expression [100]. The down-regulation of P-gp is independent of PXR protein level [95], while the decrease in BCRP level was negatively correlated with the interleukin (IL)-6 mRNA level [100]. On the contrary, UC and CD significantly increased MRP1 mRNA expression [95]. HIV infection significantly down-regulated P-gp and MRP2 protein levels in male recto-sigmoid colon, while ARV treatment up-regulated P-gp and MRP2 protein levels [109]. The HIV infection and treatment did not have obvious effect on the expression of MRP1, MRP4 and BCRP [109]. In contrast to ABC transporters, the regulation of SLC transporters by infection or ARV treatment is not well understood. Kleberg et al. reported that neoplasia up-regulated OATP2B1 and OATP4A1 mRNA levels in human colorectum [104].

In addition to the anatomical differences that influence drug absorption, distribution, and elimination between FGT and colorectal tissues, these studies indicate that the transporter expression is also significantly different. The presence of uptake transporters in colorectum could provide a differential tissue exposure, PK, and efficacy when compared to FGT. Therefore, drug delivery strategies targeting FGT and colorectal tissues need to take into account these differences and exploit respective transporter expression and functionalities for effective drug delivery. The influence of pathophysiology such as inflammation on mucosal permeability and transporter expression are additional factors that can effect drug accumulation and elimination in colorectal tissues and cells.

3.1.3. Transporters in HIV target immune cells

Due to the scarcity of human cervicovaginal and colorectal tissues, and the difficulty of purifying immune cells from these tissues, there is a lack of characterization of the transporter expression and activity of the tissue-associated submucosal immune cells. However, there have been abundant reports on peripheral blood mononuclear cells (PBMCs), PBMC-derived immune cell subsets, and immortalized immune cell lines. Numerous studies have revealed the expression profile, functionality, and regulating factors of a number of ABC and SLC transporters in PBMCs, blood-derived lymphocytes or CD4 + T cells, blood monocyte-derived macrophages (MDM), and blood-derived DCs. Table 1 lists transporters expressed in immune cells from peripheral blood, and cervicovaginal and colorectal tissues. Large inter-individual differences in P-gp level have been observed in primary lymphocytes isolated from healthy volunteers [128].

ABC transporters in human blood-derived immune cells have been demonstrated to efflux ARVs and other drugs. The expression and functionality of several transporters were investigated in immune cells using transporter inhibitors. In healthy PBMCs, Janneh et al. reported the inhibitors of P-gp, MRP1 and MRP2 significantly increased the intracellular accumulation of saquinavir in healthy PBMCs [114]. Janneh et al. demonstrated that the MRP inhibitors tariquidar, MK571, frusemide and dipyridamole, as well as protease inhibitors (PIs) ritonavir, amprenavir and atazanavir significantly increased the intracellular concentration of lopinavir [131]. In lymphocytes, Gupta et al. revealed positive efflux activity of P-gp in healthy CD4 + T cells [130]. Clemente et al. revealed that the non-steroidal anti-inflammatory drugs (NSAIDs) blocked MRP4 efflux, increased the intracellular concentration and anti-viral efficacy of several NRTIs including zidovudine (AZT) in peripheral blood lymphocytes (PBLs) [112]. Liptrott et al. revealed the nevirapine concentration in the healthy CD4 + blood cells can be increased by small interfering RNA knockdown of MRP7 [143]. Lee et al. detected MRP1 efflux activity in the CD4 + T cells isolated from healthy subjects [154]. The lymphocyte transporter has also been studied in mouse. Elliott et al. revealed the positive expression and activity of P-gp (Mdr1a/1b), Mrp1, and Bcrp in mouse blood lymphocytes [137]. Schinkel et al. examined P-gp function in mice, and found that the lymph node accumulation of intravenously administered digoxin was significantly increased in Mdr1a/1b double knockout mice, compared to the wild type mice [155]. In human MDMs, the P-gp inhibitor PSC833 and the MRP1 inhibitor probenecid increased the intracellular accumulation of AZT in HIV-infected MDM, and increased the efficacy of AZT and indinavir [148]. Liptrott et al. reported the functionality of MRP7 in effluxing nevirapine from MDMs, using small interfering RNA that specifically knocked down MRP7 [143]. Due to the observed efflux activity of immune cell transporters, the genetic polymorphisms of P-gp, MRP1, MRP4, MRP5 transporters have been shown to correlate with varying intracellular concentration and/or efficacy of substrate drugs including PIs and NRTIs, in PBMCs and blood CD4 + T cells [112,116,127,144].

The effects of disease, drug treatment, and activation status on immune cell transporter expression and activity have been evaluated. The effect of HIV infection depended on the model and cell type selected for examination. In human PBMCs, HIV infection up-regulated mRNA levels of P-gp and MRP1, 4, 5 [127]. In H9 (T cell line) and U937 (monocytic cell line) cells, HIV infection increased the expression of P-gp and increased the efflux of P-gp substrates AZT and daunorubicin [138]. In MDMs, HIV infection increased the mRNA levels of MRP1, 4, 5 [147]. However, Lucia et al. reported that viral load did not affect P-gp expression in the PBMCs of treatment-naive and -experienced patients [118]. In cynomolgus macaques, simian/human immunodeficiency virus (SHIV) infection decreased P-gp mRNA level in PBMCs and lymph node mononuclear cells [115]. Besides HIV infection, myasthenia gravis decreased P-gp efflux function of PBMCs [125], and increased intracellular cholesterol levels markedly up-regulated P-gp activity in PBMCs [126]. The effect of cytokine and ARV/anticancer drug treatment on immune cell transporters appeared to be drug-specific. IL-2 and interferon (IFN)-γ significantly increased the mRNA and protein expression of P-gp, MRP1 and MRP2, and reduced accumulation of digoxin and saquinavir, in the PBMCs of healthy volunteers [117]. In MDMs, IFN-γ increased P-gp mRNA abundance, induced polarized redistribution of P-gp protein in pseudopodia, and increased the efflux activity of P-gp [149]. The anticancer treatment of Kaposi sarcoma using the liposomal formulation of anthracycline doxorubicin up-regulated P-gp in the PBMCs of treatment-naive and -experienced patients [118]. AZT increased the MRP4 expression level in HIV infected T lymphocytes [112]. Despite these up-regulating effects, Lucia et al. reported that highly active antiretroviral therapy (HAART) did not affect P-gp expression in the PBMCs of treatment-naive and -experienced HIV-infected

patients [118]. Agrati et al. reported that ARV treatment did not affect P-gp expression in PBMCs of HIV-infected patients [110]. Bossi et al. found that the PI treatment had no effect on the P-gp expression and activity, and was not linked to the treatment outcome [136]. Chinn et al. observed no significant effect of saquinavir and atazanavir treatments on P-gp expression and activity of lymphocytes isolated from healthy subjects [128]. In addition, Lucia et al. found that treatment with PIs, including ritonavir, saquinavir, nelfinavir and indinavir, reduced the efflux of rhodamine 123 in blood lymphocytes from healthy and HIV infected patients [119]. Lee et al. reported that PIs exerted differential effects [154]. In this study, MRP1 expression and efflux activity in healthy CD4 + T cells were reduced after the treatment of darunavir/ritonavir, but not after efavirenz treatment alone. In SHIV infected cynomolgus macaques, HAART (AZT, lamivudine, and indinavir) accentuated the infection-induced decrease of P-gp mRNA level, in macaque PBMCs and lymph node mononuclear cells [115]. The chemical-induced activation was shown to increase the expression and/or activity of P-gp and ENTs in primary lymphocytes [122,130].

These studies demonstrate that drug transporters in immune cells are functional and they can influence the ARV drug uptake. Furthermore, the effect of concomitant presence of diseases and activating stimuli on transporter expression and functionality suggests possible implications for ARV drug delivery. Although few reports studied immune cells in female genital tissues and colorectum, preliminary evidence indicate expression of efflux transporters (Table 1) [94,95]. It is necessary that new drug delivery methods are developed and utilized in targeting specific immune cells relevant to HIV transmission. Targeting ARVs to specific immune cells such as CD4 + T cells, macrophages, and DCs in blood and lymphatic system has proven efficacious previously [156–159]. These targeting methods could be further improved if transporter-relevant factors are considered.

3.2. Summary

The positive expression of multiple transporters in the tissues and cells relevant to HIV transmission indicates that these transporters may play a role in drug absorption and disposition and PrEP effectiveness. Especially, some important transporters (P-gp, BCRP, MRPs) have been demonstrated to localize in multiple cell types (epithelial cells, secretory glandular cells, vascular endothelial cells) in these tissues, indicating these transporters may affect multiple processes of drug handling in the tissues and cells. The uptake transporters were generally less studied compared to efflux transporters. OCTs, CNTs, ENTs, and OATPs can be detected in some tissues, however the expression profiles of OAT1 and OAT3, which are the major uptake transporters for TFV, were found to have minimal expression in human cervicovaginal and colorectal tissues. The positively expressed transporters may be able to affect the drug PK in all the three compartments in HIV transmission (mucosal fluid, tissue, and blood).

The observation that transporters (e.g., P-gp) can be regulated by exogenous hormones, disease status, and concomitantly used drugs highlights the possibility that the transporter functionality may be variable under different pathophysiological conditions. It has been reported that many factors influence transporter expression and activity (varying level of sex hormones, age, gender, methods of lymphocyte activation, etc.) [57]. Finally, transporter information will help explain and predict the transporter-mediated drug–drug interaction, and inter-individual variability in ARV PK and PD. An important source of variability is pathophysiological factors including age, gender, and disease status, and the effects of these factors on ARV drug disposition and efficacy are mediated partially by transporters.

Contradictory reports have been observed for transporter expression and activity, especially in immune cells. There is a large heterogeneity between different subsets of immune cells, and even within the same subset (e.g., $CD4^+$ T cells) [57]. In addition to the differences in experimental approaches, the potential inherent heterogeneity in immune cells being tested may also contribute to the observed conflicting results for transporter expression and activity in immune cells. However, MRP1 was consistently reported to be highly expressed in human lymphocytes [144, 160], indicating that this transporter may play a consistently significant role in the PK of ARVs in the immune cells. Taken together, there is a clear need for a more insightful understanding of the transporters in tissues and cells relevant to HIV sexual transmission.

4. Drug transporters in the pharmacokinetics of ARVs

4.1. ABC transporters in the pharmacokinetics of ARVs

In humans, the ABC transporter superfamily is further categorized into 7 families, from ABCA to ABCG [161,162]. In this review, emphasis is given to ABCB1 (P-gp) from the ABCB subfamily, ABCC1 to 7 (MRP 1 to 7) from the ABCC subfamily, and ABCG2 (BCRP) from the ABCG subfamily, since they comprise the major efflux pumps for ARVs.

ABC transporters affect the intracellular accumulation of all classes of ARVs, such as MVC (EI), saquinavir (PI), TFV (NRTI), efavirenz (NNRTI), and raltegravir (II). Apart from being the substrates, xenobiotic and endogenous substances could also inhibit transporter activity by competitively binding to domains critical for transporter activity. For example, the PIs (e.g., ritonavir) are potent P-gp inhibitors, which exert their inhibitory effect through competitive binding to the extracellular domain of P-gp [58,63]. Besides marketed drugs, some generally-regarded-as-safe (GRAS) excipients have been shown to potently inhibit ABC transporters, by temporarily depleting intracellular ATP availability and/or reversibly modifying plasma membrane fluidity [63,163].

In tissues with high expression and/or critical localization of transporters, differential tissue distribution patterns of the substrates vs. non-substrates have been observed. In addition, genetic polymorphisms of a number of transporters are associated with inter-individual variability in ARV drug PK [164,165]. Up-regulation or down-regulation of transporter expression/activity could alter ARV drug PK profile and efficacy [22,166]. Co-administration of efflux transporter inhibitors, including ARVs and pharmaceutical excipients, has been shown to be an effective approach to enhance drug exposure in tissues with high levels of transporter expression [167]. Since many drugs can modulate transporter expression and activity, a significant portion of drug–drug interactions is mediated by transporters; more detailed information on this topic can be found in published reviews [21,63,64]. A compilation of efflux and uptake transporters and different classes of ARVs that are shown to be substrates of these transporters is listed in Table 2.

4.1.1. ABCB1 (P-gp)

Many ARVs are substrates of P-gp [58]. All PIs are high-affinity substrates of P-gp [5,168–173]. In addition, the EI, MVC, the NRTIs such as tenofovir disoproxil fumarate (TDF) and abacavir, and some IIs such as raltegravir, can be transported by P-gp. At cellular level, P-gp is localized in epithelial cells, vascular endothelial cells, as well as the immune cells. Therefore, this transporter could affect drug transport across physiologic barriers that are constructed by epithelial cells (e.g., small intestinal enterocytes) and endothelial cells (blood–brain barrier), and affect drug disposition in tissue-associated and circulating immune cells. Due to the wide tissue distribution and broad substrate specificity, P-gp has been recognized as the major efflux pump responsible for cellular resistance to antiviral and anticancer drugs. Among ARVs, some PIs and NNRTIs can inhibit P-gp [174–179]. The plasma area under the curve (AUC) after oral administration of MVC (P-gp substrate) was 3 times higher in P-gp knockout mice compared to the wild type mice [180]. In humans, the concomitant use of P-gp inhibitors atazanavir, ritonavir, saquinavir and ketoconazole have been shown to significantly increase the C_{max} and AUC of orally administered MVC up to 5 fold

Table 2
Summary of the drug transporters that efflux or uptake ARVs.

Antiretroviral drug class	Antiretroviral drugs	BCS Class	ABC transporters	SLC transporters
Entry inhibitors (EIs)	Maraviroc†	3	P-gp	OATP1B1
Nucleoside/nucleotide reverse transcriptase inhibitor (NRTIs)	Lamivudine	3	BCRP	OCT1, OCT2, CNT1
	Emtricitabine†	3	MRP1	
	Tenofovir DF†	3	P-gp	
	Tenofovir†	3	MRP4, MRP7	OAT1, OAT3
	Abacavir	1	P-gp, BCRP, MRP4	
	Zidovudine	1	BCRP, MRP4	OAT1, OAT2, OAT3, CNT1, CNT3, ENT2
	Didanosine	3	BCRP	CNT2, CNT3, ENT1, ENT2
	Stavudine	1	BCRP, MRP5	CNT1
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Efavirenz	2		
	Nevirapine	2	MRP7	
	Delavirdine	1		
	Etravirine	4		
Protease inhibitors (PIs)	Atazanavir	2	P-gp, MRP1, MRP2	
	Ritonavir	2	P-gp, MRP1, MRP2	
	Fosamprenavir	2		
	Amprenavir	2	P-gp	
	Lopinavir	2	P-gp, MRP1, MRP2	OATP1A2, OATP1B1
	Saquinavir	2	P-gp, MRP1, MRP2	OATP1A2, OATP1B1
	Darunavir	2	P-gp	OATP1A2, OATP1B1
	Indinavir	2	P-gp, MRP1, MRP2	
	Tipranavir	2	P-gp	
	Nelfinavir	2	P-gp	
Integrase inhibitors (IIs)	Raltegravir†	2	P-gp, BCRP*	

The drug-transporter interactions listed in this table are adapted from a review by Kis et al. [58]. *Obtained from reference [173]. A number of these drugs are already undergoing clinical testing (denoted by †) toward the development of PrEP products, including MVC, emtricitabine, TFV, TDF, and raltegravir. The currently available transporter inhibitors of ARVs were reviewed previously by O. Kis et al. [58].

[181]. These studies highlight that P-gp plays an important role in the PK/PD of substrate ARV drugs.

4.1.2. ABCCs (MRPs)

MRPs belong to the C family of ABC superfamily, and transport a diverse array of ARVs [58]. The MRP members most relevant to ARVs are MRP1, 2, 4 and 5. MRP1 and MRP2 are shown to transport several PIs, including ritonavir, lopinavir, atazanavir, saquinavir, and indinavir [114,182–185]. MRP1, 2, and 4 also mediate the efflux of many NRTIs. For example, MRP1 mediates emtricitabine efflux from lymphocytes. MRP2, 4, and 5 transport TFV [186–189]. Administration of TFV to Mrp4 knockout mice resulted in more than 2-fold higher TFV concentration in the kidney after intravenous administration [188]. In humans, genetic ABCC4 3463G variants lead to TFV-Diphosphate concentrations in PBMCs, which was significantly higher than that of wild type [116]. MRP5 has been shown to transport stavudine in vitro [187]. NNRTIs and NRTIs including delavirdine, efavirenz, and emtricitabine inhibit several MRPs at clinically relevant concentrations, as revealed in cell culture experiments [190]. In addition, NSAIDs such as indomethacin and ibuprofen are reported to be potent inhibitors of MRP4 [112,191–193], and have been demonstrated to enhance the antiviral efficacy of NRTIs in HIV-1-infected T-lymphocytes [112].

4.1.3. ABCG2 (BCRP)

BCRP belongs to the G family of the ABC superfamily, and transports a number of ARVs from different classes [58]. As demonstrated in lymphocytes and BCRP-overexpressed cell cultures, this transporter confers resistance to many NRTIs, including abacavir, AZT, lamivudine, didanosine, and stavudine [194–197]. In Bcrp-knockout mice, the brain accumulation of abacavir was significantly increased [194]. BCRP genetic polymorphisms exerted an impact on its activity in vitro, however no in vivo correlation was observed between BCRP polymorphisms and the concentrations of AZT- and lamivudine-triphosphates [165]. Although PIs and NNRTIs are not BCRP substrates; [97] they have been demonstrated to be potent inhibitors of BCRP. In vitro assays demonstrated that PIs and NNRTIs such as ritonavir, saquinavir, nelfinavir, lopinavir, delavirdine, efavirenz, atazanavir, and amprenavir showed BCRP inhibition with IC_{50} values in micromolar range [97,98]. It should be noted that the in vivo free plasma concentration of PIs and NNRTIs are usually in nanomolar or micromolar range, thus the in vivo effect of these drugs on BCRP-mediated transport needs further investigation [98]. The BCRP-inhibiting drugs may at least play a role in the absorption process of co-administered drugs that are BCRP substrates, such as a number of NRTIs mentioned above [98].

4.2. SLC transporters in the pharmacokinetics of ARVs

SLC transporters are widely distributed in tissues and cells including liver, kidney, intestine, and immune system cells. This review focuses on the SLC subfamilies most relevant to ARVs, which are OATs, OCTs, CNTs, ENTs, and OATPs.

4.2.1. SLC22A family members (OATs and OCTs)

OATs and OCTs belong to the SLC22 family. They transport the ionized drugs across plasma membranes. In the PK of ARVs, the most extensively studied OATs are OAT1 (SLC22A6) and OAT3 (SLC22A8), which can transport TFV. On the basolateral membrane of renal proximal tubular epithelial cells, these two OATs facilitate the uptake of TFV from blood circulation into the renal epithelial cells, which could be subsequently effluxed by MRP4 into urine. The genetic polymorphisms of renal SLC22A6 are associated with TFV-induced kidney toxicity. OCT1 and OCT2 have been shown to transport several NRTIs including lamivudine and zalcitabine [198]. Among ARVs, some PIs including indinavir, nelfinavir, ritonavir, and saquinavir, can inhibit OCT1 and OCT2 activities in vitro [199].

4.2.2. SLC28 and SLC29 family members (CNTs and ENTs)

CNTs and ENTs belong to the SLC28 and SLC29 families, respectively. They are known to transport nucleoside analogues, including NRTIs. Based on their relative affinities to cellular nucleotides, CNTs are considered as high affinity transporters while ENTs are considered as low

affinity transporters [200]. They are ubiquitously distributed in various types of tissues and cells. Among ARVs, AZT and lamivudine are transported by CNT1 [151,201], and didanosine can be transported by CNT2 [202]. AZT and didanosine can be transported by ENT3, but the affinity of this transporter is low [203]. The NRTIs are well-known substrates of ENTs. For example, didanosine can be transported by ENT1 and ENT2, AZT can be transported by ENT2 [204], and stavudine and zalcitabine can be transported by ENT3 [21].

4.2.3. SLCO family members (OATPs)

The OATPs belong to the solute carrier organic anion (SLCO) family. OATP1A2 and 1B1 are mostly studied OATPs in ARV drug PK. OATP1A2 and OATP1B1 transport PIs and EIs including darunavir, lopinavir, saquinavir and MVC in vitro [205–207]. An OATP1B1 polymorphism, 521T>C, was significantly associated with higher plasma levels of lopinavir and MVC in patients [206,207]. In addition to being transported by OATPs, some PIs are potent inhibitors of OATP1B1 [208,209].

4.3. Summary

The mentioned studies demonstrate that several subclasses of uptake and efflux transporters could affect the PK of ARVs. Furthermore, inhibition studies suggest that these transporters could be major determinants of ARVs absorption. Despite the scarcity of human studies on transporter function in FGT and colorectal tissues, it is apparent that multiple transporters, especially the ARV-relevant efflux transporters, are positively expressed in these tissues, and some are shown to affect the ARV PK and efficacy in these tissue sites. The cumulative effects of tissue and systemic transporter function dictate the drug levels in tissues and cells relevant to the sexual-transmission of HIV. Because specific ARVs have been shown to be inhibitors or inducers of transporter expression, the drug delivery methods should consider potential drug–drug interactions and the opportunities to utilize them as boosting agents. By understanding the factors that could influence drug disposition such as ARV-transporter interaction, expression of major transporters in compartments relevant to the route of delivery, and the influence of drug combinations, effective drug delivery strategies can be designed.

5. Drug delivery strategies to increase PrEP efficacy

The goal of drug delivery for PrEP-based HIV prevention is to increase drug accumulation in target tissues and cells. Studies demonstrate that drug transporters expressed in female genital tissues, colorectum, and immune cells could affect the systemic and tissue levels of ARVs. Therefore, transporter-dependent and transporter-independent drug delivery methods are needed to curb the negative impact of drug transporters. Several factors need to be considered for efficient drug delivery such as physiochemical aspects of ARVs, effect of combination drugs and excipients on transporter function, routes of administration, differential expression of transporters in cells and tissues involved in sexual HIV transmission, and influence of physiological and pathophysiological factors. The effect of drug transporters on tissue/cell uptake of drugs could be minimized by strategies that utilize inhibition, saturation, or circumvention of transporters.

Uptake and efflux transporters relevant to HIV drugs are ubiquitously expressed in the body [21,166]. Therefore, the frequency and extent of drug exposure to transporters is dependent on the route of delivery. Anti-HIV drugs delivered through the oral route can be subjected to transporters in the gastrointestinal tract, immune cells in systemic circulation, and metabolism/elimination organs such as the liver and kidneys. For example, the availability of ARVs in the central nervous system is limited by P-gp expressed in the gastrointestinal tract as well as blood–brain-barrier (BBB) [5]. Local delivery approaches to vaginal and colorectal tissues using gels, tablets, films, vaginal rings, enemas, and suppositories could overcome some of these barriers. Intravaginal and rectal delivery can surpass first pass metabolism thus reducing drug-transporter interaction in the liver. However, patient adherence and inability to deliver uniform doses are potential challenges with insertion-based administration. In addition, genetic polymorphisms of transporters are shown to affect drug PK and contribute to inter-individual variability. Development of novel transporter-independent drug delivery strategies that can maintain sustained intracellular drug concentration should be considered.

5.1. Physiochemical properties of antiretroviral drugs

Drug delivery to target tissues and cells can be governed by the physiochemical properties of ARVs, namely, solubility, permeability, and protein binding. The tissue exposure of administered drugs can be greatly affected by these properties [22]. For a given ARV drug, being an in vitro substrate of a transporter does not necessarily mean that the transporter will play a significant role in the drug's tissue absorption and disposition in vivo. The functional role of transporters is affected by the drug's permeability and solubility [21,210]. The Biopharmaceutics Classification System (BCS) categorizes different drugs into 4 classes according to drug permeability and solubility: Class 1, high permeability, high solubility; Class 2, high permeability, low solubility; Class 3, low permeability, high solubility; Class 4, low permeability, low solubility [210,211]. The BCS classification, and the interaction with efflux transporters and uptake transporters are summarized for FDA-approved ARVs in Table 2.

Class 1 drugs, due to their relatively high permeability, can enter the tissue without the aid of uptake transporters [21,210]. Three NRTIs, AZT, abacavir, and stavudine, belong to Class 1 (Table 2). These drugs can easily saturate the efflux transporters because they can quickly enter cells and accumulate high intracellular concentration. Therefore, the Class 1 drugs, including abacavir, AZT, stavudine and delavirdine, are not likely to be influenced by efflux and uptake transporters even if they are substrates (Table 2) [210].

Class 2 drugs are not likely to be affected by uptake transporters due to high permeability, but they are prone to be influenced by efflux transporters because their low solubility renders them unlikely to saturate efflux transporters [21,210]. The efflux transporters are able to pump the drug back into the lumen (topical administration) or blood stream (oral administration), and the drug can re-enter the tissue and be repeatedly exposed to intracellular metabolizing enzymes. Therefore, a feature of Class 2 drugs is that they are more likely to be subjected to transporter-enzyme interplay [210]. As shown in Table 2, all the PIs, some NNRTIs such as nevirapine, and the IIs raltegravir belong to Class 2. Therefore, these drugs will be affected by efflux transporters.

Drugs from Class 3 and Class 4 require uptake transporters to accumulate in tissues due to their low permeability [21,210]. The effect of uptake transporters is especially evident for Class 3 drugs which have relatively higher solubility [21]. These two classes also have the potential to be influenced by efflux transporters, since they are unlikely to achieve high intracellular concentrations that could saturate efflux transporters [21,210]. As shown in Table 2, five NRTIs including lamivudine, emtricitabine, TDF, TFV, and didanosine belong to Class 3. The EI MVC also belongs to Class 3. Currently only one ARV drug etravirine (NNRTI) belongs to Class 4. These drugs may be influenced by both efflux and uptake transporters.

To summarize, efflux transporters mainly affect drugs from Classes 2, 3, and 4, especially Class 2 drugs. Uptake transporters mainly affect drugs from Classes 3 and 4, especially Class 3 drugs [21,210]. In addition to solubility and permeability, protein binding is also a governing factor for tissue exposure of drugs [22]. It has been demonstrated that highly protein-bound ARVs show reduced mucosal tissue levels, i.e., in colorectal and cervicovaginal tissues. Of note, protein binding can differ between blood plasma and cervicovaginal fluid. For topical PrEP, protein binding within

FGT may impact drug distribution in cervicovaginal tissues, immune cells, and local lymph nodes. Significant protein binding could reduce tissue drug levels because only the free drug form can permeate through the mucosal layers. Therefore, the prediction of the in vivo functional role of transporters in tissue drug exposure will need to incorporate the information of drug solubility, permeability, and protein binding of ARVs, even if the drugs are proven to be transporter substrates in vitro. In addition to BCS classification, the Biopharmaceutics Drug Disposition Classification System that incorporates transporter information should be utilized [210]. This information can guide formulating effective drug delivery systems that impact solubility, permeability, and protein binding, and achieve high target concentrations of ARVs.

5.2. Formulation

It is well known that formulation ingredients impact transporter functionality. For example, pharmaceutical excipients such as cremophor, pluronic P85, Vitamin E TPGS, and polysorbate 80 are potent P-gp inhibitors [163]. Similarly, excipients such as pluronic P85, polyethylene glycol 300, and cyclodextrins are reported to inhibit MRPs [163]. Cremophor EL has shown a dose-dependent increase in oral absorption of saquinavir, which is a substrate for P-gp [212]. Thiolated chitosan, which is used as a mucoadhesive and a permeation enhancing polymer, has been shown to inhibit P-gp [213]. Such multifunctional polymers could increase intracellular drug concentration through efflux transporter inhibition, permeation enhancement, and longer retention via mucoadhesion. Therefore, currently used oral and vaginal formulations for HIV can be modified to include these excipients to modulate the transporter function and increase the efficiency of drug delivery to the target cells. However, it is important to note that formulation excipients such as surfactants could potentially increase the membrane fluidity [214,215], which could ultimately increase the probability of viral entry to the target cells. This aspect is particularly important for products designed for local application in the vaginal and rectal cavities.

Additionally, drug combinations containing transporter inhibitors can be sought to increase intracellular concentrations of drug of interest. Co-administration of multiple ARVs and drugs belonging to other classes has shown effect on transporter function and drug PK [58,216]. HAART combines several ARVs to achieve significant reduction in viral activity [217]. For topical PrEP-based prevention of HIV acquisition, combination drugs have been utilized such as TFV and emtricitabine [218] for vaginal gels, TFV and dapivirine for vaginal gel [219], ring [220], and film [221], dapivirine and MVC [222] for vaginal rings, and TFV and nevirapine [223] for vaginal rings. In addition to the additive and synergistic roles of combination drugs, their effects on transporter functionality can guide the choice of drug combinations. For example, ARVs such as atazanavir, ritonavir, and saquinavir inhibit P-gp and increase oral absorption of MVC [181]. Due to the positive expression of P-gp in tissues and cells relevant to sexual-transmission of HIV, local delivery approaches can also benefit from these drug combinations. In addition to ARVs, NSAIDs have also shown P-gp inhibitory activity [112]. The combination of hormonal contraceptives and ARVs is sought to increase the use of ARV as prophylactic strategy in women [224]. Because sex hormones have been shown to modulate transporters [225], these combinations can also be guided by their effect on transporter function. Alternatively, recent studies indicate that ARVs alter the PK of hormonal contraceptives by either increasing or decreasing AUC [226,227]. In addition to ARV effects on metabolizing enzymes, this effect could be partly influenced by transporter functions. It should be noted that some ARVs are associated with increased expression of efflux transporters or inhibition of uptake transporters, which should be considered for combination drug approach. The potential effects of these combinations on homeostatic roles of drug transporters need to be evaluated. Since the drugs can be substrates, inhibitors, or inducers of transporter function [228,229], the complexity of this interplay needs validation in ex vivo and in vitro studies.

Sustained release formulations are being actively pursued as a means to improve patient product use compliance. Such strategies could also modulate transporter function by maintaining effective concentrations for longer periods, in addition to increasing patient adherence. Formulation methods that can increase drug absorption through solubility and permeability enhancement such as prodrugs, use of surfactants and permeation enhancers could also increase target concentration of ARVs. For vaginal and colorectal delivery, the use of mucoadhesives can increase the dosage form residence time and drug delivery efficacy. These methods can be effectively combined with transporter-function modifying excipients or drugs to achieve and sustain effective drug concentration at the target locales.

5.3. Nanomedicine and targeting approaches

Nanomedicine offers several opportunities to increase and sustain target concentrations of ARVs. Several reviews have been published in this area [230–232]. Compared to drug in solution, drug loaded in nanoparticles can markedly increase the target drug accumulation and sustain drug levels for a longer period of time [233]. Several studies demonstrated the use of nanoparticles to increase oral absorption by inhibition or circumvention of P-gp transporters [234,235]. Beloqui et al. reported nanostructured lipid carriers to increase oral absorption of saquinavir by modifying physiochemical properties such as size, which effectively circumvented efflux by intestinal P-gp [234]. Nanoparticles functionalized with a cell penetrating peptide (TAT) have been utilized to evade P-gp transporters and increase permeability across cell membranes after intravenous administration [236]. This approach can be highly useful to deliver ARVs that are limited by permeability. Similar strategies could be employed to evade drug efflux from tissues and cells involved in sexual transmission of HIV. For vaginal drug delivery, nanoparticles have been shown to increase epithelial penetration and mucoadhesion [237,238].

Immune cells in blood, lymph node, and lymphoid tissues act as reservoirs for HIV and are known to express efflux transporters, which reduce target drug accumulation. Nanoparticles could be utilized to target specific immune cells to increase cellular uptake and circumvent transporters. Several in vitro studies have been reported that utilized nanoparticles as potential vaginal delivery agents to immune cells. Yang et al. investigated antibody-functionalized saquanivir loaded nanoparticles to increase uptake in CD4 + T cells [239]. Specific peptides have also been reported for active targeting of CD4 + T cells [240]. Puligujja et al. reported ritonavir-boosted atazanavir nanocrystals conjugated with folic acid to target monocyte-macrophages [158]. These nanoparticles showed enhanced accumulation in macrophage-rich areas of lymph nodes and lymphoid tissues after intravenous administration. Nanoparticles containing anti-HIV drug combinations were also reported, which showed marked increase in accumulation in mononuclear cells in peripheral blood and lymph nodes [159].

In addition to nanoparticles, efforts were made to utilize prodrugs for targeting uptake transporters or inhibiting efflux transporters. In order to increase the uptake through BBB, Namanja et al. reported a dimeric prodrug of abacavir, which is a known substrate of P-gp. This prodrug inhibited P-gp, while preserving the anti-HIV efficacy in vitro [241]. A dipeptide prodrug of lopinavir was also utilized to circumvent P-gp and MRP2 [242]. Specific transporters are involved in uptake of ARVs. Prodrugs targeting uptake transporters have been successfully implemented to increase oral absorption of drugs such as acyclovir using L-valyl ester of acyclovir (valcylovir) [243]. The prodrug approach could be potentially utilized for ARVs used in oral and topical PrEP. However, prodrugs targeting uptake transporters might be less successful for vaginal drug delivery systems, because current studies indicate that these transporters are sparingly expressed in FGT.

6. Future directions

There is a lack of systematic characterization of transporter expression and localization in the transmission-relevant human tissues and cells, in the context of PrEP. Transporter expression and functionality studies should consider influencing factors such as varying blood hormone level (menstrual cycle stages and menopause), exogenously administered hormones, contraceptives, concomitantly administered drugs, pre-existing local tissue inflammation (e.g., vaginosis), which are frequently encountered by PrEP participants. These factors have been demonstrated to impact transporter expression in metabolic organs, but almost no studies investigated their effect on cervicovaginal and colorectal transporters. The FGT is a highly dynamic tissue due to drastic change in hormone levels during the menstrual cycle. This in turn, results in cyclic change in epithelial thickness, tight junction expression and passive permeability, and may also impact the expression/localization/activity of a variety of transporters, such as P-gp. Therefore, experimental design should reduce sources of variability. For example, female animals in the same group could be synchronized to a fixed stage of their menstrual (estrous) cycle using exogenously administered hormones. The effect of gender on colorectal and immune cell transporter expression is also an important consideration. This effect, if present, may result in differential PK behavior between men and women for the same drug in the same tissue compartment (e.g., colorectum).

The role of transporters, relevant to sexual transmission of HIV, in PrEP PK remains largely unknown. Although some transporters are positively expressed, it is unclear whether they significantly impact drug PK in the critical sites of HIV transmission (lumen, tissue, lymph nodes). In addition to the blood-derived immune cells, the effect of submucosal and lymphoid immune cells on drug PK needs further investigation. The transporter function in female genital and colorectal tissues has been occasionally studied, mostly as a barrier to topically administered drugs intended for systemic absorption. However, it is more important to know whether the transporters could affect drug distribution and retention within the tissues. Moreover, most of the reported transporter functionality studies were conducted in animal models, which have well-known limitations when used in microbicide testing, in terms of interspecies differences in reproductive tract anatomy, transporter expression level, as well as substrate and inhibitor spectra of transporters. All these interspecies differences warrant future human investigations to elucidate the function of cervicovaginal and colorectal transporters in the context of PrEP.

The role of drug transporters in the physiology of transmission-relevant tissues and cells remains elusive. The efflux and uptake transporters expressed in FGT, colorectal tissue, and immune cells also transport endogenous substances (endobiotics) such as sex hormones (e.g., 17-β-estradiol) [244] and hormone derivatives [245], and immune mediators [246,247], which are critical in maintaining the physical and immunological barriers of mucosal tissues. The effect of co-administration of efflux transporter inhibitors and ARVs on tissue/cell homeostatic functions needs to be evaluated. In addition, some transporters have been suggested to directly affect HIV binding to the target cell [142], or participate in immune response such as DC migration [248]. Therefore, the net effect of transporter inhibition on the effectiveness of PrEP drugs remains unclear, and needs to be tested in efficacy models.

Utilizing the existing literature and future studies, there is a need to design drug delivery strategies that modulate transporter function and increase target drug concentrations in select tissues and cells. Although transporter inhibition has been utilized to increase bioavailability and CNS availability of ARVs in preclinical and clinical studies [249,58], it has not been applied for PrEP-based HIV prevention.

There is also lack of studies targeting uptake and efflux transporters to increase drug accumulation in FGT and colorectal tissues. Novel drug delivery approaches utilizing nanoparticles, targeting ligands, and mucoadhesive polymers that affect transporter expression and function need to be developed. Overall, the outcomes from transporter research should be diligently utilized to design next generation drug delivery strategies for prevention of HIV acquisition.

7. Concluding remarks

A number of efflux and uptake transporters are positively expressed in the FGT, colorectal tissues, and immune cells that are susceptible to HIV sexual transmission. Given their high expression levels, and their localization in multiple cell types, it is tempting to speculate that transporters affect the tissue exposure and efficacy of ARVs used in PrEP. In addition, the modulation of transporters by chemical inhibitors, many of which are ARVs or pharmaceutical excipients, may exert beneficial effects on the PK and PD of PrEP drug candidates. However, more studies are warranted to confirm this speculation, and furthermore to identify the factors that could influence transporter expression/activity in the transmission-related tissues and cells. The existing knowledge summarized in this article, combined with the proposed future research, will likely facilitate successful delivery of PrEP drug candidates. Many anti-infective and anti-cancer drugs are also transporter substrates and/or modulators [63,250]. The transporter information in cervicovaginal/colorectal tissues and immune cells will also shed light on the delivery and targeting of these drugs. Ultimately, an improved understanding of transporters in these tissues and cells will benefit large number of women and men that have various health issues.

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A Translational Pharmacology Approach to Predicting HIV Pre-Exposure Prophylaxis Outcomes in Men and Women Using Tenofovir Disoproxil Fumarate ± Emtricitabine

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Abstract

Background: A novel translational pharmacology investigation was conducted by combining an in vitro efficacy target with mucosal tissue pharmacokinetic data and mathematical modeling to determine the number of doses required for effective HIV pre-exposure prophylaxis(PrEP).

Methods: A pharmacokinetic/pharmacodynamic(PK/PD) model was developed by measuring mucosal tissue concentrations of tenofovir, emtricitabine, their active metabolites [tenofovir-diphosphate(TFVdp), emtricitabine-triphosphate(FTCtp)], and competing endogenous nucleotides(dATP, dCTP) in 47 healthy women. TZM-bl and $CD4^+$ cells were used to identify EC_{90} ratios of TFVdp:dATP and FTCtp:dCTP (alone and in combination) for protection against HIV. Monte-Carlo simulations were then performed to identify minimally effective dosing strategies to protect lower female genital tract(FGT) and colorectal tissues.

Results: Colorectal TFVdp was 10 times higher than FGT while endogenous nucleotides were 7-11 times lower. Our model predicted ≥98% of the population achieve protective mucosal tissue exposure by the third daily dose of tenofovir disoproxil fumarate+emtricitabine. However, a minimum adherence of 6/7(85%) doses/week was required to protect FGT tissue from HIV, while 2/7(28%) doses/week was required for colorectal tissue.

Conclusions: This model is predictive of recent PrEP trial results where 2-3 doses/week was 75-90% effective in men but ineffective in women. These data provide a novel approach for future PrEP investigations that can optimize clinical trial dosing strategies.

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Background:

A fixed dose, combination tablet of tenofovir disoproxil fumarate (TDF) 300mg and emtricitabine (FTC) 200mg received FDA approval for HIV pre-exposure prophylaxis (PrEP) on the basis of two trials. Partners PrEP demonstrated 75% efficacy among heterosexual serodiscordant couples[1] and iPrEx demonstrated 44% efficacy among men who have sex with men (MSM) and transgender women.[2] However, two subsequent trials investigating daily TDF±FTC in high risk women (FEM-PrEP and VOICE) failed to demonstrate efficacy.[3, 4] Analysis of plasma drug concentrations from these studies revealed only 24-30% of these women exhibited evidence of recent product use. Yet in a cohort of male participants from the successful iPrEx trial, only 28% had evidence of recent product use.[2] Subsequent analysis revealed drug exposure consistent with 2-3 doses/week achieved 75-90% protection in MSM.[5, 6] These findings pose an important question: *Why are adherence requirements for effective PrEP less strict for men?*

We recently demonstrated exposure of TDF's active metabolite (tenofovir-diphosphate; TFVdp) is 100-fold higher in colorectal tissue relative to female genital tract (FGT) tissue.[7] This may partially explain different adherence requirements in men and women. Another potential factor is the concentration of host cells' endogenous 2'deoxynucleotides analogs, deoxyadenosine triphosphate (dATP) and deoxycytidine triphosphate (dCTP). The active intracellular phosphorylated metabolites of TFV and FTC (TFVdp and emtricitabine-triphosphate (FTCtp), compete with dATP and dCTP for incorporation into the proviral DNA strand to terminate chain elongation.[8] Since dATP and dCTP can be modulated by proinflammatory signaling molecules[9, 10], and mucosal tissues may have environments with altered inflammatory states, it is possible that endogenous nucleotides differ between cervical,

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vaginal and colorectal tissue, and contribute to altered antiviral efficacy of TFVdp and FTCtp. To investigate mechanisms of efficacy within FGT and colorectal tissues, we used a quantitative systems pharmacology approach to predict the probability of protection against HIV with varying patterns of adherence to TDF, FTC, and TDF+FTC.

Methods:

Study Design

We conducted a phase I, single dose, pharmacokinetic investigation of TDF and FTC at 50, 100, or 200% of the treatment dose (NCT01330199). Blood was collected at baseline and over 48hrs and each participant provided one cervical, vaginal, and colorectal tissue sample at 6, 12, 24, or 48hrs post-dose. Drug concentration were quantified in plasma, peripheral blood mononuclear cells (PBMCs), and tissues. The study was conducted in accordance with Good Clinical Practice procedures and all participants provided written informed consent before study entry. Details about the clinical trial, sample processing and analytical methods are provided in the **Supplementary Methods**.

In vitro Concentration vs Response

TZM-bl reporter cells (NIH AIDS Reagent Program, Germantown, MD, USA)[11, 12] and human $CD4^+$ cells were used to determine HIV-1 infection in the presence of tenofovir (TFV) and FTC. 100,000 TZM-bl cells were plated in 6-well, flat bottom culture plates in 2ml of DMEM medium supplemented with 10% fetal bovine serum (FBS) and antibiotics to match the experimental conditions for drug quantification where >100,000 cells were needed. Cells were incubated in TFV (0.05-35µM), FTC (0.03-30µM), or their combination (NIH AIDS Reagent Program) for 24hrs at 37°C. 5µl of HIV-1_{JR-CSF} (NIH AIDS Reagent Program; MOI=0.006) and

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5µl DEAE-dextran were added to each well. This HIV-1_{JR-CSF} dose was selected to ensure infection, as measured by luminescence, was within linear range. After 48hrs, cells were lysed with 500µl of GloLysis Buffer (Promega, Madison, WI, USA), and relative light units (RLU) were measured using a Luciferase Assay System kit (Promega) on a Veritas™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA). Infectivity was normalized to infection in the absence of drug and reported as %inhibition.

PBMCs isolated from buffy coats obtained from New York Blood Center (Long Island City, NY, USA) were sorted for $CD4^+$ lymphocytes using an EasySep™ negative selection kit (STEMCELL Technologies Inc, BC, Canada). Typical cell yields using this kit demonstrate ~96% purity and >90% viability. $CD4^+$ cells were stimulated for 48hrs in RPMI media supplemented with 10%FBS, antibiotics, phytohaemagglutinin (PHA, 5µg/ml), and interleukin-2 (10units/ml). $1x10^6$ $CD4^+$cell/ml were resuspended and incubated in PHA-free, RPMI media as prepared above with either TFV (0.3-10µM) or FTC (0.03-30µM) for 24hrs. Pseudotyped virus was generated using NL4-3.Luc.R-.E-backbone (NIH AIDS Reagent Program)[13, 14] and transfected with *env* expression plasmid from HIV-1_{JR-CSF} using X-tremeGENE HP (Roche Life Science, Indianapolis, IN, USA). Virions were concentrated 10-fold using Lenti-X Concentrator (Clontech Laboratories, Inc., Mountain View, CA, USA). Cells were incubated for 48hrs in 25µl of pseudotyped virus (MOI=1) then lysed with 100µl of GloLysis Buffer. Infection was reported as described above.

Cellular active metabolite:endogenous nucleotide concentration ratios were quantified with an identical set of uninfected TZM-bl or $CD4^+$ cells. Cells were harvested at the time of HIV-1 challenge (24 after incubation in drug), washed, counted, lysed in 70%methanol/water, and stored at -80°C for TFVdp, FTCtp, dATP, and dCTP quantification.

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Pharmacokinetic Analysis

$AUC_{0\text{-}48hr}$ was calculated individually using the linear up/log down trapezoidal rule for plasma and PBMCs with WinNonlin® v6.3 (Pharsight Corporation, CA, USA). $AUC_{0\text{-}48hr}$ was calculated using sparsely sampled data and the linear trapezoid rule for tissue. Concentrations below the limit of detection or quantification were imputed as 10% or 50% of the lower limit of quantification, respectively.

Monte-Carlo Simulations were performed with a population pharmacokinetic model built using the clinical trial concentration data and NONMEMv7.4, ICON plc. Simulations were performed for the first 10 doses and at steady state with dosing intervals representing 1-7 doses/week. Time to steady-state was defined as the first dose at which the 24hr concentration (Ctrough) was within 10% of simulated steady-state Ctrough.

Statistical Analysis

Dose proportionality was assessed in tissues across a 4-fold dosing range to assist with pharmacokinetic modeling using the Holder method[15] and declared if the 90% confidence interval (CI) around the regression line slope ($\hat{\beta}_1$) for log transformed dose vs concentration fit within 0.64-1.36. This analysis was conducted using Rv2.14.[16] Assuming %coefficient of variation (CV)≤45%, 8 women/dosing level provided 83% power to declare dose proportionality. Between-tissue comparisons of endogenous nucleotides were made using one-way ANOVA with Bonferroni correction. Descriptive and comparative statistics were performed with SASv9.3(SAS Institute Inc., Cary, NC, USA).

For cellular data, exposure vs response was described using Emax regression (Eq 1, E_0 fixed to 0, E_{max} fixed to 1, E=Response, MR=molar ratio) for active metabolite:endogenous

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nucleotide vs fraction inhibition of HIV-1 infection. Negative values were fixed at 0. Synergy was assessed with a pharmacodynamic interaction model ($\hat{\psi}$, Eq 2)[17] for TFVdp:dATP and FTCtp:dCTP in TZM-bl cells by fixing EC_{50} and Hill slope (H) parameters from Eq 1. A 3-D goodness of fit plot was generated and visually inspected to evaluate model predictions for bias. The final fitted parameters (H_{TFV}, H_{FTC}, EC_{50TFV}, EC_{50FTC}, $\hat{\psi}$) were used to predict %inhibition from simulated concentrations, with an efficacy target of 90%.

(Eq 1) $$E = E_0 + \frac{MR^H \times E_{max}}{MR^H + EC_{50}{}^H}$$

(Eq 2) $$E = \frac{\left(\frac{TFV}{\psi \times EC_{50,TFV}}\right)^{H_{TFV}} + \left(\frac{FTC}{\psi \times EC_{50,FTC}}\right)^{H_{FTC}} + \left(\frac{TFV}{\psi \times EC_{50,TFV}}\right)^{H_{TFV}} \times \left(\frac{FTC}{\psi \times EC_{50,FTC}}\right)^{H_{FTC}}}{1 + \left(\frac{TFV}{\psi \times EC_{50,TFV}}\right)^{H_{TFV}} + \left(\frac{FTC}{\psi \times EC_{50,FTC}}\right)^{H_{FTC}} + \left(\frac{TFV}{\psi \times EC_{50,TFV}}\right)^{H_{TFV}} \times \left(\frac{FTC}{\psi \times EC_{50,FTC}}\right)^{H_{FTC}}}$$

Results:

Participant Demographics and Safety

Between April 2012 and August 2013, 49 healthy female volunteers gave consent for the clinical trial. Participant demographics are summarized in Table 1 and stratified by treatment arm. Eight participants were enrolled into each dosing group except TDF 300mg where one participant was unable to provide samples and was withdrawn and replaced. Samples from one participant dosed with FTC 200mg were not analyzed because of improper storage. Single doses

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of TDF and FTC up to 200% of the licensed treatment dose were well tolerated with no adverse events above Grade 1 (Table 1).

Drug Concentrations and Dose Proportionality

Figure 1 depicts area under the concentration-time curve vs dose for extracellular concentrations and intracellular metabolite concentrations in plasma, PBMCs, and tissues. Table 2 summarizes dose proportionality statistics. Plasma FTC increased dose proportionally. Plasma TFV did not meet dose proportionality criteria but exhibited dose linearity. PBMC concentrations of TFVdp, but not FTCtp, increased dose proportionally. Since no notable difference was observed between cervical and vaginal tissue concentrations, they were averaged for subsequent analyses. A single composite tissue $AUC_{0\text{-}48hr}$ was calculated for each dosing group and tissue type. Only FGT emtricitabine concentrations increased dose proportionally.

Median dose adjusted $AUC_{0\text{-}48hr}$ for TFV and TFVdp were 10-45 times higher in colorectal tissue (38.5µg*hr*g^{-1} and 2046.5pmol*hr*g^{-1}, respectively) compared with FGT tissue (0.83µg*hr*g^{-1} and, 188pmol*hr*g^{-1}, respectively). Although median dose adjusted FTC $AUC_{0\text{-}48hr}$ was higher in colorectal tissue (222.3µg*hr*g^{-1}) than FGT tissue (17.6µg*hr*g^{-1}), FTCtp was 140 times higher in FGT tissue (15094.3pmol*hr*g^{-1}) than colorectal tissue (108.2pmol*hr*g^{-1}). Concentration vs time profiles for the blood and tissues are provided in Supplementary Figure 1 and 2, respectively.

Human Mucosal Tissue Endogenous Nucleotides

Although dATP concentrations were similar in vaginal and cervical tissues (Figure 2A), dCTP concentrations were 50% lower in vaginal vs cervical tissue (Figure 2B). Colorectal tissue contained lower endogenous nucleotide pools compared to the FGT: dATP concentrations were

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5 times lower ($p<0.05$), and dCTP concentrations were 5-11 times lower ($p<0.05$). In all tissues, dATP and dCTP concentrations were highly correlated (cervical Pearson's r=0.84, vaginal Pearson's r=0.92, colorectal Pearson's r= 0.73; $p<0.0001$). No differences in PBMC or tissue dATP/dCTP concentrations were noted between treatment arms, dosing groups, or sample collection time. In the female genital and colorectal tissues TFVdp:dATP ratios ranged from 0.00074 to 0.14 and 0.0014 to 5.9, respectively; and FTCtp:dCTP ranged from 0.025 to 17 and 0.016 to 1.4, respectively.

Pharmacokinetic Modeling

An 8 compartment model with 7 gastrointestinal transit compartments (Supplementary Figure 3) best fit the clinical trial concentration data. Model parameters (Supplementary Table 1) and goodness of fit have been previously reported.[18] A 1000-subject Monte-Carlo simulation was performed to simulate TFVdp:dATP and FTCtp:dCTP in FGT and colorectal tissue over the dosing interval for the first 10 daily doses and under steady-state conditions for each drug alone and in combination. TFVdp reached steady-state concentrations by dose 3 in FGT and dose 9 in colorectal tissue. FTCtp reached steady-state by dose 2 in FGT and dose 6 in colorectal tissue.

In Vitro Concentration vs Response

We investigated targets for protective efficacy against HIV infection in both a cell line and primary human cells. Figure 3 illustrates the best fit Emax parameters for active metabolite:endogenous nucleotide ratios and protection from HIV infection. For TFVdp:dATP the estimated EC_{90} ratio was 0.086 (TZM-bl cells) and 0.29 ($CD4^+$ cells). For FTCtp:dCTP the estimated EC_{90} ratio was 0.27 (TZM-bl cells) and 0.07 ($CD4^+$ cells). Emax model parameters for culture medium drug concentration and intracellular active metabolite vs %inhibition are

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provided in Supplementary Table 2. TFVdp:dATP and FTCtp:dCTP demonstrated synergy in TZM-bl cells [$\hat{\psi}$(SE)=0.63(0.074); p<0.001 where $\hat{\psi}$ <1 indicates synergy]. A synergistic interaction was also noted between TFV and FTC in $CD4^+$ cells (data not shown).

Pharmacokinetic/Pharmacodynamic Simulations

EC_{90} ratios derived from the more biologically relevant activated $CD4^+$ Emax model was used as target efficacy exposure for monotherapy simulations with TDF or FTC. The $\hat{\psi}$=0.63 modeled a surface to describe all possible EC_{90} combination ratios when using TDF+FTC. To quantify the difference in predictions produced by the two pharmacodynamic models, we simulated 1000 clinically relevant TFVdp:dATP and FTCtp:dCTP ratios. The predicted proportion of the population achieving target exposure differed by ≤2% between the two models.

In colorectal tissue the maximal proportion of the population (100%) achieved target exposure for efficacy after 3 daily doses of the fixed dose combination (Figure 4A). In FGT tissue the maximal proportion of the population (99%) achieved target exposure over the entire dosing interval after 3 daily doses of the fixed dose combination (Figure 4C). In colorectal tissue, dosing twice/week with the fixed dose combination achieved target exposure in >95% of the population (in FGT, this dosing achieved target exposure in 65%; Figure 4B,D).

To predict pericoital dosing effectiveness, we simulated colorectal tissue exposure achieved by the Ipergay dosing regimen (#2 doses 2-24hrs before coitus, #1 dose 24hrs after coitus, and #1 dose 48hrs after coitus) for TDF alone and with FTC. When administered 2 and 24hrs before coitus, TDF+FTC achieved target exposure (at the time of coitus) in approximately 81% and 98% of the population, respectively (Figure 5A,B). Target exposure was sustained out to 240hrs after coitus. In FGT, when administered 2 and 24hrs before coitus, TDF+FTC achieved

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target exposure at the time of coitus in 98% and 100% of the population, respectively (Figure 5C,D). The concentrations in the FGT were short-lived compared to colorectal tissue: <85% of the population had target exposure at 120hrs following coitus.

Discussion:

Phase III clinical trials evaluating daily oral TDF±FTC for HIV PrEP have demonstrated mixed results in heterosexual women.[1, 3, 4, 19] To understand this, we generated comprehensive pharmacokinetic and biological data in vulnerable mucosal tissues and evaluated effective drug exposure with different dosing strategies. To our knowledge, this is the first biologically complete model to predict effective PrEP dosing. This analysis generated several important findings: 1) Confirmation that TFV and FTC have different tissue distribution characteristics where TFV favors colorectal tissue and FTC favors FGT tissue; 2) Characterization of the intracellular active metabolite:endogenous nucleotide ratio as an accurate pharmacodynamic target for NRTIs, whereby endogenous nucleotides were found in higher concentration in FGT tissue, and 3) Evidence that this PK/PD approach can be used to develop optimized TDF±FTC PrEP dosing strategies and predict clinical trial outcomes with different adherence patterns prior to the trials being conducted.

Previously, Patterson et. al. described 100-fold higher TFVdp concentrations in colorectal compared to FGT tissue with a single TDF+FTC dose.[7] Herein, we investigated 50% to 200% of the licensed treatment doses to develop a robust pharmacokinetic model of TFVdp and FTCtp distribution. Our findings are consistent with this previous report: TFVdp concentrations overall were 10 times higher in colorectal tissue while FTCtp concentrations were 140 times higher in FGT tissue. Between-tissue differences in drug transport systems may explain differential

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concentrations; yet blood flow to rectal and vaginal mucosa is similar.[20] Drug transport by lymph fluid into tissues has not been fully characterized. The lipid rich lymph fluid may not favorably transport hydrophilic compounds like TFV and FTC.[21, 22] However the leaky lymphatic endothelium could provide an alternative mode of absorption in colorectal tissue,[23] possibly explaining the delayed peak we observed in this tissue. We also found that dATP and dCTP concentrations were 7 and 11 times lower in colorectal tissue compared with FGT tissue, respectively. Therefore, not only were TFVdp concentrations much higher in colorectal tissue, but we found that the colorectal intracellular environment favors TFV's prophylactic activity.

A significant limitation in determining PrEP concentration targets for efficacy is the inability to easily measure the PK/PD relationship in humans. HIV prevention models rely on extrapolating data from cell culture systems, human tissue explants, or animal studies, each of which has limitations.[24, 25] Primate models utilize SIV or SHIV that may not mimic HIV's response to antiretrovirals;[26] antiretroviral exposure in humanized mouse models may not match humans;[26] and e*x vivo* tissue explants' endogenous nucleotides deteriorate by ≥80% within 24hrs of isolation.[27] Although a homogenous cell population fails to represent the 3-dimensional architecture and diverse microanatomy involved in transmission events, these models can be infected with HIV, exposed to clinically relevant antiretroviral concentrations, and sustain endogenous nucleotide pools.[28] Since active metabolite concentrations in isolated mononuclear rectal cells strongly correlate with tissue homogenate concentrations[29], we believe using isolated target cells to generate concentration-effect relationships for NRTI prevention strategies is reasonable.

A number of previous studies have highlighted the importance of endogenous nucleotides in NRTI efficacy.[30-32] Therefore, we postulated significant differences in tissue endogenous

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nucleotide concentrations may affect the PK/PD relationships for HIV prevention. Using primary (human $CD4^+$ cells) and immortalized (TZM-bl cells) culture systems with a clinically relevant HIV-1 challenge, we identified efficacy targets for TFVdp and FTCtp relative to their competing endogenous nucleotides (dATP and dCTP). Differences in the concentration-response relationship between in vitro systems were noted. However, normalizing active metabolite to endogenous nucleotide concentrations reduced the difference between TZM-bl and $CD4^+$ EC_{90} ratio values by 70% (Supplement Table 2), and resulted in a difference of <2% in clinical predictions between these model systems, highlighting the importance of the endogenous nucleotide exposure.

Combining the mathematical pharmacokinetic mucosal tissue model with the cellular target ratios, we determined the maximal proportion of the population achieved effective FGT and colorectal concentrations after 3 doses of TDF+FTC. Our data are consistent with the Cell-PrEP study.[33] These data differ from the CDC guidelines, which are built on extrapolations from PBMC data[34]. However, since they are based on tissue pharmacokinetics, we believe they better inform clinical utility. Importantly, we found 2 doses/week of TDF±FTC achieved effective colorectal concentrations in >95% of the population, which emulate iPrEx-OLE results (90% protection with 2-3 TDF+FTC doses/week).[6]

To predict colorectal protection with pericoital oral dosing, we evaluated the Ipergay dosing regimen.[35] When a double dose of TDF+FTC is taken 24hrs before coitus, and 2 doses are taken over 48hrs following coitus, we demonstrate >95% of the population achieves effective concentrations in colorectal tissue at the time of, and for ≥240hrs after, coitus. When administered 2hrs before coitus, approximately 17% of the population are below the target for the first 4hrs after coitus. However, this is likely an inconsequential delay, since it takes 4hrs for

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reverse transcription to occur after HIV binds to cellular membranes[36]. These findings reasonably emulate clinical trial data whereby this dosing regimen demonstrated 86% efficacy in MSM.[35]

Our data show daily TDF+FTC is required for 100% of the population to be protected from HIV infection within the FGT. Our efficacy predictions are in agreement with the Partners PrEP trial, which demonstrated 92% risk reduction (95% CI=19-99%) when women taking TDF +FTC for PrEP had plasma drug concentrations consistent with very high adherence.[37] We also simulated the Ipergay regimen for the FGT. Using this approach, >95% of the population achieve target exposure in FGT tissue at the time of, and for 72hrs after, coitus. However, the effect is comparatively short lived, as by 120hrs following coitus <85% are still at target. Administering TDF+FTC 2 or 24hrs before coitus did not change the proportion achieving target exposure in the FGT tissue across this time frame. To match the percent of the population achieving target exposure in colorectal tissue with Ipergay dosing, women would need to take 9 daily doses of TDF+FTC following coitus. These data suggest this may not be a strongly protective regimen for women vaginally exposed to HIV and are consistent with recent preliminary reports from clinical trials where more women using intermittent dosing acquired HIV compared to those using daily dosing.[38]

Our approach has certain limitations. First, our in vitro methods necessitated the use of PHA/IL-2 stimulation which may increase the phosphorylation of NRTIs and endogenous nucleotides in $CD4^+$ cells.[9] However, direct measurement of active metabolites and normalization to endogenous nucleotides helped minimize confounding. Although the exposure-response relationship was defined under conditions of static drug exposure, given the long intracellular half-lives of TFVdp and FTCtp (~48 and 39 hours, respectively)[39, 40] marked

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fluctuation of active metabolite concentrations in tissues is not expected, and has not been identified. The concentration data used to build the pharmacokinetic model was obtained over 48 hours. However, there is precedent for making steady state drug exposure predictions from similarly developed models.[41] Further, our predicted steady state plasma concentrations were consistent with literature values, and our predicted time to steady state in colorectal tissue (9 days for TFVdp) was consistent with previous reports.[33] The use of tissue biopsy homogenates to measure mucosal tissue drug concentrations assumes uniform distribution of drug and endogenous nucleotide concentrations across the biopsy, which is unlikely.[42] However, isolating HIV target cells from vaginal and cervical tissue biopsies has resulted in incomplete pharmacokinetic data sets due to small, inconsistent cell yields[39], and previous publications have demonstrated linear relationship between isolated mucosal cells and tissue homogenate concentrations.[29]

Although we used pharmacokinetic data collected only from women, parent and metabolite colorectal drug concentrations overlapped with previously published data in men,[7] suggesting mucosal tissue differences are driven primarily by the drugs' distribution characteristics rather than inherent sex differences. Since the risk of HIV transmission per act of unprotected receptive anal intercourse does not differ between MSM and heterosexuals[43], it is unlikely that biological differences alter HIV infectability in male vs female colorectal tissue. Thus, we believe it is reasonable to substantiate our colorectal model predictions with PrEP clinical trial results from MSM only populations. Lastly, our population pharmacokinetic model was generated using data from healthy volunteers in the absence of sexually transmitted diseases or conditions causing inflammation in the lower gastrointestinal tract and FGT. Active metabolite and endogenous nucleotide concentrations may be altered in a setting mucosal tissue

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inflammation,[44] along with altered susceptibility to HIV infection and warrant further investigation.

In summary, this study is the first of its kind for HIV oral PrEP. By pairing phase I tissue concentration data with in vitro systems of HIV infection, we created a predictive population PK/PD model for PrEP which is consistent with clinical trial outcomes. Here we demonstrate proof of concept that a more complete characterization of the achievable and effective concentrations can be used to generate PK/PD models to optimize drug dosing prior to the initiation of phase III trials and quantify the effect of incomplete adherence on protective efficacy. We believe this approach can be applied to other drugs and combinations to inform clinical trial design and optimize resources within the HIV prevention field.

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Footnote Page

Conflict of Interests/Disclosures: Angela Kashuba and her laboratory are part of the study teams for CAPRISA 004 and 008, FACTS 001, MTN 006, HPTN 066, FEM-PrEP, and CONRAD 113, 114, and 117. Grant funding from Gilead Sciences Inc. has been received by UNC and Angela Kashuba. No other authors have commercial interests that might pose a conflict of interest to disclose.

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Figure Legends

Figure 1 Dose Proportionality in Blood Plasma, PBMCs, and Mucosal Tissues

Figure 1. Mean (±Standard Error) $AUC_{0\text{-}48hr}$ for tenofovir (A), tenofovir diphosphate (TFVdp; B), emtricitabine (C), and emtricitabine triphosphate (FTCtp; D) in the female genital tract (FGT) tissue(Δ); colorectal tissue(○); or plasma(□) for tenofovir and emtricitabine, and (□)peripheral blood mononuclear cells (PBMCs) for TFVdp and FTCtp.

Figure 2 Tissue Endogenous Nucleotide Concentrations

Figure 2. Tissue endogenous nucleotide concentrations are plotted as median (solid line within each box), 25th to 75th percentile (box edges), and 10th to 90th percentile (error bar whiskers) for 47 women. (*) denotes $p<0.05$. Median deoxyadenosine triphosphate (dATP) concentrations (A) are 85% lower in colorectal tissue compared to cervical and vaginal tissue ($p<0.05$; N=47). Median deoxycytidine triphosphate (dCTP) concentrations (B) in colorectal tissue are 90% lower than cervical tissue and 80% lower than vaginal tissue ($p<0.001$; N=47).

Figure 3= *In Vitro* Concentration vs Response for TZM-bl and $CD4^+$ Cells

Figure 3. TZM-bl and stimulated, primary $CD4^+$ cells were incubated for 24hr in 0.03-35µM concentrations of tenofovir and emtricitabine. Intracellular active metabolite and endogenous nucleotide (EN) concentrations were quantified at the time of challenge with HIV-$1_{JR\text{-}CSF}$ (TZM-bl cells) or psuedovirus with HIV-1 $_{JR\text{-}CSF}$ envelope ($CD4^+$ cells). For TZM-bl cells N=34 TFVdp:dATP and 41 FTCtp:dCTP samples collected across at least 2 independent experiments (6 of 72 samples were below the limit of quantification; BLQ). For $CD4^+$ cells N=14 TFVdp:dATP and 27 FTCtp:dCTP samples collected across at least 2 independent experiments (2 of 42 samples were BLQ). Solid lines represent the median regression line of the Emax model.

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The dashed reference line indicates 90% inhibition of HIV infection and the dotted represents 50% inhibition. The fifty percent effective concentration (EC_{50}) ratio for tenofovir diphosphate (TFVdp):dATP (A) was 0.010 (SE=0.001, p<0.001) with a Hill slope (H) of 1.02 (SE=0.09, p<0.001) in TZM-bl cells (○) and 0.086 (SE=0.011, p<0.001) with an H of 1.81 (SE=0.39, p<0.001) in CD4+ cells (▲). The EC_{50} ratio for emtricitabine triphosphate (FTCtp):dCTP (B) was 0.059 (SE 0.004, p<0.001) with an H of 1.42 (SE=0.11, p<0.001) in TZM-bl cells (○) and 0.022 (SE=0.005, p<0.001) with an H of 1.88 (SE=0.67, p<0.05) in CD4+ cells (▲).

Figure 4. Time to Protection and Minimally Effective PrEP Dosing

Figure 4 reports pharmacokinetic/pharmacodynamic (PK/PD) simulations for each tissue type: colorectal (A,B) and lower female genital tract (FGT; C,D). The percent of the simulated population achieving the EC_{90} TFVdp:dATP, FTCtp:dCTP, or combination ratios derived from the in vitro PK/PD relationship in $CD4^+$ cells is plotted over the dosing interval for the first 10 daily doses (A,C) of tenofovir disoproxil fumarate (TDF; dashed line), emtricitabine (FTC; dotted line), or the fixed dose combination (TDF+FTC; solid line). The percent of the population achieving these EC_{90} ratios at the end of the dosing interval under steady state conditions with 1 to 7 doses/week of TDF, FTC, or TDF+FTC are stratified by tissue (B,D). We predict that the maximal percent of the population achieved EC_{90} ratios by the third daily dose of TDF+FTC in colorectal and FGT tissue after beginning PrEP. Consistently using 7 doses/week of TDF+FTC will achieve EC_{90} ratios in 100% of the population in the FGT and colorectal tissue. Only 65% of the population using 2 doses/week of TDF+FTC achieve target exposure in the FGT tissue, whereas ≥95% using 2 doses/week of either TDF or TDF+FTC achieve target exposure in colorectal tissue.

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Figure 5 Protection with Pericoital PrEP Dosing

Figure 5 reports pharmacokinetic/pharmacodynamic (PK/PD) simulations for the Ipergay dosing regimen (#2 tablets 2 to 24hrs before coitus (solid vertical line), #1 tablet 24hrs after coitus, #1 tablet 48hrs after coitus) for tenofovir disoproxil fumarate (TDF; solid line) and the fixed dose combination of tenofovir disoproxil fumarate+emtricitabine (TDF+FTC; dashed line). The percent of the simulated population achieving the EC_{90} TFVdp:dATP ± FTCtp:dCTP derived from the in vitro PK/PD relationship in $CD4^+$ cells over 14 days following a single act of coitus in colorectal (A,B) and lower female genital tract (FGT) tissue (C,D). PK/PD simulations are reported assuming the first dose was administered 24hrs (A,C) or 2hrs (B,D) before coitus. We predict the maximal percent of the population achieving EC_{90} ratios in colorectal tissue over a 240hr postcoital window is achieved by initiating the Ipergay dosing 24hrs before coitus. Dosing at 24hrs or 2hrs before coitus did not appear to alter the percent of the population achieving target exposure in the FGT tissue over a 72hr postcoital window.

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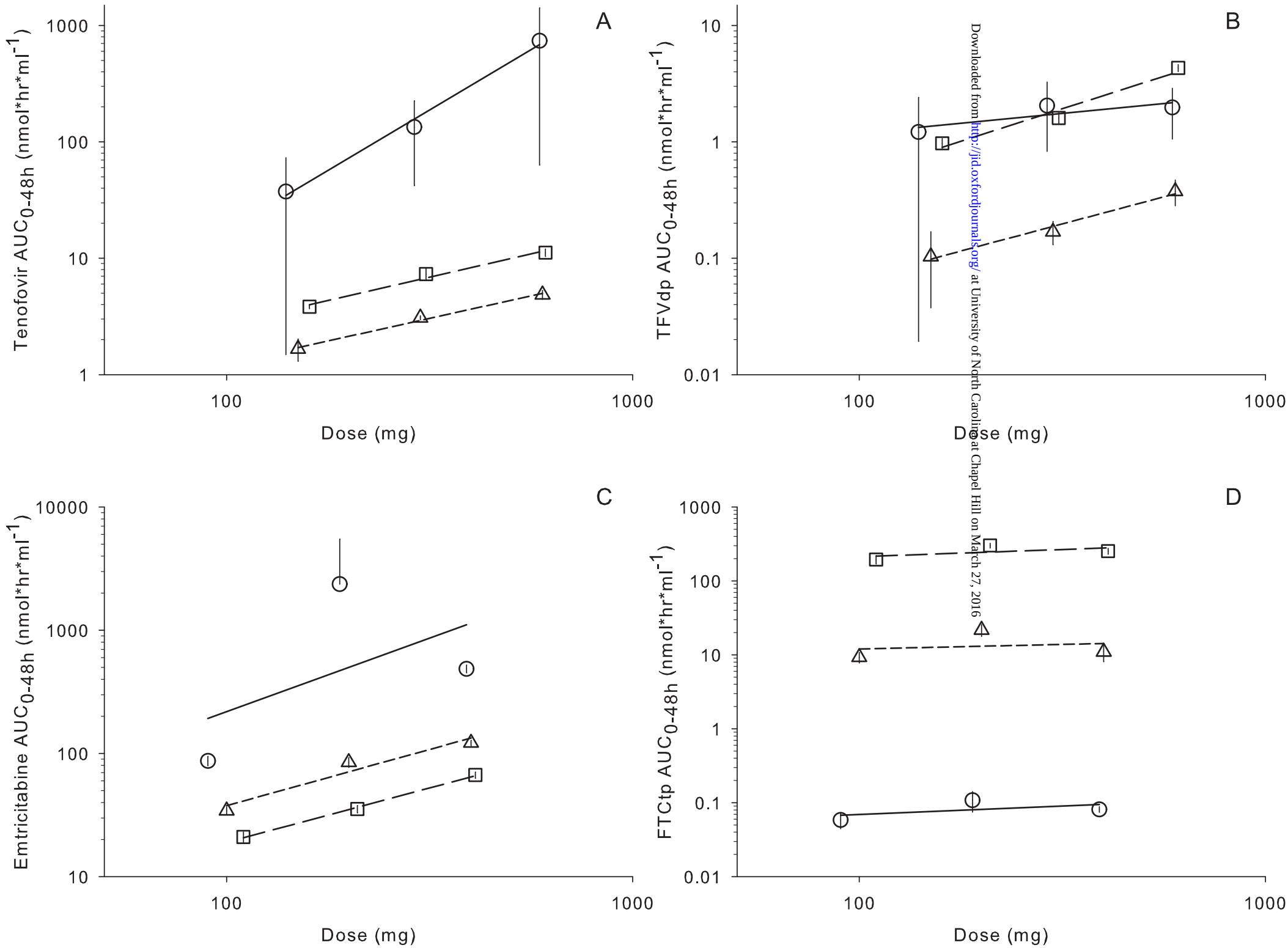

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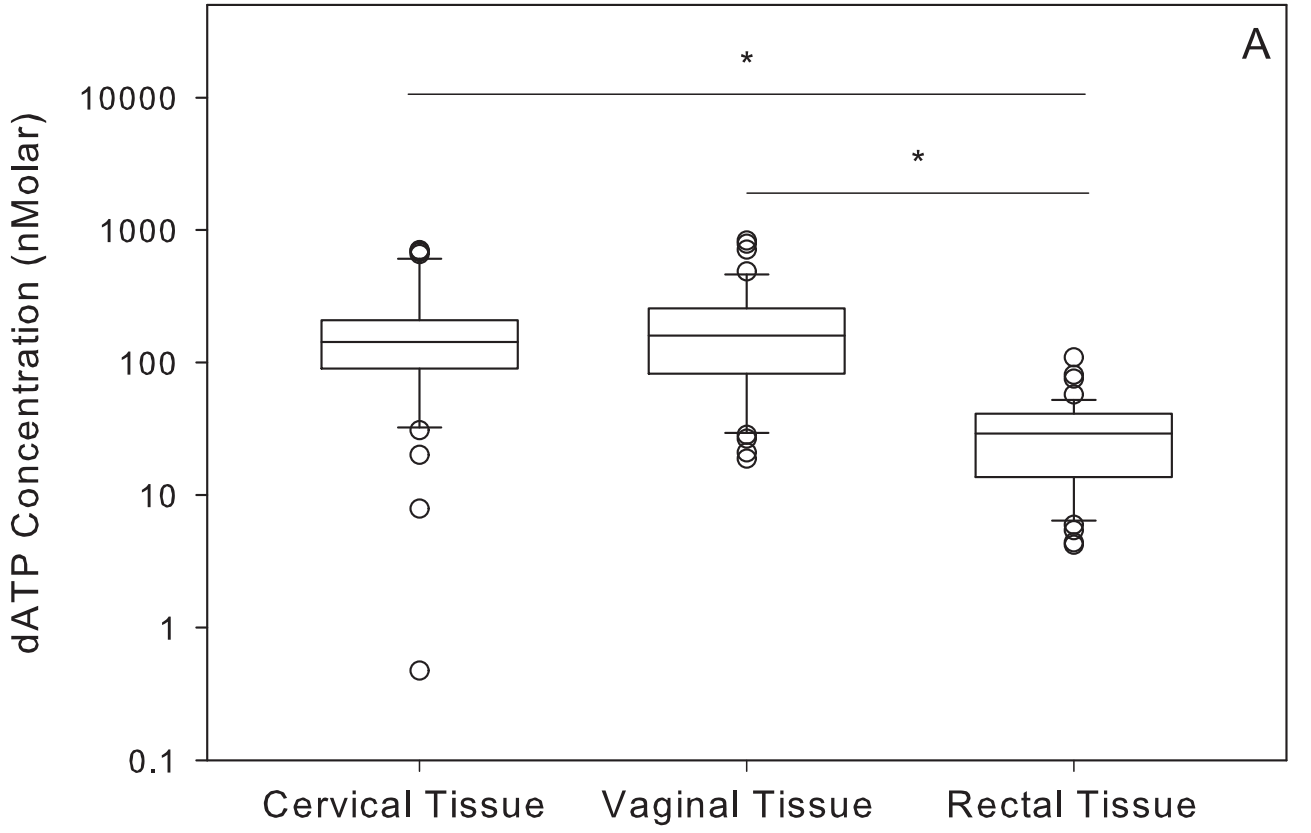
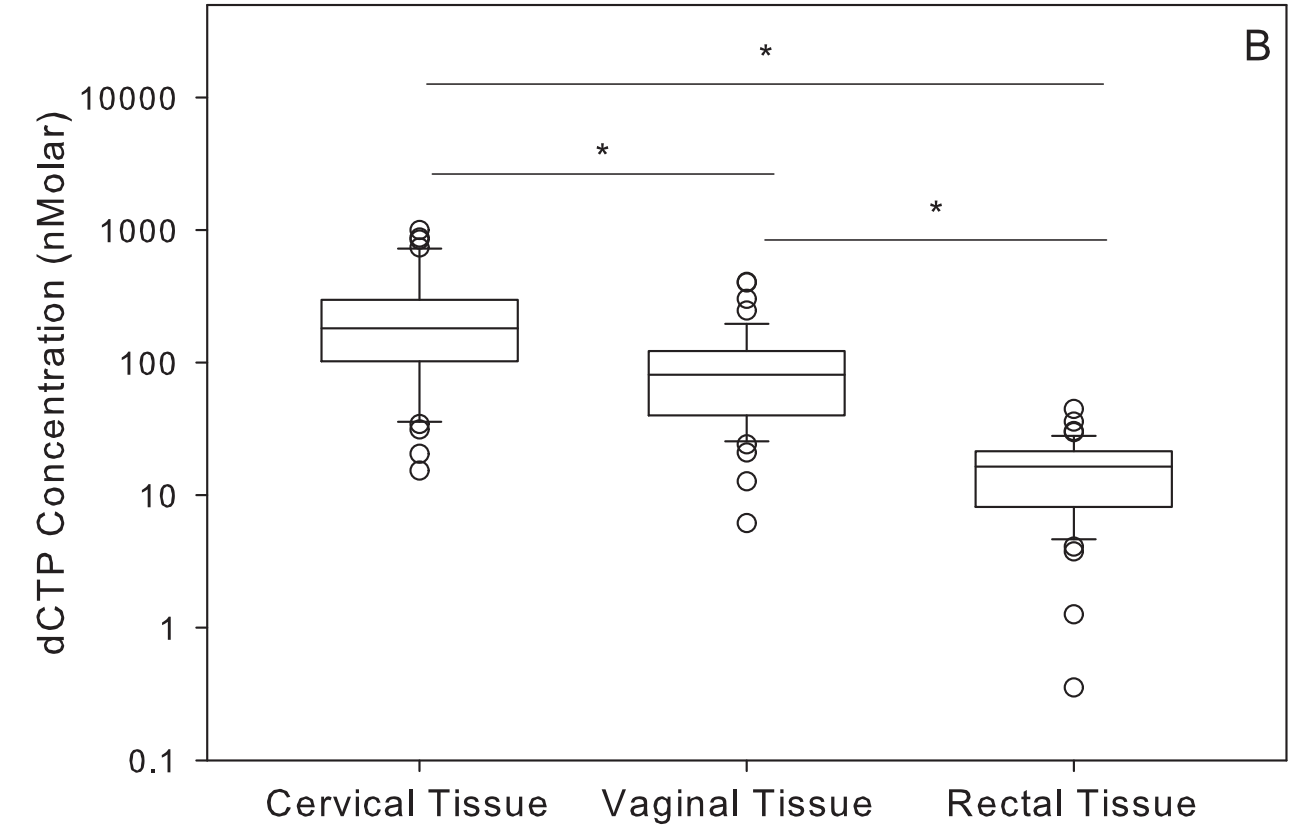
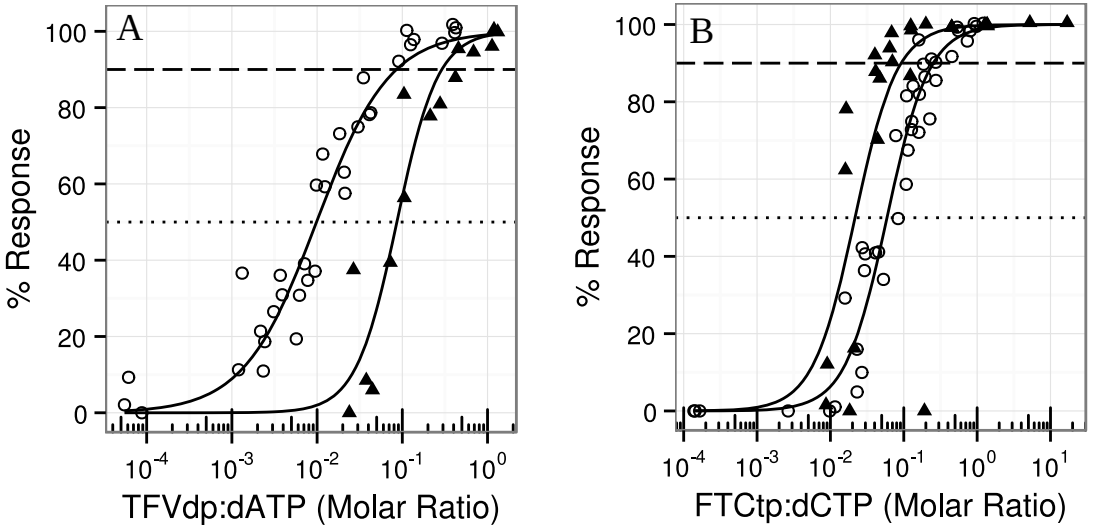
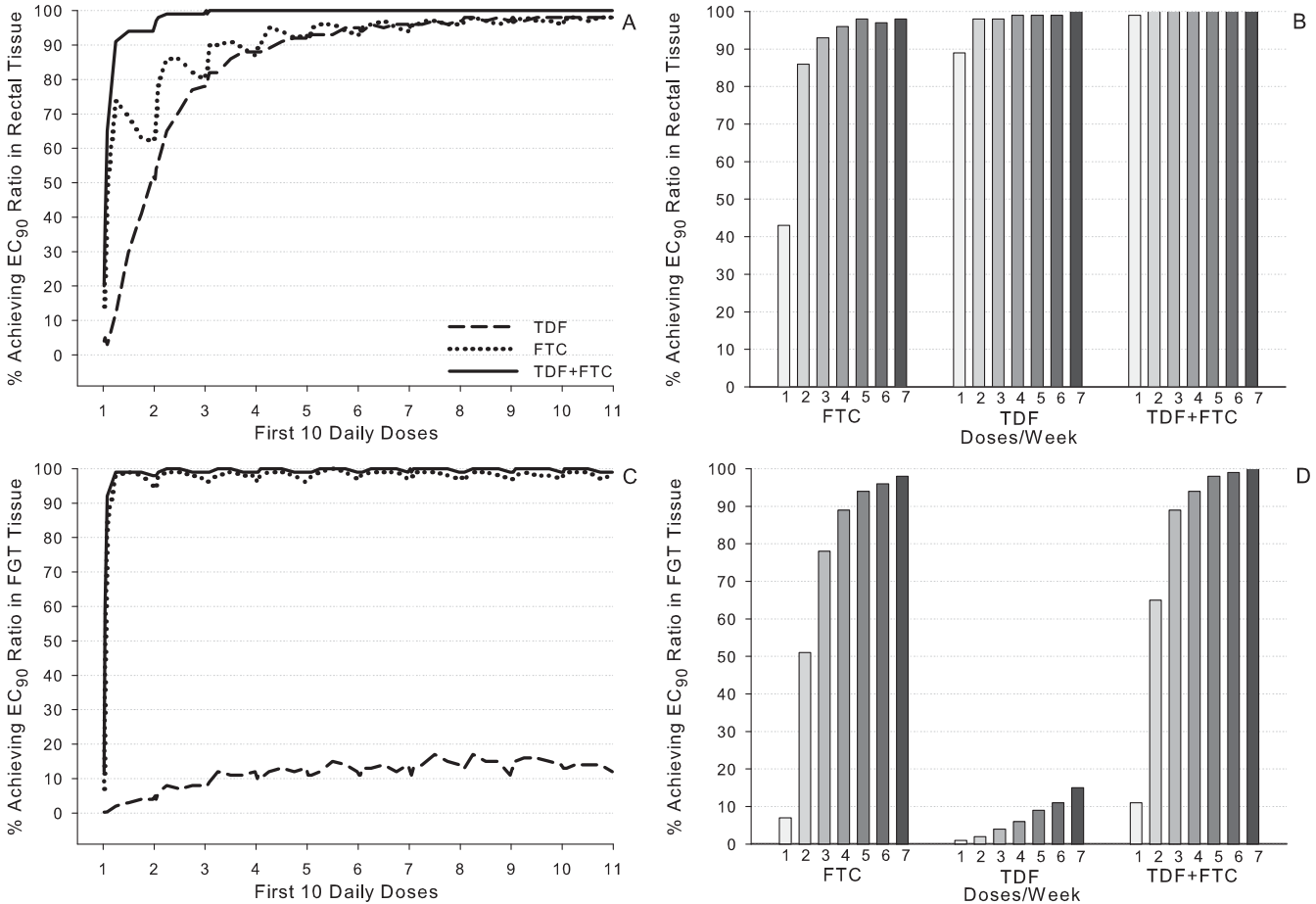

A
dATP Concentration (nMolar)
10000
1000
100
10
1
0.1
*
*
Cervical Tissue
Vaginal Tissue
Rectal Tissue


B
dCTP Concentration (nMolar)
10000
1000
100
10
1
0.1
*
*
*
Cervical Tissue
Vaginal Tissue
Rectal Tissue


A
B
% Response
100
80
60
40
20
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
TFVdp:dATP (Molar Ratio)
FTCtp:dCTP (Molar Ratio)


A
% Achieving EC90 Ratio in Rectal Tissue
First 10 Daily Doses
TDF
FTC
TDF+FTC
B
% Achieving EC90 Ratio in Rectal Tissue
FTC
TDF
TDF+FTC
Doses/Week
C
% Achieving EC90 Ratio in FGT Tissue
First 10 Daily Doses
D
% Achieving EC90 Ratio in FGT Tissue
FTC
TDF
TDF+FTC
Doses/Week

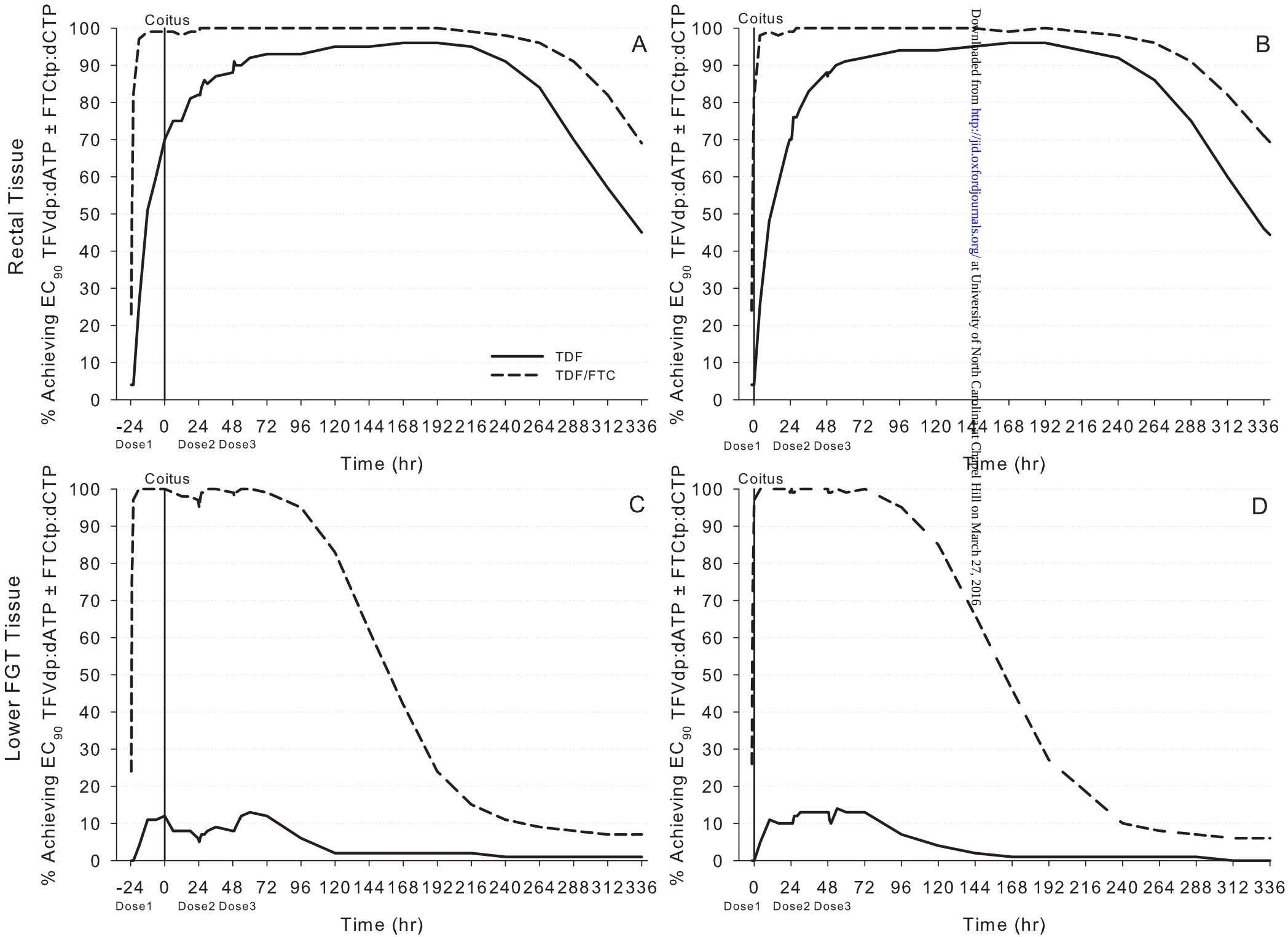


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Table 1. Demographics and Adverse Events

Demographic	**Tenofovir disoproxil fumarate (N=25)**	**Emtricitabine (N=24)**
Age (years)[a]	27 (22.75-31.25)	22 (21-27)
Weight (kg)[a]	67.4 (60.4-76.8)	62.8 (57.7-72.3)
BMI (kg/m^2)[a]	24.1 (21.6-26.9)	22.5 (20.8-26.5)
Race[b]		
Caucasian	16 (64)	18 (75)
African American	8 (32)	4 (16.7)
Asian American	1 (4)	1 (4.2)
American Indian	0 (0)	1 (4.2)
Adverse Event[b]		
Headache	1 (4)	5 (20.8)
Nausea	2 (8)	0
Early menses	1 (4)	0
Fatigue	1 (4)	0
Bowel disturbances	1 (4)	1 (4.2)
Elevated transaminases	0	1 (4.2)
Pelvic cramps	0	1 (4.2)
Post nasal drip	0	1 (4.2)
Vaginal dryness	0	1 (4.2)
Ear infection	1 (4)	0
Viral pharyngitis	1 (4)	0

[a] Data summarized as median (Interquartile range)
[b] Data summarized as number (percentage)

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Table 2. Dose Proportionality Statistics

Matrix	Analyte	Slope ($\hat{\beta}_1$)[a]	90% Confidence Interval	r^2 (p value)	Dose Proportional[b]
Plasma	Emtricitabine	0.84	0.70, 0.97	0.85 (<0.0001)	Yes
Plasma	Tenofovir	0.77	0.63, 0.92	0.80 (<0.0001)	No
PBMC	FTCtp	0.19	0.012, 0.37	0.13 (0.09)	No
PBMC	TFVdp	1.07	0.91, 1.24	0.83 (<0.0001)	Yes
FGT	Emtricitabine	0.91	0.78, 1.05		Yes
FGT	FTCtp	0.12	-0.29, 0.53		No
FGT	Tenofovir	0.77	0.46, 1.09		No
FGT	TFVdp	0.93	0.14, 1.72	Not applicable; Sparsely sampled data	No
Colorectal	Emtricitabine	1.24	-0.29, 2.77		No
Colorectal	FTCtp	0.22	-0.23, 0.67		No
Colorectal	Tenofovir	2.15	0.31, 4.00		No
Colorectal	TFVdp	0.35	-1.10, 1.80		No

[a] $\hat{\beta}_1$=regression line slope for log transformed $AUC_{0\text{-}48}$ vs log transformed dose.
[b] Perfect dose proportionality $\hat{\beta}_1 = 1$ therefore dose proportionality declared if the 90% confidence interval within 0.64, 1.36
FGT=female genital tract; FTCtp=emtricitabine triphosphate; PBMC=peripheral blood mononuclear cells; TFVdp=tenofovir diphosphate

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Pharmacokinetics and Pharmacodynamics in HIV Prevention; Current Status and Future Directions: A Summary of the DAIDS and BMGF Sponsored Think Tank on Pharmacokinetics (PK)/Pharmacodynamics (PD) in HIV Prevention

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on Behalf of the Antiretroviral Pharmacology in HIV Prevention Think Tank Participants

Abstract

Thirty years after its beginning, the HIV/AIDS epidemic is still raging around the world. According to UNAIDS, in 2011 alone 1.7M deaths were attributable to AIDS, and 2.5M people were newly infected by the virus. Despite the success in treating HIV-infected people with potent antiretroviral drugs, preventing HIV infection is the key to ending the epidemic. Recently, the efficacy of topical and systemic antiviral chemoprophylaxis (i.e., pre-exposure prophylaxis or "PrEP"), using the same drugs used for HIV treatment, has been demonstrated in a number of clinical trials. However, results from other trials have been inconsistent, especially those evaluating PrEP in women. These inconsistencies may result from our incomplete understanding of pharmacokinetics (PK)/pharmacodynamics (PD) at the mucosal sites of sexual transmission: the male and female gastrointestinal and reproductive tracts. The drug concentrations used in these trials were derived from those used for treatment; however, we still do not know the relationship between the therapeutic and the preventive dose. This article presents the first comprehensive review of the available data in the HIV pharmacology field from animal models to human studies, and outlines gaps, challenges, and future directions. Addressing these pharmacological gaps and challenges will be critical in selecting and advancing future PrEP candidates and strategies with the greatest impact on the HIV epidemic.

Introduction

Over the past 2 years, results from multiple clinical trials have been published evaluating the efficacy of different antiretroviral (ARV)-based strategies for the prevention of human immunodeficiency virus (HIV) transmission (Table 1). Unfortunately, results from these studies have been inconsistent. For example, 1% tenofovir (TFV) vaginal gel demonstrated a 39% reduction in HIV transmission to women in the Phase 2b Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004 study conducted using the coitally related "BAT-24" dosing regimen.[1] In contrast, evaluation of once-daily use of 1% TFV gel was discontinued in the Microbicide Trials Network 003 Vaginal and Oral Interventions to Control the Epidemic (VOICE) trial due to a lack of effect relative to placebo.[2]

Results from trials evaluating oral ARV strategies have been similarly divergent. Daily oral use of Truvada [200 mg emtricitabine (FTC)–300 mg tenofovir disoproxil fumarate (TDF)] was shown to be efficacious in a population of men who have sex with men (MSM) in the Preexposure Prophylaxis (PrEP) Initiative (iPrEX) study,[3] as well as in serodiscordant couples in the Partners PrEP and TDF2 studies.[4–7] Although efficacy results for these studies were statistically significant, the confidence intervals were quite wide, making the determination of efficacy not definitive.

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Table 1. Recent HIV-Prevention Product Efficacy Trial Results

Study	*Product (dosing frequency)*	*Population*	*Efficacy result*
CAPRISA 004[1]	1.0% TFV Gel ("BAT-24")	HIV-negative women	39%, $p = 0.017$; 95% CI: 6–60%
iPrEx[3]	Oral Truvada (daily)	HIV-negative MSM	44%, $p = 0.005$; 95% CI: 15–63%
Partners PrEP[4]	Oral Truvada (daily)	Serodiscordant couples	62%, $p = 0.0003$; 95% CI: 34–78%
	Oral TDF (300 mg) (daily)		73%, $p < 0.0001$; 95% CI: 49–85%
TDF2 Study[7]	Oral Truvada (daily)	HIV-negative women and men	63%, $p = 0.013$; 95% CI: 22–83%
Fem PrEP[5]	Oral Truvada (daily)	HIV-negative women	Early termination
VOICE[2,6]	Oral TFV (daily) 1.0% TFV gel (daily)	HIV-negative women	Early termination

MSM, men who have sex with men.

Conversely, oral TDF/FTC failed to protect HIV-negative women in the FEM-PrEP trial and VOICE.[5,6] Although oral TFV (300 mg TDF) was shown to be efficacious in the Partners PrEP study,[4] its evaluation was discontinued in the VOICE study due to futility.[6]

Possible reasons for these discordant results with the same products in different studies could include differences in dosing regimens, trial designs, trial populations, behavior and sexual practices, and different levels of adherence in the trial populations. Thus, the need for more robust assessments of efficacy potential for next-generation, high-impact, HIV prevention products earlier in clinical development remains a priority for the field.

Although proper preclinical development and clinical safety assessment of ARV-based prevention products have been achieved for a number of products, meaningful evaluation of the efficacy potential of such products, prior to Phase 3 trials, remains elusive. The efficacy of prevention products can be influenced by a number of factors including dose and product exposure, target cell bioavailability, tissue permeation in the compartments of HIV exposure, *in vivo* half-life, protein binding, effects of biological fluids, local immune responses, and perhaps most complex of all, the behaviors affecting consistent use of the product. Unfortunately, typical Phase 2 proof-of-concept study designs based on products for treatment indications are not feasible for prevention products, given the number of participants required to establish the statistical significance of a prevention outcome. Consequently, the prevention field has relied on limited, alternative means of assessing efficacy potential earlier in the drug development pipeline, which include the use of animal models[8] or *ex vivo* tissue explant systems.[9] At best, these systems have provided limited insight to assess safety as well as efficacy potential as influenced by dose, dosing regimen, and *in vivo* properties of the drug candidates. None of the models has yet been conclusively validated against clinical outcomes in human Phase 3 trials. Improving the robustness and accurate assessments of product pharmacokinetics (PK) and pharmacodynamics (PD) in humans and animal models may provide a relevant, useful, and earlier means of identifying safe and effective HIV prevention products.

Determination of *in vivo* ARV concentrations in various compartments after clinical dosing has occasionally been included in early prevention product trials, but typically it has progressed in parallel with larger efficacy studies. Often, it has been considered as a follow-up analysis of samples collected from efficacy studies. In most instances, these samples are plasma and occasionally include peripheral blood mononuclear cells (PBMCs), whole tissue biopsies, or HIV target cells isolated from target mucosal sites. Even vaginal/rectal secretions are rarely collected. Also, when included as part of early clinical evaluation, such trials typically involved sexually abstinent participants, which negated the ability to determine the effects of sexual intercourse on drug levels in the gastrointestinal and genital tract. While these limited PK measurements may be useful in understanding outcomes posttrial, the field needs improved pre-Phase 3 methods to better inform efficacy trial design and more accurately predict efficacy outcomes. The development of more robust human and animal PK/PD assessment systems will provide a better understanding of the relationship between drug concentrations and activity, as well as the effects of dose and drug exposure on clinical outcome.

To define the critical issues relevant to the development of effective PK/PD models for use in HIV prevention, the National Institute of Allergy and Infectious Diseases (NIAID), Division of AIDS (DAIDS), Prevention Sciences Program (PSP) at the National Institutes of Health (NIH) in collaboration with the Bill and Melinda Gates Foundation (BMGF) sponsored an Antiretroviral Pharmacology in HIV Prevention Think Tank. This article outlines the major issues addressed at this meeting with particular focus on the details of conventional derivation and use of PK/PD models and data in drug development, the current state of such models and their use in the HIV prevention field, and the PK/PD knowledge and capability gaps in HIV prevention that need to be addressed.

PK/PD in Drug Development

The determination of drug-candidate PK and PD, and the relationships between the two (i.e., a particular concentration of drug *in vivo* yields a specific level of effect/efficacy), is a fundamental component of drug product development. Typically, PK/PD evaluations are initially conducted in animal models. However, PK/PD relationships can be complicated by species differences between animal models and humans [e.g., differences in absorption, distribution, metabolism, and excretion (ADME), plasma to effect site ratios, and protein binding, immune responses]. Consequently, clinical studies are the definitive determination of the PK/PD relationship in humans. Once drug concentrations over a particular time course are derived for specific doses in animals, exposure–response relationships can be analyzed in various ways. Given the lack of a proof of concept outcome in phase 2 PrEP studies, there is increased need to appropriately study PK/PD relationships with prevention products in order to inform development decisions for such products.

This type of analysis is routinely performed for antimicrobials, where a dose-fractionation study design is commonly implemented first. With this approach, a fixed total daily dose of antimicrobial is administered as a single dose or fractionated into smaller doses administered using different dosing intervals. Based on such studies, the antibacterial drugs can be classified according to the correlation between the effect (most often defined as the bacterial count after 24 h of treatment) and three main PK/PD indices: (1) the ratio of the maximal unbound (free) drug concentration (C_{max}) to the minimum inhibitory concentration (MIC) (fC_{max}/MIC), (2) the ratio of the area under the unbound drug concentration–time curve (AUC) to the MIC [f(AUC)/MIC], or (3) the percentage of a 24-h time period that the unbound drug concentration exceeds the MIC (fT > MIC) (Table 2).[10–12] Typically, the approach closest to a linear relationship of drug concentration and effect (generally, a decrease in colony-forming units for bacterial infection) is identified as having the most predictive value for measuring the biological effect of the drug on the pathogen. Although this approach is routinely used to study antimicrobial agents, such an evaluation of anti-HIV drugs would be more challenging given the lack of robust assays for measuring pharmacodynamics in samples from clinical trials.

Importantly, individual drugs (or classes of drugs) with different physicochemical properties or mechanisms of action may display different efficacy outcomes with these alternative means of analysis. Thus, until proven, it should not be assumed that the relationships observed with one particular anti-HIV drug will be the same as another such drug. Until more data are available, it is recommended that each drug in development be individually evaluated by these PK/PD assessment strategies.

There are a number of important variables to consider in the application of these PK/PD assessment strategies as a step toward identifying optimal prevention interventions. First, knowing the amount of free drug and the amount of total drug in the test system is relevant to understanding the outcome. Presumably, a drug that is bound to another molecule in a model system is not available to exert a biological effect, at least not as potently as a free drug. Differences in protein-binding affinity of different ARVs have been well documented[13] and would likely be relevant in the application of such drugs for prevention. Thus, methods of measuring drug concentrations that do not account for free versus total drug concentrations, particularly in the compartments of effect, may lead to biased drug requirement recommendations.

Table 2. Pharmacokinetic/Pharmacodynamic Relationships Used to Predict Antibiotic Efficacy

AUC/MIC	AUC obtained from plotting *in vivo* drug concentrations over a fixed time period, divided by the MIC of drug against a specific pathogen
C_{max}/MIC	The C_{max} measured *in vivo* over a specific time period, divided by the MIC of a drug against a specific pathogen
Time > MIC	The duration of time that *in vivo* drug concentrations are measured to be greater than the MIC of drug against a specific pathogen

AUC, area under the curve; MIC, minimum inhibitory concentration.

Second, it is also important to understand the effects of the immune system on biological outcomes, particularly in the evaluation of drugs targeting infectious agents. If measured effects in an *in vivo* system are a composite of drug effects and innate or adaptive immunity, then assessment of true drug effect alone could be confounded. This issue can complicate results further since immune responses are not typically uniform across different species, or even between individuals of the same species. The use of immune-suppressed, animal-model systems may also complicate effect outcomes. Thus, understanding both protein binding and the immune response in PK/PD model systems is relevant to interpreting the overall findings from such studies. Several different types of immune responses have been described.[14] However, their contribution to the measured efficacy is further complicated by the absence of a clear immune correlate of protection for HIV.

Other variables are also relevant to the application of PK/PD models in drug candidate evaluation. Since a drug distributes to different body sites at different rates, single time-point-based assessments can be misleading. Therefore, it is important to assess exposure across the entire dosing interval using multiple time points. Aside from potentially providing a higher degree of accuracy, assessments over time support the derivation of dosing regimens that are optimized for the duration of effect. This is particularly relevant for the evaluation of chronic, intermittent, or episodic use of oral or topical products. For the purpose of this article, we define episodic as the use of PrEP in an on-demand fashion relative to sexual intercourse. We define chronic dosing as a dose schedule that may include either daily use or sustained release of drugs. Intermittent dosing is the use of a PrEP product for one or more days followed by a variable period of nonuse. In the case of infectious disease indications, studies over time may also be relevant to assessing the risk of resistance since selection of resistance can occur at subtherapeutic concentrations of inhibitory drugs.[15]

It is also important to understand the relationship between drug plasma concentrations (the typical target in PK assessments) and the concentrations in tissue or cell compartments where prevention is mediated. Blood may serve as the means of compartment supply; however, it is not necessarily the case that the amount of drug in plasma at a given time point is the same as that in a particular compartment of effect. This is relevant for an HIV prevention indication (particularly at the earliest time points after dosing), which will rely on specific drug concentrations in compartments of viral exposure—as opposed to a treatment effect, which depends more on chronically established plasma concentrations necessary to suppress ongoing viral replication in the periphery.[16]

When constructing dose-fractionation or escalation studies in these model systems, it is important to be sure that the full dynamic range of effect is evaluated from a drug concentration perspective. Establishing a concentration curve that spans the range of no effect up through maximal effect is critical to dose evaluation and selection. It is also important to understand the elimination kinetics of a drug. The decaying exposure over time could be relevant to medical management of individuals using the product, the understanding of potential risks associated with prescribed product use, as well as potential risks associated with variable adherence. Drug concentrations below the protective target could allow for

HIV infection, and the presence of these suboptimal concentrations in an HIV-positive individual could lead to the *in vivo* selection of resistant virus.

Another major challenge confronting PK assessments in tissue samples for a prevention indication is the fact that tissues themselves are multicompartmental, and only very specific subcompartments are susceptible to HIV infection (e.g., the $CD4^+/CCR5^+$ cell populations). Tissues are comprised of multiple cell types (immune, nonimmune, etc.), blood, and interstitial fluids. However, drug distribution may not be homogeneous throughout these distinct compartments and the cells within them. Thus, the simple homogenization of tissue samples followed by drug concentration determination may not be reflective of the true potential for target cell protection against HIV.

Intratarget cell drug concentrations are particularly important for prodrugs such as TFV, which require intracellular conversion to an active form and to other agents that may be substrates of uptake and efflux transporters. Similarly, drugs that inhibit active HIV replication also need to establish inhibitory intracellular concentrations. In cases of topical dosing, tissue sampling (e.g., genital or rectal tract samples after gel dosing) is further complicated by a risk of cocollecting the dosed drug present in the extracellular fluids associated with these tissue samples. The presence of drug in these fluids may result in an overestimation of drug exposure associated with the tissue sample. However, if a consistent relationship between these fluids and tissues can be documented, and the same sampling method is utilized across preclinical and clinical studies, the concentration obtained may be a viable surrogate of efficacy. Therefore, sample collection and processing methods are extremely relevant in the use of PK/PD model systems and must be carefully controlled and implemented in order to avoid obtaining biased results.

Results from studies involving different sample collection, processing, and analysis procedures may not be readily compared due to the potential variability inherent in the individual methods. The pharmacology of HIV prevention is further complicated since the target cells (such as tissue-based activated $CD4^+/CCR5^+$ lymphocytes) regularly circulate in and out of the compartments of exposure. Thus, the drug needs to be available at all times to those cells entering the compartment to achieve protective levels in those target cells. In the absence of validated processes for isolating cells from tissue, which ensures that intracellular concentrations remain unaltered during laboratory processing, it may be necessary to consider the contribution of blood and extracellular fluid concentrations to the results obtained in the analysis of gross sample homogenates.

The relevance of drug concentrations in specific compartments (or subcompartments) at the site of HIV exposure may be informed by the study of antibiotics in specific compartments and their antimicrobial effects. It is clear from multiple studies that curing of a bacterial infection likely involves more complicated phenomena than simply reaching a specific drug concentration at the site of infection. For example, the presence of drugs in the epithelial lining fluid (ELF) of lung tissue is relevant to the treatment of lower respiratory tract infections since ELF is often the site of extracellular infections.[17] Thus, for a specific indication, the appropriate biological targets for drug measurement cannot be assumed; they must be determined. Once the relevant compartment of effect has been identified, it is necessary to develop methods for specifically measuring exposure to understand the kinetics of penetration in these target subcompartments.[18] For drugs requiring intracellular activation (or drugs that inhibit HIV intracellularly) it will be necessary to determine their concentrations in specific target cells (e.g., activated versus resting $CD4^+/CCR5^+$ T cells), which is particularly complex from an analytical perspective. Moreover, measuring clinical intracellular concentrations of drugs at the time of viral exposure is an even more complex challenge.

Many of the types of assessments described above could be achieved with HIV prevention products through the use of appropriate animal models. Potential advantages of animal models include reasonable and workable sample sizes due to efficient (e.g., 80–100%) infection rates with a fixed number of viral exposures, the ability to conduct diverse virus challenges, and the capacity to comprehensively collect samples. However, animal models for a prevention indication could be more challenging to develop and optimize than those used for a treatment indication. To correlate findings in animal models to humans, it is necessary to understand the complex variables that control drug distribution and metabolism at a specific tissue site, as well as physiological and biological differences between systems. The following section summarizes the details of some of the animal models used to study HIV infection and evaluate candidate-prevention products. The challenges and gaps with these models are also described.

Animal Models for HIV Infection

In vivo *models for efficacy: nonhuman primate (NHP)*

The NHP model is one of the earliest established *in vivo* animal systems used to evaluate the efficacy of oral and topical prevention products. This model allows the assessment of blood/plasma and mucosal fluid/tissue drug concentrations in both cervicovaginal and rectal transmission.[8] The NHP system has some important limitations as an infection model. First, it typically requires challenge with an engineered virus [R5 or X4 tropic env simian/human immunodeficiency virus (SHIV), or RT SHIV]. Thus, viral challenge involves a single virus species, unlike the quasispecies that occur in human exposure, and the virus inoculum in this model is higher than human exposure with sex. Second, primarily due to costs, the number of animals routinely used per study arm is low, limiting the derivation of statistically meaningful results. Finally, viral challenge is not usually achieved in the presence of semen, nor does it involve coitus or coinfection with other sexually transmitted infections. The model has been developed primarily to support challenge with free virus, and most studies do not involve challenge with infected cells.

There are two versions of the vaginal challenge model currently being used, a *multidose* and a *single dose*. The *single-dose* virus challenge can be applied after preliminary treatment of rhesus macaques with medroxyprogesterone (or Depo-Provera), which enhances infection potential,[8] and prior treatment with the test product (e.g., ≥ 30 min before challenge). The *multidose* viral challenge model involves repeated viral exposures at a lower multiplicity of infection over time in pigtailed macaques (no Depo-Provera pretreatment) after application of the test product.[19] Neither of these approaches is a direct parallel to human exposure, and both are potentially complicated by the anatomical, histological, and

physiological differences between the macaque and the human female reproductive tracts. Despite these issues with both NHP models, they have been used in a number of studies to evaluate ARV-based prevention products and strategies (Table 3). Both *single-dose* and *multidose* versions of the challenge model are also available for rectal exposure.

A number of laboratories support the use of the *single-dose* model, and several studies have been reported for a variety of microbicide-candidate products in a variety of formulations.[20–23] This model has been used to examine polyanion-type microbicide products, such as PRO-2000[24] and Carraguard-based formulations.[25] In the case of the polyanion products, low-level efficacy was reported. However, neither of these products proved efficacious in human studies. The Depo-Provera pretreatment serves to render the animals more susceptible to infection (likely due to epithelial and mucus thinning) and synchronizes the menstrual cycle. In this model, reproductive hormones may also influence drug absorption into genital tissues.[26]

Evaluation of HIV prevention products with the *multidose* viral challenge model in pigtail macaques has gained attention. For example, the *multidose* macaque model was used to demonstrate protective effects of both 1% TFV gel and 1% TFV/5% FTC combination gel after vaginal application and subsequent challenge with SHIV.[19] This study demonstrated 100% efficacy for both products. PK evaluations were restricted to plasma concentrations. Although the study demonstrated 100% efficacy, only 76% of plasma samples contained detectable TFV and/or FTC. A subsequent study in the same model examined the window of protection of 1% TFV gel and its relationship to the more relevant drug concentrations in vaginal tissues.[27] This study demonstrated a wide window of protection ($\sim$75% efficacy) when gel was applied 3 days before SHIV challenge and suggested that TFV-DP concentrations in vaginal lymphocytes that are above the *in vitro* IC_{95} may be good predictors of protection.[27] Protection in macaques with 1% TFV gel was higher than what was observed in the CAPRISA and VOICE trials, highlighting the contribution of other factors including adherence and inflammation to lack of protection.[2]

More recently, this *low-dose* model was also used to examine the efficacy of orally dosed ARV. In a study involving various oral-dosing regimens of TDF/FTC followed by repeat rectal exposure to virus, protective effects were determined. In a separate set of animals, concentrations of TFV and FTC were measured in rectal fluid, and the active intracellular metabolite of TFV diphosphate (TFV-DP) was measured in rectal tissue and PBMCs after a single dose.[28] Because the PK was performed in a set of animals that was different from the set challenged, PK/PD relationships could not be established. However, several dosing regimens involving intermittent use of the drug were found to be as effective as daily use in protection against rectal challenge. It was concluded that this finding was due to the long intracellular persistence of TFV-DP seen in macaque rectal cells. Further PK/PD correlations in the same animal, and to human study results, are still needed for currently investigated products and beyond.

In vivo *models for efficacy: humanized mouse*

In 2002, a model involving severe combined immunodeficiency (SCID) mice engrafted with human peripheral blood leukocytes was developed in which cell-associated HIV was transmitted through vaginal exposure.[29] Although the *in vivo* relevance of cell-associated HIV transmission in humans is unknown, this model was used to demonstrate the protective effects of several candidate microbicides.[29,30]

Recently, more elaborate humanized mouse infection models have been developed [the RAG-hu mice;[31,32] bone marrow/liver/thymus (BLT) mice[33]]. In the BLT humanized mouse model, SCID mice are implanted with human thymus and liver tissue followed by irradiation and infusion with human $CD34^+$ hematopoietic progenitor cells.[33] This results in immune reconstitution of humanized lymphoid cells in both the female reproductive tract and gastrointestinal tract, which can then be infected with HIV. Although relatively high doses of virus are needed to achieve infection, there is a clear advantage to being able to use clinical HIV isolates for vaginal and rectal challenge, rather than the engineered strains of SHIV used in the NHP model. Advantages of the humanized murine models include the continuous production of human immune cells, which allows their use in long-term experiments, that multiple human donors can be used to produce multiple cohorts of mice, and that multiple prevention (oral and topical) strategies can be evaluated.[33]

Costs to generate humanized mice can rival the costs of NHPs, as they require long periods of time for their production. However, there is a relatively unlimited supply of this model compared to the NHP. The anatomical restrictions of the mouse may limit the volume and dosage forms that can be evaluated with this model. Nonetheless, assays for quantitation of drug in various matrices have been developed and applied to this model,[34] and they may be used more easily for PK/PD assessments. Thus, humanized mouse models offer a potential means of studying prevention efficacy in ways that parallel but also differ from the NHP model. Furthermore, having multiple models for comparison with the human system helps address the limitations associated with them individually.

The relevance of both NHP and humanized mice models to the human system is as yet unclear. Without continued development and characterization, the biological limitations, as well as further benefits, of the models will be difficult to address. Improvements needed to make these models more robust for PK/PD use include (1) characterization and understanding of histological and biological similarities and differences with the human system, (2) optimization and standardization of both sample collection and processing methods, (3) standardization of drug treatment and virus-challenge techniques, (4) understanding if any differences exist in the PK/PD relationship of a given compound compared to humans, and (5) expanding the data set with different drugs.

Human PK/PD Assessments of HIV Prevention Product Candidates

Drug concentrations in human plasma, PBMCs, genital and colorectal tissues, and genital and colorectal fluids have been measured for a number of HIV prevention candidates after clinical dosing. For example, TFV and TFV-DP were measured in plasma, cervicovaginal fluid (CVF), vaginal tissue, and cervical cells after vaginal dosing with 4 ml of 1% TFV gel.[35] High concentrations of TFV and TFV-DP could be found

Table 3. Preclinical Animal Studies with Emtricitabine, Tenofovir, and Tenofovir Disoproxil Fumerate

Drugs and dose	*Interventions*	*Animal model*	*Main findings*
TFV, 20 mg/kg (subcutaneous)	TFV initiated −48 h and continued for 4 weeks	Long-tailed macaques	Full protection
TFV, 30 mg/kg (subcutaneous)	Two doses given at −4 h/+24 h	Rhesus macaques	Full protection
TDF, 0.01–0.02 mg/kg (oral)	TDF initiated −24 h and maintained during virus inoculations	Rhesus macaques	No protection; low doses of TDF
TDF, 10 mg/kg (oral)	Repeated cycles of daily TDF initiated 1–2 days before exposure	Rhesus macaques	Partial efficacy; infection associated with low drug levels
TDF, 22 mg/kg (oral)	Daily or weekly TDF	Rhesus macaques	Infection delayed in treated animals
FTC, 3.5 mg; TDF, 5.2 mg (intraperitoneal)	Daily FTC/TDF initiated 48 h prior to exposure and continued for 7 days	Humanized mice	Complete protection
FTC, 20 mg/kg; TDF, 22 mg/kg; TFV, 22 mg/kg (oral or subcutaneous)	Daily FTC (subcutaneous); daily oral Truvada; daily or intermittent FTC/TFV (subcutaneous)	Rhesus macaques	Risk of infection reduced with FTC and Truvada; no infection with high TFV doses
FTC, 20 mg/kg; TDF, 22 mg/kg (oral)	Intermittent PrEP: fixed dosing and event-driven	Rhesus macaques	Efficacy with two weekly doses of Truvada given at different intervals
FTC, 3.5 mg; TDF, 5.2 mg (intraperitoneal)	Daily FTC/TDF for 7 days	Humanized mice	Protection regardless of the route of exposure
FTC, 20 mg/kg; TDF, 22 mg/kg (oral)	Two doses given 24 h prior and 2 h after virus exposure	Rhesus macaques	Full protection
1% TFV (rectal gel)	Gel applied 15 min to 2 h before exposure	Rhesus macaques	Partial (6/9) protection
1% TFV; 1% TFV and 5% FTC (vaginal gel)	Gel applied 30 min before virus exposure	Pigtail macaques	Full protection
1% TFV (vaginal gel)	Gel applied 4 h before and 4 h after virus exposure (BAT24)	BLT mice	High protection (7/8)
1% TFV gel (vaginal gel)	Gel applied 3 days before virus exposure	Pigtail macaques	Partial (4/6) protection

Adapted from Garcia-Lerma JG and Heneine W: Animal models of antiretroviral prophylaxis for HIV prevention. Curr Opin HIV AIDS 2012;7(6):505–513. Copyright Lippincott Williams & Wilkins.
TFV, tenofovir; TDF, tenofovir disaproxil fumerate; FTC, emtricitabine; PrEP, preexposure prophylaxis.

in the genital tract over 24 h after single and multiple dosing. In a retrospective analysis, both TFV CVF concentrations >1,000 ng/ml and detection of TFV in blood plasma were associated with reduced HIV infection in the CAPRISA 004 study.[36,37] Recently, the MTN 001 study demonstrated that vaginal dosing achieved much lower plasma concentrations and higher vaginal tissue concentrations than those achieved with oral dosing.[38] Based on these results, vaginal dosing with TFV should provide higher concentrations of active drug in the compartment of viral exposure than oral dosing. However, results from the VOICE trial suggest issues beyond the antiviral effect of TFV are relevant to efficacy/effectiveness.

Plasma, genital secretions, and tissue concentrations of TFV, TFV-DP FTC, and FTC- triphosphate (TP) have been similarly evaluated after a single oral dose of TDF/FTC.[39] Interestingly, the investigators noted divergent penetration of FTC and TFV in vaginal/cervical and colorectal tissues. TFV exposure in the female genital tract was similar to blood plasma, whereas TFV exposure in colorectal tissue was 100 times higher. The same pattern was seen with intracellular TFV-DP; concentrations were 100-fold higher in colorectal tissue than in vaginal/cervical tissue. The opposite trend was seen for FTC and FTC-TP; exposure in the female genital tract was approximately five times higher than exposure in colorectal tissue.

Plasma and PBMC drug concentrations have been evaluated in efficacy studies involving TDF/FTC. Generally, drug exposure is lower in those who become infected with HIV than those who remain protected.[3,4] It is difficult to extrapolate findings from one study to another, as different populations of subjects were followed at different time intervals and had different sampling strategies. However, it does appear that the divergent pharmacology of TDF/FTC in mucosal tissues may explain the different rates of efficacy in the face of suboptimal adherence.[40] For example, the iPrEX study demonstrated 44% protection in men who were only sporadically taking their daily doses of TDF/FTC; only 18% of uninfected men in a case-cohort analysis had evidence of daily drug taking behavior, and 52% were using <2 doses of TDF/FTC per week.[41] Yet no efficacy was noted in the FemPrEP study[5] where 20–30% of women had evidence of recent drug taking behavior. Similar to what was noted in macaques,[27] higher colorectal penetration of TFV may not necessitate TDF/FTC's daily use for efficacy in MSM, whereas lower cervical/vaginal penetration may require consistent daily adherence for efficacy.

Recently, investigators have been testing various approaches to *ex vivo* PD assessments in patients receiving HIV prevention products. For example, infection was inhibited after an *ex vivo* HIV challenge of rectal biopsies obtained from men who dosed rectally with 0.25% UC-781 gel.[42] A similar effect was noted with rectal application of 1% TFV gel.[43] Rectal tissue biopsies collected 30 min after single and multiple rectal dosing, as well as a single oral dosing, were analyzed for drug concentrations, and evaluated in the *ex vivo* HIV challenge model.[43] An increased level of protection against HIV challenge correlated with higher tissue concentrations of TFV-DP. In a cell culture model, a significant increase over baseline endogenous anti-HIV activity was noted in CVL from women who had previously applied TFV gel, and this activity correlated with TFV concentration.[44] Moreover, application of a single dose of TFV topically or systemically to ectocervical explants was effective at preventing HIV infection.[45]

Although these *ex vivo* clinical studies have suggested a correlation between drug concentration and efficacy, these methods and models are relatively new and complex and require further development and optimization.[46] As noted earlier, methods for collection and processing of tissue samples are extremely relevant to outcomes in terms of accurate measurement of drug exposure in tissues. Although the typical method for processing tissue samples is homogenization, specific cell isolation may be more informative. However, despite technical feasibility, quantifying intracellular drug concentrations of lipophilic compounds that accurately reflect the original *in vivo* concentrations at the end of a lengthy cellular isolation process is complex. Moreover, current analytical instrumentation require large numbers (>100,000) of cells. Although these cell numbers can be isolated from 20 biopsies of colorectal tissue, the number of biopsy samples that can be collected from the female genital tract[2–4] is significantly less. Cervical cytobrush sampling also yields small numbers of mononuclear cells.[47] Consequently, tissue collection represents a technical and quantitative challenge to the implementation of these human PD models.

The majority of the *ex vivo* clinical PK/PD effort to date has involved TFV and TDF/FTC. Therefore, the performance of these model systems with drugs that differ in terms of physicochemical properties or mechanisms of action needs to be investigated. Also, the types of PK/PD assessments that have been applied in human systems (i.e., dose, biopsy, *ex vivo* challenge) have not been extensively evaluated in any of the animal model systems available for the study of HIV prevention. Determining if an animal tissue explant system can predict animal efficacy would assist in interpreting and extrapolating human tissue data to clinical outcomes. Expanded study of animal models, which afford more flexibility in terms of use, may provide significant insight into the relationships between drug exposure and biological effect. However, the clinical relevance of animal model data will require a calibration between animal models and humans. It may be that certain correlations are drug specific. The evaluation of more drugs of different classes is essential to understand these relationships and determine the overall utility of animal models as predictors of effective HIV prevention strategies.

Finally, the study of PK and PD in animal or human systems for HIV prevention ideally should go beyond simple correlations of drug concentrations in different compartments with efficacy. Efforts should be made to understand the types of relationships described earlier for the study of antimicrobials (e.g., AUC/MIC, C_{max}/MIC, and time>MIC) for HIV prevention candidates. Different drugs may have different parameter relationships, and understanding those relationships may be crucial to determining the efficacy potential of the drug. The lesson from other systems and indications is that the simple measure of concentration and effect (or the assumption of effect) will likely be inadequate to predict human efficacy outcomes *in vivo*. Multiple and more sophisticated relationships will need to be studied.

Regulatory Aspects of PK/PD

At present, there is no regulatory requirement for animal model data to support the efficacy claims of a product.

Regulatory agencies recognize the utility of animal models in the study of mechanism of action and in efforts to determine dose and dosing regimen, but it is also clear that animal efficacy data are not accepted as representative of efficacy in humans. In the case of HIV prevention, the utility of animal models from the regulatory perspective is dependent on the degree to which the model resembles the human system. Of fundamental importance is whether the animal model is representative of infection in humans. As outlined earlier, we still do not know how current animal infection models correlate to natural HIV infection in humans. Similarly, it is important to demonstrate that the PK profile in the relevant compartment(s) of the animal model can mimic human exposure. It is also essential to demonstrate that a model can be used to characterize drugs with different mechanisms of action and routes of administration. Thus, addressing these issues with available models is important in establishing regulatory significance. Even if models closely parallel human systems, regulators will still require clinical trials.

Therefore, the ultimate benefit of such models resides in their ability to better inform and streamline clinical study designs. Perhaps more relevant for regulatory purposes than animal models is the accurate assessment of product adherence in clinical trials. Understanding actual product use as opposed to prescribed product use is crucial in interpreting the outcomes of clinical trials. Inadequate adherence to product in trials can lead to underestimation of efficacy, and a lack of accurate adherence data can confound the understanding of PK and efficacy outcomes in trials.[48]

Summary

To select those new HIV prevention products capable of having the highest impact on the epidemic, it will be necessary to assess their efficacy potential *prior* to Phase 3 studies with strategies that are more robust and predictive than those used in the past. Thus, with the overarching goal of identifying the most appropriate new products for development and enhancing our ability to interpret results from clinical trials, a better understanding of PK/PD is a key requirement. The joint DAIDS/BMGF Antiretroviral Pharmacology of HIV Prevention Think Tank reached the consensus that one of the most critical needs in the field of HIV prevention is the delineation of the PK relationship between human and animal models of HIV infection. The Think Tank participants also defined a number of key studies that could potentially address this specific need (Table 4).

Table 4. Key Issues and Studies for Pharmacokinetics/Pharmacodynamics in HIV Prevention

1. Complementary, codesigned animal and human studies for PK/PD model development and comparison
2. Dose-ranging, dose-fractionation and time-course studies in humans and animal models
3. PK/PD assessments for defining prevention vs. treatment dose
4. Tissue targets, tissue sampling, process and method definition, optimization, and standardization
5. Expanded PK/PD studies in animal models (humanized mice, NHP)

PK/PD, pharmacokinetics/pharmacodynamics; NHP, nonhuman primates.

A fundamental understanding of the relationship between drug concentration and effect (or potential effect) is critically needed. To generate these data, assessments of antiretroviral PK/PD in animals need to be applied over wider dose ranges, and need to be based on accurate assessment of drug exposure. Discriminating between free (e.g., protein-unbound) and total (e.g., protein-unbound + protein-bound) drug concentrations, as well as accurately assessing drug exposure in subcompartments, will require the development of sophisticated sample collection, processing, and analysis techniques. A wider array of drugs and drug classes needs to be evaluated in these systems. Both animal and human PD models need further development, optimization, and standardization; there needs to be a better understanding of correlations, if any, between these model systems.

NIAID/DAIDS and BMGF are committed to addressing the critical priorities identified at the Think Tank so that our ability in selecting candidate products with the highest potential for impact on the epidemic, as well as understanding results from clinical trials, may be significantly enhanced. Under the auspices of the NIAID/DAIDS Comprehensive Resources for HIV Microbicides and Biomedical Prevention (CRMP) contract, an HIV Pharmacology Best Practices Working Group has been established from a group of interested Think Tank members. This group will focus their initial effort on the key issues described above with an emphasis on planning studies that will attempt to establish a PK/PD relationship between different animal models and between animal models and humans.

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Non-ARV-Based Products for HIV Prevention

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Prevention of vaginal SHIV transmission in macaques by a live recombinant *Lactobacillus*

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Abstract

Most HIV transmission in women occurs through the cervicovaginal mucosa, which is coated by a bacterial biofilm including *Lactobacillus*. This commensal bacterium plays a role in maintaining healthy mucosa and can be genetically engineered to produce anti-viral peptides. Here, we report a 63% reduction in transmission of a chimeric simian/human immunodeficiency virus ($SHIV_{SF162P3}$) after repeated vaginal challenges of macaques treated with *Lactobacillus jensenii* expressing the HIV-1 entry inhibitor cyanovirin-N. Furthermore, peak viral loads in colonized macaques with breakthrough infection were reduced 6-fold. Colonization and prolonged anti-viral protein secretion by the genetically engineered lactobacilli did not cause any increase in proinflammatory markers. These findings lay the foundation for an accessible and durable approach to reduce heterosexual transmission of HIV in women that is coitally independent, inexpensive, and enhances the natural protective effects of the vaginal microflora.

INTRODUCTION

Halting the global HIV/AIDS epidemic requires interventions that have practical and achievable goals in the developing world setting. Topical prevention strategies designed to

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Conflict of interest: LAL, XL, YL, and PPL work for Osel Inc., a company developing this approach as a topical microbicide.

DISCLOSURE

LAL, XL, YL, and PPL work for Osel Inc., Mountain View, CA, a company developing this approach as a topical microbicide. BIOQUAL (BES) and ABL (RP) are for-profit institutions and are subawardees to Osel, Inc.

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interdict infection at the mucosal surface represent one approach. Early prevention trials examined non-specific microbicides, such as detergents and polyanions (e.g., Nonoxynol-9 and cellulose sulfate), which were wholly ineffective or in some cases may have increased the risk of HIV acquisition due to the inflammatory and viral-enhancing properties of the compounds employed [1–7]. These clinical trials also revealed that coitally-dependent products, such as Carraguard, were used less frequently than women reported [8], highlighting the difficulty of achieving the level of adherence required for efficacy of such microbicides. Recent results from the CAPRISA 004 trial provided a much-needed proof of concept that a topical agent, the specific antiretroviral drug tenofovir delivered as a gel, can prevent the acquisition of HIV in women [9]. However, the efficacy of this approach was modest (39%), probably in large part because it is a coitally dependent regimen and the gel must be applied within 12 hours both before and after sexual intercourse for maximum efficacy. Concluded from the trial results was that the main goals are to improve adherence and to develop coitally independent microbicide strategies.

In many cultural settings, women need a product that can be used covertly without obtaining the permission of their sexual partner. Additionally, the cost of the HIV prevention must be affordable to the developing world. Thus, there is still a need for products that block HIV transmission, are safe and easy to use, and are coitally independent, discreet, as well as cost effective.

The majority of viral transmissions in women occur during unprotected vaginal intercourse on the mucosal surfaces of the endocervix, cervix and vagina. In macaques [10] and human vaginal explant tissues [11,12], Langerhans cells and intraepithelial CD4+ T cells are the targets of HIV-1 viral entry and initial replication [12]. Mucosal surfaces of the lower female reproductive tract are normally coated with a biofilm of commensal bacteria including lactobacilli, which are an important component of the vaginal microbiome and provide the first line of defense against invading pathogens [13]. Lactobacilli acidify the vagina by producing lactic acid [14] and may reduce the risk of acquiring HIV-1 and other sexually transmitted infections [15,16].

One practical and cost-effective approach to protect the vaginal epithelium is to colonize it with a recombinant *Lactobacillus* expressing an anti-viral protein, thus transforming the vaginal microflora into a “live” bioshield [17]. This is a novel approach to preventing the transmission of HIV, and eventually other mucosal pathogens, and thereby represents a platform approach to intervention of disease transmission that is both discreet to the user and partner. Importantly, it is also inexpensive to manufacture.

Cyanovirin-N (CV-N), a protein originally isolated from the cyanobacterium, *Nostoc elipsosporum*, was selected as a model HIV inhibitor for this approach. CV-N has broad cross-clade potency against HIV [18,19] including primary isolates [20,21]. Previous studies showed that CV-N protein, formulated in a gel, protected macaques from vaginal and rectal challenge [22,23].

We previously described a *Lactobacillus* strain, *L. jensenii* 1153–1666, that is genetically engineered to secrete an amino-terminally modified CV-N protein (P51G) [24]. CV-N protein

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isolated from the culture media of this strain has potent anti-HIV activity against CCR5- and CXCR4-tropic viruses *in vitro* whereas control media from the parental strain, *L. jensenii* 1153 OSEL 175, is inactive [24]. Compared to the parental strain, *L. jensenii* 1153–1666 displays no loss of fitness, grows to the same levels and produces similar levels of lactic acid and hydrogen peroxide [24]. Although *L. jensenii* 1153–1666 persisted in the estrogenized-CD1 mouse model [24], mice do not support *Lactobacillus* colonization as they have significantly different vaginal biology from primates. Therefore, to advance this platform technology, a non-human primate model was developed.

The rhesus macaque model is commonly used for microbicide and vaccine studies, but has not previously been used for testing live microbicides; therefore we performed extensive characterization to determine whether rhesus macaques were suitable as a model. We found that Chinese and Indian-origin rhesus macaques typically harbor abundant endogenous vaginal *L. johnsonii* as their predominant species, and, like humans, generally have only one vaginal *Lactobacillus* strain at a time [25]. We examined the DNA fingerprints and biochemical profiles of *L johnsonii* strains and found that most are strong H_2O_2 producers and are similar to the human strain *L. gasseri* [25]. We further characterized changes in the vaginal microbiome during menses. Unlike humans, the endogenous *Lactobacillus* flora of the macaque varies more throughout the menstrual cycle, with the lowest levels found at or around menstruation [25,26]. Vaginal pH in macaques is also higher than in humans with the average value around 6.3 among the animals in this study. Higher vaginal pH is also found in women with bacterial vaginosis (BV), which predisposes them to increased risk of HIV infection [16]. Thus it has recently been suggested that the macaque may be a useful model for HIV protection studies because they resemble women with BV [27]. Displacement of endogenous lactobacilli followed by long-term colonization of rhesus macaques with human *L. jensenii* 1153 strain has been achieved, making this animal model useful for pre-clinical evaluation of live *Lactobacillus*-based anti-viral approaches [25].

Here, we used the macaque model to test colonization of recombinant lactobacilli, CV-N protein expression and efficacy of the recombinant *L. jensenii* 1153–1666 in a repeated vaginal SHIV challenge model, and demonstrate that colonized animals show a 63% reduction in acquisition of the virus. We also examined vaginal pH, histology and biomarkers of inflammation to assess the safety of this approach.

RESULTS

Colonization and protein expression of recombinant *Lactobacillus* in macaques

First we examined the duration of colonization and *in situ* protein expression using *L. jensenii* 1153–1666 in macaques. Vaginal inoculation of two macaques, M1 and M2, with live *L. jensenii* 1153–1666 delivered in hydroxyethylcellulose HEC on five successive days led to sustained colonization and secretion of full length CV-N that was detected in cervical vaginal lavage (CVL) as early as 24 hr post inoculation, and persisted at three and at six weeks post inoculation (Fig.1). Decreases in the CV-N levels in CVL, as seen in animal M1 at week 3, appear to be due to lower levels of *Lactobacillus* colonization during menses [25]. CV-N in CVLs collected from macaques was measured at a concentration of 83–160 ng/ml.

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Histology and immunohistochemistry were performed on vaginal biopsies taken from control macaques or macaques colonized with *L. jensenii* 1153–1666. Colonization by the recombinant *Lactobacillus* did not lead to observable inflammation or disruption of squamous epithelial cell integrity as indicated by examination of vaginal histology sections from *L. jensenii* 1153–1666 colonized macaques compared to controls (Fig. 2a). To detect CV-N *in situ*, biopsies were incubated with control serum or anti-CV-N serum. The CV-N specific serum revealed co-localization of CV-N protein with *Lactobacillus* bacteria on the vaginal mucosa and in the vaginal lumen (Fig. 2b).

Examination of mucosal markers of inflammation, vaginal pH and immunogenicity

To determine the effects of the recombinant *Lactobacillus* on the vaginal environment, we performed sequential measurements of proinflammatory cytokines and pH in CVL of animals in the absence of detectable *Lactobacillus*, in the presence of spontaneously occurring endogenous *L. johnsonii*, and in the presence of introduced recombinant *L. jensenii* 1153–1666. The cytokine measurements focused on four pro-inflammatory mediator cytokines that are often used as biomarkers of inflammation and indicators of mucosal barrier function: IL-1β, IL-1ra, IL-6, and IL-8 [28,29]. As shown in Fig. 3 a–d, there was a high variation from animal to animal in the measured values of these cytokines, but no consistent or statistically significant differences as a function of bacterial colonization status. The average vaginal pH value of the macaques used in this study, as determined by seven sequential measurements, was 6.3 ± 0.78 (mean ± SEM). Comparison of uncolonized animals to those harboring endogenous *L. johnsonii* or introduced *L. jensenii* 1153–666 did not reveal any significant variations (Fig. 3e). Additionally, macaques that were repeatedly exposed to *L. jensenii* 1153–1666 had no detectable IgG, IgA or IgM response to either CV-N or killed *Lactobacillus* measured in CVL whereas macaques immunized via the intramuscular route with 100 μg/ml purified CV-N delivered in adjuvant exhibited a robust mucosal and systemic immune response to CV-N (data not shown).

Inhibition of HIV-1 infection in an EpiVaginal tissue model

The MatTek EpiVaginal™ Tissue Model, was used to assess the activity of the *L. jensenii* 1153 (parental strain) vs. *L. jensenii* 1153–1666 (producing CV-N). Infection was monitored by p24 release at day 3, 5 and 8-post infection. It was shown that co-cultivation of the tissue model with the *L. jensenii* 1153 parental strain reduced infection by 23%, compared to a control with no added bacteria ($p=0.1$), whereas co-cultivation with CV-N-producing *L. jensenii* 1153–1666 inhibited infection by 72% ($p<0.008$) (Fig. 4).

SHIV challenge protocol and results

To determine the efficacy of the *L. jensenii* 1153–1666 live prevention strategy in the macaque model, we designed a repetitive vaginal challenge protocol using the well-characterized CCR5-tropic virus $SHIV_{SF162P3}$. The repeated challenge model was chosen because it more closely resembles human infection than does a single challenge using either a very high viral dose or pretreatment with DepoProvera®. The study protocol is shown in Fig. 5a. Twelve menstrually cycling adult female macaques were given vaginal azithromycin suppositories to reduce endogenous *Lactobacillus* flora. They were then

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colonized with *L. jensenii* 1153–1666 for five days initially, and again at 48 and 24 hrs prior to each of six weekly challenges. Each challenge was performed a minimum of 24 hr after the bacterial inoculation, a period sufficient for CV-N protein accumulation to occur (Fig 1a) as well as to demonstrate the potential of coital independence. The challenge contained 300 median tissue culture infectious doses ($TCID_{50}$) of $SHIV_{SF162P3}$; this amount of virus was previously determined to infect approximately 1 of 3 animals after each challenge. We confirmed the presence and quantity of *L. jensenii* 1153–1666 by culture and the expression of CV-N by analysis of recovered *L. jensenii* 1153–1666 (Table 1).

The results of the challenge study are shown as a Kaplan-Meier analysis (Fig. 5b). Twelve control macaques were infected at a rate of 35.4% following each vaginal $SHIV_{SF162P3}$ challenge; 31 challenges were required to infect 11 controls (11/31 = 35.4%), with one control macaque remaining uninfected following six challenges. By contrast, 12 macaques treated with *L. jensenii* 1153–1666 (LB-CVN) were infected significantly less frequently: 61 challenges were required to infect eight LB-CVN treated macaques (8/61 = 13.1%), with four macaques remaining uninfected following six challenges. Thus, this live prevention strategy reduced the infection rate by 62.9% ($p = 0.0039$).

The data were further analyzed by separately considering the three sub-groups of the 12 control animals that received no exogenous *Lactobacillus*: those that received no treatment of any type (n=4), HEC only (n=4) or azithromycin + HEC (n=4). Fig. 5c shows that each of these subgroups has a greater infection rate than the experimental animals, and this difference was significant for the non-treatment group ($p = 0.008$) and the HEC only group ($p = 0.002$).

Unexpectedly, we also observed that the peak SHIV RNA plasma viral levels in macaques with breakthrough infection were 6-fold lower in experimental animals that had been colonized with *L. jensenii* 1153–1666 compared to infected non-colonized control animals (Fig. 6). Despite variability in the peak viral loads, this difference was significant ($p = 0.029$).

The serological response to SHIV was evaluated by testing all plasma samples for seroconversion and quantifying antibody titers to p27, a simian immunodeficiency virus (SIV) core antigen. All macaques with detectable plasma viral load also generated antibodies to SIV specific antigens with a prominent p27 protein band in Western blots and positive titers to p27 by ELISA, whereas all protected animals had no detectable SIV-specific antibodies by either Western blot or ELISA (data not shown).

In addition, we measured TRIM5α alleles for 20/24 macaques used in the study, including all 12 Lactobacillus inoculated animals and 8 control animals (Supplemental Table 1). We found no correlation between TRIM5α resistant or sensitive phenotype and rate of infection or viral load.

DISCUSSION

This study examined a live recombinant *Lactobacillus* producing an HIV-entry inhibitor on the cervical/vaginal mucosa as a means to prevent transmission of $SHIV_{SF162P3}$ to

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macaques. Genetically engineered lactobacilli, which are capable of forming a colonizing biofilm on the cervical/vaginal mucosa, can provide a continuous delivery system for anti-viral proteins. Furthermore, they provide beneficial protective effects of a normal vaginal microbiome, which helps to control a variety of vaginal infections through multiple mechanisms including H_2O_2 and lactic acid production [13–16].

Given the multifactorial nature of this approach, the present study was designed to evaluate the combined, natural and engineered antiviral properties of L. *jensenii* 1153–1666 rather than to focus only on anti-viral protein expression. Control animals were not experimentally colonized with wild-type lactobacilli, but. two control subgroups did receive either HEC placebo gel, which was used as a carrier for the *L. jensenii* 1153–1666, or HEC plus azithromycin, an antibiotic that was administered to reduce endogenous flora. There was a statistically significant difference in protection between *L. jensenii* 1153–1666 colonized animals and the control group as whole and between *L. jensenii* 1153–1666 colonized animals and the control non-treated or HEC only subgroups. However, the number of animals in the individual subgroups was too small for a definitive sub-group comparison.

Now that efficacy of *L. jensenii* 1153–1666 has been established, further experiments are required to differentiate the effects of the host organism as compared to the produced protein. *Lactobacillus* is associated with good vaginal health, and may have some degree of indigenous protective activity against a variety of pathogens including HIV. Initial *in vitro* experiments using HIV infection of a human vaginal tissue model showed that co-cultivation with the *L. jensenii* 1153 parental strain reduced infection by 23%, (but did not reach statistical significance $p=0.10$) compared to a control with no added bacteria, whereas co-cultivation with CV-N-producing *L. jensenii* 1153–1666 inhibited infection by 72% ($p<0.008$). By contrast, in a MAGI cell attachment assay, total conditioned media from the parental strain showed no activity compared to strong inhibition by material from CV-N-producing cells, suggesting that whatever inhibition is due to the innate activity of *Lactobacillus* occurs through a mechanism distinct from the fusion inhibiting activity of CV-N (data not shown).

An essential criterion for a successful microbicide is that it not cause inflammation, which is well known to increase susceptibility to HIV infection [16,30–32]. In our study, we found no evidence for *L. jensenii* 1153–1666-induced inflammation either by microscopic examination of vaginal biopsy samples or by measurement of multiple pro-inflammatory mediator cytokines. In addition, CV-N did not appear to be immunogenic.

In many women, lactobacilli are the dominant flora and contribute to the average pH value of around 4 through lactic acid production [14]. The pH of the vagina may also influence HIV transmission, with low pH values acting in a protective fashion. But it's important to note that HIV-1 is introduced in semen, thus vaginal pH can transiently fluctuate to neutral or even basic pH for several hours post coitus [33]. CV-N protein is active in a range from pH 4–8.2 [17].

In this model, we show that macaques have a more dynamic and complex flora, which is more similar to women with BV. Thus, the macaque study may underestimate the potential

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efficacy of recombinant lactobacilli in humans, in whom the bacteria will play a more dominant role. Importantly, a Phase 2a study has shown that 61–78% of women with BV can be re-colonized with an introduced *L. crispatus* strain [34].

CV-N protein was used in this study because it has broad activity against a wide range of HIV-1 isolates, it is stable in the vaginal environment, and it is potent at achievable concentrations. Huskins *et al.* [35] have questioned the safety of CV-N as a topical microbicide because they observed mitogenic activity and enhanced expression of T cell activation in peripheral blood mononuclear cells exposed to certain preparations of the protein. However, the clinical relevance of the Huskins study is unclear since it is not known whether the proteins used in their analysis were monomeric (P51G) [36] or dimeric (P51) or whether they were contaminated with endotoxin. By contrast, a study by Buffa *et al*. found low levels of T-cell proliferation only during prolonged exposure to extremely high CV-N concentrations (360,000 nM) with no tissue toxicity [20]. We observed that expression of the low 1–2 μg/ml (92–183 nM) levels of monomeric CV-N (P51G) produced by *L. jensenii* 1153–1666 did not enhance expression of typical markers associated with mucosal barrier function or inflammation. While additional biomarkers evaluation is needed to determine any toxicity that may be seen with long-term use, these preliminary studies suggest that this low level is safe.

With the recent characterization of the early HIV-1 infection events and findings that a majority of HIV transmissions involve a single founder virus [37], there has been a re-examination of SHIV and SIV models in an attempt to mimic the physiologic dosage seen in natural infection. This has led to the use of lower doses with repeated challenges, requiring more prolonged studies but being more physiologically relevant than high dose models. For example, low-dose rectal inoculations of macaques with SIV were shown to more closely recapitulate human mucosal infection by HIV-1 [38] in terms of the number of viral variants that were transmitted. Repeated challenge approaches also increase the statistical power achieved [26,39]. Perhaps most importantly, lower challenge doses may be responsive to clinically achievable doses of microbicides; protection against high doses typically requires extraordinarily high and potentially toxic doses of the tested compounds [22,23,40–42]. For example, when tested in a cynomolgus macaque model, 0.5–2% CV-N, equivalent to 5–20 mg/ml (454 μM-1.8 mM) was required to inhibit 83% of infections in a very stringent vaginal challenge of 5000 $TCID_{50}$ on epithelium that was thinned with medroxyprogesterone [22]. Such early studies lead to the belief that vast excesses of microbicide were needed to provide efficacy and led to concerns about toxicity of the products used at such high levels.

These factors led us to the selection of a repeated low-dose vaginal challenge model for testing *L. jensenii* 1153–1666. The $SHIV_{SF162P3}$ stock was diluted to a level that infects untreated macaques at approximately 30% per challenge. This infection rate is still higher than that for women infected heterosexually, thus providing a stringent test of protection, yet more closely mimics natural infection than use of single high dose.

The reduction in viral load in macaques receiving *L. jensenii* 1153–1666 was an unexpected finding. It has been hypothesized that small founder populations of virus undergo local

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expansion prior to systemic dissemination [43]. It is possible that these initial foci of infection are still accessible to the mucosally produced CV-N, providing some level of viral control after the initial infection event. Certain virus strains, such as SIV_{E660}, are restricted by TRIM5α alleles. We found no correlation between susceptibility related to TRIM alleles. Restrictive MHC alleles, such as MamuA01, are rare in Chinese-origin macaques, but more common in Indian-origin macaques [44,45].

In this study, macaques were repeatedly colonized with *L. jensenii* 1153–1666 at least 24 hrs prior to each viral challenge. However, because this *Lactobacillus* strain was shown to colonize and secrete CV-N for at least six weeks following administration, it likely has the capacity to provide considerably more durable protection with relatively infrequent re-colonization. The ability of the recombinant *Lactobacillus* to continually colonize also suggests that our introduction of a single gene into the bacterial chromosome did not have a deleterious effect on its growth and survival *in vivo*. Importantly, because healthy women have 10-fold higher levels of *Lactobacillus* colonization in the cervical and vaginal mucosa than do female rhesus macaques, this approach may actually lead to even greater protection in humans than observed in this model.

The live *L. jensenii* 1153–1666 reduced the rate of $SHIV_{SF162P3}$ acquisition by 63% in the macaque model. Statistical modeling predicts that a reduction in the rate of HIV acquisition by 50% using either a partially effective vaccine or microbicide could have a significant impact on the global HIV epidemic [46,47]. Our data suggest that this approach to HIV prevention is effective and safe. It is also highly flexible since the modular nature of the *Lactobacillus* expression system readily allows the expression of other HIV inhibitor proteins, such as the CCR5 inhibitor C1C5 RANTES [48], or even the production of multiple inhibitors. Furthermore, because lactobacilli are inexpensive to ferment and manufacture, this approach is eminently suitable for large-scale manufacturing for delivery to women in the resource-limited settings of the developing world.

METHODS

Study animals, procedures, and pH analysis

Twenty-four normal cycling female Chinese-origin rhesus macaques (*Macaca mulatta*), ages 3.5–7 yrs were used in this study. Animals were housed either at BIOQUAL, Inc. in Rockville, MD, or at Advanced Biosciences Laboratories (ABL), Kensington, MD. Nonhuman primate housing, care, and treatments were performed in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), the Animal Welfare Act as amended, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002 and the NIH guidelines for Research Involving Recombinant DNA Molecules. In all studies animals were sedated with 10 mg/kg ketamine and 1 mg/kg acepromazine when required. Vaginal pH was monitored using a portable HI 99181 Skin pH Meter Hanna Instrument® (Woonsocket, RI). We monitored menses by inserting a clean saline dampened cotton-tipped applicator into the monkey's vagina. The swab was then removed and a recording from 0 to 3 was made (0=no blood, 1=slight, 2=moderate, 3=heavy bleeding). The monkeys were trained for this procedure by giving food as a reward. Cervicovaginal lavages (CVLs) were taken by

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instilling 2 mL of PBS spiked with complete, EDTA-free protease Inhibitor (Roche Applied Science, Indianapolis, IN) into the vagina, flushing it back and forth four times, and then collecting the fluids on ice. Samples were clarified by centrifugation at 820×g at +4°C and snap frozen on dry ice, prior to storage at −80°C until analysis. Samples were collected in all phases of the menstrual cycle.

Genetic modifications of *Lactobacillus*

Lactobacillus jensenii 1153–1666 used in the study was created at Osel, Inc. and is described in Liu *et al.* [24]. Briefly, the nucleotide sequence of the CV-N gene was recoded for optimal *Lactobacillus* codon usage. In addition, proline at amino acid 51 was changed to glycine (P51G) by site-directed mutagenesis to stabilize CV-N in a properly folded monomeric form. A 4-amino-acid peptide containing, alanine, proline, valine, and threonine (APVT), corresponding to the amino terminus of the mature collagen-binding S-layer protein A (CbsA) protein of *Lactobacillus crispatus*, was inserted downstream of the CbsA signal sequence to resemble the native signal peptidase cleavage site [24]. An expression cassette containing an *L. jensenii* promoter for the ribosomal protein subunit (P_{rpsU}) directing expression of APVT-CV-N (P51G) was integrated by homologous recombination in a single copy into the *pox1* site of the genome of *L. jensenii* 1153. The resolved strain, *L. jensenii* 1153–1666, was used in the macaque studies described in this paper. *L. jensenii* 1153–1666 when cultured to ~10^8 CFU, secretes modified CV-N at concentrations of approximately 1.5–2 μg/ml [24]. APVT-CV-N has similar anti-viral activity against CCR5 and CXCR4 viruses as the original CV-N protein [24].

Lactobacillus growth conditions, colonization and sampling

Lactobacilli were cultured using Mann Rogosa Sharp (MRS) broth or agar purchased from Difco (Becton Dickinson, Franklin Lakes, NJ). A colonization model using the Chinese rhesus macaque for testing a live *Lactobacillus*-based microbicide was established prior to this study [25]. Lactobacilli were delivered to the macaques in a 3 ml volume with equal volumes of MRS and 2.7% hydroxyethylcellulose (HEC) (Hercules Incorporated, Hopewell, VA). The vaginal microflora of each animal was sampled using the Port-A-Cul™ swab/tube collection system (Becton Dickinson, Cockeysville, MD, USA). Vaginal swabs were taken two days prior to each challenge and plated to MRS agar (Difco, BD) and PRAS Brucella plates (Anaerobe Systems, Morgan Hill, CA). Lactobacilli were identified using Gram stain, and API Microorganism Identification Strips (Biomerieux, Inc, St. Louis, MO). *Lactobacillus sp.* identity was confirmed by DNA extraction and 16S ribosomal DNA (rDNA) sequencing and BLASTn DNA sequence analysis.

Detection of CV-N in CVL

Macaques were colonized with live *L. jensenii* 1153–1666 in HEC without the use of antibiotic to determine the extent and duration of colonization. CVLs were performed to detect secreted CV-N protein collected at 24 hr, 3 and 6 weeks post *Lactobacillus* inoculation. To detect CV-N, CVL samples were incubated with a rabbit pAb against CV-N (Cocalico Biologicals, Inc., Reamstown, PA). The mixture was then coupled to the pre-washed and pre-equilibrated protein-A sepharose beads (Sigma-Aldrich, St. Louis, MO) at

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4°C for four hours under agitation. The beads were washed with lysis buffer, and the immunoprecipitated proteins were separated on 4–12% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes (PVDF) using an IBlot system (Invitrogen, Carlsbad, CA). CV-N present in the CVL was visualized with a goat anti-CVN pAb (Cocalico Biologicals) and WesternBreeze® Chromogenic kit (Invitrogen). Control CV-N used in Western blots did not contain the N-terminal APVT sequence and had a slightly faster mobility on SDS-PAGE gels.

Vaginal biopsies and *in situ* detection of CV-N

Vaginal pinch biopsies were collected from *L. jensenii* 1153–1666 colonized macaques and either formalin fixed and stained with hematoxylin and eosin or flash frozen for immunohistochemistry (IHC). Samples for IHC were microsectioned and reacted with goat anti-CV-N antibody diluted 1:4000 in PBS containing 1% bovine serum albumin (BSA). Secondary antibody was anti-goat conjugated with immunoperoxidase diluted 1:500 in PBS-BSA. DAB (3,3'-diaminobenzidine) substrate (Sigma) was used to visualize the CV-N in tissue. All IHC procedures were performed by Histoserv Inc., Germantown, MD. The sections were visualized using light microscopy on a Leica SP5 Confocal Microscope (Leica Microsystems, Mannheim, Germany). Slides were viewed and photographed using either a 10×-low power objective or 63×NA1.4 oil immersion objective.

Cytokine and immunoglobulin measurements

The levels of IL-1β, IL-1ra, IL-6, and IL-8 in CVLs were determined from 16 of the macaques used in the challenge experiment, with a multiplexed fluorescent microsphere immunoassay using the Luminex 100 system (Luminex Corporation, Austin, TX) using Milliplex™ MAP Non-human primate cytokine kit (Millipore). Data were collected and analyzed with Bioplex Manager v3.0 software (Bio-Rad, Hercules, CA) using a 5-parameter fitting algorithm. To account for possible differences between tests, samples were analyzed in overlapping batches testing all the time points from the individual animals at the same time. CV-N specific and *L. jensenii* specific IgG, IgA and IgM from macaque CVL were measured by ELISA. NUNC Maxisorp (Thermo Scientific, Rochester, NY) plates were coated with CV-N protein or *L. jensenii* 1153 whole bacteria, blocked with 1% casein (Thermo Scientific) and 10% heat inactivated goat serum. CVL and serum samples were tested for the presence of antibodies to CV-N and *Lactobacillus* using goat anti-monkey IgA, IgG and IgM (Rockland Immunochemicals Inc., Gilbertsville, PA) and visualized with p-Nitrophenyl Phosphate, Disodium Salt (Sigma) substrate at 405 nM. Control macaques were immunized with CV-N delivered in Titermax® Gold Adjuvant (Sigma). Immunized macaques reached serum titers against CV-N of up to 1:36,000 following immunization and antibodies to CV-N were detected in CVLs collected after the second immunization.

SHIV stock

For expansion of $SHIV_{SF162P3}$, a naïve Indian rhesus macaque, which was shown to be free from SRV, STLV, B and foamy viruses, was infected intravenously with 1 ml of cell-free $SHIV_{SF162P3}$ stock. The infected macaque was monitored for plasma viremia and when the RNA load reached ~10^8 copies/ml, PBMCs were harvested, depleted of CD8+ T cells and

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activated with PHA for 72 hours. Activated CD4+ T- cell-enriched PBMCs were then cocultured with PHA-activated PBMCs pooled from three macaques. Culture media was replenished as needed with fresh medium every 2–3 days and fresh PHA-activated rhesus PBMCs were added after five days. The culture was monitored for the presence of SIV Gag p27 protein by antigen capture ELISA kit (ABL) and when the SIV p27 level reached >50 ng/ml, cell free supernatant was harvested, filtered, aliquoted into 1.0 ml aliquots, and stored in the vapor phase of liquid nitrogen.

MatTek culture

The MatTek EpiVaginal™ Tissue Model VLC-100-FT (MatTek Corporation, Ashland, MA) was used to assess the effect of *L. jensenii* 1153–1666 vs. *L. jensenii* 1153 (parental strain) on HIV-1 replication. Epivaginal tissue VLC-100 FT, which includes vaginal-ectocervical epithelial cells, fibroblast-containing lamina propria and dendritic cells, was infected with HIV-1_{SF162} at a p24 concentration of 3 ng/ml in 100 µl, in the presence of 10^7 CFU *L. jensenii* 1153–1666 or 10^7 CFU *L. jensenii* 1153 (parental strain). Two assays were performed with duplicate tissue replicates for each sample. Samples of tissue culture media were harvested for p24 analysis at day 1, 3, 5 and 8-post infection. HIV-1 p24 concentrations were determined using ELISA (PerkinElmer, Inc., Waltham, MA).

SHIV challenge study

In the SHIV challenge study, azithromycin suppositories were administered vaginally to 12 macaques to clear their *Lactobacillus* flora. After a 1-week rest, the 12 macaques in the experimental arm were colonized with *L. jensenii* 1153–1666 in approximately 3 ml of a 1:1 ratio of MRS broth and HEC daily 5 times in the first week and 2 times each following week with SHIV challenge 24–48 h later. Control animals (n=12) received the following, azithromycin treatment (as described above) followed by 2.7% HEC (as described) (n=4), 2.7 % HEC (as described) (n=4) or no treatment (n=4).

SHIV challenges were performed 24–48 hours after *Lactobacillus* or HEC inoculation. One ml of RPMI containing 300 $TCID_{50}$ $SHIV_{SF162P3}$ in RMPI was intravaginally inoculated using a 3cc syringe in an atraumatic manner. Animals were challenged up to 6 times or until infection was established by a positive plasma viral load.

Viral load determination and seroconversion

Quantitative SHIV RT-PCR was performed as described previously [49]. Protection was defined as absence of plasma viremia (RNA viral load) at weekly time points from 7 to 70 days after challenge. Infection was defined by consecutive positive viral RNA detections >50 copies SHIV RNA/ml. Plasma samples obtained from all macaques were tested for seroconversion and antibody titers to p27, a simian immunodeficiency virus (SIV) core antigen, using an ELISA (performed at ABL). In addition, all plasma samples were tested by Western Blot Assay (ZeptoMetrix Corporation, Buffalo, NY) to detect antibodies to SIV antigens using a modified procedure recommended by the manufacturer.

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Trim5α allele testing

TaqMan RT-PCR was used to amplify Trim5α alleles from PBMCs in 15 macaques (BIOQUAL, Inc). In five macaques genomic sequence was determined from blood (Harvard NEPRC, Southborough, MA). Four macaques were not tested due to insufficient numbers of PBMCs available.

Statistical analysis

Paired Student's T tests were used to analyze the vaginal pH data. The cytokine data and HIV-1 inhibition in MatTek cultures were analyzed with nonparametric Wilcoxon matched-pair signed-rank test using Prism Version 5 (GraphPad Software, La Jolla, CA). The proportion of uninfected vs infected animals was analyzed using Kaplan-Meier survival analysis, and peak viral loads compared using the Student's T test, in JMP Version 8 (SAS, Cary, NC). All the hypothesis tests were two-tailed, and a p value of ≤ 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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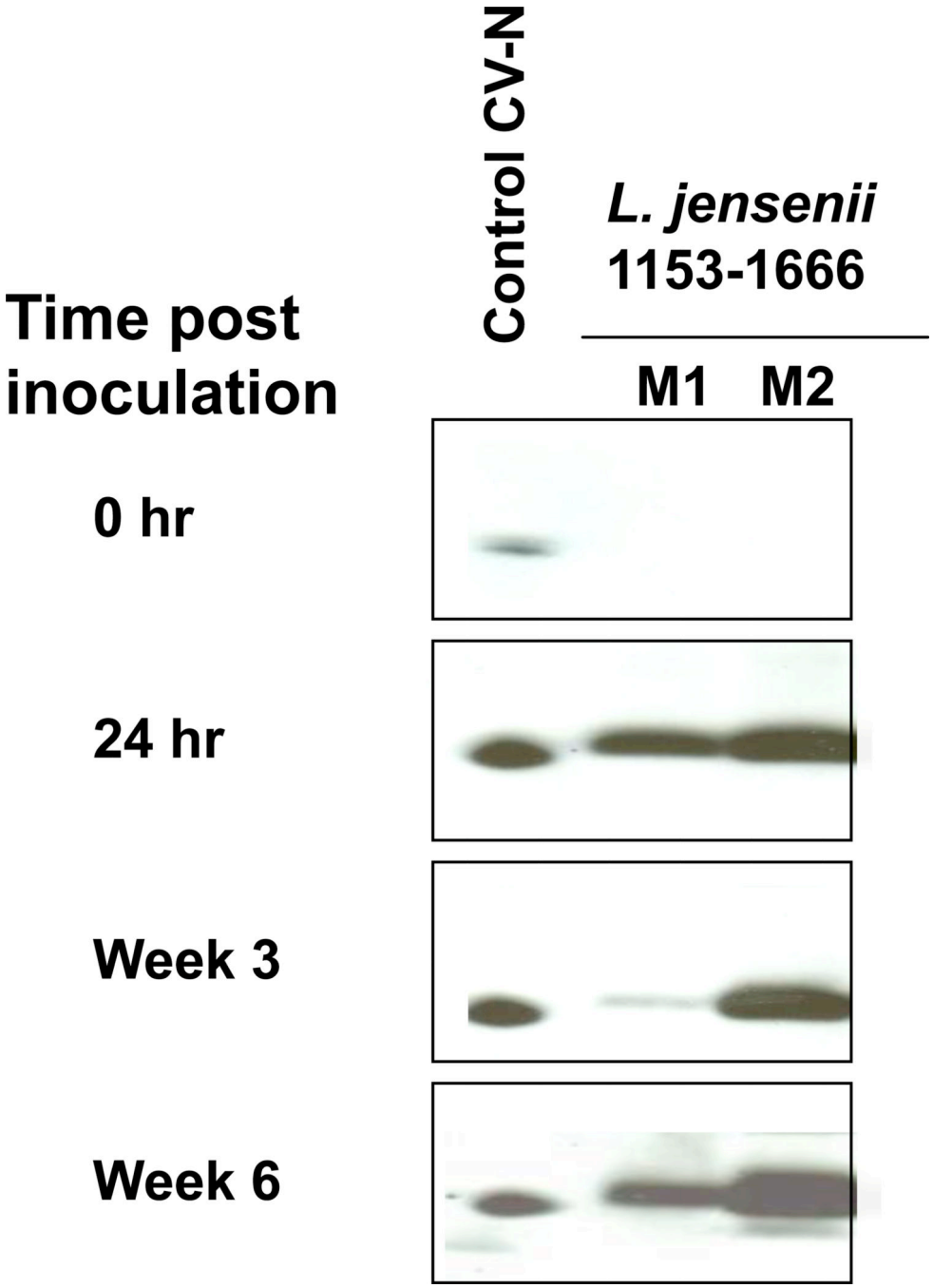

Fig. 1.

Expression and detection of CV-N *in situ* in the macaque vagina. *L. jensenii* 1153–1666 was used to colonize macaques for a 5-day period. Following this dosing regimen, cervicovaginal lavages (CVLs) were taken at 24 hr, 3 weeks and 6 weeks. Samples of each CVL were immunoprecipitated and then visualized by Western blot. Expression of CV-N was constitutive for up to 6 weeks post inoculation. At week 3, monkey 1 (M1) had a very low but detectable CV-N protein band, likely caused by reduced levels of CV-N-producing lactobacilli due to menstruation. *L. jensenii* 1153–1666, measured in CVL, grew on vaginal

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epithelial cells *in vivo* at concentrations up to 7×10^6 CFU and yielded CV-N at 83–160 ng/ml.

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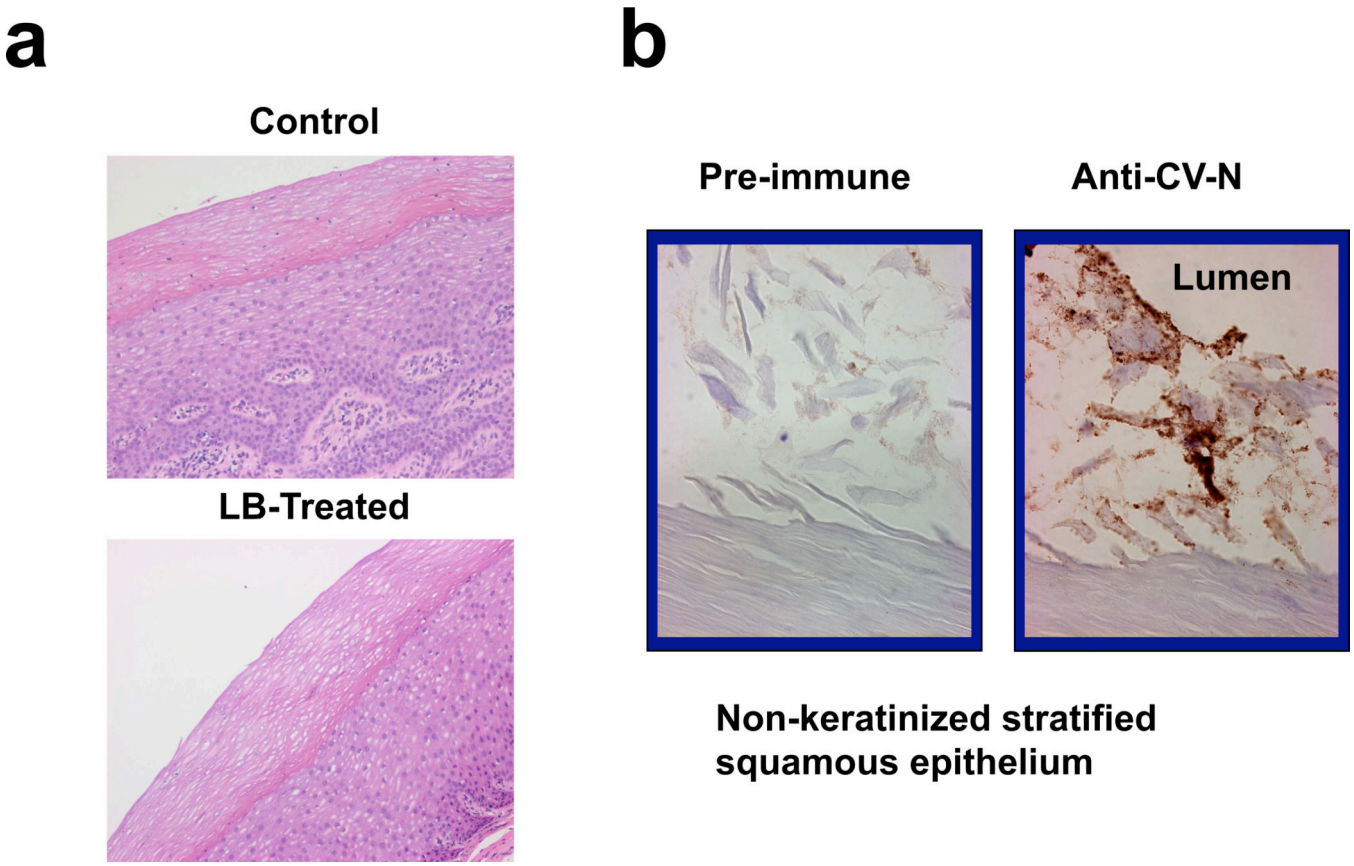

Fig. 2.

a) Histology of vaginal biopsies. A biopsy from a macaque colonized with *L. jensenii* 1153–1666 for one month (bottom) and one from a control macaque (top) were compared. Formalin-fixed sections were stained with hematoxylin and eosin and examined microscopically. There was no evidence of loss of epithelial cell integrity and no leukocytic infiltration noted. Two representative biopsies are shown at 20× magnification. b) Immunohistochemical analysis of CV-N in a vaginal biopsy. The biopsy on the left is reacted with pre-immune serum and shows no staining. In the biopsy on the right, the CV-N staining co-localized with the *Lactobacillus* bacteria on the vaginal mucosal surface and in the vaginal lumen.

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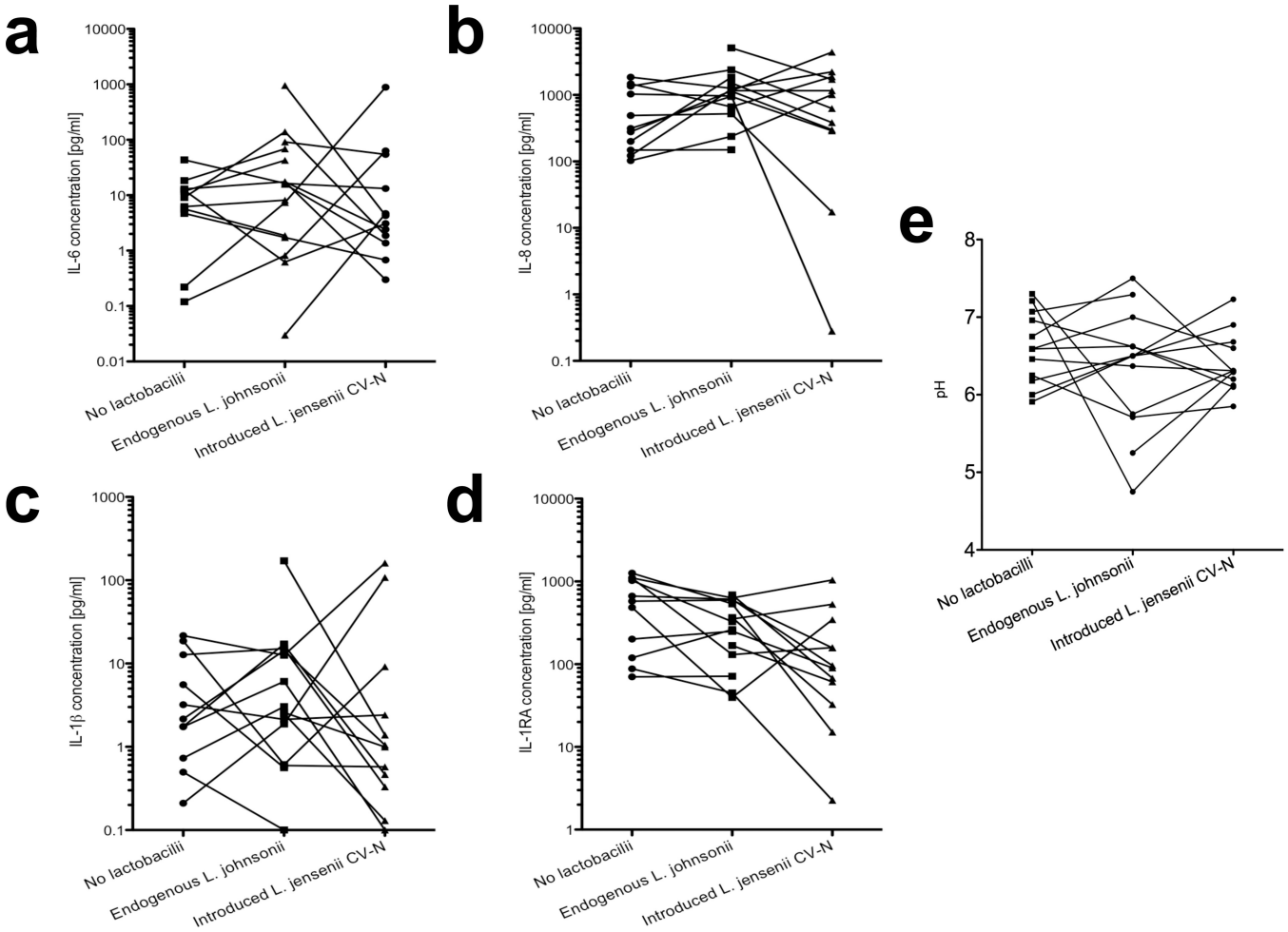

Fig. 3.

Measurements of biomarkers of inflammation in CVL and pH. The levels of IL-6 (a), IL-8 (b), IL-1β (c), and IL-1ra (d), were determined in CVLs of 16 macaques with no lactobacilli, or colonized with either endogenous strains of *L. johnsonii* or with introduced *L. jensenii* 1153–1666. Comparisons were made within the same macaque. e) Vaginal pH values were determined from 12 macaques at multiple time points using a portable Skin pH Meter. Comparisons were made for the same macaque with no lactobacilli or colonized either with endogenous strains of *L. johnsonii* or with *L. jensenii* 1153–1666.

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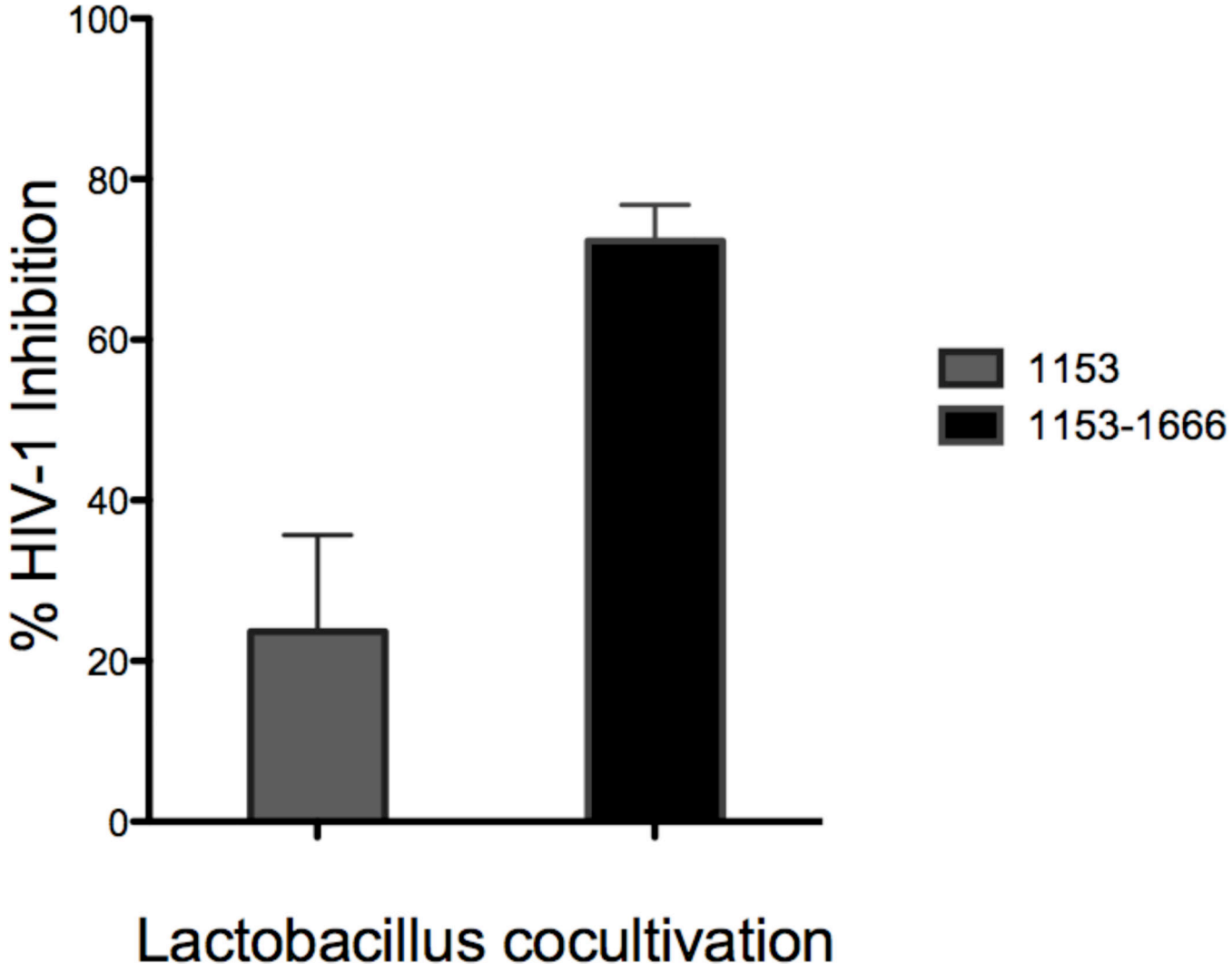

Fig. 4.

Inhibition of HIV-1 infection in a MatTek EpiVaginal tissue model. Initial *in vitro* experiments were performed with HIV-1_{SF162} infection of the MatTek EpiVaginal™ Tissue Model VLC-100 FT. We show that co-cultivation of the tissue model with the *L. jensenii* 1153 parental strain reduced infection by 23%±12.1 (mean ± SEM), compared to a control with no added bacteria, whereas co-cultivation with CV-N-producing *L. jensenii* 1153–1666 inhibited infection by 72% ±4.5 (mean ± SEM), (Fig. 4). Supernatants were harvested for p24 analysis and HIV-1 infection was determined by p24 ELISA (PerkinElmer, Inc., Waltham, MA).

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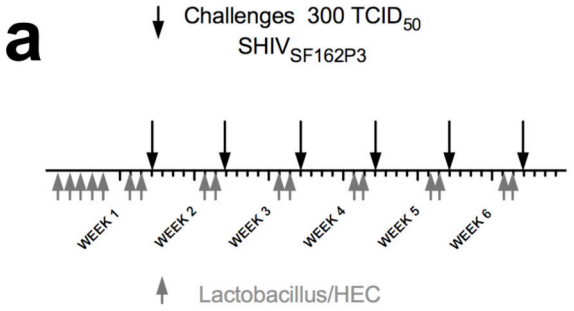

Macaque	Azithromycin	L. jensenii 1153-1666	2.7% HEC	No treatment
Experimental	12	12	12	
Control	4		4	
Control			4	
Control				4

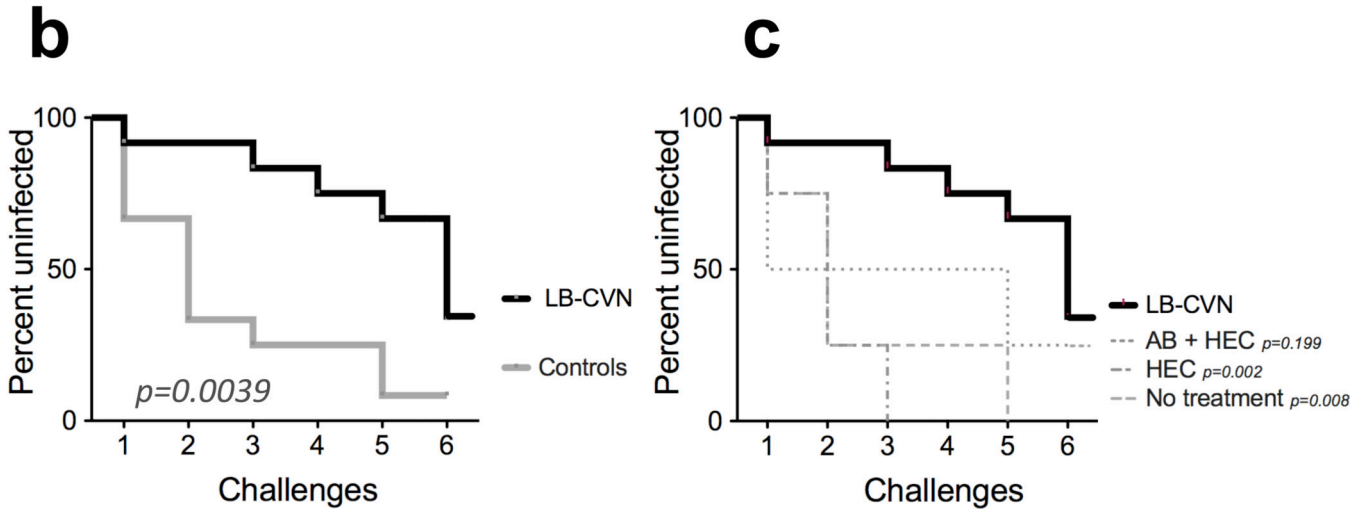

Fig. 5.

L. jensenii 1153–1666 protected rhesus macaques against repeated vaginal challenge by $SHIV_{SF162P3}$. (a) Study protocol. Adult female rhesus macaques (n=12) were treated with the antibiotic azithromycin vaginally one week prior to the study. Animals were inoculated with *L. jensenii* 1153–1666 in 2.7% HEC for a 5-day period, and then again for 2 days prior to each challenge. A total of 12 control macaques were used. Control macaques received the following; azithromycin treatment (as described above) followed by 2.7% HEC (as described) (n=4), 2.7 % HEC (as described) (n=4) or no treatment (n=4). Macaques were challenged once weekly up to six challenges. (b) Kaplan-Meier analysis of time to detectable $SHIV_{SF162P3}$ infection. On the left, all controls were combined; the difference in protection was highly significant (p = 0.0039). (c) On the right, each subgroup within the controls was separately analyzed; the difference was significant for the non-treatment subgroup (p = 0.008) and the HEC only subgroup (p = 0.002).

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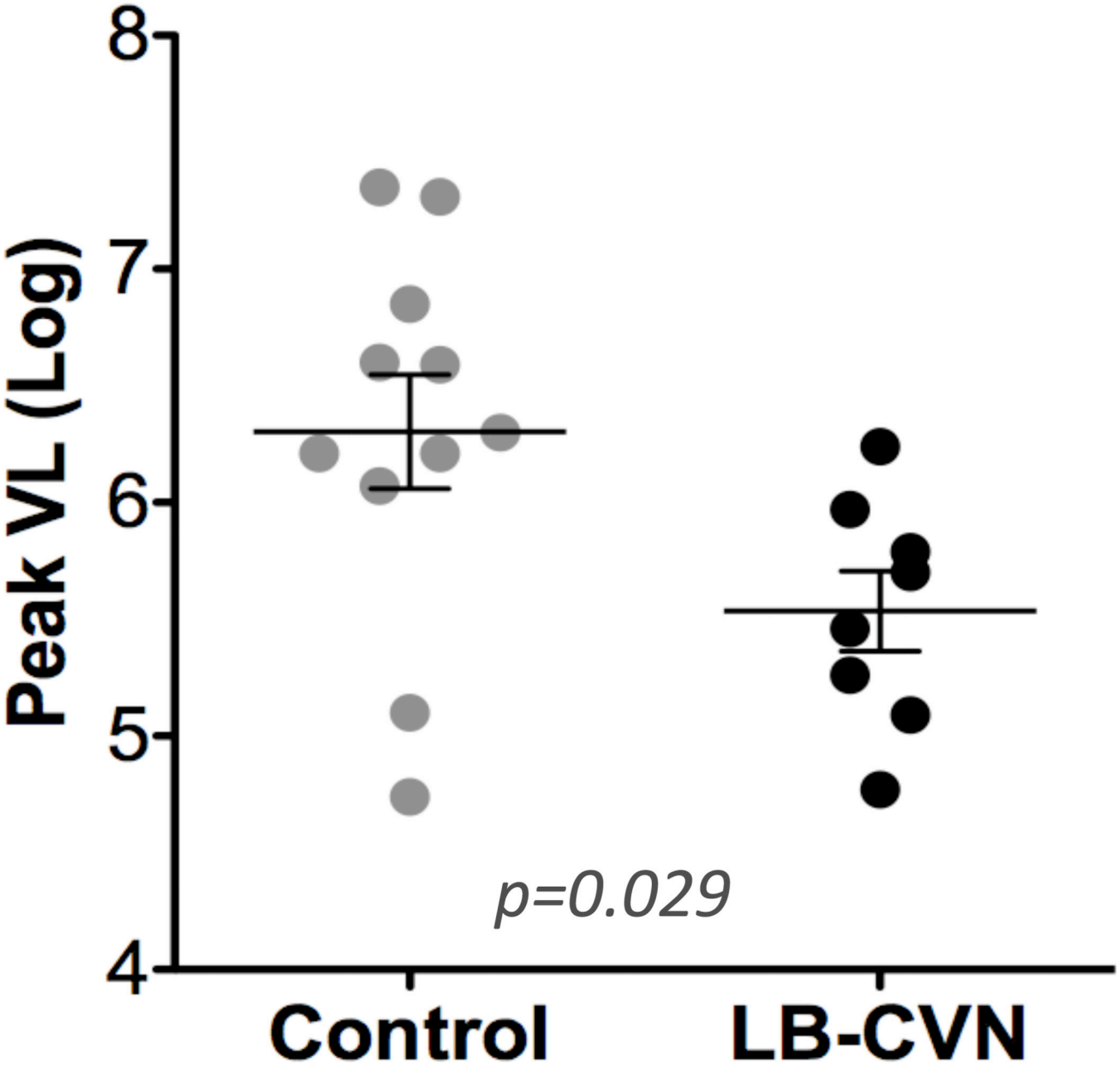

Fig. 6.

Comparison of peak viral loads in *L. jensenii* 1153–1666 colonized (LB-CVN) and control animals with breakthrough infection. Average $\log_{10}$ peak plasma viral levels were 5.5 ± 0.17 (mean ± SEM) for animals colonized with *L. jensenii* 1153–1666 vs 6.3 ± 0.24 for control animals (p = 0.029).

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Table 1

L. jensenii 1153–1666 and CV-N protein detection in *L. jensenii* 1153–1666-colonized macaques during the course of the challenge study.

Animals	Challenge 1		Challenge 2		Challenge 3		Challenge 4		Challenge 5		Challenge 6	
	L j	CV-N	*L j*	CV-N	*L j*	CV-N	*L j*	CV-N	*L j*	CV-N	*L j*	CV-N
M4698	5	+	6	+	6	+	4	+	6	+	5	+
M4699	6	+	5	+	4	+	0	ND	6	+	6	+
M4700	0	ND	0	ND	5	+	5	+	6	+	5	+
M4701	5	+	5	+	4	+	6	+	6	+	0	ND
M4704	5	+	0	ND	0	ND	6	+	6	+	5	+
M4705	5	+	4	+	6	+	0	ND	4	−	0	ND
M4706	5	+	0	ND	6	+	5	+	6	+	4	+
M4707	5	+	5	+	5	+	5	+	4	+	5	+
M4708	5	+	5	+	0	ND	6	+	6	+	5	+
M4709	5	+	5	+	5	+	6	+	6	+	0	ND
M4711	5	+	5	+	6	+	6	+	6	+	5	+
M4713	5	+	5	+	4	+	5	+	6	+	5	+

L j = *L. jensenii* 1153–1666, 6 =10^6 colony forming units CFU/swab, 5 =10^5 CFU/swab, 4 =10^4 CFU/swab, 0 =no lactobacillus detected, ND = not determined

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Broadly Neutralizing Anti-HIV Antibodies Prevent HIV Infection of Mucosal Tissue *Ex Vivo*

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Broadly neutralizing monoclonal antibodies (nAbs) specific for HIV are being investigated for use in HIV prevention. Due to their ability to inhibit HIV attachment to and entry into target cells, nAbs may be suitable for use as topical HIV microbicides. As such, they would present an alternative intervention for individuals who may not benefit from using antiretroviral-based products for HIV prevention. We theorize that nAbs can inhibit viral transmission through mucosal tissue, thus reducing the incidence of HIV infection. The efficacy of the PG9, PG16, VRC01, and 4E10 antibodies was evaluated in an *ex vivo* human model of mucosal HIV transmission. nAbs reduced HIV transmission, causing 1.5- to 2-$\log_{10}$ reductions in HIV replication in ectocervical tissues and ≈3-$\log_{10}$ reductions in HIV replication in colonic tissues over 21 days. These antibodies demonstrated greater potency in colonic tissues, with a 50-fold higher dose being required to reduce transmission in ectocervical tissues. Importantly, nAbs retained their potency and reduced viral transmission in the presence of whole semen. No changes in tissue viability or immune activation were observed in colonic or ectocervical tissue after nAb exposure. Our data suggest that topically applied nAbs are safe and effective against HIV infection of mucosal tissue and support further development of nAbs as a topical microbicide that could be used for anal as well as vaginal protection.

With circulating drug-resistant human immunodeficiency virus (HIV) on the rise in many communities where preexposure prophylactics (PrEP) will be used (1), the risk of transmitting virus with reduced susceptibility to antiretrovirals (ARVs) is possible. To circumvent this risk, several non-ARV microbicide candidates are being considered. Previous investigations of non-ARV microbicides used non-HIV-specific formulations of compounds, such as surfactants, polyanions, and buffering agents; however, all were ineffective at preventing HIV acquisition. Among them, the surfactant nonoxynol-9 (N-9), which is commercially available as a spermicide, was shown to have anti-HIV activity *in vitro* (2). However, clinical evaluation of N-9 was stopped due to increased HIV incidence in women using an N-9 vaginal gel (3). Additionally, N-9 was shown to cause tight junction disruptions in epithelial cells *in vitro* (4) and to be harmful to beneficial vaginal flora (5). Other candidates, such as BufferGel (6), the carrageenan derivative Carraguard (7), and polyanionic gels (PRO 2000 [6] and cellulose sulfate [4]), were all unsuccessful as HIV microbicides. However, the new non-ARV microbicide candidates being investigated are HIV-specific agents and include broadly neutralizing monoclonal antibodies (nAbs).

While nAbs have been investigated extensively in the development of HIV vaccines, an increased focus has been placed on their development for HIV prevention. Originally isolated from chronically HIV-infected individuals, nAbs were shown to retain potent neutralizing activity across a broad range of HIV clades (8). These highly cross-reactive antibodies are found in only a small subset of HIV-infected individuals (9, 10), and they develop their broad cross-reactivity through a process of somatic hypermutation over 2 to 4 years (10, 11). They bind epitopes on key regions of the HIV envelope and directly inhibit the ability of virions to engage entry receptors on target cells, thereby reducing viral infection. nAbs such as VRC01, b12, and NIH 45-46 exert their HIV-inhibitory activity by binding to the CD4 binding site (12), while 4E10, 10E8, and 2F5 bind to the membrane-proximal external region (MPER) at the base of the envelope spike (12), and others, such as PG9 and PG16, recognize quaternary glycosylation motifs on the exposed variable loops of gp120 (12). nAbs have shown efficacy in reducing HIV transmission *in vitro* (8, 13) and in animal models of HIV transmission *in vivo* (14, 15). This ability to inhibit viral transmission is particularly important in the context of HIV prevention, as it is better to prevent HIV acquisition than to overcome the complications inherent to treating an established HIV infection. nAbs also bridge the gap between non-HIV-specific compounds and ARV drugs in the spectrum of HIV microbicide candidates. The antibodies are specific for HIV, but their activity has not been shown to affect viral sensitivity to HIV enzyme inhibitors. Conversely, viral neutralization by nAbs is not expected to be hampered by drug resistance mutations in traditional ARV drug targets—HIV reverse transcriptase, protease, and integrase. This is because HIV envelope proteins are not under the selective pressure of HIV enzyme inhibitors. Hence, nAbs are expected to retain efficacy against viruses that are ARV resistant.

In this study, the efficacy of nAbs—PG9, PG16, VRC01, and 4E10—was evaluated in human mucosal tissue *ex vivo*. While studies of nAb efficacy have been conducted using animal models, this study is the first to evaluate the efficacy of topically applied nAbs by using a relevant human mucosal model of HIV transmis-

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sion. Individual nAb potencies were assessed in the absence or presence of semen, in addition to assessments of their safety in mucosal tissue. Our findings support nAbs as a viable alternative to ARV-based HIV preventatives for dual-compartment use.

MATERIALS AND METHODS

Reagents. Unless otherwise noted, culture media were purchased from Mediatech, Inc. (Manassas, VA), and serum and medium supplements were purchased from Gemini BioProducts (West Sacramento, CA). Whole human semen from pooled donors was purchased from Lee BioSolutions (St. Louis, MO).

Antibodies. VRC01-N, 4E10-N, and HSV-8-N monoclonal antibodies were generously provided by Kevin Whaley (Mapp Biopharmaceuticals Inc., San Diego, CA). These antibodies were produced in genetically engineered *Nicotiana benthamiana* plants, a species closely related to tobacco (16); hence, in this paper they have been given an "-N" designation. PG9 and PG16 monoclonal antibodies, produced in Chinese hamster ovary (CHO) cells, were provided by the International AIDS Vaccine Initiative (La Jolla, CA). All antibodies used are of the IgG1 isotype. The HSV-8-N antibody has specificity for a herpes simplex virus 1/2 (HSV-1/2) envelope epitope and was used as a non-HIV-specific isotype control.

Virus. The pYK-JR-CSF molecular clone was purchased from ATCC (Manassas, VA). 293T cells were a gift from Christina Ochsenbauer-Jambor and John C. Kappes of the University of Alabama, Birmingham. 293T cells were cultured in complete Dulbecco's modified Eagle's medium (cDMEM; 1× DMEM supplemented with 10% [vol/vol] heat-inactivated fetal bovine serum [FBS] and 1% penicillin–streptomycin–L-glutamine [Pen-Strep-L-Glut]). 293T cells were transfected with proviral DNA by using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 48 h, mature HIV virions were harvested from the culture medium by filtration through a 0.45-μm-pore-size syringe filter. The 50% tissue culture infective dose ($TCID_{50}$) was determined in activated human peripheral blood mononuclear cells (Central Blood Bank, Pittsburgh, PA) by using the Reed-Muench method (17).

Assessment of nAb neutralization activity *in vitro*. TZM-bl cells (18) were obtained through the NIH Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. and were cultured in cDMEM. Individual wells of a 96-well plate were seeded with 1×10^4 cells, which were allowed to adhere overnight. The culture media in the wells were replaced with 100-μl aliquots of 2× nAb dilutions, or with cDMEM in untreated control wells, and cells were incubated for 1 h at 37°C. Treated cells and untreated controls were inoculated with 3,000 $TCID_{50}$ HIV-1_{JR-CSF} in 100 μl and cultured for 48 h. Tat-activated luciferase expression was detected using Bright-Glo luciferase assay reagent (Promega, Madison, WI), and luminescence was measured using a SpectraMax M3 plate reader (Molecular Devices, LLC, Sunnyvale, CA). The 90% inhibitory concentrations (IC_{90}) were determined using SigmaPlot software, version 11.0 (Systat Software, Inc., San Jose, CA).

***Ex vivo* tissue culture.** Normal human ectocervical tissues were obtained from premenopausal women undergoing routine hysterectomy after informed consent (IRB permit PRO09110431) or were purchased from the National Disease Research Interchange (NDRI) (http://ndriresource.org/) and transported overnight on wet ice. Polarized explants were prepared from the specimens as described elsewhere (19). Briefly, the ectocervical epithelium was trimmed of the muscularis, and 5-mm-diameter explants were mounted with the epithelium upward in 12-mm permeable Transwell supports and sealed in position with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA). Ectocervical explants were activated with 0.5 μg/ml phytohemagglutinin-P (PHA-P) and cultured in DMEM supplemented with 10% (vol/vol) human A/B serum, 1% Pen-Strep-L-Glut, 100 U/ml human interleukin-2 (hIL-2) (Roche Diagnostics, Indianapolis, IN), and 1% nonessential amino acids (Lonza, Walkersville, MD).

Human colonic tissue was obtained after informed consent (IRB permit PRO09110431) from individuals undergoing scheduled colon resection for noninflammatory conditions (20). The epithelium and lamina propria were trimmed of excess adipose tissue and muscle, and 5-mm-diameter explants were mounted with the epithelium upward on gel foam inserts in 12-mm permeable transwell supports and sealed with Matrigel. Colonic explants were activated with 0.5 μg/ml PHA-P and cultured in RPMI 1640 supplemented with 5% (vol/vol) human A/B serum, 1% Pen-Strep-L-Glut, 0.5 mg/ml piperacillin-tazobactam (Zosyn; Wyeth, Collegeville, PA), 100 U/ml hIL-2 (Roche Diagnostics, Indianapolis, IN), and 2.5 mM HEPES (HyClone, Logan, UT).

Efficacy evaluations in human tissue *ex vivo*. Paired polarized explants were treated with nAbs 24 h (colon) or 48 h (ectocervix) after setup. nAbs were applied at 2 times the final concentration to the apical surface of the appropriate explants in duplicate and cultured for 1 h at 37°C. Controls were treated similarly with either medium only or the HSV-8-N antibody. After 1 h, each explant was inoculated apically with 50,000 $TCID_{50}$ (ectocervix) or 10,000 $TCID_{50}$ (colon) HIV-1_{JR-CSF}, suspended in RPMI 1640 or 50% (vol/vol) pooled whole human semen (Lee BioSolutions, St. Louis, MO). Tissues were incubated for 24 h, after which the basolateral medium was collected and the explants were washed with 1× Dulbecco's phosphate-buffered saline (DPBS; Mediatech, Inc., Manassas, VA). The basolateral medium was replenished with fresh medium supplemented with 100 U/ml hIL-2 and subsequently collected every 3 to 4 days for up to 21 days postinfection and stored at −80°C. Viral replication was monitored by measuring HIV p24 in the basolateral medium by using an Alliance HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (PerkinElmer, Waltham, MA). Individual ectocervical explants were fixed in formalin at 21 days postinfection and processed for immunohistochemical (IHC) analysis of intracellular HIV p24 antigen (19).

Evaluation of immune activation and tissue viability *ex vivo*. Nonactivated polarized ectocervical and colonic explants were treated on the apical surface with 1.5 μM and 0.03 μM nAbs, respectively. For controls, tissues were treated with medium only (negative control) or with 0.5 μg/ml PHA-P and 100 U/ml hIL-2 in the basolateral medium (positive control). All treatments were performed in duplicate, and tissues were cultured for 24 h at 37°C and 5% CO_2. The basolateral medium was sampled 24 h after treatment and used for quantitative comparison of inflammatory cytokines (gamma interferon [IFN-γ], IP-10, IL-1β, IL-6, IL-8, tumor necrosis factor alpha [TNF-α], granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-12, macrophage inflammatory protein 1β [MIP-1β], IL-18, and IL-15) by using a Milliplex human cytokine/chemokine magnetic bead panel (EMD Millipore Corp., Billerica, MA). For viability assays, nonactivated polarized explants were treated with nAbs as described above for 24 h. Control tissues were treated apically with medium only or with a 1:5 dilution of Options Gynol II Extra Strength gel (Caldwell Consumer Health LLC, Madison, NJ), containing 3% N-9. After 24 h of treatment, explants were processed for histology and viability determination by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay.

Statistical analysis. All variables were inspected using descriptive statistics and graphical methods. For analyses, raw HIV p24 values were log transformed because their distribution was heavily skewed. Linear mixed models were used to investigate the effects of semen, nAb treatment, and different nAb doses on the trajectory of HIV p24 during the 21-day culture period. For all models, random effects for explants and tissue donors were included to adjust for any clustering effect of repeated measures within each explant and tissue donor. Separate models were used for ectocervical and colonic data.

The model used to evaluate the effects of different nAbs on HIV p24 included dummy variables for nAbs, day of culture, and the interaction between day of culture and individual nAbs as fixed effects. To evaluate the effects of semen on nAb potency, the model included the presence or absence of semen, day of culture, and the interaction between these factors as fixed effects. The effect of semen on HIV p24 was also investigated while adjusting for the effect of nAbs by using a model that included the pres-

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TABLE 1 Comparative *in vitro* efficacies of nAbs against HIV_{JR-CSF} infection of TZM-bl cells

nAb	IC_{90} (μM)[a]	SD	*P* value
PG16	0.000435	0.000268	
PG9	0.00410	0.003981	
VRC01	0.0734	0.059379	0.165[c]
VRC01-N	0.0298	0.019791	
4E10[b]	0.727	Not applicable	0.350[d]
4E10-N	1.19	0.375102	

[a] Results are the means for 4 or more experiments.
[b] Results of 1 experiment are reported.
[c] This *P* value corresponds to VRC01 and VRC01-N.
[d] This *P* value corresponds to 4E10 and 4E10-N.

ence or absence of semen and dummy variables for each nAb, day of culture, and the interactions between day of culture and semen or nAb treatment as fixed effects. The effects of individual nAbs at each dose were investigated with dose and day of culture as dummy variables for each nAb and with interactions between day of culture and dose or each nAb as fixed effects. A model using dose, day of culture, and their interaction as fixed effects was used to interrogate the effect of nAb dose on HIV p24. Finally, the effects of semen and nAb dose were investigated separately for each antibody, using a model that included the effects of semen, nAb dose, and their interaction separately for each antibody. All analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria).

Comparisons between treatments and viability outcomes from MTT viability experiments were made by one-way analysis of variance (ANOVA). The effects of nAb treatment on cytokine concentrations were compared using two-way ANOVA and the Holm-Sidak correction for multiple comparisons. Colon and ectocervix data were analyzed separately, and analyses were performed using GraphPad Prism, version 6.05 (GraphPad Software, La Jolla, CA).

RESULTS

Assessment of nAb efficacy *in vitro*. Using TZM-bl cells, the IC_{90}s of all nAbs against $HIV\text{-}1_{JR-CSF}$ infection *in vitro* were derived. The potencies of nAbs VRC01-N and 4E10-N, produced in the transgenic *N. benthamiana* expression system, were also compared to those of the same nAbs produced in the traditional CHO cell system (VRC01 and 4E10) and were shown to have equivalent inhibitory activities ($P > 0.05$) (Table 1). The IC_{90} values indicated that nAb potencies had the following order: PG16 > PG9 > VRC01/VRC01-N ≫ 4E10/4E10-N. Due to their equivalence to CHO cell-produced nAbs and their greater availability, the *Nicotiana*-produced antibodies were used in the subsequent work.

nAbs reduce HIV transmission in human mucosal tissue *ex vivo*. To define nAb potencies in mucosal tissue, equivalent molar concentrations of IgG were used for all nAbs. Since the IC_{90} value of VRC01-N was intermediate in the range of those for the other nAbs used in this study (Table 1), the doses of all nAbs were standardized to the effective concentrations of VRC01-N. For ectocervical tissue, 1.5 μM and 0.3 μM IgG concentrations were used, which were the doses of IgG equivalent to 50× and 10× the IC_{90} of VRC01-N, respectively. Similarly, for colonic tissue, concentrations of 0.03 μM and 0.003 μM were used, which were equivalent to 1× and 0.1× the VRC01-N IC_{90}, respectively. The HSV-8-N isotype control antibody was used at a concentration of 0.3 μM in ectocervical tissues and 0.003 μM in colonic tissues.

nAbs were applied to the apical surface of tissues for 1 h before inoculation with virus to simulate pericoital application of a topical microbicide preparation. Using this strategy, treatment of ectocervical tissues with 1.5 μM and 0.3 μM doses of VRC01-N, PG9, and PG16 caused significant reductions in HIV p24 over the 21-day culture period ($P < 0.0001$) compared to the levels in the untreated controls. Treatment with 1.5 μM VRC01-N, PG9, or PG16 caused a median reduction of 1.5, 1.9, or 1.8 $\log_{10}$ pg/ml HIV p24 at day 21 of culture, respectively, compared to the level in the untreated control (Fig. 1a; Table 2). Conversely, treatment with 4E10-N did not significantly reduce HIV p24 ($P = 0.5797$) (Fig. 1a; Table 2), as the concentrations used were below the *in vitro* IC_{90} of this nAb (Table 1). However, tissues pretreated with doses of 59.5 μM and 11.9 μM 4E10-N in ectocervical tissues and 1.19 μM and 0.119 μM in colonic tissues showed reductions in HIV transmission (see Fig. S1 in the supplemental material). These doses correspond to 50×, 10×, 1×, and 0.1× the IC_{90} of 4E10-N (Table 1), respectively, and also reflect 8- to 40-fold higher concentrations than the effective doses of nAbs used in this study. Treatment with the isotype control antibody, HSV-8-N, also did not affect HIV replication ($P = 0.2830$) (Table 2). There was also a greater reduction in HIV p24 with the use of 1.5 μM PG9 ($P = 0.0171$) and PG16 ($P = 0.0302$) at day 21 than with a 0.3 μM concentration of these nAbs (Table 2). While there was a trend, the high dose of VRC01-N was not significantly different from the low dose ($P = 0.0810$). This suggests that the neutralizing capacity of 0.3 and 1.5 μM doses of VRC01-N may have been saturated by the inoculum used in the ectocervical model. The dose effect observed with the PG9 and PG16 antibodies in the same model supports the observation that these nAbs are more potent and is concordant with the nAb hierarchy of potency observed *in vitro* (Table 1).

nAbs provide differential protection between human colonic and ectocervical tissues *ex vivo*. Using 0.03 or 0.003 μM nAbs (Fig. 1b), treatment with PG16 ($P < 0.0001$), PG9 ($P < 0.0001$), or VRC01-N ($P < 0.0001$) caused significant reductions in HIV p24 over the treatment period. Pretreatment of colonic tissues with 0.03 μM PG16, PG9, and VRC01-N yielded reductions of 3.0, 3.3, and 3.3 $\log_{10}$, respectively, in the median HIV p24 level by day 21 of culture compared to the levels in untreated controls (Fig. 1b). A dose effect was observed in colonic tissues, as tissues treated with the low dose (0.003 μM) of VRC01-N, PG9, or PG16 had decreases in median HIV p24 of 1.2 $\log_{10}$ or less at day 21 ($P < 0.0001$) compared to tissues treated with 0.03 μM nAbs (Table 2). However, treatment with 4E10-N had no effect on HIV p24 at any of the concentrations used ($P = 0.1994$). The reductions of median HIV p24 of <0.5 $\log_{10}$ observed at day 21 for tissues treated with 4E10-N did not reach statistical significance and support the lack of potency observed with 4E10-N in this study. Infection curves for tissues treated with 0.003 μM PG9 or PG16 showed a delay in infection that was not observed with VRC01-N, with virus production expanding from 14 to 17 days postinfection (Fig. 1b). These results support the observation that PG9 and PG16 demonstrate superior potency in colonic tissue compared to ectocervical tissue (Fig. 1a) and are also concordant with the order of nAb potency described in our *in vitro* assays (Table 1). Higher doses of nAbs showed greater responses than lower doses of the same nAbs in colonic tissues, with the exception of 4E10-N.

nAbs retain potency in the presence of semen. During *in vitro* evaluations of some non-ARV microbicides, such as the polyanionic gel PRO 2000, semen was shown to abrogate anti-HIV activity (21). The counteractive effect of semen on microbicides has been attributed to an activity of seminal proteins on microbicide

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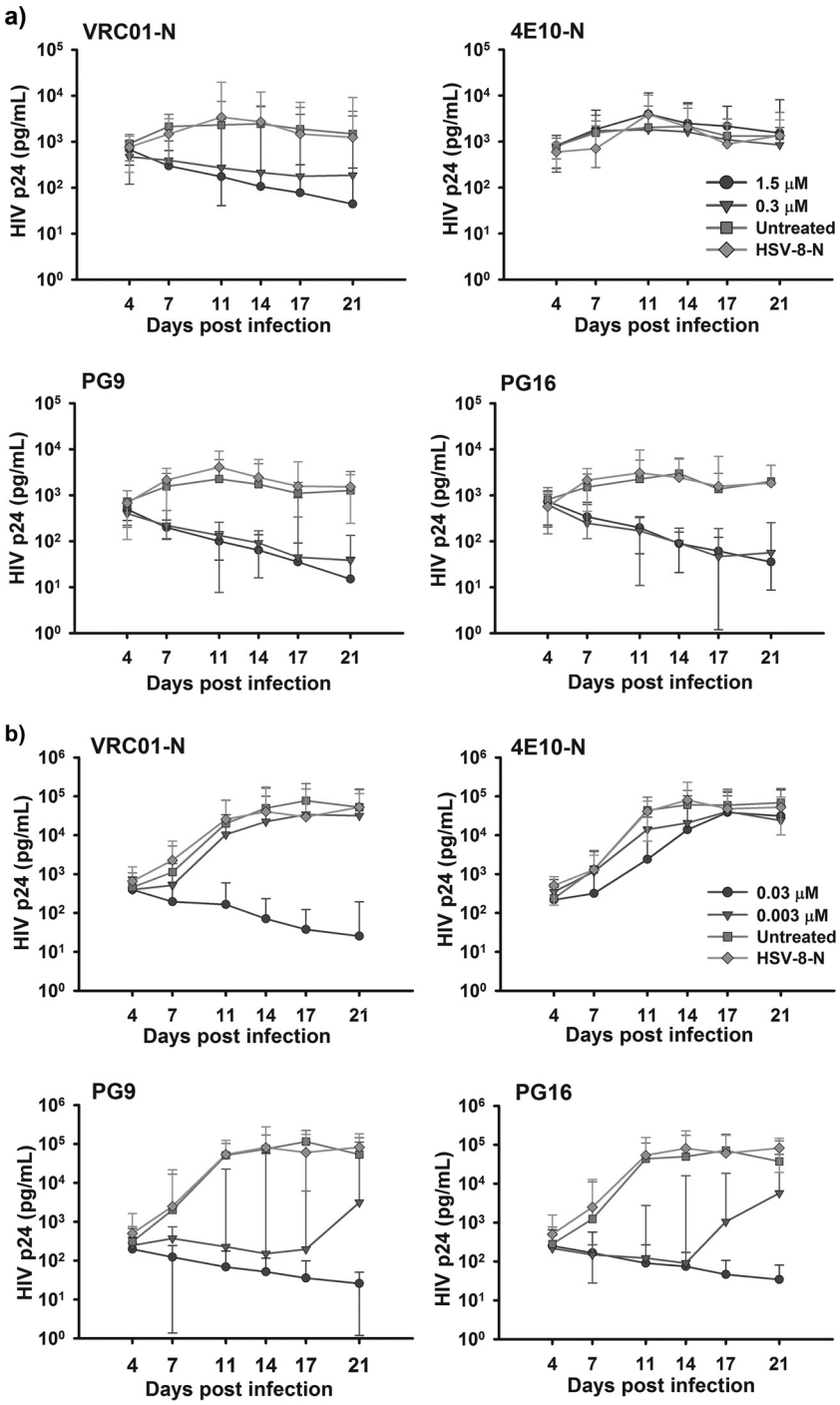

FIG 1 nAb efficacies in human ectocervical (a) and colonic (b) tissues *ex vivo*. Viral replication was monitored by p24 antigen ELISA on basolateral culture supernatants collected at 4, 7, 11, 14, 17, and 21 days postinfection. Data points represent the medians and interquartile ranges for ≥5 tissues from individual donors.

moieties (21); the formation of amyloid fibrils that enhance HIV attachment to target cells (22); and the neutralizing effect of semen that lowers the vaginal pH, prolonging the survival of HIV virions (23–26). Hence, the potencies of nAbs in ectocervical (Fig. 2a) and colonic (Fig. 2b) tissues were compared in the presence and absence of whole human semen. Semen had no effect on HIV p24 concentrations ($P = 0.2382$) or on nAb potency in ectocervical tissue ($P = 0.0670$) compared to no-semen controls. In colonic tissues (Fig. 2b), the presence of semen had no effect on nAb potency during the 21-day culture period ($P = 0.0940$), and comparison of HIV p24 levels also showed similar levels of HIV replication in the presence or absence of semen ($P = 0.1177$). These data show no enhancement of HIV infection or effect on nAb activity in the presence of semen and collectively support the hy-

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TABLE 2 Effects of nAbs on HIV p24 at day 21 postinfection in human tissues *ex vivo*

Treatment	Cervix			Colon		
	Dose (μM)	Log_{10} ΔHIV p24	*P* value[a]	Dose (μM)	Log_{10} ΔHIV p24	*P* value[a]
VRC01-N	1.5	−1.523	<0.0001	0.03	−3.315	<0.0001
	0.3	−0.898		0.003	−0.221	
4E10-N	1.5	+0.067	0.5797	0.03	−0.342	0.1994
	0.3	−0.192		0.003	−0.458	
PG9	1.5	−1.924	<0.0001	0.03	−3.319	<0.0001
	0.3	−1.516		0.003	−1.233	
PG16	1.5	−1.752	<0.0001	0.03	−3.037	<0.0001
	0.3	−1.547		0.003	−0.813	
HSV-8-N	0.3	−0.005	0.2830	0.003	+0.102	0.3117

[a] *P* values represent comparisons of changes in p24 over time in tissues treated with each nAb, regardless of dose, relative to the levels in untreated tissue. Each *P* value, except those for HSV-8-N, corresponds to the values for each set of 2 rows.

pothesis that nAbs applied topically would be effective at preventing sexual transmission of HIV.

Safety of nAbs. The safety of nAb treatment was assessed in ectocervical and colonic tissue explants by using the MTT assay and histology. Tissue viability after 24 h of treatment with nAbs was compared to that of untreated tissues and tissues treated with N-9 (Fig. 3). There was no loss of viability with nAb treatment compared to the viability of untreated tissues. However, treatment with N-9 decreased tissue viability to a mean of 13% ($P = 0.0007$) relative to that of untreated tissues for ectocervical tissue (Fig. 3a) and 17% ($P = 0.0002$) for colonic tissue (Fig. 3b). Colonic and ectocervical tissues treated with nAbs were histologically comparable to untreated tissues after 24 h of exposure to treatment. However, N-9-treated tissues showed considerable sloughing of the epithelium and focal necrosis (Fig. 3a and b). Inflammatory cytokine levels were measured in the culture supernatants of tissues treated for 24 h with 1.5 μM nAbs for ectocervical tissues and 0.3 μM nAbs for colonic tissue. In ectocervical tissues, concentrations of GM-CSF, IFN-γ, IL-1β, MIP-1β, and TNF-α were significantly elevated ($P < 0.01$) by PHA-P plus hIL-2 compared to those in the untreated ectocervical tissues (Fig. 3c). Cytokine concentrations between nAb-treated and untreated ectocervical tissues were not significantly different, with the exception of IP-10, which had significantly lower concentrations in tissues treated with VRC01-N than in untreated tissues ($P < 0.01$). In colonic tissues, 24 h of treatment with nAbs did not cause significant differences in inflammatory cytokine concentrations compared to those in untreated tissues (Fig. 3d). However, there were significant increases in the concentrations of IL-6 and IL-8 between untreated tissues and tissues treated with PHA-P plus hIL-2 ($P < 0.0001$). These data suggest that 24 h of treatment with nAbs does not adversely affect viability or expression of inflammatory cytokines in ectocervical or colonic tissues *ex vivo*.

DISCUSSION

Early microbicide development was focused on vaginal products. However, recent efforts have expanded to optimize products to safely prevent HIV transmission during receptive anal intercourse as well. While these new products are directed at reducing the incidence of HIV among men who have sex with men (MSM), it has also been acknowledged that heterosexual anal intercourse may be underreported due to social taboos and other factors (27–29). This issue has shifted the paradigm of microbicide development toward a new generation of dual-compartment microbicides that are safe for both vaginal and rectal use (30, 31). Hence, in this study, we used models of rectal and vaginal mucosal transmission to evaluate the potential of topically applied nAbs. This study presents the first comprehensive preclinical evaluation of nAbs as a topical microbicide by use of a human *ex vivo* model of sexual HIV transmission. The data show that nAbs are potent and effective at reducing mucosal HIV transmission in human ectocervical and colonic tissues *ex vivo*. These results are concordant with animal studies where nAb preparations applied to the vaginal lumen before vaginal inoculation with simian-human immunodeficiency virus (SHIV) were protective (14, 32). Importantly, nAb potency was not affected by the presence of whole semen. In addition, this study shows that nAbs have a good safety profile, with no loss of tissue viability or immune activation that could preclude their use as an effective topical HIV microbicide.

The effective nAb dose for ectocervical tissue (1.5 μM) was 50 times higher than the effective dose for colonic tissue (0.03 μM) (Fig. 1). It is currently unclear why there are different effects of nAbs between colonic and ectocervical tissues. Pharmacokinetic studies of intravenously, intramuscularly, and orally administered ARVs have shown a differential deposition of drugs in female genital and rectal compartments. Higher drug levels are typically found in the rectal tissue than in the female genital tract (33, 34), and topically applied drugs are metabolized differently in the vaginal and rectal mucosae (35). However, in the context of a topically applied preparation of nAbs, where pharmacokinetic coverage is limited to the lumen, those observations do not explain why higher concentrations of topically applied nAbs are required to prevent viral transmission in cervical tissue than in colonic tissue. The greater potency of nAbs observed in this model of rectal mucosal transmission is not easily explained by differences in the size of inoculum used and merits further exploration. nAb potencies in the order PG16 > PG9 > VRC01-N ≫ 4E10-N were resolved *in vitro*. However, in the *ex vivo* model, the distinction between the potencies of PG9 and PG16 was not well defined. Lower doses of both of these nAbs caused a delay in infection

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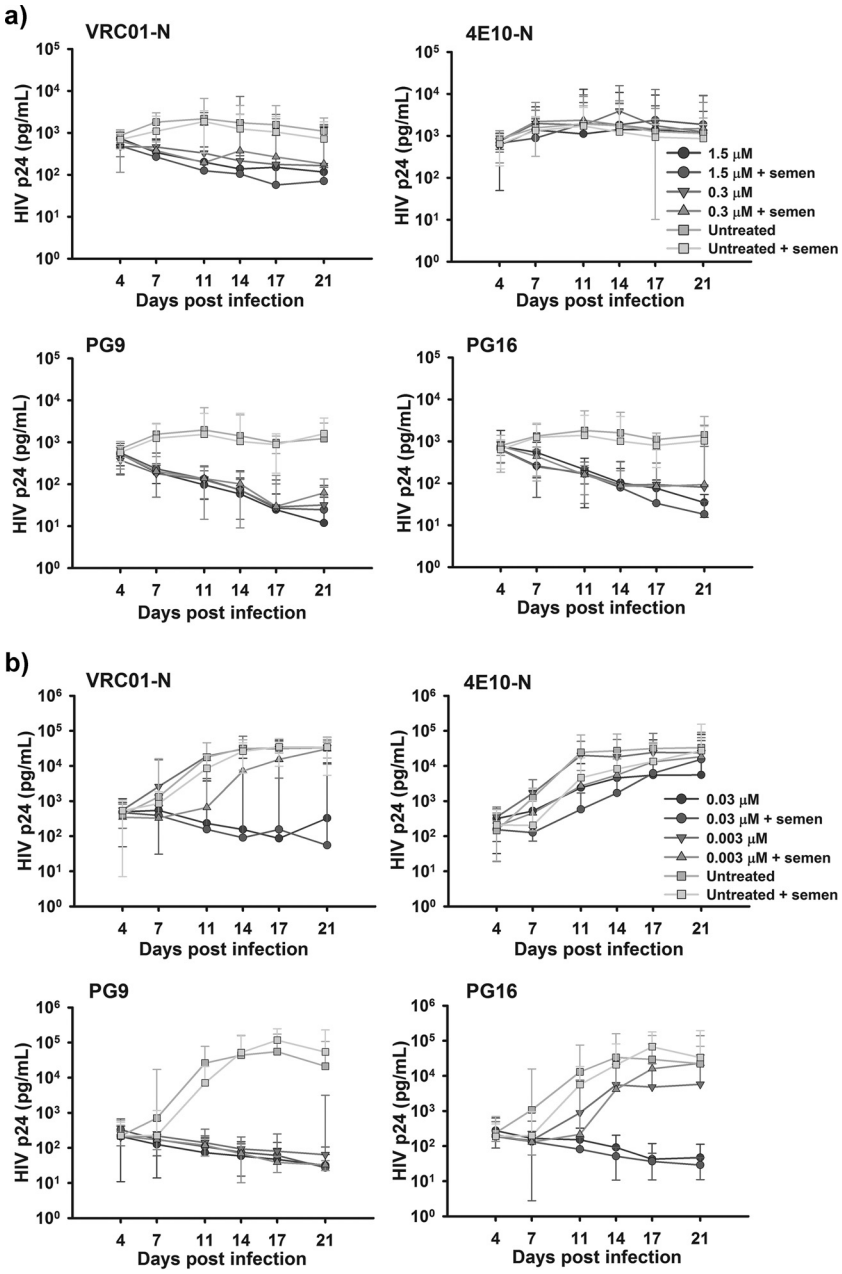

FIG 2 Potencies of nAbs in the presence of semen in human ectocervical (a) and colonic (b) *ex vivo* models. Tissues were treated with nAbs for 1 h before inoculation with HIV_{JR-CSF} in the presence or absence of 50% human semen. Viral replication was monitored by HIV p24 antigen ELISA on supernatants collected at 4, 7, 11, 14, 17, and 21 days postinfection. Data points represent the medians and interquartile ranges for ≥5 tissues from individual donors.

(Fig. 1), further attesting to the greater potency of these nAbs than that of VRC01-N or 4E10-N. This may suggest that further dilutions of PG9 and PG16 may have been needed in both tissue models to better define the comparative potencies of these antibodies.

The delay in infection observed with PG9 and PG16 treatment (Fig. 1) was likely due to a small portion of the inoculum not being neutralized by nAb treatment due to insufficiency of the antibody dose used. This low-level infection would have taken some time to expand enough to generate p24 concentrations that were above the ELISA limit of detection, hence the observed outgrowth. It is unlikely that this outgrowth of virus was due to the emergence of escape mutants, as exposure to a single dose of nAbs would not have provided sufficient selective pressure in this model. However, it must be acknowledged that infection by viruses with natural polymorphisms in envelope glycoproteins that make them resistant to neutralization by a single antibody is possible and may be prevented more effectively by using combinations of anti-HIV antibodies that simultaneously target multiple key envelope epitopes.

The relative lack of potency demonstrated by 4E10-N was not surprising, as this antibody, although broadly neutralizing, has

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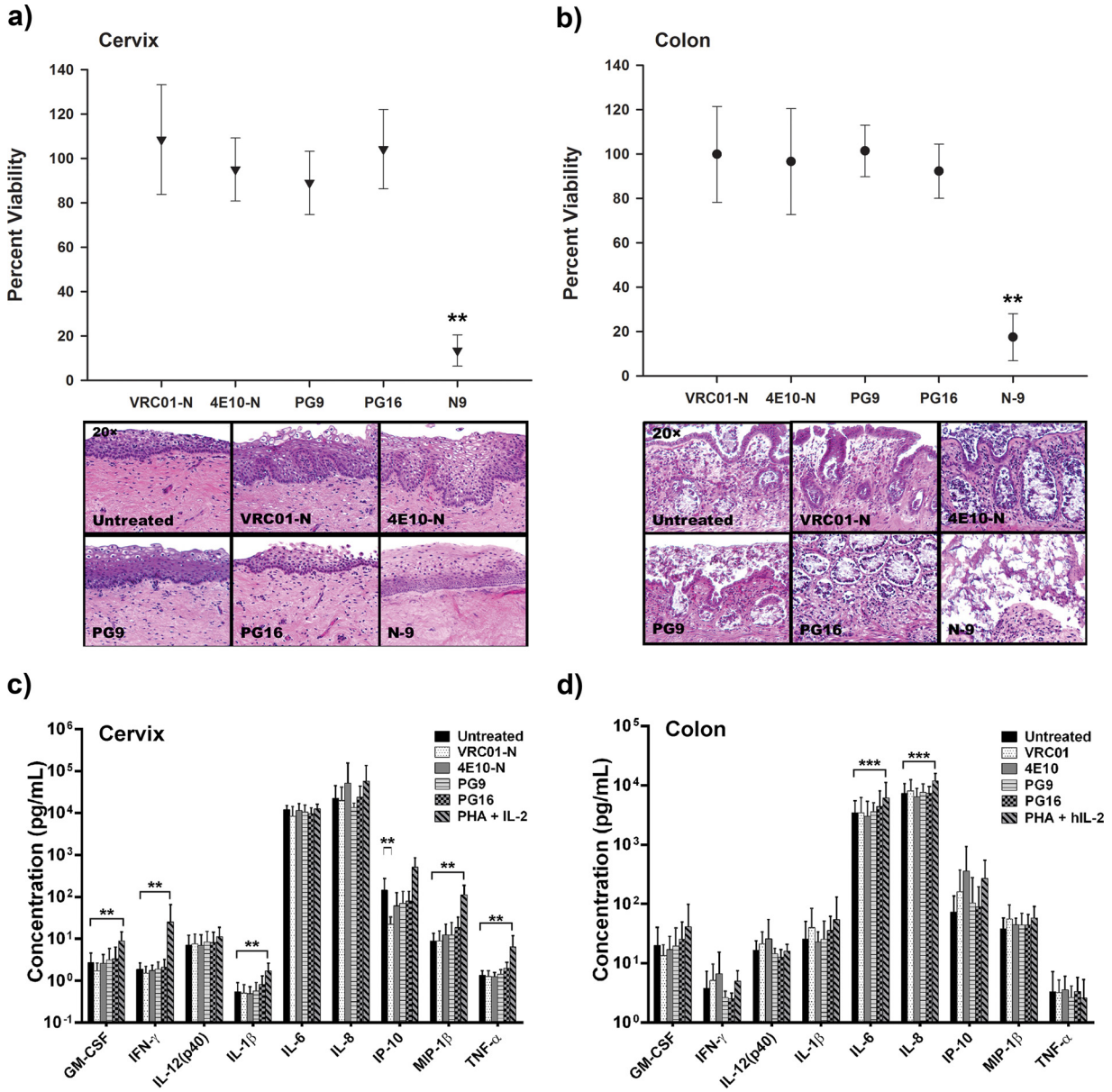

FIG 3 nAb safety in mucosal tissue. Ectocervical (a) or colonic (b) explants were treated for 24 h with 1.5 μM (ectocervix) or 0.03 μM (colon) nAbs or N-9 for 24 h. Viability was measured using the MTT viability assay and is presented as the mean percent viability relative to that of untreated tissues, with the standard deviation indicated by error bars ($n = 5$). Histologic panels show images of comparative hematoxylin and eosin staining of representative tissues after 24 h of treatment. Images were captured at a magnification of ×20. Quantitative Luminex analysis was performed on ectocervical (c) or colonic (d) tissue culture supernatants after 24 h of treatment with nAbs or an immune activation cocktail containing PHA-P plus hIL-2. The histograms present the mean cytokine concentration (pg/ml) and standard deviation for each treatment ($n = 3$ to 5 tissues). Statistical comparisons were made using one- or two-way ANOVA with an α value of 0.05. **, $P < 0.01$; ***, $P < 0.0001$.

been characterized as being only moderately potent (36). 4E10 exerts its main HIV-inhibitory activity by MPER binding; however, this antibody is also polyreactive, engaging in relatively short-lived, low-avidity interactions with other hydrophobic moieties (37). These may contribute to the decreased potency of 4E10 in HIV neutralization. The observed lack of potency may also be attributed to slower neutralization kinetics seen for MPER antibodies, such as 4E10 and 2F5. These antibodies require an epitope conformation that is thought to only be realized post-receptor engagement in more neutralization-resistant HIV-1 isolates, such as HIV-1_{JR-CSF} (38).

Seminal plasma contains factors that enhance HIV transmission (22, 39) and others that may interfere with microbicide activity (21). Previous microbicide evaluations showed that PRO 2000 gel protected mice from HSV-2 transmission. However, in the presence of semen, the product showed decreased efficacy *in vitro* (21). It was postulated that this effect may have been due to seminal protein interactions with the polyanion that blocked binding to HSV-2. This effect of semen essentially precludes the use of PRO 2000 for prevention of mucosal HIV transmission, where the putative inoculum is HIV-infected semen. In contrast, evaluation of nAb potency in the presence

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of semen showed that nAb activity in this model was not affected.

Preliminary safety data on the use of topically applied nAbs (Fig. 3) suggest that they are suitable for use as a topical on-demand prophylactic. In experiments to determine if there was immune activation due to nAb treatment, nAbs generally caused no changes in concentrations of inflammatory mediators, suggesting their suitability as topical microbicides. Additional safety evaluations may be necessary to determine the safety of other nAb applications. Previous nonantiretroviral microbicide candidates were shown to cause immune activation. Increased expression of IP-10 in the female genital tract has been associated with increased HIV acquisition (40) and is indicative of an inflammatory milieu. Treatment of ectocervical tissue with VRC01 was associated with decreased expression of IP-10 (Fig. 3c), suggesting that these nAbs do not increase production of soluble proinflammatory mediators.

Regarding a nAb microbicide, antibodies are highly specific for their cognate HIV epitopes and are native to the human body, hence nAbs applied luminally are expected to have negligible adverse effects that would preclude a therapeutic benefit. Traditional pharmaceutic antibody production uses transgenic mouse or human cell systems, both of which can be cost prohibitive to producing an affordable antibody-based topical microbicide. The cost of producing a pharmaceutic-grade supply of antibodies by using mammalian cell or animal systems was estimated to be $5 to 6 million over 18 months. In comparison, the use of transgenic plant production systems has made large-scale production of antibodies feasible and relatively cheap. Production of a pharmaceutic-grade supply of antibodies in transgenic plant production systems was estimated to cost $0.5 to 0.8 million over 12 months (16). These time and cost savings are important factors for making a nAb microbicide accessible for use in resource-limited settings.

The data reported here show that nAbs are effective in models of both rectal and vaginal transmission and may be considered for formulation as a dual-compartment microbicide product. Hence, an antibody-based microbicide could possibly expand microbicide application beyond vaginal use in high-risk women to rectal application and use in other vulnerable populations. A nAb microbicide may also be recommended for use by heterosexual couples engaging in both vaginal and anal sex. nAb efficacy is not expected to be affected by HIV mutations selected as a result of suboptimal ARV use, hence a nAb microbicide could be used by HIV-serodiscordant couples. In addition, nAbs may provide a non-ARV microbicide option for individuals who choose to avoid the side effects of using an HIV drug. Our findings obtained using unformulated nAbs suggest that these antibodies can prevent mucosal transmission of HIV when applied pericoitally and would likely be safe and effective as topical vaginal or rectal microbicides.

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Investigation of Griffithsin's Interactions with Human Cells Confirms Its Outstanding Safety and Efficacy Profile as a Microbicide Candidate

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Abstract

Many natural product-derived lectins such as the red algal lectin griffithsin (GRFT) have potent *in vitro* activity against viruses that display dense clusters of oligomannose N-linked glycans (NLG) on their surface envelope glycoproteins. However, since oligomannose NLG are also found on some host proteins it is possible that treatment with antiviral lectins may trigger undesirable side effects. For other antiviral lectins such as concanavalin A, banana lectin and cyanovirin-N (CV-N), interactions between the lectin and as yet undescribed cellular moieties have been reported to induce undesirable side effects including secretion of inflammatory cytokines and activation of host T-cells. We show that GRFT, unlike CV-N, binds the surface of human epithelial and peripheral blood mononuclear cells (PBMC) through an exclusively oligosaccharide-dependent interaction. In contrast to several other antiviral lectins however, GRFT treatment induces only minimal changes in secretion of inflammatory cytokines and chemokines by epithelial cells or human PBMC, has no measureable effect on cell viability and does not significantly upregulate markers of T-cell activation. In addition, GRFT appears to retain antiviral activity once bound to the surface of PBMC. Finally, RNA microarray studies show that, while CV-N and ConA regulate expression of a multitude of cellular genes, GRFT treatment effects only minimal alterations in the gene expression profile of a human ectocervical cell line. These studies indicate that GRFT has an outstanding safety profile with little evidence of induced toxicity, T-cell activation or deleterious immunological consequence, unique attributes for a natural product-derived lectin.

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Competing Interests: The authors have read the journal's policy and have the following conflicts: Author Barry O'Keefe is listed as an inventor on patent applications relating to the use of Griffithsin as an antiviral and owned by the National Institutes of Health/Public Health Service. Author Kenneth Palmer holds equity interest in a company that previously licensed the National Institutes of Health Griffithsin technologies. Dormeur Investment Service Ltd. partly funded the study. The following patents have been granted: US Patent No. 7,884,178, and related international filings under the PCT (application number WO/2005/118627), including Canadian Patent 2567728, Australian Patent 2005250429, European Patent 2005804849, Japanese Patent 2007515398, New Zealand Patent 551375 and South African Patent 2006/09573. The parent patent US 7,884,178 was issued on February 8th, 2011. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

HIV-1 is the prototype example of a virus that utilizes an oligomannose-rich "glycan shield" to occlude functionally important domains of the envelope glycoproteins from antibodies, and evade the immune response [1,2]. Recently Doores *et al.* [3] showed that previous measurement of the proportion of oligomannose NLG relative to complex NLG on recombinant HIV envelope glycoproteins underestimated the representation of oligomannose NLG on the native envelope spikes of HIV-1, which appear to display NLG that are almost exclusively mannose-terminal Man_{5-9}-$GlcNAc_2$ structures. It is likely that limited access to the high density of NLG presented on the HIV-1 trimeric glycoprotein spike by Golgi and endoplasmic reticulum (ER) $\alpha 1 \rightarrow 2$ mannosidases results in an atypical preponderance of oligomannose glycans rather than complex NLG on HIV-1 surface glycoproteins [3,4,5]. Given that Man_{5-9}-$GlcNAc_2$ structures are present on less than 4% of the normal human N-glycome [6,7], dense clusters of oligomannose NLG appear to be a feature specific to viral envelope glycoproteins, particularly those of HIV-1 and other immunodeficiency lentiviruses [3]. Consequently, clusters of oligomannose NLG may be attractive molecular targets for antiviral drugs and vaccines that act to interrupt HIV-1 infection of target cells by: (i) binding on the virus envelope and thereby interfering with the structural transitions involved in receptor and co-receptor docking and virus entry into T-cells and (ii) blocking access to viral envelope oligomannose NLG targeted

by C-type lectin receptors DC-SIGN and MMR on dendritic cells and macrophages [5,8,9].

It has long been known that a variety of oligomannose-specific lectins have potent *in vitro* HIV-1 inhibitory activities, and therefore have been proposed as microbicide candidates for topical prophylaxis of HIV-1 infection, and as potential therapeutics [8,9]. However many lectins possess lymphocyte mitogenic activities incompatible with their use as pharmaceuticals, and some are known human and animal toxins, although the pharmacological basis for their toxicity is poorly characterized [10,11]. The antiviral potency of lectins has been correlated to their capacity to bind multiple glycans simultaneously, often facilitated by their ability to form dimers and higher order multimers [12,13,14].

The most extensively characterized antiviral lectin is CV-N, a small protein that exists in both monomeric and homodimeric configurations and has exceptionally potent anti-HIV activity, in the low nanomolar range [15]. CV-N targets the Manα1→2Man terminating glycans displayed in the $Man_{6-9}GlcNAc_2$ structures on the surface of many viral envelope glycoproteins. Each monomer of CV-N has the capacity to bind two oligomannose structures [16]. When formulated into a carboxyethylcellulose gel matrix, CV-N provided almost complete protection against a single high dose intrarectal or intravaginal challenge with a pathogenic simian-human immunodeficiency virus (SHIV) [17,18]. However, subsequent *in vitro* toxicity studies have raised concerns about the safety of CV-N based microbicides, finding that *in vitro*, CV-N has the capacity to promote secretion of pro-inflammatory cytokines and chemokines from human peripheral blood mononuclear cells (PBMC), activate quiescent $CD4^+$ T-cells, and promote T-cell proliferation [19,20,21,22]. Similar results were also reported for other lectins such as microvirin (MVN) (17) and concanavalin A (Con A) (18). It should be noted that the toxicities of CV-N were much milder in treated cervical explants in comparison with PBMC [20,21]. The possible pathogenic consequences associated with these off-target activities have raised concerns about all other members of the natural product-based class of antiviral lectins [19,20,21].

GRFT has the most potent and broad spectrum HIV-1 inhibitory activity yet described for any antiviral lectin [15,23,24,25]. It is a 25 kDa domain-swapped homodimer, with the first 16 amino acids of each 12.7 kDa monomer completing the β-prism fold of the other [26]. The homodimer has six carbohydrate binding pockets, 3 located at each of the opposite ends of the double-prism homodimer. Atomic resolution crystal structures of an engineered monomeric GRFT showed that each monomer can bind to two different nonamannoside molecules through all three carbohydrate binding sites [13,26]. The antiviral activity of monomeric GRFT is substantially lower than that of the homodimeric form, confirming that the GRFT potency is dependent on its ability to bind multiple oligomannose structures simultaneously, with strong avidity [13]. We showed recently that GRFT causes no mitogenic stimulation of PBMC exposed to the drug [23]. GRFT is fully active in the presence of macaque vaginal secretions [25], and was shown to have a good safety profile in the rabbit vaginal irritation model, the Gold Standard preclinical safety test for vaginal products [23]. Moreover, treatment of human cervical explants with GRFT induced minimal alterations in the expression profile of a panel of proinflammatory chemokines and cytokines. GRFT also strongly inhibited HIV-1 infection of the cervical explants, and dissemination of HIV-1 infection from cells resident in the explants to donor T-cells [23]. In the present study, we performed a comprehensive set of experiments to interrogate the molecular response of cultured human cervico-vaginal cells and PBMC to GRFT exposure. Our investigations employed comparisons between the biological activities of GRFT, which binds mannose-terminal Man_{5-9}-$GlcNAc_2$, with other lectins of well-defined carbohydrate binding specificity: (1) CV-N, which binds Manα1→2Man terminating glycans on Man_{6-9}-$GlcNAc_2$ structures; (2) phytohemagglutinin A (PHA), targeting D-galactose and N-acetyl-D-galactosamine on gycan structures; (3) ConA, specific for terminal (tri) mannose on high mannose glycans and (4) Pokeweed agglutinin (PKM), which binds N-acetylglucosamine. Our studies reveal clear distinctions in biological and toxicological properties of these lectins, and confirm GRFT's superior safety profile for use as a topical microbicide.

Results

GRFT and CV-N binding to human cervical epithelium, cultured human cervicovaginal cells, and PBMC

We used paraffin-embedded cervical epithelial sections from a 21-year old donor to evaluate the binding pattern of fluorescently labeled GRFT and CV-N to human mucosal epithelia. $GRFT^{Lec-}$, a mutant form of GRFT where we eliminated the lectin activity through mutation of all six mannose binding sites, was used as a control to help distinguish binding mediated by the GRFT carbohydrate binding pockets versus binding associated with other GRFT structures. The light micrograph in Figure 1A shows an H&E stained cervical tissue section to orient the observer to the microanatomy of the human cervical epithelium. Different layers of the squamous epithelium starting at the basement membrane (basal, parabasal, intermediate, superficial) are evident; cervical connective tissue or stroma is beneath the basement membrane. Tissues incubated with labeled GRFT, $GRFT^{Lec-}$ and CV-N are shown in Fig. 1B, C and D, respectively. Minimal fluorescence seen in tissues exposed to $GRFT^{Lec-}$ (Fig. 1C) compared with GRFT-stained tissues (Fig. 1B) confirmed that the binding of GRFT to the outermost layer of the squamous epithelium was via its carbohydrate binding activity. There were distinct differences evident in the binding pattern of GRFT (Fig. 1C) relative to CV-N (Fig. 1D), which bound far more extensively than GRFT throughout all layers of the squamous epithelium, basement membrane and underlying stromal tissue. Additional fluorescence micrographs are provided in Fig. S1. In cultured cervicovaginal epithelial cells we also observed binding of both GRFT and CV-N, but not $GRFT^{Lec-}$ to Ect1/E6E7 cells (compare Fig. 1E through H), as well as End1/E6E7 and VK2/E6E7 cells (data not shown). We used flow cytometry to evaluate binding of fluorescently labeled GRFT, $GRFT^{Lec-}$ and CV-N to human PBMC. Clear shifts in fluorescence intensity show that GRFT (Fig. 1 I) and CV-N (Fig. 1K) efficiently bind the surface of human PBMC relative to $GRFT^{Lec-}$ (Fig. 1J), for which we observed only minimal binding. Binding of GRFT to the surface of PBMC was significantly reduced when occluding the glycan binding pockets by pre-incubation with yeast mannan (Fig. 1I). Interestingly, the binding of CV-N to PBMC was reduced, but not eliminated, by mannan binding, which implies a second mode of binding between CV-N and the cell surface (Fig. 1K). We assume that distinct populations of differentially labeled cells seen in the flow histograms reflect differences in the amount of labeled protein that binds different subpopulations of leukocytes in the unfractionated PBMC samples.

Griffithsin bound to PBMC retains its antiviral activity

When freshly-isolated PBMC were pre-incubated for 24 hrs with GRFT at various concentrations, washed and then infected with HIV-1 R5 strain BaL (without adding new compound), GRFT inhibited viral replication for 9 days of cell culture (Fig. 2). As a control maraviroc (MVR) at 2 μM was included and this also showed anti-HIV activity after 9 days in culture, as this compound is known to bind specifically to the CCR5 receptor. However,

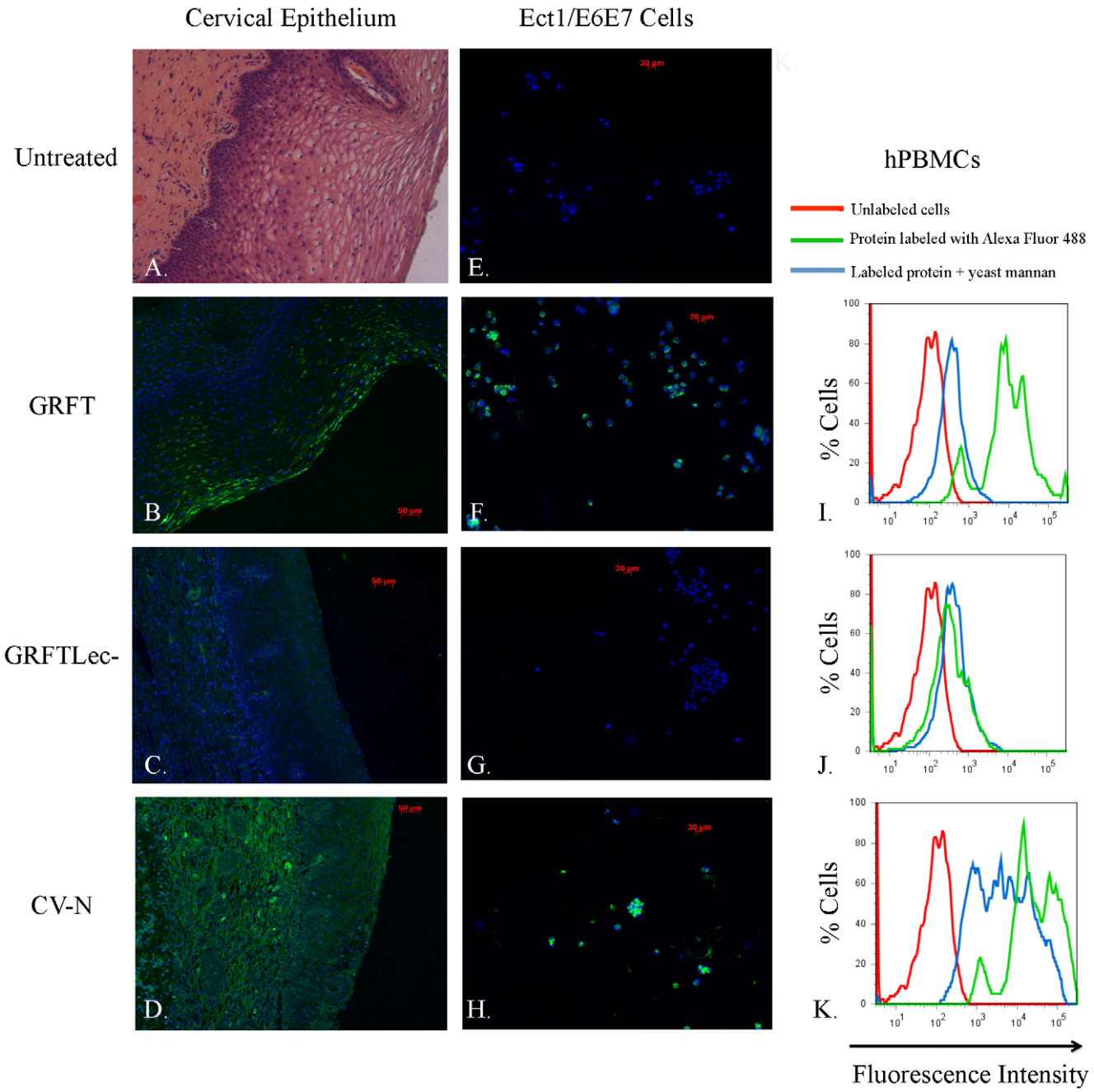

Figure 1. Analysis of binding specificity of GRFT in comparison with GRFT^{lec-} and CV-N. In the first column we depict fluorescence micrographs that show binding of AlexaFluor 488-labeled GRFT (B.), GRFT^{Lec-} (C.) and CV-N (D.) to paraffin-embedded cervical tissue sections from a 21-year old female. In (A.) we show a hematoxilin and eosin-stained light micrograph of cervical epithelial tissue, showing the general micro-anatomy of the cervical epithelium. In the second column, we show fluorescent micrographs of cultured human ectocervical cell line Ec1/E6E7 untreated (E.), and treated with AlexaFluor 488-labeled GRFT (F.), GRFTLec- (G.) and CV-N (H.). The third column shows histograms that depict flow cytometric analysis of hPMBCs treated with fluorescently labeled GRFT (I.), GRFT^{Lec-} (J.) and CV-N (K.). The X-axis shows increasing fluorescence intensity from left to right, the Y-axis shows percentage of labeled cells in the sample.
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lower concentrations of maraviroc (at 0.4 μM) did not retain its antiviral activity in this assay protocol (data not shown).

High concentrations of GRFT are not cytotoxic to cervico-vaginal cell lines

An MTT assay was used to assess the effects of GRFT on End1/E6E7, Ec1/E6E7 and VK2/E6E7 cell viability by measuring the metabolic activity of treated cells. In these experiments (Fig. 3) we observed no loss in cell viability after a 3 day exposure of the endocervical and ectocervical cell lines to concentrations of up to 1 mg/ml (84 μM) GRFT, at least 10-times more concentrated than a likely microbicide formulation [23]. High doses of GRFT did, however, slightly reduce viability of the vaginal keratinocyte (VK2) cells. In marked contrast to GRFT, the mannose-specific mitogenic

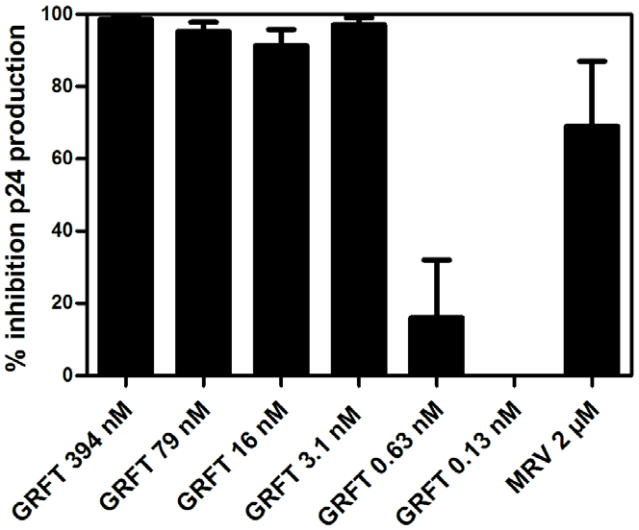

Figure 2. Antiviral activity of GRFT-pretreated PBMC for R5 HIV-1 BaL infection. Freshly isolated PBMC from 3 different donors were pretreated with GRFT (394-0.13 nM) and maraviroc (2 μM) for 24 hrs and then washed and infected with the R5 HIV-1 BaL strain for 9 days. No compounds were added during the infection and viral replication was measured by p24 Ag specific ELISA in the collected supernatants.
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lectin ConA showed clear concentration-dependent cytotoxicity towards all three cell lines. As shown in Fig. 3, treatment with 1 μM ConA killed approximately 94% of the ectocervical cells and vaginal keratinocytes, and 80% of the endocervical cells. PHA, another lectin known to be mitogenic in vivo and in vitro also caused dose-responsive cell death, but to a lesser extent than ConA.

GRFT does not stimulate cell proliferation

We previously showed that GRFT exhibits no mitogenic stimulatory effect in human PBMC, using incorporation of tritiated thymidine as a marker for cell proliferation [23]. In the present study we investigated the mitogenic activity of GRFT on PBMC by flow cytometry, evaluating changes in size and morphology of cells treated with GRFT in comparison with cells treated with the vehicle (PBS). Cells treated with 1 or 4 μM GRFT had flow cytometry profiles similar to the control cells (Fig. 4A, B and C). In contrast treatment with lectins ConA and PHA at doses (0.37 μM ConA and 10 μg/ml PHA) that do not negatively affect cell viability, resulted in completely different flow cytometric plots, with emergence of a subpopulation composed of larger cells (higher forward scatter FSC) with perceptibly higher side scatter (SSC) values gated in Region R2, as shown in Fig. 4D and E. In addition, a clear decrease in cell number was observed in region R1 after treatment with ConA and PHA, as quantified in Fig. 4F. In Fig. 5, we show that GRFT also does not induce cell proliferation in any of the three cultured human cervical and vaginal epithelial cells. Cell division was assessed by monitoring BrdU incorporation in newly synthesized DNA of actively dividing cervicovaginal cell lines. Treatment with 1 or 8 μM GRFT did not induce proliferation of any of the cell lines since BrdU counts were not elevated in comparison with control cells treated with vehicle alone (PBS). In contrast Pokeweed agglutinin (PKM), a well characterized mitogen, caused concentration-dependent increase in cell division, especially in the cervical cell lines End1/E6E7 and Ect1/E6E7.

Effects of GRFT treatment on PBMC activation markers

To evaluate the effect of GRFT on cell surface markers of immune activation, we measured expression of the following membrane proteins: (i) CD69, a marker of activated T-lymphocytes, considered an "early" marker of T-cell activation; (ii) CD25, the alpha chain of the IL-2 receptor, upregulated on

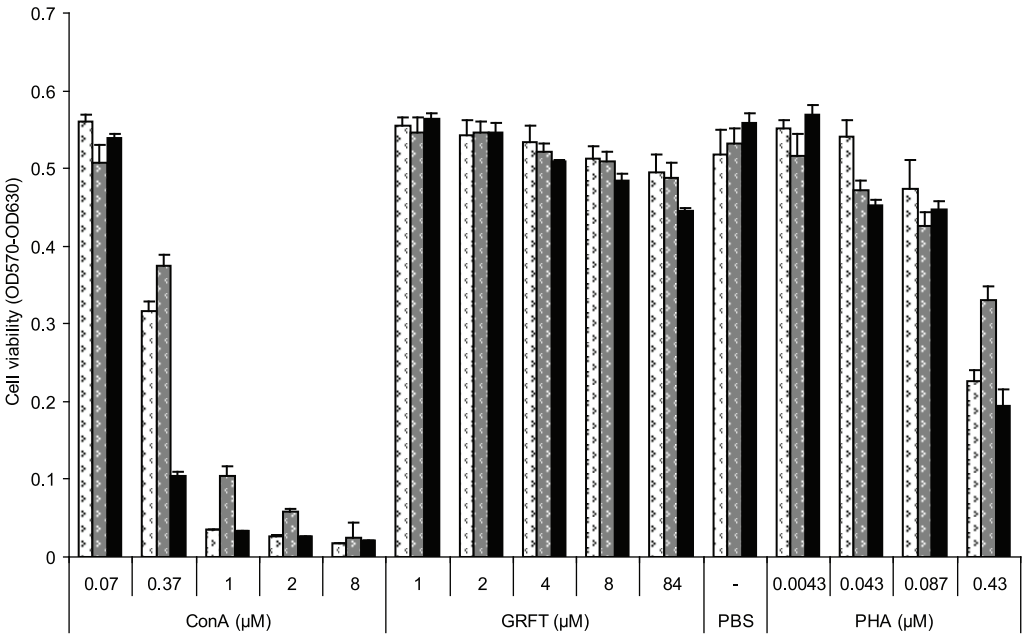

Figure 3. Effect of GRFT on cervico-vaginal cell viability. Cell viability was evaluated in an MTT assay after 72 hours treatment of Ect1/E6E7 (white), End1/E6E7 (grey) and VK2/E6E7 (black) with different test compounds listed on the X-axis together with their concentrations. Values are given as mean ± standard deviation of OD570 – OD630.
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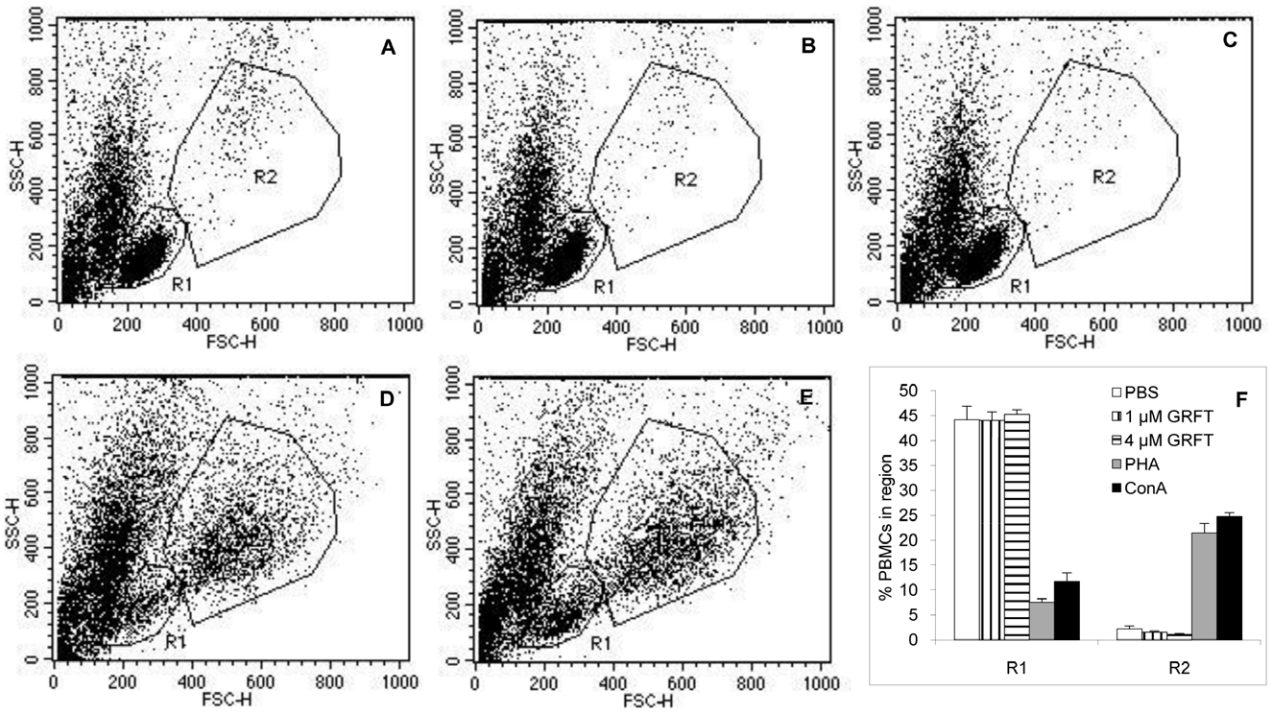

Figure 4. Flow cytometric evaluation of the mitogenic effect of GRFT on PBMC. Cells were analyzed flow-cytometrically after three days treatment with PBS (A), 1 μM GRFT (B), 4 μM GRFT (C), 10 μg/ml PHA (D) and 0.37 μM ConA (E). Typical live PBMC population were gated in region R1 and a subpopulation with increased size and higher SSC was gated in region R2. Quantitation of cells in these regions was plotted in F for each test compound.
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activated T-cells, B-cells, and some thymocytes and myeloid precursors, considered a "middle" marker of T-cell activation; and (iii) HLA-DR, a component of the type II major histocompatibility (MHC) complex, and a "late" marker of T-cell activation. Activated T-cells are highly susceptible to HIV-1 infection, and hence induction of these markers indicates an undesirable side effect of lectin treatment of PBMC. PBMC were incubated in the presence of the test compounds for 72 hours. The vehicle control was PBS, and positive controls were 10 μg/ml PHA [20], as well as 0.37 μM ConA, chosen as this concentration was not cytotoxic. In PBS treated cells, 1.2% PBMC in average were double positive ($CD4^+/CD25^+$) and a non significant increase of this population was observed after incubation in presence of 1 or 4 μM GRFT (Fig. 6, left panel and Fig. S2A). Treatment with PHA and ConA resulted in an impressive increase in the number of $CD4^+/CD25^+$ cells (Fig. 6, left panel and Fig. S2A). In addition, the numbers of $CD4^-/CD25^+$ cells were elevated when PBMC were cultured in presence of PHA or ConA compared to their PBS and GRFT counterpart (Fig. 6, left panel and data not shown). Similarly, GRFT treatment did not affect significantly the proportion of $CD4^+/CD69^+$ PBMC compared with PBS treatment, whereas ConA and PHA treatment resulted in an increase of more than 10 times in this sub-population (Fig. 6, middle panel and Fig. S2B). The numbers of $CD4^-/CD69^+$ were also elevated after treatment with ConA and PHA (Fig. 6, middle panel, and data not shown). In unstimulated cells, about 15% of CD4 positive PBMC expressed the late activation marker HLA-DR and treatment with GRFT and ConA did not affect this number in a significant fashion (Fig. 6, right panel and Fig. S2C). PHA treatment of cells yielded more than twice the number of $CD4^+/HLA\text{-}DR^+$ cells (33.4%) compared with unstimulated PBMC.

Cytokine and Chemokine Profile of GRFT-incubated PBMC

Activation of PBMC may also be reflected by the production of cytokines and chemokines. To investigate potential activating properties of GRFT more thoroughly, PBMC were cultured in the presence of 10 μg/ml GRFT (788 nM) for 72 h. In the culture supernatant, the concentrations of IL-1α, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF were determined. A detailed overview of the cytokine profiles of GRFT-treated PBMC from multiple blood donors is given in Fig. 7. For reference, we provide previously published data where PBMC with the same donor origins were treated with CV-N at 2 μg/ml (182 nM), lower than the GRFT concentration tested, since higher concentrations of CV-N proved too toxic to PBMC [19]. The concentration of the separate cytokines was compared with that of the untreated PBMC and calculated as a fold increase value. In the previous studies on PBMC treatment with CV-N and MVN, considerable variability in the lectin-induced cytokine profile was observed between the different PBMC donors. Therefore, the fold increase values obtained from the different donors were divided over different ranking groups (*i.e.* 1–3-, 3–10-, 10–100-, 100–500-, and >500-fold increase), and the number in each rank is given as a percentage of the total and indicated by a specific *color* (Fig. 7). Confirming our data in Fig. 6 that GRFT has very little effect on lymphocyte activation markers, we also see minimal alterations in the cytokine and chemokine release for the majority of markers, in most donor PBMC. This profile indicates that GRFT induces significantly less response from PBMC than has been previously reported for CV-N, MVN and ConA [19]. The only chemokine

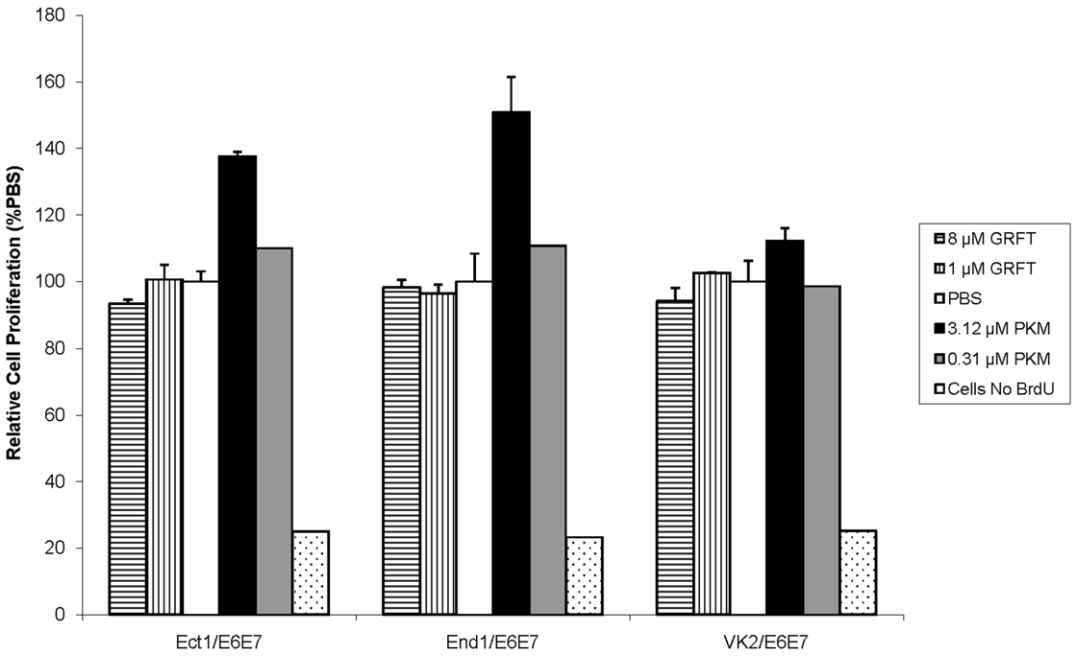

Figure 5. Effect of GRFT on cervico-vaginal cell proliferation as measured by BrdU incorporation. Cell proliferation was measured by the incorporation of BrdU in newly synthesized DNA using a colorimetric assay after treatment of Ect1/E6E7, End1/E6E7 and VK2/E6E7 with GRFT (1 or 8 µM), PKM (3.12 or 0.31 µM) or PBS. Cells without BrdU were assessed in the assay as a negative control according to the manufacturer's intructions. Values are given as mean ± standard deviation of the relative cell proliferation rate derived from BrdU incorporated as a percentage of that found in PBS.
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induced weakly by GRFT in the majority of donors (75%) was MCP-1 [19]. Although these studies were performed at a later date than the previously published CV-N and MVN studies, we used exactly the same donor panel, and the assays were performed in the same laboratory (D. Schols), justifying comparison between the experiments.

Validation of the utility of cultured cervico-vaginal cell lines for detection of off-target effects of GRFT treatment

In our previously published studies, we showed that treatment of human cervical explants with a range of GRFT concentrations had no significant effects on expression of a panel of chemokines and cytokines [23]. In the present studies, we used cultured human endocervical, ectocervical and vaginal cell lines for more detailed analysis of potential "off-target" effects of GRFT treatment, since the cell lines are easier to procure than fresh human cervical tissues, and experiments with the cell lines are more easily reproduced without question of variability in genetic background and physiological conditions of the donor. To validate the utility of these cell lines (which are immortalized by induction of papillomavirus E6 and E7 oncogenes) for analysis of any "off-target" effects of GRFT treatment, we wished to confirm that the cell lines behaved similarly to cervical explants after treatment with GRFT, and ConA, which induced inflammatory responses in our PBMC studies. We assessed the secretion of many key mediators of inflammatory responses, including Il-1b, IL-2, IL-6 and IL-8 by all three cervico-vaginal cell lines. ELISA kits specific for these cytokines were used for their detection in End1/E6E7, Ect1/E6E7 and VK2E6E7 cell culture supernatants after 24 h of incubation in presence of the test compounds. After treatment with 8 µM GRFT, the supernatants showed barely detectable levels of IL-1b and IL-2, similar to the cells treated with PBS (Fig. 8A and B). In contrast, all three cell lines produced impressive amounts of these cytokines upon incubation in presence of ConA (Fig. 8A and B). Treatment of cervical epithelial cell lines End1/E6E7and Ect1/E6E7 with GRFT (8 µM), ConA (2 µM) or PBS resulted in similar levels of IL-6 secretion (Fig. 8C), but in VK2/E6E7, IL-6 release was approximately three times higher after ConA treatment compared to PBS, whereas GRFT did not induce the secretion of this cytokine (Fig. 8C). In the case of IL-8, each of the three cell lines showed a distinct pattern of secretion of this cytokine, although no induction was observed after treatment with 8 µM GRFT: VK2/E6E7 showed barely detectable levels of IL-8 regardless of the treatment; IL-8 levels in Ect1/E6E7 were relatively high after PBS and GRFT treatment and these amounts were about ten times reduced after treatment with ConA; GRFT treatment resulted in a slight decrease of IL-8 in End1/E6E7 cell culture supernatants whereas the concentrations of this cytokine were significantly increased following treatment with ConA (Fig. 8D). In addition, GRFT treatment did not alter the production of IL-10, IP-10, MIP-1β and TGF-α in the cervico-vaginal cells studied (data not shown). These data indicated that all 3 cell lines behaved as predicted in response to GRFT and ConA treatment. Since the ectocervical epithelium is an important point of mucosal transmission of HIV-1, we focused on the Ect1/E6E7 cell line to derive a more comprehensive assessment of the effect of GRFT treatment on cytokine production by the cultured ectocervical cells. A multiplexed Luminex assay of the Ect1/E6E7 cell line treated with GRFT confirmed that the Ect1 cells behaved very similarly to human cervical explants (data not shown), and validated our use of these cells in our microarray studies.

Effect of GRFT Treatment on Gene Expression in Ect1/E6E7 cells

Gene expression in Ect1/E6E7 cells in response to treatment with GRFT was compared with vehicle (PBS), $GRFT^{Lec-}$, CV-N

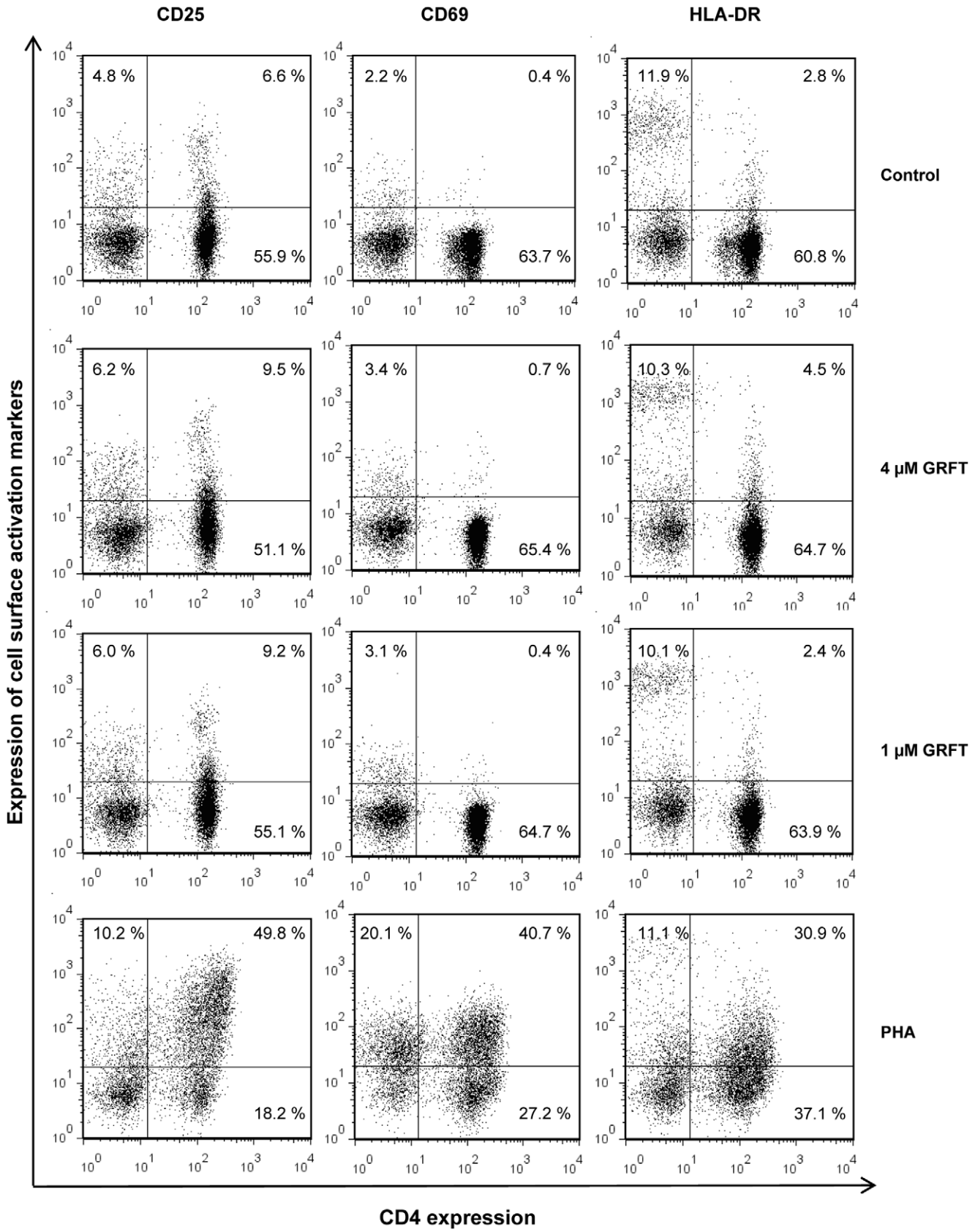

Figure 6. Effect of GRFT on PBMC activation. Cells were incubated for three days in culture medium alone, with 1 µM GRFT, with 4 µM GRFT and with 10 µg/ml PHA and dual stained using FITC-conjugated anti-CD4 mAb in combination with PE-conjugated anti-CD25 (left panel), anti-CD69 (middle panel) and anti-HLA-DR (right panel). The percentages of receptor positive cell populations are indicated in the individual dot plots.
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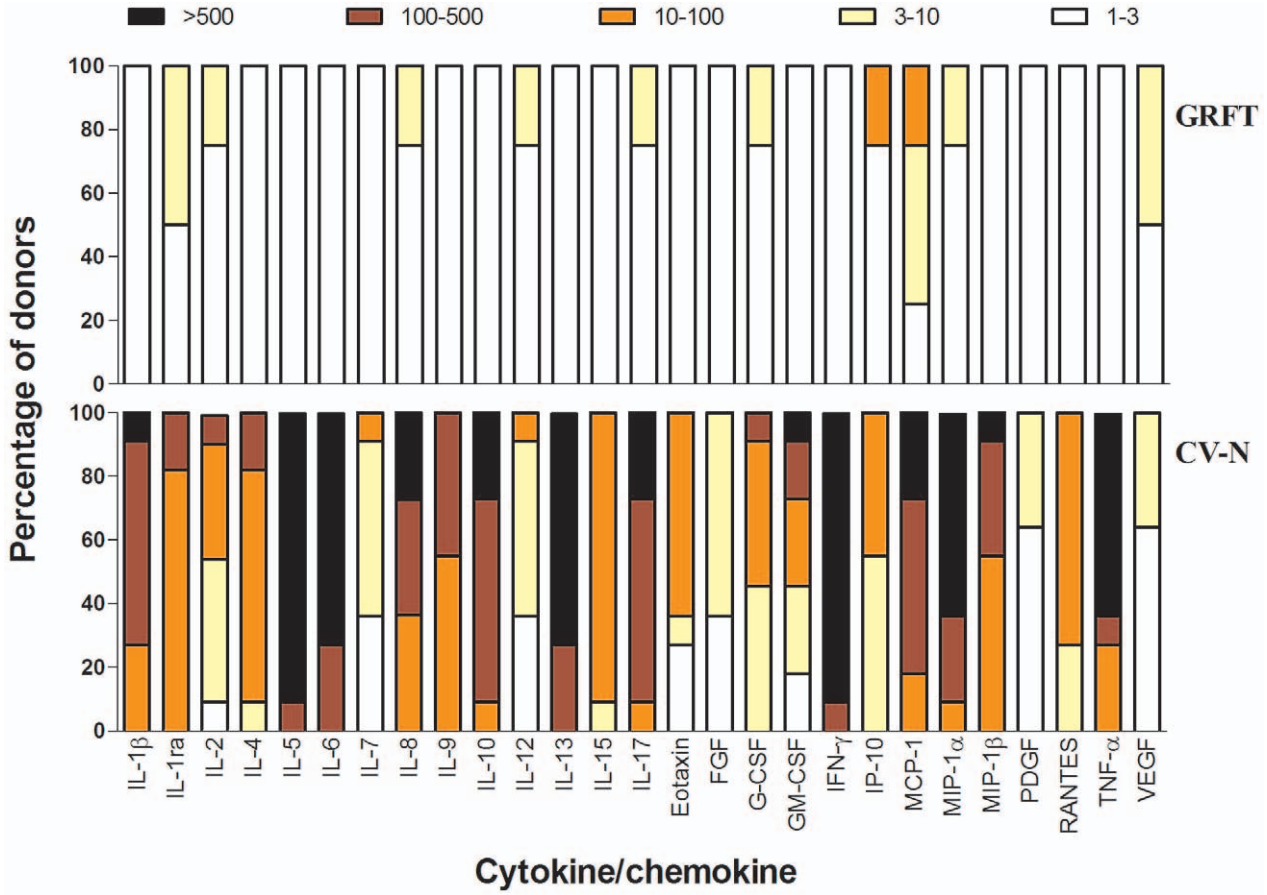

Figure 7. Effect of GRFT on the induction of cytokines/chemokines in PBMC. PBMC from healthy donors were incubated for 72 h with medium only, GRFT or CV-N at 788 nM and 182 nM, respectively. Supernatants were collected and cytokine levels were measured by the Bio-Plex array system. The fold-increase values of the cytokine concentrations in the supernatant of stimulated PBMC with respect to the concentrations in the supernatant of untreated cells were determined from 4 and 11 different donors for GRFT and CV-N, respectively. The fold-increase values are divided in subgroups: 1–3 fold-increase (white squares), 3–10 fold-increase (yellow squares), 10–100 fold-increase (orange squares), 100–500 fold-increase (dark red squares) and >500 fold-increase (black squares). The amount of fold-increase values for each chemokine/cytokine is given as percentage in the total amount of donors (Y-axis).
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and ConA treatments (all qualified with endotoxin levels less than 0.05 EU per milligram). This was studied using a microarray experiment in which the whole human genome was represented by the 41,000 known genes and transcripts [27]. The microarray data were deposited in the Gene Expression Omnibus database, under accession number GSE28584. The heat map shown in Fig. 9A indicates that cells exposed for 24 hours to $GRFT^{Lec-}$ (1 and 8 μM), and low concentrations of GRFT (0.1 μM) and CV-N (0.05 μM) showed comparable gene expression profiles to those that were incubated in presence of PBS alone. Treatment of Ect1/E6E7 with 1 μM GRFT resulted in minor changes in the expression profile while 4 μM GRFT appeared to alter the expression of many genes but to a much lesser extent compared to CV-N (0.5 and 4 μM) and ConA at 1 μM (Fig. 9). Thus, the microarray studies confirm the data showing that GRFT has substantially lower "off-target" effect in comparison with CV-N and ConA, although there are clearly several genes that are regulated by the carbohydrate binding activity of GRFT (compare with $GRFT^{Lec-}$). As predicted from the heat map (Fig. 9A), no gene was identified as regulated by 0.1 μM GRFT, even when the cutoff was brought to 1.0. However, a first analysis using Benjamini-Hochberg low stringency correction with a cutoff of 2.0 yielded 107 and 35 entries as differentially expressed in samples treated with 1 and 4 μM GRFT, respectively (Fig. 9B). Treatment with CV-N (0.5 and 4 μM) or ConA (1 μM) resulted in regulation of impressive numbers of human genes (Fig. 9B). We then employed stricter criteria by keeping only the positive entries that showed GRFT concentration dependent gene expression. This yielded 2 and 32 genes for 1 and 4 μM GRFT, respectively. The entries which showed an increased gene expression after treatment with 1 μM GRFT included a gene annotated as immunoglobulin-like and fibronectin type III domain containing 1 (IGFN1). The entries that were found to be affected by 4 μM GRFT are summarized in Table S2. Among the 26 genes mapped by the Ingenuity database, there was overrepresentation of genes in the following canonical pathways: NRF2-mediated oxidative stress response (MAF, HMOX1, SOD2), phospholipid degradation, glycerophospholipid metabolism and Endothelin-1 Signaling (HMOX1, WISP2), cAMP mediated signaling (CALML5, PKIB) and acute phase response signaling (HMOX1, SOD2). Furthermore using the Ingenuity software we identified five toxicological functions with an overrepresentation of genes including liver hyperbilirubinemia and steatohepatitis, cardiac arteriopathy, renal and liver necrosis (data not shown). None of these is relevant to mucosal treatment with GRFT. We used quantitative RT-PCR (Q-RT-PCR) to validate the microarray results. Expression of 14

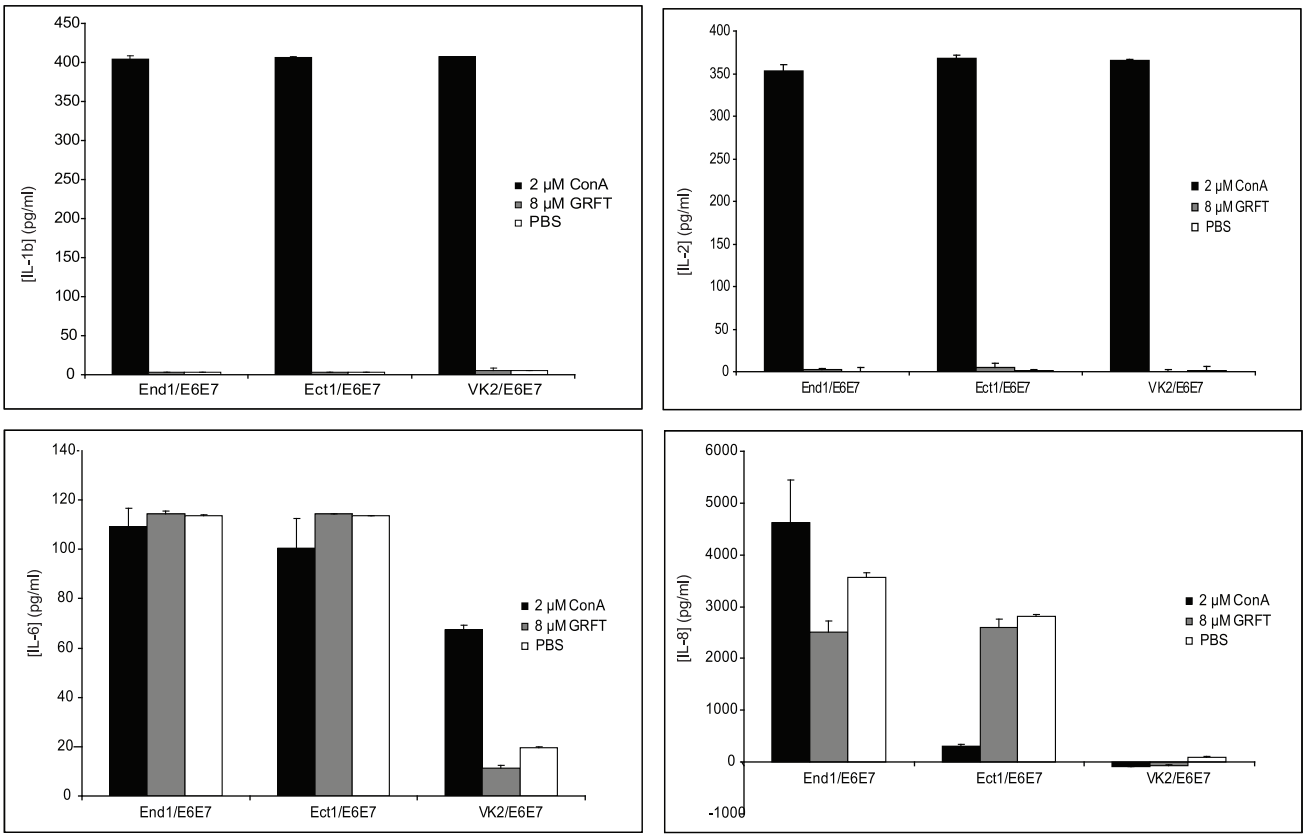

Figure 8. Effect of GRFT on the secretion of selected key cytokines in cervico-vaginal cell lines. Individual ELISA experiments for detection of IL-1b (A), IL-2 (B), IL-6 (C) and IL-8 (D) after 24 hours treatment of cervico-vaginal cells with 2 μM ConA (black), 8 μM GRFT (grey) and PBS (white).
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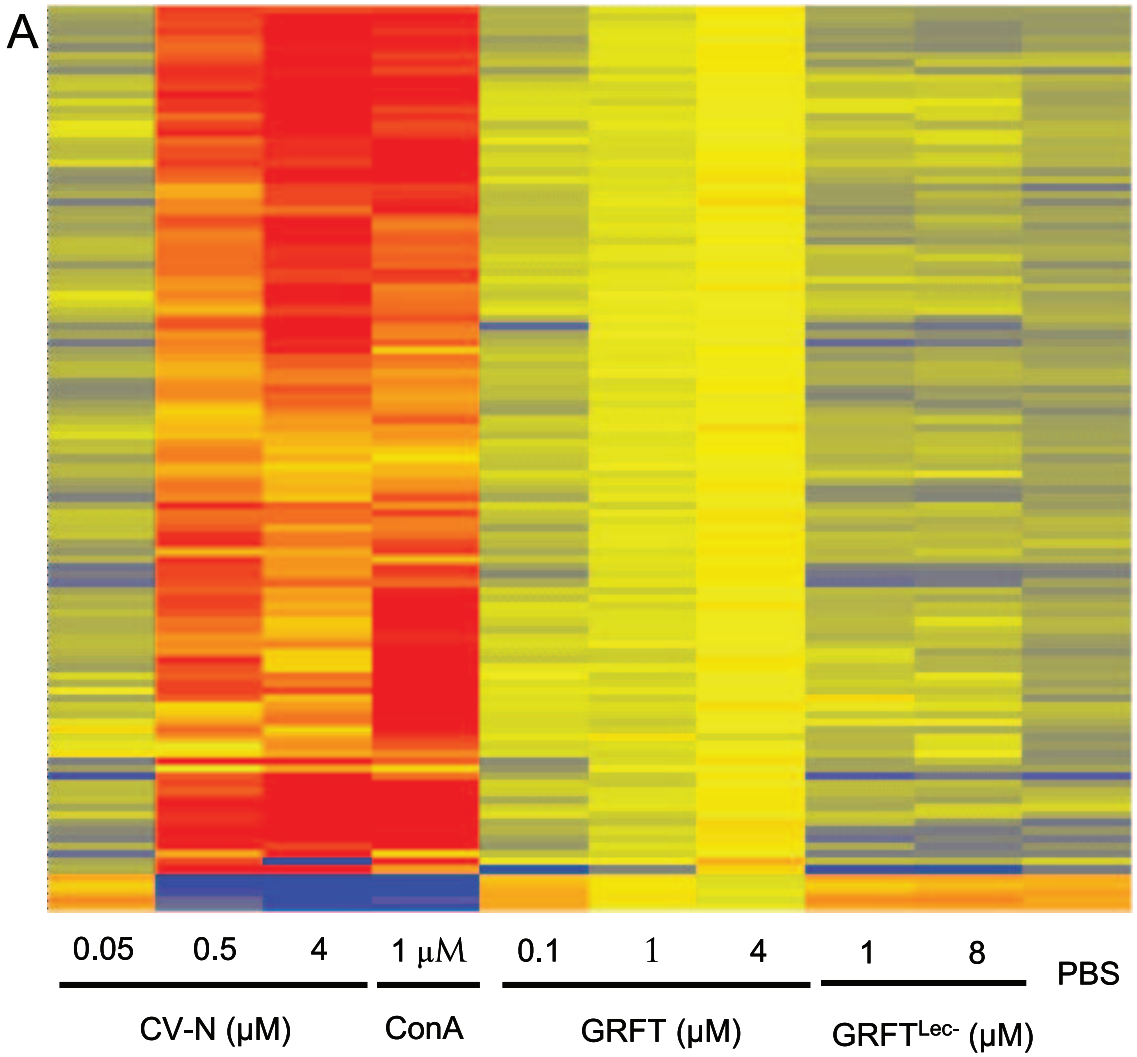
genes was studied using 18S RNA and β-actin mRNA as controls. With the exception of MYCN, all genes studied showed comparable expression levels in both experimental systems including microarrays and Q-PCR (Table S3).

Discussion

Natural product lectins have received considerable attention as potential antiviral drugs, particularly in the context of prevention of HIV-1 transmission via mucosal surfaces (reviewed recently in [8]). Although the volume of literature supporting their use as antivirals *in vivo* is dwarfed by a comprehensive set of data showing potent *in vitro* antiviral activity, there are reports of impressive *in vivo* efficacy of CV-N in animal models of HIV-1 prevention [17,18], influenza prevention and treatment [28], and Ebola virus prophylaxis and treatment [29], and of GRFT in prevention of SARS-CoV infection [30]. Despite the myriad of potential prophylactic and therapeutic applications of antiviral lectins, enthusiasm for their development as pharmaceuticals is tempered by a long history of research into natural product lectins, which characterizes many members of this broad class as erythrocyte agglutinins, lymphocyte mitogens, and potentially lethal toxins [10,11]. The pharmacological basis of natural product lectin toxicity is generally poorly understood, but at a basic mechanistic level is thought to reside in the lectins' capacity for multivalency of binding to cell surface glycans, resulting in cell agglutination and/or cross-linking of cell surface receptor molecules with consequent activation of signaling pathways. Why different lectins that bind identical glycan moieties have quite distinct biological effects *in vitro* remains a paradox that is well illustrated by our data which, show very different *in vitro* activity profiles of four different oligomannose-binding lectins: GRFT, CV-N, MVN [19] and ConA.

Characterization of GRFT is the primary focus of our studies, but understanding the molecular pharmacology and toxicology of this potent antiviral lectin is informed by comparison to CV-N, for which a rich set of *in vitro* and *in vivo* data is available. Both molecules have comparable HIV-1 neutralization activities, with mean IC_{80} values against Clade C viruses of 42.7±4.4 nM for GRFT and 77.0±18.2 for CV-N [15]. Both proteins bind oligomannose glycans, with GRFT targeting terminal mannose residues found on Man_{5-9}-$GlcNAc_2$ [13] and CV-N specific for the Manα1→2Man linkages found on Man_{6-9}-$GlcNAc_2$ [16,31,32]. They thus share overlapping binding specificities, and should target identical cell surface and viral glycans. If anything, GRFT would be predicted to bind to a larger number of glycan targets than CV-N since it can bind pentamannose structures that lack the α1→2 mannose linkages that CV-N targets [13]. In this context, it is surprising that CV-N appears to bind more promiscuously than GRFT throughout the cervical epithelium and sub-epithelial stroma (Fig. 1A–D, and Fig. S1). Given that $GRFT^{Lec-}$ (the carbohydrate-binding deficient form of GRFT) hardly bound the epithelial sections or cultured cervical and vaginal epithelial cells (Fig. 1), and GRFT binding to cultured epithelial cells was blocked by mannan, we conclude that GRFT's binding activity to the cell surface is exclusively via its


A
0.05
0.5
4
1 μM
0.1
1
4
1
8
PBS
CV-N (μM)
ConA
GRFT (μM)
GRFTLec- (μM)

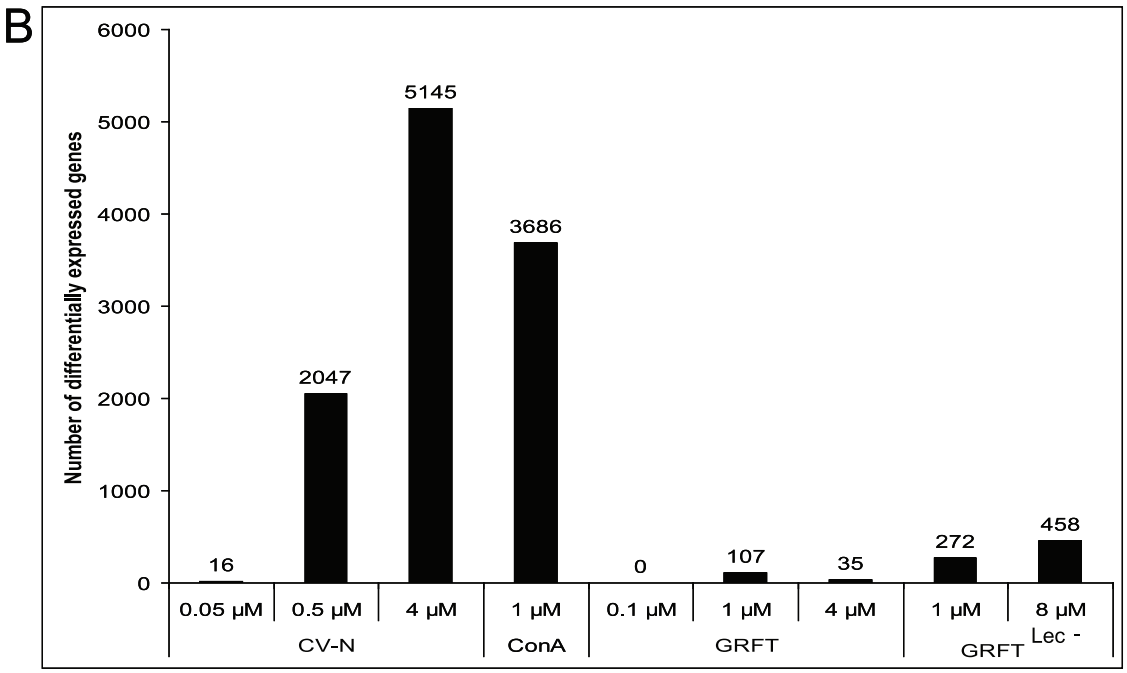

B
Number of differentially expressed genes
6000
5000
4000
3000
2000
1000
0
16
2047
5145
3686
0
107
35
272
458
0.05 μM
0.5 μM
4 μM
1 μM
0.1 μM
1 μM
4 μM
1 μM
8 μM
CV-N
ConA
GRFT
GRFT Lec -

Figure 9. Effect of lectin treatment on gene expression profiles. Cells were treated with CV-N (0.05, 0.5 and 1 μM), ConA (1 μM), GRFT (0.1, 1 and 4 μM), $GRFT^{Lec-}$ (1 and 8 μM) or PBS before RNA isolation, and hybridization with Agilent total human genome microarrays. The heat map (A.) was derived from normalized data using GeneSpring GX 10 software. B. A graphical representation of the total number of genes detected as differentially expressed using Benjamini-Hochberg low stringency correction with a cutoff of 2.0, are reported for CV-N (0.05, 0.5 and 1 μM), ConA (1 μM), GRFT (0.1, 1 and 4 μM), $GRFT^{Lec-}$ (1 and 8 μM) and PBS samples.
doi:10.1371/journal.pone.0022635.g009

carbohydrate binding activity. It is very interesting to note that CV-N binding to the surface of PBMC is not entirely eliminated by blocking its carbohydrate binding sites with mannan (Fig. 1K). This implies that its capacity to bind and induce signaling in cells may not reside entirely in its lectin activities, and supports prior studies with CV-N [21]. Another important result showed that GRFT seems to bind somewhat selectively to terminally differentiated keratinocytes on the epithelial surface, presumably reflecting presence of optimal glycoprotein binding targets on the surface of those cells only in the intact epithelium (Fig. 1C and D; and Fig. S1).

The data presented in Fig. 2 strongly support GRFT's candidacy as a microbicide and antiviral, since they show that GRFT retains antiviral activity even when complexed to the surface of PBMC. This contrasts with many other lectins or compounds we have tested in this assay. The most plausible mechanism that might explain GRFT's retention of HIV-1 entry inhibition activity is that fewer than all six carbohydrate binding sites are occupied when the lectin is docked on cell surface glycoprotein/s, leaving sites available for binding to the viral envelope glycoprotein. The crystal structure of GRFT suggests that the three glycan-binding pockets of each GRFT monomer are on opposite ends of the double prism homodimer [13], suggesting that only one monomer of the homodimer is engaged in binding the cell surface, leaving the "free" monomer competent to bind and cross-link two oligomannose structures on the surface of HIV-1. Since the PBMC were washed extensively 24 hours after treatment and prior to addition of the HIV-1 inoculum, this suggests that cell surface-bound GRFT irreversibly inactivated the inoculum, with no evidence of breakthrough infection at 9 days post infection. This duration of antiviral activity is unprecedented. Conventionally, antiviral potencies of virus-targeted entry inhibitor drugs are measured without washing the cells prior to addition of viruses. In traditional antiviral assays the IC_{50} of GRFT against HIV-1 BaL is 0.2 nM in PBMC, and 0.1 nM against HIV-1 BaL in monocytes/macrophages (data not shown). Remarkably, the IC_{50} for GRFT in the washed PBMC assay (Fig. 2), when the test agent is applied 24 hours prior to cell washing and infection, is 0.78 nM, showing quite exceptional antiviral activity of GRFT. In the same assay we show that activity of CCR5 antagonist maraviroc persists for 24 hrs, although a high concentrations, but GRFT's activity persists substantially longer, a property that may facilitate non-coitally linked administration of microbicides containing GRFT as an active ingredient.

The cardinal rule in development of an anti-HIV-1 microbicide must be "first, do no harm". Randomized controlled preclinical and clinical studies with detergent-based microbicides such as nonoxynol-9 showed a trend towards evidence of harm, with increased incidence of not only HIV-1 infection, but also HSV-2 and HPV seen in the experimental arms [33,34,35,36,37]. The microbicides field therefore requires stringent and extensive *in vitro* and *in vivo* safety studies before human trials initiate. Initial studies showed that GRFT was not cytotoxic; had no mitogenic activity; did not induce secretion of chemokine and cytokine-mediators of inflammation in treated cervical explants; and showed a good safety profile in the rabbit vaginal irritation test [23,24,25].

The first tissues that GRFT would be exposed to in the human vagina are keratinocytes in the outer surface of the vaginal and cervical epithelia. GRFT would also encounter HIV-1 target cells such as dendritic cells and macrophages resident in the epithelium and submucosal stroma as well as the primary HIV-1 target cells, mucosal CD4+ T-cells. In this work we expanded the safety studies to include analyses of off-target effects derived from GRFT binding cell surface oligomannose glycans on human PBMC as well as characterized ectocervical, endocervical and vaginal keratinocyte cell lines End1/E6E7, Ect1/E6E7 and VK2E6E7, which were originally established from normal human endocervical, ectocervical, and vaginal epithelia, respectively, and immortalized by expression of human papillomavirus 16/E6E7 [38]. The immortalized cell lines were shown to have close resemblance to those of their respective tissues of origin and primary cultures in their morphological and immunocytochemical characteristics and therefore proposed for studies dealing with testing pharmacological agents for intravaginal application [38].

First we evaluated the effects of GRFT on cell viability and we showed that up to 84 μM GRFT did not decrease cell viability in the cervical cell lines End1/E6E7and Ect1/E6E7 whereas VK2/E6E7 was somewhat sensitive to GRFT at high concentrations of 8 μM and above. These results are consistent with the pioneering work of Mori and coworkers in which no significant cellular toxicity was found when a variety of human cell types were treated with GRFT at concentrations of up to 0.783 μM [24]. Since many lectins have been reported to be mitogenic [39,40], we examined if GRFT would have an effect on the cervico-vaginal cell proliferation. Using a colorimetric assay for detection of BrdU in newly synthesized DNA, we showed that high concentrations of GRFT did not increase the proliferation of either End1/E6E7, Ect1/E6E7 or VK2/E6E7. We then evaluated the concentrations of a panel of cytokines and chemokines in cervico-vaginal cell culture supernatants using ELISA. We found that high concentrations of GRFT had little to no effect on the secretion of cytokines/chemokines studied including Il-1b, IL-2, IL-6, IL-8, IL-10, IP-10, MIP-1b and TGFA in all the three cervico-vaginal cell lines including End1/E6E7, Ect1/E6E7 and VK2/E6E7. Therefore, our results, taken together suggest that GRFT at these concentrations does not alter the secretion of the immune system mediators by cervico-vaginal cells in a significant fashion.

Using the Ect1/E6E7 cell model, we tested GRFT in order to evaluate its effects on gene expression after over night incubation. To our knowledge, this is the first study evaluating the effect of an anti-HIV lectin on gene expression using human genome microarrays. Our data revealed that exposure to GRFT at 0.1 μM did not affect gene expression whereas treatment of Ect1/E6E7 with 1 μM GRFT resulted in minor alteration of the gene expression profile, showing only 2 genes that were considered as significantly upregulated (cutoff of 2.0). Of note our working concentrations were very high. For instance, 1 μM corresponds to about 1,600 times and more than 23,000 times the EC_{50} found for the most resistant and the most sensitive HIV-1 strain, respectively. At higher concentrations of 4 μM GRFT, 32 genes were considered as differentially expressed (cutoff = 2.0). The fold changes found were less or equal to 3.2. Q-RT-PCR evaluation of

the expression of selected genes validated the microarray data presented here. It is unclear what is the biological relevance and significance of this level of regulation. Using the same cell line (Ect1/E6E7) Sharkey et al. classified a gene as differentially expressed when the fold change was more than 2.0 and found a total of 444 probe sets that fell in this category after treating the cells 12 h with 10% human seminal plasma [41]. This is in sharp contrast to treatment with 4 μM GRFT, a level at least 1,000 fold greater than the average antiviral EC_{50}, which regulated expression of only 32 genes.

In summary, our data provide further evidence that GRFT, an exceptionally potent antiviral lectin, has very minor effects on the molecular physiology of human cells. At this point, the molecular basis for the distinct biological activities of different antiviral lectins is uncharacterized, we propose that the specific spatial arrangement of the carbohydrate binding sites may determine the nature and extent of cross-linking of cell surface glycoproteins. GRFT clearly has superior binding and cross-linking activity with the HIV-1 envelope glycoprotein, which displays dense clusters of oligomannose NLG, but does not induce off-target cellular signaling to the extent that other lectins do. We believe this provides further data in strong support of focused clinical development of HIV-1 microbicides containing GRFT as an active ingredient.

Materials and Methods

Lectin reagents

Recombinant GRFT was produced in *Nicotiana benthamiana* plants. Recombinant CV-N was produced in *Escherichia coli*. Methods for expression and purification of both products have been described previously [23,42]. A synthetic cDNA encoding a lectin activity-deficient mutant of GRFT, termed $GRFT^{Lec-}$, was designed with a conservative amino acid substitution of aspartic acid to asparagine in each of the 3 carbohydrate binding pockets identified in the primary amino acid sequence and crystal structures of GRFT [24,26]. $GRFT^{Lec-}$ was expressed in *N. benthamiana* and purified exactly as described for GRFT [23]. Proteins were purified to >99% purity, and formulated in phosphate buffered saline (PBS), pH 7.4 at 10 mg/ml protein concentration. Endotoxin was removed from GRFT, $GRFT^{Lec-}$ and CV-N protein samples using Detoxi-Gel endotoxin-removing gel gravity flow columns (Thermo Scientific). Endotoxin levels were measured using the ToxinSensor™ chromogenic LAL endotoxin assay kit from GenScript (Piscataway, NJ). Only products with final Endotoxin readings less than 0.05 Endotoxin Units (EU) per milligram were used in the *in vitro* studies, and all dilutions were performed in Endotoxin-free buffers. GRFT, $GRFT^{Lec-}$ and CV-N were fluorescently-labeled with amine-reactive Alexa Fluor 488 carboxylic acid, succinimidyl ester using a kit from Molecular Probes/Invitrogen, according to the manufacturer's specifications. Control lectins Concanavalin A (ConA), phytohemagglutinin A (PHA) and Pokeweed agglutinin (PKM) were purchased from Sigma.

Lectin activity measurements using HIV-1 gp120-binding ELISA

Immobilized HIV-1 gp120 (Protein Sciences Corporation) was used to measure the lectin activity of purified GRFT, CV-N and fluorescently labeled conjugates thereof, and to confirm that $GRFT^{Lec-}$ lacked gp120 binding activity. Nunc Maxisorp ELISA plates were coated overnight with 1 μg/ml gp120 (strain IIIB, Protein Sciences) diluted in PBS. The wells were blocked with 5% (w/v) non-fat dry milk in PBS+0.05% Tween (PBS-T; Immunowash, Bio-Rad) and washed before addition of serial dilutions of lectin analyte (GRFT, $GRFT^{Lec-}$, CV-N or Alexa-Fluor 488-labelled conjugates thereof) diluted in 1× PBS for 1 h. After three washes with PBS-T, a primary polyclonal antiserum (rabbit anti-GRFT or CV-N or Alexa-Fluor 488 (Invitrogen), as appropriate) diluted 1:10,000 in PBS was added for 1 h at room temperature. The wells were again washed before goat anti-rabbit IgG-HRP (Southern Biotech) was added at a 1:10,000 dilution. Colorimetric values reflecting HRP activity were derived using KPL SureBlue TMB Microwell Peroxidase Substrate, with the reaction stopped by addition of 1 N H_2SO_4. The plates were read at 450 nm on a BioTek Synergy HT reader with data collected using Gemini Software. We confirmed that the labeled products retained lectin activities comparable to the unlabeled product by gp120-binding ELISA. We used ELISA with anti-AlexaFluor 488 detection to measure the total amount of label conjugated to GRFT, $GRFT^{Lec-}$ and CV-N, which displayed quantitatively similar labeling efficiency.

Cervico-vaginal cells lines and human PBMC (PBMC)

End1/E6E7, Ect1/E6E7 and VK2/E6E7 are well characterized immortalized cell lines derived from normal human endocervical, ectocervical and vaginal epithelia, respectively [38]. All 3 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cervico-vaginal cell lines were grown as previously described [38] in keratinocyte serum-free medium (KSFM) supplemented with recombinant human epidermal growth factor (0.1 ng/ml), bovine pituitary extract (50 μg/ml), calcium chloride (0.4 mM) and an antibiotic cocktail composed of penicillin and streptomycin at final concentrations of 100 U/ml and 100 μg/ml, respectively. Reagents were obtained from Invitrogen (SanDiego CA, or from Sigma Chemical Company). Cryopreserved human PBMC used in assays of inflammatory cytokines and chemokines were purchased from SeraCare life Sciences Inc. (Milford, MA) and were immediately cultured for the experiments in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and the penicillin-streptomycin antibiotic cocktail (to 100 U/ml and 100 μg/ml final concentrations, respectively).

Microscopical analysis of lectin interaction with human cervical tissues and cultured cervicovaginal cells

Slides with paraffin embedded human cervical tissue sections from a healthy 21 year old female (US Biomax, Inc.) were deparaffinized and rehydrated. Alexa-Fluor 488-labelled proteins of interest were added to the slides and incubated overnight at 4°C in a humidity chamber. The slides were rinsed with PBS twice for 10 minutes and coverslipped using VectaShield Mounting Media with DAPI (Vector Laboratories, Burlingame, CA). For light microscopy, tissue sections were stained with hematoxylin and eosin by standard methods. Ect1/E6E7, End1/E6E7 and VK2/E6E7 cultured cells were seeded onto eight-well Lab-Tek chamber slides (Nalgene Nunc) in duplicate at 10,000 cells per well and allowed to incubate at 37°C with 5% CO_2. After eight hours the fluorescently labeled proteins of interest were added to the wells and incubated overnight at 37°C with 5% CO_2. The slides were washed twice for 10 minutes with PBS and coverslipped using VectaShield Mounting Media with DAPI (Vector Laboratories, Burlingame, CA). Slides were visualized using the Axio Observer Z1 microscope with ApoTome assembly (Carl Zeiss, Thornwood, NY).

Analysis of lectin interaction with PBMC surface molecules by flow cytometry

Human PBMC (SeraCare Life Sciences, MD) were thawed and seeded onto 48-well culture plates (CellTreat, MA) at 2.5×10^5 cells per well. Three dilutions of each Alexa-Fluor 488-labeled protein were

added to the cells for overnight incubation at 37°C with 5% CO_2. Samples were also prepared with 5 and 10 mg of mannan (Sigma, St. Louis MO) at each protein dilution. All samples were analyzed in duplicate. Following incubation, cells were briefly trypsinized (TrypLE Express, Gibco) and placed in 5 mL polystyrene tubes (BD Falcon, MA). Cells were washed twice with PBS and analyzed using the BD FACSAria (BD Biosciences, NJ) flow cytometer.

Antiviral activity assay in PBMC

Freshly isolated PBMC were cultured in the presence of GRFT, CV-N and maraviroc for 24 hrs. Then the cells were collected, washed in culture medium, suspended in RPMI medium with 2 ng/ml IL-2 and seeded in a 48-well flat bottom plate (5×10^5 cells in 450 µl) and 50 µl of the CCR5-tropic clade B HIV-1 BaL stock was added at 100 $TCID_{50}$. The supernatant of each sample was collected after 9 days and viral replication measured by a specific p24 Ag ELISA (Perkin Elmer, Zaventem, Belgium).

Cell viability assays

Viability of cervico-vaginal cell lines was measured using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] assay kit from BIOTIUM Inc. (Hayward, CA) following the manufacturer's instructions. Briefly, 10^4 cells per well were seeded (96 well plates), the test reagents added and the cultures incubated for three days in a humid environment with 5% CO_2 at 37°C. Afterwards, 10 µl of MTT solution were added to each well followed by 4 hours incubation at 37°C. Then the medium was gently removed and the insoluble purple formazan product dissolved in DMSO to yield a colored solution which absorbance was read at 570 nm with a background at 630 nm.

Mitogenicity assays

Proliferation of cervicovaginal cell lines was evaluated by bromodeoxyuridine (BrdU, a thymidine analog) incorporation in newly synthesized DNA using a Cell Proliferation ELISA kit from Roche, according to the manufacturer's instructions. For human PBMC, cells were treated with GRFT for three days and analyzed by flow cytometry for any changes in size and/or morphology using forward scatter (FSC) and side scatter (SSC) with a FACSCalibur (BD, San Jose, CA) counting 10,000 events per sample. Data were acquired and analyzed using CellQuest Pro from BD. ConA (0.37 µM), PHA (10 µg/ml) and PBS were used as controls.

Evaluation of cellular activition markers

Three day old PBMC were analyzed flow-cytometrically after dual fluorescent staining with anti-mouse antibodies purchased from BD Pharmingen (San Diego, CA). Briefly, cell cultures were transferred from plates to a 5 ml round bottom tubes and washed with PBS containing 5% inactivated FBS (washing solution). After 10 min blocking with purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block), cells were incubated in dark with FITC-conjugated anti-CD4 mAb in combination with PE-conjugated anti-CD25, anti-CD69 or anti HLA-DR mAb for 30 min on ice. Finally PBMC were washed and analyzed with a FACSCalibur (BD, San Jose, CA), counting 10 000 events per sample. Data were acquired and analyzed using CellQuest Pro from BD. ConA (0.37 µM) and PHA (10 µg/ml), and PBS were used as positive and negative controls, respectively.

Immunoassays for cytokine detection in cultured cervicovaginal cell supernatants

Multiplex immunoassays were carried out on a Bio-Plex instrument (Biorad) using a Milliplex Human Cytokine/Chemokine 42-plex Luminex bead-based assay (Millipore), following the manufacturer's instructions. Each luminex immunoassay was repeated three times. The kit allows simultaneous quantification of human- epidermal growth factor (EGF), eotaxin, fibroblast growth factor-2 (FGF-2), fms-like tyrosine-kinase 3 ligand (Flt-3 ligand), fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), GRO, interferon-α2(IFN-α2), IFN-γ, interleukin-10 (IL-10), IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-1 receptor antagonist (IL-1Rα), interferon-inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), MCP-3, macrophage-derived chemokine (MDC), macrophage inflammatory protein-1α (MIP-α), MIP-1β, platelet-derived growth factor-AA (PDGF-AA), PDGF-AB/BB, regulated upon activation normal T-cell expressed and secreted (RANTES), soluble CD40 ligand (sCD40L), soluble IL-2Rα (sIL-2Rα), transforming growth factor α (TGF-α), tumor necrosis factor-α (TNF-α), TNF-β and vascular endothelial growth factor (VEGF). Supernatants collected from Ect1/E6E7 after 24, 48 and 72 hours of culture in presence of test compounds were studied in the multiplex-immuno assays and data were analyzed using the Luminex ×PONENT^R software. Individual ELISA experiments were performed on End1/E6E7, Ect1/E6E7 and VK2E6E7 cell culture supernatants collected after 24 hours of incubation in presence of GRFT or controls. ELISA Ready-SET-Go! Kits designed for accurate and precise measurement of human IL-1β, IL-2, IL-6 and IL-10 were purchased from eBioscience, Inc. Quantikine® ELISA kits from R&D Systems, Inc. were used for the detection of IL-8, IP-10 and TGF-α. MIP-1β was quantified using an ELISA kit from Cellsciences. All ELISAs were performed according to the manufacturer's specifications.

Bio-Plex Cytokine Assay in human PBMC supernatants

PBMC from multiple blood donors [19] were cultured for 72 h in presence of 10 µg/ml GRFT (788 nM). In the culture supernatants, the concentrations of IL-1α, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF were determined by the Bio-Plex 200 system (Bio-Rad) and Bio-Plex Human Cytokine 27-plex assay according to the manufacturer's instructions. Data were generated with Bio-Plex Manager 4.1 software.

Gene expression analysis in Ect1/E6E7 cells

Cultured Ect1/E6E7 cells were incubated with dilutions of GRFT, GRFT^{Lec-}, CV-N, ConA, or vehicle only (PBS, pH 7.4) for 16 hours. The cells were lysed using a Qiagen Qiashredder, and total RNA was extracted using a Qiagen RNeasy Mini Kit. Total RNA was quantified spectrophotometrically and the sample quality was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, De). 200 ng RNA were used to generate cyanine 3 (Cy3) cRNA with the aid of Low RNA Input Linear Amplification kit, one-color (Agilent Technologies, Wilmington, De) according to the manufacturer's instructions. 1.65 ug of each labeled cRNA sample were fragmented at 60°C for 30 min using an Agilent Gene Expression Hybridization kit (Agilent Technologies, Wilmington, De) followed by hybridization to a whole human genome Agilent oligonucleotide slide containing four high-definition 44 K microarray (Agilent Technologies, Wilmington, De) at 65°C for 17 hours. After hybridization, the slides were washed with Agilent gene expression wash buffers (Agilent Technologies, Wilmington, De) and scanned using an Agilent G2565BA microarray scanner system (Agilent Technolo-

gies, Wilmington, De). Then, one-color microarray images were extracted with the Feature Extraction software v 9.5.1 (Agilent Technologies, Wilmington, De) and the raw data imported into GeneSpring GX 10 software (Agilent Technologies, Wilmington, De) for normalization and further analysis to yield a list of differentially expressed genes. Benjammini and Hocheberg correction controlling the false discovery rate FDR [43,44,45] was used and we considered only a fold change of at least 2.0 compared to the PBS treated samples to represent a meaningfully altered gene expression [41,46,47]. The raw microarray data were uploaded into the Gene Expression Ominbus (GEO) database, a MIAME-compliant database as detailed on the MGED Society Website http://www.mged.org/Workgroups/MIAME.html. The GEO accession number is GSE28584. Further analysis of the regulated genes was carried out using the Ingenuity knowledge database (Ingenuity Systems Inc., Redwood City, CA) yielding putative toxicological functions, and canonical pathways. Quantitative RT-PCR was carried out in order to validate the microarray results. First strand cDNA was reverse transcribed from 250 ng RNA employing a High Capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's instructions. Optimal amounts of template cDNA were added to a reaction mixture containing 10 µl of 2×TaqMan® Gene Expression Master Mix (Applied Biosystems) and water to 20 µl and this mixture was used to set the PCR reactions in TaqMan® Array Standard 96 well Plates (Applied Biosystems). These plates contain pre-spotted individual TaqMan® Gene Expression Assays for detection of human cysteine-aspartic acid protease (caspase14, CASP14), beta defensin 103A (DEFB103A), immunoglobulin-like and fibronectin type III domain containing 1 (IGFN1), interleukin-1β (IL-1β), IL-2, IL-6, IL-8, IL-10, IL-33, IP-10, *V-Myc* Myelocytomatosis Viral Related Oncogene (MYCN), thyroglobulin (TG), TGFA, tripartite motif-containing 63 (TRIM63) as well as the house keeping genes 18 S and beta actin (ACTB). Individual probes used in these experiments are described in Table S1. PCR amplification was performed in an 7900HT Fast Real-Time PCR System (AppliedBiosystems) as follows: initial activation step (95°C, 20 min), 40 cycles (95°C, 1 min) and 20 min at 60°C. SDS 2.3 or RQ Manager 1.2 software from Applied Biosystems was used to evaluate the cycle threshold (Ct) for each reaction, which is the cycle number at which 50% maximal amplicon synthesis is achieved. Ratios were derived as proposed elsewhere [48].

Statistical analyses

Group means and standard deviations were derived from the values obtained in three individual replicates. Statistical significance was analyzed by a one-way analysis of variance (ANOVA) and student's *t*-test unless otherwise stated, using GraphPad software (San Diego, CA). Differences were considered statistically significant if $p<0.05$. For microarray data, the statistical analysis was carried out as described in the *Gene expression analysis* methods above.

Supporting Information

Figure S1 Analysis of binding specificity of GRFT in comparison with GRFT^{lec-} and CV-N. In the first column we depict the identical fluorescence micrographs to Fig. 1, these show binding of AlexaFluor 488-labeled GRFT (B.), GRFT^{Lec-} (C.) and CV-N (D.) to paraffin-embedded cervical tissue sections from a 21-year old female. In (A.) we show a hematoxilin and eosin-stained light micrograph of cervical epithelial tissue, showing the general micro-anatomy of the cervical epithelium. We have provided additional fluorescence micrographs of tissues stained with labeled GRFT (B2 and B3), GRFT^{Lec-} (C2 and C3) and CV-N (D2 and D3).
(TIF)

Figure S2 Quantitation of the effect of test compounds on the expression of activation markers in CD4 positive PBMC. Percentages of double positive PBMC are reported for cells after 3 days treatment with PBS, 1 µM GRFT, 4 µM GRFT, 10 µg/ml PHA and 0.37 µM ConA and dual staining using FITC-conjugated anti-CD4 mAb in combination with PE-conjugated anti-CD25 (A), anti-CD69 (B) and anti-HLA-DR (C).
(TIF)

Table S1 Genes used in Q-PCR experiments with corresponding TaqMan assay identities.
(DOC)

Table S2 List and fold changes (FC) of mapped genes differentially expressed after treatment with 4 µM GRFT.
(DOC)

Table S3 Relative expression of selected genes after treatment with GRFT (1 and 4 µM) and 1 µM ConA as assessed by Q-PCR and microarrays (µArrays).
(DOCX)

Author Contributions

Conceived and designed the experiments: JCK DH DS AJ SKR NM BRO KEP. Performed the experiments: JCK DH AJ SRK WW JMW KEP. Analyzed the data: JCK DH DS AJ SKR NM BRO KEP. Contributed reagents/materials/analysis tools: KEP. Wrote the paper: KEP JCK.

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Long Acting ARVs

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Drug delivery strategies and systems for HIV/AIDS pre-exposure prophylaxis and treatment

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ABSTRACT

The year 2016 will mark an important milestone — the 35th anniversary of the first reported cases of HIV/AIDS. Antiretroviral Therapy (ART) including Highly Active Antiretroviral Therapy (HAART) drug regimens is widely considered to be one of the greatest achievements in therapeutic drug research having transformed HIV infection into a chronically managed disease. Unfortunately, the lack of widespread preventive measures and the inability to eradicate HIV from infected cells highlight the significant challenges remaining today. Moving forward there are at least three high priority goals for anti-HIV drug delivery (DD) research: (1) to prevent new HIV infections from occurring, (2) to facilitate a functional cure, i.e., when HIV is present but the body controls it without drugs and (3) to eradicate established infection. Pre-exposure Prophylaxis (PrEP) represents a significant step forward in preventing the establishment of chronic HIV infection. However, the ultimate success of PrEP will depend on achieving sustained antiretroviral (ARV) tissue concentrations and will require strict patient adherence to the regimen. While first generation long acting/extended release (LA/ER) DD Systems (DDS) currently in development show considerable promise, significant DD treatment and prevention challenges persist. First, there is a critical need to improve cell specificity through targeting in order to selectively achieve efficacious drug concentrations in HIV reservoir sites to control/eradicate HIV as well as mitigate systemic side effects. In addition, approaches for reducing cellular efflux and metabolism of ARV drugs to prolong effective concentrations in target cells need to be developed. Finally, given the current understanding of HIV pathogenesis, next generation anti-HIV DDS need to address selective DD to the gut mucosa and lymph nodes. The current review focuses on the DDS technologies, critical challenges, opportunities, strategies, and approaches by which novel delivery systems will help iterate towards prevention, functional cure and eventually the eradication of HIV infection.

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1. Introduction

The year 2016 will mark an important milestone — the 35th anniversary of the first reported cases of HIV/AIDS. Antiretroviral Therapy (ART) drug regimens are widely considered to be one of the greatest achievements in therapeutic drug research. In fact, ART has transformed healthcare for HIV-infected people from a terminal illness where patients quickly progress from HIV infection to AIDS and serious opportunistic infections to today, where HIV infection is widely regarded as a chronic disease. Unfortunately, the discontinuation of ART or the development of drug resistance results in rapid viral rebound. The lack of widespread successful preventive measures and the inability to eradicate HIV from infected cells highlight the significant healthcare challenges and DD opportunities that remain today.

The World Health Organization (WHO) estimates that approximately 35.0 million people were living with HIV and 2.1 million people became newly infected in 2013 [1]. Due to the global spread of HIV/AIDS it is considered a pandemic with an estimated 39 million deaths from AIDS-related causes, including 1.5 million in 2013. Further, the impact of HIV/AIDS on society and the challenges of curing it are highlighted by two facts. First, the severity of the HIV/AIDS pandemic is comparable to the plague (*Yersinia pestis* infection, over 75 million deaths) [1]. Second, the only infectious virus ever eradicated in humans is smallpox [2]. Fortunately, unlike past pandemics with high mortality rates, ART enables HIV-infected patients to have a near-normal lifespan and quality of life.

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There is still no cure or vaccine available for HIV/AIDS. While ART regimens are considered to be highly successful, significant limitations to current approaches persist. These include the need for chronic administration, patient non-adherence to therapy, which is often exacerbated by side effects of current medications and the continued threat of drug resistance. Treating HIV during the early stages of infection is likely to be more effective than at later stages due to the vulnerability of the virus to drugs [3]. Early treatment of patients may limit the establishment of viral reservoirs and the emergence of resistant viral mutations, while preserving immune responses for controlling infection. Early treatment could move ART to the next level — a functional cure where the body is able to control the disease without drugs, despite the continued presence of the virus [4,5]. Pre-exposure prophylaxis (PrEP), the treatment of non-infected individuals prior to HIV exposure, has become a high priority strategy/regimen that provides early treatment to prevent post-transmission establishment of HIV infection [6,7]. PrEP was first used successfully in HIV-infected pregnant women undergoing ART thereby protecting the fetus from infection during pregnancy and labor/delivery [8]. Unfortunately, Truvada, the only drug product approved for chronic oral PrEP, suffers from the same limitations as chronic oral ART in terms of posology, adherence, side effects and the risk of resistance if an individual is infected in the intervening time. It is important to note that the goal of HIV eradication has thus far categorically not been met.

New DD systems and strategies are needed to facilitate a functional cure and/or enable HIV eradication. Achieving efficacious drug concentrations in HIV reservoir sites using targeting approaches and reducing cellular efflux and metabolism to prolong effective drug concentrations in target cells remain significant DD challenges. In addition, the urgent need for long acting/extended release (LA/ER) treatments to reduce patient compliance issues and complement those technologies in late stage development has become clear. LA/ER strategies for PrEP have numerous desirable attributes that include infrequent dosing and long dosing intervals making administration convenient for patients; the possibility of directly observed therapy and better long-term adherence; use in difficult to treat populations such as adolescents or those with ongoing substance abuse; use in patients reporting pill fatigue; and protection of patient privacy by eliminating the risk of disclosing pill taking to family and co-workers. The current review focuses on DD technologies, critical challenges, opportunities, strategies, and approaches that will help iterate towards infection prevention, functional cure and eventually the eradication of HIV infection.

1.1. Importance of understanding HIV pathogenesis to enable effective anti-HIV drug delivery

Unlike traditional pathogens that coevolved with mankind for millions of years, HIV is an exotic pathogen that crossed species from African primates to humans in at least five independent events (hence HIV-1 groups M, N, O and P and HIV-2) starting approximately a century ago [9]. Consequently, humans have not had sufficient time to develop an adequate immune response. While broadly neutralizing antibodies eventually develop in 10 to 30% of patients, the overwhelming majority of these antibodies (~99%) are not potent enough to stop the progression of disease. In addition, the induction of these antibodies through vaccination has been unsuccessful [10].

HIV is a member of the lentivirus genus in the retroviridae family. The high mutation rate allows HIV to evade destruction by host immune responses. The provirus form of HIV can hide in the genome of latently-infected CD4+ memory T cells for years without revealing any sign of infection to host immune surveillance. It also does not provide an obvious target for eradicative treatment. Natural eradication of any pathogen relies on host immunity. However, HIV is unique among retroviral pathogens because its main target is CD4+ helper T cells, which serve as key coordinators within the immune system. As the CD4+ T cell pool is progressively depleted, host immune function becomes weaker, making eradication increasingly difficult. Compounding the reduction in CD4+ T-cells is the establishment of cellular HIV reservoirs, defined in the context of eradication as a cell type or anatomical site that allows persistence of replication-competent HIV-1 on a timescale of years in patients on optimal ART [11–13]. Anatomically, HIV reservoirs are mainly located in the mucosa of the alimentary, respiratory and genital tracts, the brain/central nervous system (CNS), and some lymph nodes (LNs) [12,14]. Cellular reservoirs include latently infected CD4+ memory T cells but may also include other infected cells such as hematopoietic stem cells and macrophages [11]. Some reservoir sites are also considered viral sanctuary sites where drug concentrations are suboptimal due to biological barriers (e.g., blood–brain barrier) that protect the anatomical sites (e.g., brain/CNS). This allows HIV to replicate unhindered [14].

HIV establishes persistent infection by two mechanisms: reservoir formation and ongoing replication. The gut mucosa plays a pivotal role in HIV pathogenesis. The gut mucosa harbors approximately 60% of the body's immune cells, including the majority of CD4+ T cells. Many of the CD4+ T cells are activated due to the bacteria present in the gut lumen, enabling viral replication. Furthermore, these CD4+ T cells express the HIV co-receptor CCR5, which is needed for HIV entry [15,16]. Accordingly, at all stages of HIV infection, viral replication and CD4+ T cell depletion predominantly occurs in the gut mucosa, even under apparently successful ART [17,18]. Following CD4+ T cell depletion, bacterial products breach the gut epithelial barrier. This leads to further local and systemic CD4+ T cell activation, and the acceleration of viral replication and immune cell depletion [19]. Very recently, whole-body immunoPET scans of simian immunodeficiency virus (SIV)-infected macaques confirmed that persistent viral replication occurs in strategic mucosal sites, such as the gut, irrespective of ART [20]. Thus, ART cannot suppress low level, persistent viral replication in mucosal sites, making them high priority targets for DD.

Targeting HIV mucosal transmission earlier is likely to be more effective than treating at later stages of infection [3]. CD4+ T-cells are scattered throughout the colorectal and vaginal submucosa in small numbers. Innate immunity suppresses initial infection, resulting in a small founder population of infected cells that is highly vulnerable to ART [21]. During the first few days after transmission, there is a dynamic balance between the shrinkage of the infected founder cells resulting from viral and host innate immunity killing and expansion of these cells by spreading of infection to uninfected cells. The basic reproductive rate of the founder cells is the ratio of expansion to shrinkage and when the ratio falls below 1 the founder population of the infected cells shrinks, resulting in aborted infection. Once locally expanded HIV spreads to the draining LNs, systemic infection is established. The viral vulnerability creates prevention opportunities since it would not be necessary to completely inactivate all HIV or kill all infected cells. Rather, reducing the founder population to below the critical threshold value may suffice. This window of opportunity lasts about one week from the time of vaginal exposure to the establishment of HIV infection whereas after colorectal exposure, it may be significantly shorter (Fig. 1) [3]. Although this estimate is based on data from non-human primates and vaginal SIV challenge, the mechanism provides a logical basis and rationale for the concept of a window for establishing HIV infection in humans, post exposure to HIV, and an opportunity for PrEP DDS to deliver ART and prevent the establishment of HIV infection.

In a critical proof-of-concept study, Li et al. [22] demonstrated that inhibition of the recruitment of CD4+ T cells alone was sufficient to prevent vaginal transmission in a SIV-macaque model. They found that mucosal inflammatory signaling in conjunction with the innate immune responses to infection greatly fueled CD4+ T cell recruitment. The signaling involves macrophage inflammatory protein-3α cytokine, plasmacytoid dendritic cells (pDC) and pDC-produced CCR51 chemokine. The surfactant glycerol monolaurate is an antimicrobial compound that has no effect on the SIV life cycle per se but is known to inhibit immune activation and chemokine/cytokine production *in vitro*. Topical

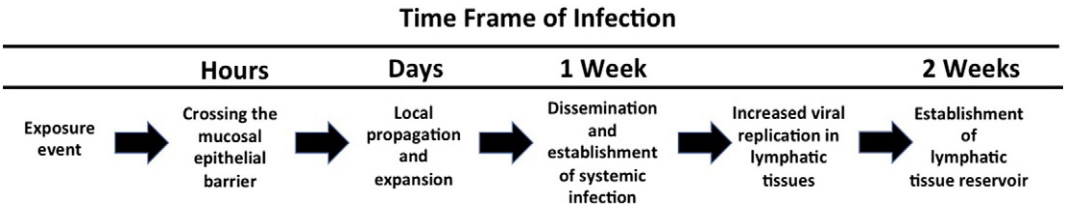

Fig. 1. Approximate timeline for establishing HIV infection after vaginal exposure to HIV [3]. This timeline suggests a window of opportunity for PrEP therapy. Given the difficult logistics of diagnosing and initiating treatment immediately post transmission, PrEP delivers effective drug concentrations for at risk populations prior to exposure.
Adapted from [3].

application of glycerol monolaurate to the vaginal mucosa protected rhesus macaques from acute infection despite repeated intra-vaginal exposure to high doses of SIV. The data thus suggest that limiting local expansion to below a critical threshold alone can be effective in preventing viral infection. The concept of early treatment for prevention has also been supported in other studies using non-human primate models. For example, agents that block viral binding, co-receptor-mediated entry and reverse transcription have been shown to protect against SHIV and SIV vaginal and rectal challenges in the rhesus macaque model [3]. The implications for DD are clear: (1) constant, therapeutically effective drug concentrations need to be maintained in the vaginal or colorectal mucosa of high risk individuals prior to viral exposure, (2) methods to inactivate HIV prior to mucosal entry may have value, and (3) approaches to control the number and rate of responding immune cells to the colorectal and vaginal mucosa after HIV exposure are warranted.

1.2. Drugs for prevention and treatment of HIV infection

There are currently 37 Food and Drug Administration (FDA)-approved ARV drug products used clinically in the US for the treatment of HIV/AIDS [23]. Although a myriad of highly potent drugs have been approved and widely used, the HIV drug pipeline still shows robust growth in volume as well as in molecular target diversity [24]. Existing ARVs can be classified by the site of action (i.e., extracellularly in the vaginal/colorectal lumen or mucosa or intracellularly in HIV-susceptible immune cells). Extracellular acting ARVs, which interfere directly with the entry process, target either the HIV co-receptor (CCR5) [25,26] on host cells or gp41, the viral transmembrane glycoprotein on HIV. The vast majority of ARVs act inside host cells by preventing viral proliferation. Four classes of intracellular acting ARVs have been introduced including Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI), Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI), Protease Inhibitors (PI), and HIV Integrase Strand Transfer Inhibitors (InSTI). The vast majority of ARVs have solubility, stability and/or permeability limitations that ultimately lead to a high degree of pharmacokinetic (PK) variability in humans [27–31]. National Institute of Allergy and Infectious Diseases (NIAID) — supported researchers established ART regimens, a combination of at least two drugs, in order to reduce the incidence and rate of viral resistance. These treatment regimens are multi-class drug combination products. Truvada, an orally administered fixed combination drug product containing tenofovir disoproxil fumarate (TDF) and emtricitabine, represents the first potent combination drug product approved for preventing HIV infection.

Intensive ARV research has resulted in drugs with high potency and the discovery of new mechanisms of action (Table 1). Besides introducing newer generations of NRT, NNRT InSTI and protease inhibitors, novel mechanisms of action are being explored, e.g. therapeutic reclamation of apoptotic proficiency-based (TRAP) are moving to the clinical trials stage. As of today, 59 HIV PrEP clinical trials are listed in the AVAC database (avac.org). However, most trials involve Truvada. Even though new potent ARVs are needed, it appears that a paradigm shift from drug discovery to drug delivery is occurring in the field of HIV therapy.

1.3. Drug delivery systems for HIV prevention

Currently, there are few options for preventing HIV infection. PrEP DDS in development are administered either locally through rectal or vaginal administration, or systemically by means of oral or parenteral administration, providing therapeutically relevant drug concentrations in vaginal and colorectal tissues [7,40–45]. By any route of administration, the key requirements are the delivery and maintenance of effective ARV concentrations at vulnerable mucosal sites during periods of HIV exposure since HIV transmission through the colorectal and vaginal mucosae is responsible for the vast majority of new HIV infections [46,47].

In 1995, Tsai et al. [48] published a seminal paper demonstrating that subcutaneous administration of TFV could prevent SIV transmission in macaques. Following this report, the use of vaginally- and orally-administered TFV and TDF, the orally bioavailable prodrug of TFV [49], to prevent HIV infection in high-risk populations became the focus of many investigations [42]. To date, numerous clinical trials have been conducted investigating TDF and other ARVs as potential oral PrEP agents. Of these studies, one treatment regimen was successful, leading to the only FDA-approved and licensed treatment for HIV prevention. This product, Truvada, consists of two drugs, TDF and emtricitabine (FTC) in a once a-day oral dose form [42].

One early investigation involving Truvada was the Partner's PrEP study where it was found to reduce HIV acquisition with a 75% rate of effectiveness. However, in the FEM-PrEP and VOICE (Vaginal and Oral Interventions to Control the Epidemic) clinical trials that followed, it was not found to be effective. A lack of patient compliance was determined to be one of the primary factors in the failure of these studies.

Table 1
Recent drugs in use or development (MOA: mechanism of action; TRAP: therapeutic reclamation of apoptotic proficiency).

Name	MOA	Status	Source (reference, trial number)
Tenofovir disoproxil fumarate (TDF)	RT inhibition	Approved 2012, Truvada	[32]
Emtricitabine, FTC	RT inhibition		
Tenofovir alafenamide (TAF, GS-7340)	RT inhibition	Investigational prodrug of TFV	[33]
Rilpivirine (TMC-278)	NNRT inhibition	Recruiting	[34]
Cabotegravir (GSK 744) (S/GSK1265744)	InST inhibition	Not yet recruiting	[35,36]
Maraviroc	CCR5 antagonism	Ongoing trial	NCT01505114 [37]
Ciclopirox	TRAP	Recruiting	[38,39]
Deferiprone			

Similar results were seen in other clinical trial including the iPrex study in which Truvada reduced HIV transmission by only 42% overall [42,50]. It was determined that only 18% of patients were taking the drug at the prescribed daily regimen [42]. When the analysis was narrowed to only patients with high adherence to the regimen (i.e., where patients steady-state plasma TFV concentrations >40 ng/mL), the therapies were found to be 88% and 91% effective for the groups that received TDF and TDF/FTC, respectively [51]. Therefore, while a number of trials have reported that systemic oral PrEP is only 39% to 75% effective, subsequent correction of the data for medication regimen adherence, as indicated by measureable drug blood levels, showed that effectiveness was greater than 90% in patients that took their medication consistently [40,41,43,52,53]. This and other pharmacokinetic (PK) studies show a clear relationship between PrEP efficacy and patient adherence as well [42,52]. For rectal and vaginal PrEP formulations there is also a relationship between the size, texture and color of a formulation, perceptibility (user sensory perceptions including leakiness/messiness) and patient adherence [54,55].

This body of work highlights the two major challenges influencing patient adherence to PrEP regimens: the chronic nature of administration/frequency of use (oral, rectal and vaginal) and perceptibility (rectal and vaginal). Chronic oral PrEP also raises concerns of toxicity and the potential for ARV resistance due to widespread systemic exposure. Finally, additional studies have also revealed suboptimal drug concentrations in the target mucosal tissues even though relatively high drug concentrations are found in the blood [56]. Alternatively, microbicides are applied topically to the vagina or rectum and act locally to prevent HIV transmission. Therefore, the development of DD strategies and DDS for colorectal and vaginal mucosal PrEP that maximize local tissue drug concentrations and minimize systemic exposure are a high priority.

Microbicides have been extensively investigated as potential preventative options in numerous DDS/dose forms such as gels, creams, films, foams, quick-dissolving tablets and intravaginal rings (IVR) [41, 42,44,57–59]. Highlights of significant microbicide/local PrEP DDS are described below.

1.3.1. Vaginal gels

Microbicides were first explored as a means of offering broad protection against most or all sexually transmitted infections in the early 1990s. The first generation of microbicides, were membrane solubilizing surfactants including the nonionic spermicide nonoxynol-9 (N-9), an OTC vaginal contraceptive known to destroy the membrane of cells [7,40,44]. Unfortunately, N-9 failed at the clinical trial stage where it was found to increase HIV transmission due to the development of inflammatory genital tract lesions in the epithelial layer of the vagina. Additionally, the product did not adequately cover the vaginal mucosa evenly, pointing to major formulation challenges for the future for vaginal PrEP products [41,43,52]. Since the publication of these reports, N-9 and similar surfactants have been excluded, with few exceptions, from the developmental pipeline [41].

Second generation microbicides incorporated polymers into the application design. Early formulations of this period were aimed at reducing the toxicity of nonoxynol-9. Polymers were used to minimize mucosal irritation and prevent environmental changes in the vagina that may increase risk of infection. However, sufficient efficacy was not achieved. Thus with the widely accepted dismissal of surfactants, efforts focused on polyanionic compounds. This group of polymers was specifically chosen because of their ability to act as entry inhibitors to prevent HIV from entering target immune cells in the vagina [7]. Several polyanionic polymer-based microbicides advanced to clinical trials including Carraguard, Cellulose Sulfate and PRO2000. Unfortunately, all were proven ineffective [60].

Third generation topical PrEP focused on microbicides using intracellularly acting ARVs to prevent HIV infection [42]. Karim et al. [61] presented the results of a study (CAPRISA 004), which was carried out to assess the safety and effectiveness of a 1% TFV gel for HIV prevention. They demonstrated that the gel, if applied both before and after sex, reduced HIV incidence by 39% overall and by greater than 54% for those who used the gel consistently [57]. TFV was the first ARV found to be effective as a microbicide. However, although it was found to be safe and effective in preventing HIV infection, patient adherence was once again an obvious concern with these treatments [61].

Haaland et al. [62] reported another clinical trial studying an ARV microbicide candidate, UC781. UC781 is a NNRTI with poor oral bioavailability. Although it was unsuccessful as a potential oral agent, it was believed to be a promising choice as a topically applied microbicide. A carbopol gel formulation of 0.1% UC781 was found to be effective *in vitro* and *in vivo* in a macaque model. When evaluated in a phase III clinical trial, the study was quickly terminated due to the insolubility and instability of UC781; similar limitations were reported after oral administration. This study showed the importance of evaluating microbicide effectiveness after exposure to the human female genital tract and to semen. It is possible that semen may inhibit the antiviral activity of UC781 *in vivo* albeit this interaction with semen plasma was investigated *in vitro* during preclinical studies and found to have no significant effect.

1.3.2. Intravaginal rings (IVR)

In order to overcome patient adherence issues, IVRs have been proposed to deliver LA/ER ART [42,59]. IVRs are typically formed from elastically deformable polymers such as thermostat silicones, poly(ethylene-co-vinyl acetate), or polyurethanes. The drug is usually mixed or dissolved in the polymer matrix during the formulation process and incorporated into the injection molding or hot-melt extrusion. Once the DDS is exposed to the vaginal lumen, a concentration gradient is initiated, allowing for the surface drug to diffuse into the contacting tissue. The rate of drug release depends on numerous factors such as drug solubility, partition coefficient, and diffusion coefficient of drug in IVR polymer and of drug in vaginal fluid.

Nel et al. [63] reported a clinical trial investigating the safety and PK of an IVR that delivered the NNRTI dapivirine. Twenty-four women were treated with dapivirine (25 mg) silicone elastomer matrix IVR, dapivirine (25 mg) silicone elastomer reservoir IVR, or a silicone elastomer placebo IVR. IVRs were used for 28 consecutive days and plasma and vaginal fluid samples were collected on day 1 and day 28 of the trial. The matrix IVR and reservoir IVR were able to achieve significant drug levels in vaginal fluid with maximum drug levels of 6 mM and 42 μM respectively. This data is encouraging because the reported vaginal fluid and mucosal tissue drug concentrations surrounding the IVR location were more than 1000-times the 50% effective concentration (EC_{50}) against the wild-type HIV-1. This suggests that the IVR investigated may be able to achieve sufficient drug concentrations within vaginal mucosal tissue to prevent HIV infection [59,63].

Several other studies have been conducted investigating IVRs incorporating dapirivine [64–66]. Nel et al. presented results of a clinical trial for the Dapivirine Vaginal Ring-004 (25 mg dapivirine) at the 22nd Conference on Retroviruses and opportunistic infections. Patients wore the IVR for periods between 4 to 12 weeks at a time. For patients that wore the IVR for 12 weeks consistently, mean vaginal fluid concentrations were found to be more than 4000-times the *in vitro* IC_{99} in cervical tissues at the end of the study. This ring is currently being evaluated for safety and efficacy in phase III clinical trials (International Partnership for Microbicides website: last accessed May 30, 2015).

Recently, a 90-day TFV reservoir ring inspired by the success of the 1% TFV gel was investigated [67]. They hypothesized that the IVR would be able to provide a more controlled and sustained vaginal drug concentrations in the cervicovaginal area. Polyurethane tubings of various hydrophobicities were filled with high densities semisolid paste of TFV, glycerol and water. *In vitro*, a more rapid release of TFV was found with increasing polyurethane hydrophilicity allowing for systematic control of drug release. IVRs were evaluated in two 90-day

in vivo sheep studies where pharmacokinetics and safety of TFV was evaluated. The polyurethane IVR was found to be capable of delivering 10 to 30 mg of TFV daily for up to 90 days consistently. Previous matrix IVRs typically had high fractions of undissolved drug that was never released from the ring. They also commonly showed a decrease in drug release rates with time as well as a decrease in material stiffness as a result of drug release. This study is significant because it highlights a tunable membrane that can be controlled to achieve and maintain a desired TFV loading, release rate and ring stiffness by altering material properties. The polyurethane TFV IVR also showed no significant toxicological effects. However, in comparison to the control, there was slight to moderate increase in inflammatory infiltration within vaginal epithelium. Although clinical studies are needed to confirm the safety and potential efficacy of this therapy, it shows promising results for the future of IVR for HIV PrEP [67].

Patient adherence to PrEP regimens remain a concern and developing better ways of determining adherence are needed. Boyd et al. [68] describes the development of a technology to address the pivotal issue of inaccurate self-reporting of patient adherence during clinical trials. They report a temperature-monitoring microbicide-releasing vaginal ring that uses DST nano-T temperature loggers to monitor user adherence. While still in the pre-clinical stages, the IVR was tested in macaques where it showed high sensitivity to fluctuations in vaginal temperatures and the ability to detect periods of IVR removal with accuracy. This introduces the concept of multipurpose IVR to treat patients as well as monitor their use and significant events that may affect efficacy such as sexual arousal, vaginal intercourse, and menstrual cycle. Such technologies would markedly enhance the accuracy of product evaluation during clinical testing.

1.3.3. Vaginal films

Another type of mucosal PrEP DDS are vaginal films [66,67,69,70]. Polymeric vaginal films are a solid dosage form that offers several additional advantages over other microbicide dose forms such as gels and creams. Some of these advantages are (1) accuracy of dose administration in the absence of an applicator; (2) rapid drug release upon exposure to vaginal fluid; (3) discreet use and minimal product volume; (4) improved drug stability; (5) convenient storage; and (6) minimal leakage. In addition, when compared to soft-gel capsules and tablets (not discussed in this review), an acceptability study reported that greater than 80% of participants surveyed, found the films to be acceptable forms of treatment. 77% to 91% of women in the study also stated that they would definitely use any of these products if they were confirmed to be effective at preventing HIV transmission [6,69,70].

Until recently, most published studies proved the feasibility of formulating polymeric vaginal films containing a single anti-HIV drug candidate. For example, a vaginal film reported by Akil et al. [71] comprised of only dapivirine was shown to be effective in blocking HIV-1 both *in vitro* using P4/R5 and MT-2 cells as models of HIV infection. The film was also shown to be effective *ex vivo* in polarized and cervical explant models. Recently, research has given increased focus on developing films capable of co-delivering multiple drugs [70,72]. Another paper by Akil et al. [70] describes the utilization of vaginal films to deliver different combinations of dapivirine, maraviroc, and TFV. Films were manufactured by a solvent casting method and solid phase solubility was used to select appropriate polymers to be used in the formulation. Cellulose polymers and polyvinyl alcohol were used in the development of three combinational film products. These vaginal films were determined to be stable for up to 12 months at ambient temperatures with rapid drug release reported (>50% of each drug) within the first 30 min *in vitro*. The results from this study show the potential of vaginal films to incorporate multiple antiretroviral agents, which is of great importance considering the prevalence in use of ART for both prevention and eradication. The vaginal films were also well tolerated by both the women and their partners, which makes them promising candidates for microbicide therapies.

A comprehensive summary of clinical trials for topical vaginal microbicides can be found in a recent paper by Antimisiaris et al. [73]. To date, there have not been any products approved for use in humans. This demonstrates a great need for new technologies and innovative ideas moving forward. Recently, there has been an increased interest in LA/ER ARV drug combinations for microbicides to enhance efficacy and user adherence. Nanotechnology-based drug delivery systems are a promising option to advance the field of microbicides by their ability to (1) facilitate drug/virus interactions, (2) increase synergy for drug cocktails (3) penetrate mucosa tissue, (4) provide sustained and triggered drug release, (5) target HIV susceptible cells, (6) improve drug solubility and permeability, and (7) serve as a drug barrier along the epithelial cell lining [74–76]. Recent review articles published earlier this year discuss the benefits and potential drawbacks of exploring nanotechnology-based solutions to improve the efficacy of vaginal microbicides [73,77].

1.3.4. Colorectal microbicides

Until recently, most efforts at microbicide development and initiated clinical trials focused on vaginal microbicides despite the major role colorectal transmission plays in the establishment of new infections. Men and women who practice unprotected receptive anal intercourse (RAI) are at a much higher risk of contracting HIV from an infected partner than those who engage in unprotected vaginal sex. Unprotected RAI is estimated to result in 10–100 times more incidences per exposure than unprotected vaginal intercourse [78]. A major cause of this is the structure of the colorectal mucosa (shown in Fig. 2) [79]. The colorectal epithelial barrier is a thin single-cell columnar layer above the lamina propria that is much easier to penetrate than the multi-layer barrier found within the vagina. Once HIV traverses the epithelial layer and

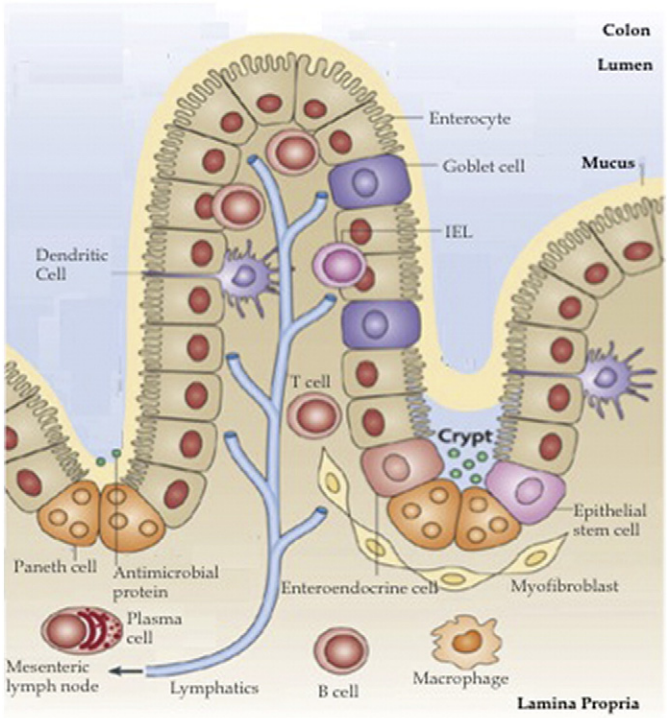

Fig. 2. Colorectal Mucosal Barriers and HIV Transmission Pathway. A single epithelial layer provides a barrier between the intestinal lumen and the lamina propria. The cells most susceptible to/or responsible for propagating early HIV infection, $CD4^+$ T-cells, macrophages, dendritic cells, Langerhans cells and M-cells are located in the Lamina Propria. The lymphatics play an important role in the dissemination of HIV into the systemic circulation.
Image adapted from [79] with permission.

enters the lamina propria, it has direct access to an extensive population of activated resident CD4+ T cells, macrophages, dendritic cells, and other lymphocytes that are highly susceptible to HIV. There are also individual LNs that serve as a primary route for HIV to enter the systemic circulation [56]. Due to distinct anatomical differences between the vagina and colorectum, microbicide technologies developed specifically for one route of administration cannot be incautiously translated to an application for the other.

Since colorectal microbicides were recently reviewed in this journal, a summary of the formulation factors, DDS issues and challenges will be presented and the reader is referred elsewhere for a more general discussion of broader issues [56]. Colorectal microbicide dose forms commonly include gels, enemas and suppositories. The use of these traditional dose forms present numerous delivery challenges to PrEP treatment and this is an area that would greatly benefit from the development of new DDS technologies and techniques. The challenges can be generally categorized as follows: (1) administration methods as it relates to mucosal coverage, (2) patient acceptability and adherence as it relates to volume administered and retention, (3) coital dependence and persistence of therapeutic effect, and (4) chronic safety/toxicity of the DDS/formulation.

1.3.4.1. Administration methods as it relates to mucosal coverage

Complete coverage of the colorectal musoca is important for providing protection from HIV transmission. It is suggested that a colorectal microbicide should cover the entire rectum, sigmoid colon and descending colon to up to the splenic flexure to provide sufficient protection. Careful consideration must be taken to choose the ideal delivery vehicle and applicator to ensure the spread of therapeutic agent to all areas that may become exposed to the virus [80–82].

1.3.4.2. Patient acceptability and adherence as it relates to volume administered and retention

While there is no standard volume of product that is used for colorectal treatments, the volume should be sufficient enough to cover the entire area that may become exposed to HIV during intercourse. One study [83] reported that participants tolerated up to 35 mL of a rectal microbicide gel, and once this volume was exceeded, there is increased anal leakage and discomfort. The smallest effective volume for treatment is preferred [56,83]. As has been proven many times, patient acceptability will have a major influence on efficacy [54,56].

1.3.4.3. Coital dependence and persistence of therapeutic effect

Coital dependence of an application (requiring dosing close to the time of sexual intercourse) may be a disadvantage when it relates to adherence. It requires that the users anticipate when they will have sex and be able to privately apply the treatment in advance. If the therapy is only active for a few hours, this may place a limitation on the timespan in which a person has to use the application. Ideally, a LA/ER PrEP approach with sustained drug activity would allow for more flexibility in application and increase adherence and efficacy [44].

1.3.4.4. Chronic safety/toxicity of the DDS/formulation

Microbicide formulations need to preserve the activity and stability of drugs while also ensuring that the excipient concentrations used pose no significant toxicity. Product acceptability is strongly dependent on the safety and comfort so it is important that products are nontoxic especially for chronic treatment.

In parallel to these four primary challenges, the physicochemical properties of the delivery vehicle play a key role in addressing these overcoming these challenges and increasing overall therapeutic efficacy. The ideal delivery vehicle will differ depending on the route of administration and vehicle type. In order to achieve sufficient levels of coverage, retention, safety, drug release and therapeutic efficacy, many properties of the formulation have to be optimized to compliment the biological environment that is being treated. Important properties to consider include pH, viscosity, osmolality, mucoadhesivity, yield stress and shear rate [56,84,85].

Although it has not always been a main focus of PrEP research, interest in colorectal PrEP has existed since the development of the first generation of microbicides. Nonionic surfactant N-9 was one of the first microbicides tested for colorectal application. Similarly to the results for vaginal administration, N-9 was found to cause significant epithelial sloughing and a potential increase in the risk of infection [56,86]. Since then, five ARV phase 1 clinical trials have been completed for rectal microbicides. Most of these trials have also focused on TFV gel formulations and one study investigated the UC781 gel that was also explored as a vaginal application [42]. Anton et al. [86] described the first phase 1 double-blind, placebo-controlled randomized clinical trial for UC781 gel as a rectal microbicide. Men and women were treated rectally with two concentrations of UC781 (0.1% and 0.25%) in gels formulated with Carbomer 974P, methylcellulose, glycerin, purified water and paraben preservatives. This class of gels is similar to thickened solutions with a limited ability to spread evenly or be retained on the mucosa. Both dosages were found to be safe with no epithelial sloughing from rectal lavage and no changes in histology.

Another gel investigated as a rectal microbicide was the vaginal formula of the 1% TFV gel used in the CAPRISA 004. This gel was evaluated in a phase 1 rectal study [RMP-02/MTN-006] where it was shown to induce mild to moderate gastrointestinal side effects such as pain, bloating and diarrhea. These symptoms were believed to be caused by the osmolality of the vaginal formulation (3111 mOsmol/kg) [87,88]. Following this study, a second gel was developed to address the osmolality of the 1% TFV gel. The gel formulation included a reduced amount of glycerin compared to the 1% TFV vaginal formulation and was studied in the MTN-007 phase 1 rectal microbicide trial. With a lower osmolality (836 mOsmol/kg), it was determined to be better tolerated by the participants of the trial [58,88]. Recently, McGowan et al. [88] reported a randomized phase 1 clinical trial comparing the safety, acceptability, PK, pharmacodynamics of the 1% TFV gel, the reduced glycerin gel, and a third gel never tested before in a phase 1 rectal study (Charm-01). The third gel was a rectal-specific formulation made with an even lower concentration of glycerin than the already reduced glycerin formulation. An additional carbopol was also added to create a nearly iso-osmolar product (479 mOsmol/kg). After a single dose of each treatment, all three gels were found to be safe and acceptable to participants. *Ex vivo* colorectal studies were also performed and all formulations were found to result in a significant suppression of HIV-1 viral replication. However, due to low participation and limited product availability, Charm-01 was not fully enrolled and significant conclusions regarding which product should advance to later stage trials as a rectal microbicide could not be made. Currently the reduced glycerin gel is being evaluated in an International Phase 2 expanded safety study expected to be completed by early 2016.

Nano-sized DDS are showing promise for colorectal microbicide development. The majority of information published involves Vivagel® (Starpharma Pty Ltd). Vivagel is a carbomer-based gel loaded with SPL7013, a dendrimer reported to have inhibitory activity against both X4 and R5 strains of HIV-1. A 5% SPL703 gel reduced HIV-1 infection by greater than 85% in colorectal explants *ex vivo* but it was also shown to cause epithelial shedding. The toxicity of the treatment was attributed to the vehicle and not the dendrimer [56,89]. Das Neves et al. reports the use of poly(ethylene oxide)-modified poly(ε-caprolactone) (PEO-PCL)-based nanoparticles (NPs) as a potential nanocarrier (NC) for the NNRTI dapivirine. 200 nm PEO-PCL NPs loaded with dapivirine were shown to increase drug uptake into Caco-2 intestinal cells *in vitro* [56,90,91]. The development of colorectal microbicides is in the very early stages. An editorial published by Sarmento and das Neves called for more research in this area and describes the growing field in more detail [92].

There are numerous rectally administered products and excipients that have been collectively approved for human use for literally

hundreds of years. However, given the unique application to rectal microbicides, the use of traditional rectal products is not very promising. A technology covering the concept of a colorectal dose form/DDS that could spread uniformly along the descending colon from the splenic flexure to the rectum and be retained for a reasonable period of time is virtually nonexistent. Even if such a technology existed, the coital dependence of efficacy due to physical abrasion of the colorectal tissue and the persistence of effect would be questionable. The obvious modifications to traditional rectal dose forms/administration such as large volume enemas to deliver adequate volumes to ensure initial complete colorectal mucosal coverage would not be well received by patients. This will certainly result in unacceptable leakage volume and extremely poor patient use/adherence no less a transient effect due to poor mucosal retention. Hence, the dependence on old, approved rectal formulations that were not designed for the purpose of rectal microbicides is a critical issue facing the DD field.

1.4. Drug delivery systems for HIV treatment

Although a variety of DDSs have been explored for treating HIV/AIDS, they have been, for the most part extensively covered in previous reviews [76,93–97]. In the following sections, DDSs that focus on resolving the remaining DD challenges are highlighted.

1.4.1. Gut mucosa and lymph nodes: targeting the critical sites of HIV infection

The current understanding of HIV pathogenesis informs future DD strategies for eradicating HIV infection from the gut and LNs. Viral replication and CD4 + T cell depletion predominantly occurs in the gut mucosa at all stages of HIV infection even under apparently successful ART making DD to the gut mucosa a high priority [17,18]. In a 2012 study [98], 30 acute HIV infected patients (viremia^{+}/HIV IgG^{-}) were identified/recruited from screening 24,430 high-risk persons and treated with mega-HAART (five drugs at the entry, RT and integration steps) for 24 weeks. The mega-HAART regimen resulted in a viral reservoir reduction and partial CD4 + T cell count recovery in the gut mucosa. However, even in these patients, the viral reservoir persisted emphasizing the need for a gut-targeted DDS to achieve a functional cure for early diagnosed patients.

The mucosal mucus layer is the first barrier that a DDS will encounter. Mucus properties, function, barrier to NP delivery and mucoadhesive and mucopenetrating polymeric NPs have been extensively reviewed [99]. The mucus layer covering the alimentary and reproductive tracts consists of interwoven fibers and water-filled spaces in between. NPs must penetrate through this layer to reach the mucosa. The Saltzman group has found that certain polymers (e.g., PEG and PVP) are capable of altering the mucus structure to facilitate polymeric NP, or even monocyte, penetration of the mucus layer [100]. While the Hanes group reported that short (~2 kDa) PEG polymers displayed on NPs are able to the penetrate mucus layer, apparently mimicking the surface proper of viruses that infect human epithelia (reviewed in [99]), the Sinko group demonstrated size dependence of particle translocation through mucus [101,102]. Mucoadhesive NPs can be used in HIV topical PrEP while mucopenetrating NPs are more suitable for oral DD. In the latter case, however, mucopenetrating capability alone is not sufficient; the NP must also be able to penetrate the gut epithelial barrier. In this regard, an oral NP targeting the neonatal Fc receptor has achieved some degree of success [103].

Lipid-formulated DDS that exploit the gut lymphatic route for absorption are becoming increasingly popular [104]. Lipids are cost effective in comparison to synthetic polymers and the relative ease of manufacture, which avoids the use of organic solvents, collectively this makes this technology worthy of further investigation [105]. In separate rats dosed intraduodenally with a solid lipid NP formulation of lopinavir, cumulative lopinavir concentrations in lymph fluid were 9.68 μg for the nanoformulation compared to 1.97 μg for the conventional formulation 6 h after administration [106]. Negi et al. reported similar observations using another solid lipid NP of lopinavir that was fabricated using a hot self nano-emulsification technique [107,108]. Although the gut lymphatic-targeting mechanism of lipid-formulated drugs is not clear, it is believed to be similar to dietary fat absorption. Dietary fats translocate across the gut in the form of nano-sized chylomicrons and since the chylomicrons are too big to enter the blood microvasculature they by default enter the close-ended/one way terminal capillary lymphatics (i.e., the lacteals) through endothelial intercellular gaps. This also suggests that lymphatic-targeted NPs have the potential to be retained in gut lamina propria if their size exceeds the size of the chylomicrons. Solid lipid NPs have been reported to exhibit low drug loading capacity and low lipid chemical stability, which may limit their use for some drug delivery applications [109]. Other technologies that result in the formation of micellar suspensions have also been shown to improve the plasma concentrations of various ARVs after oral administration but lymph penetration was not assessed as the potential mechanism [110,111].

Unfortunately, with the exception of a very early attempt in 2001 [112], literature reports suggesting or proving the feasibility of specifically targeting the gut mucosa using DDS are lacking. This 2001 study achieved a four-fold higher AZT gut mucosa concentrations 90 min after oral dosing in rat using a poly(isohexylcyanoacrylate) NP formulation compared to free AZT. The enhancement mechanism was proposed to be mucoadhesion. DD to the LNs has proven to have a disproportionally larger effect on controlling HIV as compared to the blood compartment (i.e., systemic therapy). Critical progress made in targeting LNs is described in the following sections.

Since LNs are an important immune induction site, a HIV replication site and a reservoir, DD to LNs is also a high priority. The Ho lab pioneered LN targeted HIV DD. They found that in HIV-1 positive patients, indinavir (IDV) concentrations in LN mononuclear cells were about 25–35% that of mononuclear cells in blood. LN targeting enhanced IDV delivery in a HIV-2-infected macaque model [113]. The DDS consisted of a lipid-associated IDV complex NP (50–80 nm in diameter). The subcutaneously (SC) injected lipid/drug NPs became trapped in the draining and systemic LNs where they were taken up by resident macrophages. The LN macrophages served as a drug depot slowly releasing IDV. IDV concentrations in both peripheral and mesenteric LNs were 250–2270% higher than plasma while in humans soluble lipid-free drug administration reached a concentration <35% compared to the plasma. SC injection at IDV-equivalent 20 mg/kg daily for 30–33 weeks resulted in significantly reduced viral RNA load and increased CD4 + T-cell counts. Although the exact fate of SC-injected lipid-associated IDV NPs is not well characterized, it is likely that lipid–IDV NPs were trapped in LNs throughout the lymphatic system. The Ho group further optimized a lipid NP formulation [114]. Compared to previous formulations, new lipid-IDV NP provided 6-fold higher IDV concentrations in LNs of the macaque/SIV model and enhanced drug exposure in blood. Recently, they reported that they had further developed a lipid NP formulation incorporating newer HIV drugs [115]. With the new generation of lipid-drug formulation, they reported a lipid-drug NP containing a combination of lopinavir, ritonavir, and TFV [116]. These NPs produced over 50-fold higher intracellular lopinavir, ritonavir and TFV concentrations in LNs compared to free drug. Enhanced plasma and intracellular drug levels persisted for 7 days after a single SC dose, exceeding that achievable with current oral therapies. These results underscore the benefit of targeting an anatomic site critical in HIV infection as compared to systemic administration.

1.4.2. Targeting cell receptors on HIV infected T-cells and macrophages

As evidenced by the lack of progress in HIV vaccine development, targeting HIV proteins has been difficult. Host cell surface receptors are more readily targeted regardless of HIV infection status. The major roadblock for targeting CD4 + T cells and macrophages is the limited availability of targeting ligands. Most potential targeting ligands are

small molecule chemicals whereby conjugation to a DD carrier tends to reduce the ability of the ligand to bind to a target cell receptor. In addition, there is a tendency to "decorate" rather than engineer NC surfaces. Ligand display configuration (spacing, linker dimensions, density, etc.) will impact NC-receptor binding and cellular uptake. Little has been published in this area in general and specifically as it relates to HIV/AIDS. A priority must be put on the discovery of new cell targeting ligands for NCs, their optimal display on drug-loaded NCs. In addition, techniques to avoid endosomal entrapment and the avoidance of off-target uptake and effects will have to be carefully considered. While there have been some reviews regarding active DDS targeting in HIV/AIDS, this area remains largely undeveloped and, from a critical *in vivo* perspective, feasibility is largely unproven [93,96,117].

There are numerous potential cell targets on CD4 + T-cells and macrophages. However, only a few have been exploited due to concerns about the potential inference with host immune function or the lack of suitable ligands that can be conjugated to NCs. Most targeting ligands have been peptides as the peptidyl nature makes it amenable to conjugation to a NC. DV3 is a 10-mer, D peptide derived from a natural CXCR7 ligand vMIP-II. It has been shown to be able to bind to CXCR4 and to exhibit an anti-HIV activity of EC_{50} 0.439 μM [118]. Recently, a derivative of DV3 named DV1-K-(DV3) was reported [119]. It was created by chemically linking DV1 (a 21-mer peptide also derived from vMIP-II) and DV3. This peptidyl ligand of CXCR4 shows an anti-HIV activity of EC_{50} 4 nM, which is more potent than the natural CXCR4 ligand SDF-1a. T140 is a 14-mer peptide derived from a horseshoe crab protein named polyphemusin that is a CXCR4 antagonist with anti-HIV activity of EC_{50} 3.3 nM [120]. Remarkably, a cyclic pentapeptide derivative of T140 named FC131 retains most T140's potency and one of FC131 analogs is even more potent than T140 with an EC_{50} 1.4 nM [121]. Floudas' group has identified a few short (10 to 15 residue) peptides as HIV CXCR4 entry and HIV fusion inhibitors, respectively [122,123]. New and better performing derivatives of these peptides are being tested in our lab (to be published). Tuftsin is a tetrapeptide with the sequence of TKPR that utilizes an unidentified receptor on macrophages. It was displayed on efaviranz-packed dendrimers and the resultant dendrimer demonstrated macrophage targeting specificity and moderate anti-HIV activity [124]. Other potential ligands for NCs have also been explored, including a three-residue short peptide fMLF [125] and the monosaccharide mannose [126,127].

1.4.3. Cell depot-based DDS

Significant progress has been made in using human cells as DD depot sites. These approaches have utilized macrophages as drug carriers. HIV drugs were first loaded into NCs [128] or red blood ghost cells [129] that become engulfed by macrophages *ex vivo* or *in vivo*. The macrophages then migrated to LNs or additionally to other reticuloendothelial system (RES) tissues, acting as a cellular depot in these critical sites of HIV infection where the drugs slowly diffuse out over a period of days to weeks resulting in sustained high local drug concentrations in the LNs or other RES tissue.

In a feline immunodeficiency virus (FIV) model [129], the membrane-impermeable HIV drug zalcitabine-TP was first loaded *ex vivo* into autologous red blood cells and the plasma membrane of these red blood cells was then chemically modified so macrophages would recognize and engulf them. In a 7-month experimental FIV infection, zalcitabine-loaded erythrocytes protected the majority of peritoneal macrophages and reduced the infection of circulating lymphocytes. The Gendelman group has developed LA/ER nanoformulations of ritonavir (RTV), indinavir (IDV) and efavirenz (EFV) (nanoART) [128]. They reasoned that circulating macrophages traveling across the blood–brain barrier could enhance nanoART brain delivery [130]. An HIV-1 encephalitis (HIVE) rodent model was used. IDV NPs (a nanoART) were loaded into murine bone marrow macrophages (BMM, IDV-NP-BMM) *ex vivo* and the IDV-NP-BMM was administered intravenously. Rhodamine-labeled IDV-NP showed up in areas of HIVE. Continuous IDV release was observed for 14 days. IDV-NP-BMM treatment led to reduced HIV-1 replication in HIVE brain regions. Mechanistic studies [131] showed that nanoART-macrophage interactions enhanced phagocytosis, secretory functions, and cell migration, which could be exploited to increase macrophage nanoART loading capacity. Aouadi et al. [132] studied anti-inflammatory siRNA delivery utilizing yeast ghost cells. Yeast ghost cells were made by chemically treatment of yeast cell wall so that the cell surface was left with only beta1 3-D-glucan, for which macrophages have a special receptor. siRNA was then loaded into the ghost cells. The ghost cells can be efficiently absorbed orally through M-cells and, once crossed M-cells, avidly phagocytosed by macrophages in the Peyer's Patches. Interestingly, macrophages in the Peyer's Patches were able to migrate into blood circulation and settle at various LNs. Oral gavage of mice with the siRNA-loaded ghost cells containing as little as 20 μg/kg of siRNA directed against tumor necrosis factor alpha (TNF-α) depleted its messenger RNA in macrophages recovered from the peritoneum, spleen, liver and lung, and lowered serum TNF-α levels. Although not developed an HIV application, this approach is readily translatable to the delivery siRNA against HIV infection.

In addition to treating HIV/AIDS using pharmacotherapy, vaccination and even gene delivery have been attempted. Steinbach has extensively reviewed protein and oligonucleotide DDSs for vaginal microbicides against viral sexually transmitted diseases [133]. These biological agents include antibodies for passive immunization in the vagina, various proteins or plasmid DNA encoding viral proteins as antigens for induction of vaginal immunity, siRNA to downregulate the expression of viral and host proteins involved in infection, and other proteins and peptides as antagonists in virus-host interactions. Often, these agents are non-covalently or covalently complexed/linked to polymers or other molecules in vaginal formulations and the formulations are incorporated into various vaginal devices. These studies are generally at the early stages but they have established proof-of-concept. Typically, natural infection is the most effective vaccination. The fact that not a single patient among millions infected with HIV has spontaneously recovered, underlines the inefficiency of a conventional vaccination strategy for HIV. Current vaccine development focuses on new approaches, such as broadly neutralizing antibodies and unconventional vaccines with recombinant DNA technology [134,135]. In 2009, a report of a cure in the so-called "Berlin Patient" by using bone marrow transplantation of stems cells homozygous for a deletion in the CCR5 gene proved for the first time that eradication of HIV is possible [136]. Unfortunately, this strategy has proven to be unrealistic for most patients leading to efforts to develop gene therapy based strategies to disrupt the gene encoding CCR5 or other host proteins, as well as to disrupt the provirus [137]. Another strategy being explored involves purging the latent reservoir by activating latent provirus while eliminating new infections with ART [138,139]. So far none of these strategies has achieved eradication in patients.

1.5. Long-acting/extended-release (LA/ER) parenteral (injectable) DDS for PrEP and treatment

The development of LA/ER injectable ARV DDS that are administered infrequently (i.e., monthly or less), either in the clinic or at home represents an important potential solution to many of the problems associated with chronic ART and PrEP. There are numerous challenges facing LA/ER injectable DDS including identifying the optimal dosing interval, number and volume of injections per visit, the classes and properties of drugs that can be combined, and what to do if a patient becomes pregnant after the drug is administered. However, benefits to patients include infrequent dosing and long dosing intervals making administration convenient; the possibility of directly observed therapy and better long-term adherence; use in difficult to treat populations such as adolescents or those with ongoing substance abuse; use in patients reporting pill fatigue; and protection of patient privacy by eliminating the risk of disclosing pill taking to family and co-workers. There is also a clear rationale for the benefit of using LA/ER ARV formulations in

pregnant women, postpartum women (including those who are breast feeding), and infants/children who cannot take typical adult oral formulations. In fact, patient surveys disclose great enthusiasm for trying LA/ER injectable nanoformulations for treatment. One recently published survey found that 84% of patients currently taking oral therapy definitely or probably would try such an intervention if the dosing frequency was once-monthly or less frequent [140]. Additional applications that would benefit from LA/ER ARV formulations include their possible temporary use as parenteral ART for those unable to take oral medications. This could include patients requiring gastrointestinal surgery, those who are comatose and/or intubated, those with significant nausea and vomiting, patients developing severe mucositis from cancer chemotherapy, and very young pediatric patients unable to reliably swallow pills. Treatment options for such patients are extremely limited at present, resulting in significant risk of treatment failure as a consequence of intercurrent illness [141].

Injectable nanoformulation technologies previously applied to approved LA/ER treatments for chronic schizophrenia, such as paliperidone [142] or LA/ER injectable contraception, are now being evaluated for treating HIV [143]. The LA/ER formulation consists of a solid drug particle nanosuspension produced by wet bead milling of large fragments of drug in aqueous solution in the presence of a surfactant until the particles are within the nanometer size range. Since these NPs are composed of pure drug and not a mixed matrix including polymers and other excipients, which typically comprise 50% or more of the NP formulation, higher loading into the formulation of up to 200 mg/ml is possible along with a reduced injection volume. A formulation of the nanosuspension is administered to form an IM depot. Given typical IM injection volumes once monthly dosing is feasible for potent drugs. Due to the physicochemical and dosing limitations as well as insufficient antiviral potency of currently used ARVs, it was not feasible to apply this approach until the development of two potent ARVs, rilpivirine and cabotegravir (S/GSK1265744). Both compounds are poorly water-soluble but possess sufficient potency and favorable PK properties to support once monthly dosing. The combination subnanomolar potency and a plasma elimination half-life of approximately 40 h makes cabotegravir an ideal candidate for this approach. One concern with IM depots of poorly soluble drugs is the relatively high variability in bioavailability that has been observed in other applications. This would be a concern if the lower end of the deviation in blood/tissue concentrations falls below the IC_{90} of HIV-1.

Two formulations, a LA/ER injectable NNRTI rilpivirine (LA-RPV), and an InSTI, LA/ER injectable cabotegravir (LA-744), have been developed and are currently undergoing clinical testing [45,144,145]. The target-dosing interval for LA-RPV, based on its PK properties, is once monthly, and for LA-744 is once every three months for prevention applications and once monthly if combined with LA-RPV. Although LA-RPV and LA-744 represent important first steps in developing LA/ER ARV's, they are not well matched in terms of PK properties. Differences in half-life mean that missed dosing may negatively impact resistance since LA-RPV has a shorter half-life.

In the first study [146] investigating LA-RPV, the reported plasma and genital tract concentrations in 60 women and six men given intramuscular (IM) depot injections of three different doses of LA-RPV (300, 600, and 1200 mg) showed that RPV concentrations in females were between 26 and 77% of plasma concentrations in cervico-vaginal fluid and between 45 and 379% of plasma concentrations in cervicovaginal tissue, while in males, they were between 33 and >77% of plasma concentration in rectal fluid and tissue. In a second study [45], LA-744 (200 mg/ml) was evaluated after abdominal SC (100 mg, 200 mg, and 400 mg) or gluteal IM (100 mg, 200 mg, 400 mg and 800 mg) administration. The highest doses for each route were split due to injection volume considerations. Due to the low solubility of the NPs and inherent low tissue perfusion of muscle, the mean absorption half-times ranged from 21 to 50 days as compared to 40 h following single dose oral administration. With the exception of the 100 mg doses, all treatment groups remained above the IC_{90} of wild-type HIV-1 for at least 6 months. In two additional cohorts, (400 mg and 2 × 200 mg IM) were studied in 4 males and 4 females. Median individual tissue:plasma drug concentration ratios ranged from 16 to 25% in the female genital tract and were 8% in rectal tissue in men suggesting that at the dose tested the formulation would fall short as a PrEP agent. Furthermore, a recent study conducted in rhesus macaques showed that two IM doses 1 month apart protected eight out of eight animals from multiple rectal exposures to simian/human immunodeficiency virus (SHIV), while all eight animals receiving placebo injections acquired infection [147–149].

Since all currently used ART regimens involve the administration of at least three drugs in combination, there is an urgent need for other LA/ER technologies to complement those in late stage development. Additionally, LA/ER technologies that do not require injection need to be developed. It is clear that high drug potency is a prerequisite for compatibility with the LA/ER approach since it determines the plasma concentrations that need to be achieved (and sustained). Ultimately, dose is the limiting factor for LA/ER delivery because it directly influences the volume of the depot required to provide sufficient drug concentrations, which is limited by anatomical considerations as well as patient acceptability. However, release rate is also a key determinant of success since too fast a release rate will result in "dose dumping" where high initial concentrations that are rapidly cleared, whereas too slow a release rate will not provide sufficiently high plasma concentrations for therapeutic effect. A recent study employed physiologically-based pharmacokinetic modeling to determine which release rate/dose combinations would be required to provide either weekly or monthly therapeutic concentrations for a range of commonly used existing ART drugs [150]. Importantly, these data do not determine that the predicted dose/release characteristics are achievable, but they do set a benchmark to inform the target product profile for developing LA/ER medicines with other ART drugs. The National Institutes for Health have recently funded a Long-Acting/Extended Release Antiretroviral Resource Program (LEAP), which once launched will aim to foster further development of such medicines. LEAP will also provide services, which include a PBPK modeling core to help guide development of LA/ER drugs and/or regimens.

1.6. Future perspectives, gaps and opportunities

The tripartite goals of preventing new HIV infections, achieving a functional cure and complete eradication have become even more important since ART transformed HIV infection into a chronically managed disease. In recent years, great strides have been made in understanding how novel DDS may benefit HIV therapy in a disease-specific manner. Central to this has been the explicit demonstration in pre-clinical species of pharmacologically beneficial behaviors. Indeed, some of these benefits have also been translated to studies in humans and this is true for PrEP and LA/ER development discussed in this review. However, there remain a significant number of unmet needs and this is particularly true for those technologies that are at early stages of translation. In broad terms, considerations for successful future development can be divided into those that relate to the drug delivery system itself, as well as those that relate to the drug, pharmacological rationale and the ultimate clinical application.

While numerous benefits have been achieved and demonstrated pre-clinically, there remain significant challenges in terms of the intricacies of manufacture. The development of complex DDS such as NCs is challenging at the bench no less in scale for manufacture. Translation at all stages represents the biggest hurdle for large-scale fabrication, testing and manufacture of DDS. For many technologies, there is a current paucity of knowledge regarding how the physical properties and constituents of the DDS specifically relate to the pharmacological behavior. Drug loading potential will be a key determinant of success for many technologies because of its impact upon format size or volume required for injectable depot medicines. Moreover, many technologies are

only compatible with drugs that exhibit specific physiochemical properties and this may pose challenges to holistic development of enabled-regimens consisting of drugs from different classes. For conventional oral medicines, the framework within which drugs are developed has been extensively researched over the past decades. This framework has resulted in the Biopharmaceutics Classification System (BCS) for oral drugs and a solid understanding of the chemical space that is optimal for oral drug delivery that is provided by Lipinski's rule of 5 (and variations thereof). However, there are gaps in knowledge that specifically relate to many newer DDS as well as the chemical compatibility of drugs with the DDS and with specific applications (e.g. LA/ER depot delivery).

One of the significant gaps in HIV/AIDS DDS research is targeting to sites of infection in order to achieve effective ART concentrations. Another significant gap is the ability to prolong ART effectiveness in order to reduce the frequency of administration and burden on patients. Classic sustained release technologies currently being utilized in LA/ER PrEP have proven the feasibility of the approach. However, significant improvements are needed. This suggests a major opportunity for DD research — the marriage of targeting technologies and techniques to sustain not just the release of ART but, more importantly, the effect.

Since the overwhelming burden of HIV disease continues to fall upon individuals in resource-limited settings, there are societal implications that need to be considered if novel technologies are to impact significantly on the HIV pandemic. Some novel DDS challenge convention in terms of route of delivery, and consideration needs to be given to compatibility with existing global healthcare systems and acceptability of the approach to patients with different cultural backgrounds. Clearly, it is imperative that early technological development and selection of technologies for further development consider cost, and compatibility of the platform with sterile production of the medicine at scale.

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ORIGINAL RESEARCH ARTICLE

Physiologically Based Pharmacokinetic Modelling to Inform Development of Intramuscular Long-Acting Nanoformulations for HIV

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Abstract

Background and Objectives Antiretrovirals are currently used for the treatment and prevention of HIV infection. However, poor adherence and low tolerability of some existing oral formulations can hinder their efficacy. Long-acting (LA) injectable nanoformulations could help address these complications by simplifying antiretroviral administration. The aim of this study is to inform the optimisation of intramuscular LA formulations for eight antiretrovirals through physiologically based pharmacokinetic (PBPK) modelling.

Methods A whole-body PBPK model was constructed using mathematical descriptions of molecular, physiological and anatomical processes defining pharmacokinetics. These models were validated against available clinical data and subsequently used to predict the pharmacokinetics of injectable LA formulations

Results The predictions suggest that monthly intramuscular injections are possible for dolutegravir, efavirenz, emtricitabine, raltegravir, rilpivirine and tenofovir provided that technological challenges to control their release rate can be addressed.

Conclusions These data may help inform the target product profiles for LA antiretroviral reformulation strategies.

Key Points

This study investigated the feasibility of applying mathematical models to candidate long-acting (LA) formulations for antiretrovirals in order to predict drug concentrations over time.

Six antiretrovirals have been identified as potential therapeutic options for once-monthly intramuscular administration, providing theoretical optimal dose and release rates.

This modelling approach constitutes a new paradigm for the rational design of LA formulations, considering a thorough evaluation of the drug pharmacological properties and defining an innovative platform for the optimisation of future nanoformulations.

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1 Introduction

HIV is a global pandemic affecting 75 million people to date, with 2.3 million new cases in 2012 [1]. Modern antiretroviral therapy is based on the targeting of specific stages of the viral replication, with optimal suppression

obtained through the use of two or more antiretroviral [2] classes in combination. Antiretrovirals can also be used for pre-exposure prophylaxis (PrEP) strategies, preventing the transmission of HIV to uninfected individuals who are at high risk of acquiring the infection [3].

Currently available oral formulations necessitate lifelong daily dosing and subjects often encounter pill fatigue as a consequence. This has the potential of producing suboptimal adherence, which has emerged as one of the main causes of therapeutic failure and low rates of PrEP protection [4]. The introduction of long-acting (LA) formulations could revolutionise treatment and PrEP by enabling administration once monthly or even less frequently. This can simplify antiretroviral administration and improve adherence [5]. LA formulations are most commonly administered parenterally, decreasing inter- and intra-patient variability in drug exposure. Additional potential advantages include a decrease in overall drug consumption and cost of treatment [6]. LA injectable contraceptives and antipsychotics are widely used and have excellent efficacy and tolerability compared with oral routes of administration [4].

Recently, injectable LA formulations of the antiretrovirals rilpivirine and S/GSK1265744 (cabotegravir) have been developed with the potential for once-monthly, or less frequent, dosing [4]. The development of the first injectable LA antiretroviral formulations has led to considerable interest amongst patients; a recent study found that 84 % of surveyed patients would definitely or probably try a once-monthly injectable LA antiretroviral [7]. The LA formulations are based on solid drug nanoparticles (SDNs) which release the drug from the depot over a protracted period of time. The optimisation of SDNs for LA administration is complicated by several pharmacokinetic factors and has to take into account the optimal dose and drug release into the circulation to achieve therapeutic or prophylactic plasma concentrations for the entire dosing interval.

The pharmacokinetics of a drug are determined by processes that can be simulated through physiologically based pharmacokinetic (PBPK) models. PBPK models are based on the mathematical description of anatomical, physiological and molecular processes defining drug distribution, integrating information on drug characteristics and patient-specific factors [8]. This modelling approach can be applied to the development and optimisation of novel formulations to identify optimal pharmacokinetic characteristics [8]. PBPK models have been used extensively in different disease areas for the prediction of pharmacokinetics for novel drugs, optimisation of existing therapies and simulation of different clinical scenarios. Specifically, PBPK can be used to predict pharmacokinetics as a function of formulation and route of administration [9]. These predictions can then inform the design of LA formulations, identifying optimal characteristics in terms of dose and depot release rate. For antiretrovirals, this modelling approach has been applied to identify factors influencing the pharmacokinetics of novel formulations [10, 11], the effect of genetics on drug distribution [11], pharmacokinetics in special populations [12] and drug–drug interactions [13].

The aim of this study was to simulate the pharmacokinetics of antiretrovirals after administration of virtual intramuscular LA formulations using PBPK modelling. The PBPK models were first validated against clinical data for oral counterparts, to assess the accuracy of the simulation. Theoretical target dose and release rate combinations for once-weekly and once-monthly intramuscular administration were then identified.

2 Methods

Virtual patients between the ages of 18 and 60 years were generated. All organs and tissues were represented as individual compartments. The model was based on the following assumptions: (1) well-stirred compartments with instant distribution of the drug; (2) no absorption of the drug from the colon; and (3) the model is blood flow limited. The PBPK model was designed using SimBiology® version 4.3.1, a product of MATLAB® version 8.2 (MathWorks, Natick, MA, USA 2013). The PBPK approach has been developed for key antiretrovirals [two nucleoside reverse-transcriptase inhibitors (NRTIs), tenofovir and emtricitabine; three non-NRTIs (NNRTIs), efavirenz, rilpivirine and etravirine; two integrase inhibitors, raltegravir and dolutegravir; and an unboosted protease inhibitor, atazanavir].

2.1 Anatomy

The age, body mass index, body surface area, height and weight of the individual were defined as previously described [14]. These initial values were used for the calculation of organ and tissue volumes through allometric equations [14]. The blood circulation was represented by considering the cardiac output and regional blood flows as previously described [14].

2.2 Intestinal Absorption

A compartmental absorption and transit model was integrated to simulate for oral drugs as previously described [15]. The absorption rate was evaluated using the apparent permeability obtained from Caco-2 cells or the polar surface area and hydrogen bond donor (HBD) values as previously described [16].

2.3 Intestinal Metabolism

The clearance of drugs in the gut (CL_{gut}) was calculated considering the in vitro intrinsic clearance (CL_{int}) and the abundance of cytochrome P450 (CYP) family 3, subfamily A-CYP3A in the intestinal tissue (Ab_{CYP3A}) (Eq. 1) [16, 17]:

$$CL_{gut}(L/h) = \frac{CL_{int} \times Ab_{CYP3A} \times 1,000 \times 60}{1,000,000} \quad (1)$$

The fraction of the drug transiting to the liver after metabolism in the gut (F_g) was computed using the following equation (Eq. 2):

$$F_g = \frac{Q_{gut}}{Q_{gut} + f_{u,gut} \times CL_{gut}} \quad (2)$$

where Q_{gut} represents the blood flow to the gut and $f_{u,gut}$ is the fraction unbound in the gut.

2.4 First-Pass Metabolism

The total intrinsic clearance (TCL_{int}) of an enzyme was calculated as follows (Eq. 3):

$$TCL_{int} = \frac{CL_{int} \times \text{Abundance} \times \text{Liver weight} \times \text{MPPGL} \times 1,000 \times 60}{1,000,000} \quad (3)$$

where Abundance is the amount of enzyme present in a milligram of microsomal protein and MPPGL is amount of microsomal protein per gram of liver (Eq. 4) [18]:

$$\text{MPPGL} = 10^{1.407 + 0.0158 \times \text{Age} - 0.00038 \times \text{Age}^2 + 0.0000024 \times \text{Age}^3} \quad (4)$$

Then, the sum of the CL_{int} values of all the enzymes that are metabolising the drug in the liver gives the total apparent clearance (CL_{app}) (Eq. 5):

$$CL_{app} = \sum_{n=1}^{n} TCL_{int} \quad (5)$$

The systemic clearance (CL) is calculated from the blood, and not the liver, and is given by Eq. 6:

$$CL = \frac{Q_{hv} \times f_u \times CL_{app}}{Q_{hv} + CL_{app} \times f_u} \quad (6)$$

where Q_{hv} is the hepatic flow rate and f_u is the fraction unbound in plasma.

The fraction of the active drug that is available after first-pass hepatic metabolism (F_h) is given as follows (Eq. 7):

$$F_h = 1 - CL/Q_{hv} \quad (7)$$

2.5 Systemic Distribution

The volume of distribution and systemic circulation was calculated using previously published equations [19, 20].

2.6 Intramuscular Depot Compartment

A compartment was created to virtually represent the depot with a maximum volume of 6 mL since it is the maximum volume of injection that has previously been used for intramuscular administration, although lower volumes are usually needed for optimal tolerability [21]. The blood flow to this area was calculated accordingly. The release of the drug from the depot to the blood capillaries present in the muscle was given as follows (Eq. 8) [9]:

$$\frac{dA}{dt} = k \times A \quad (8)$$

where A is the amount of drug released from the depot and k is the first-order release rate. A variability of ±10 % for the release rate values was considered.

The highest single dose that has been tested clinically is 1,500 mg with cefuroxime [21]. Therefore, any strategy for LA antiretroviral development needs to address depot volume and dose in order to successfully translate.

2.7 Model Validation

The physicochemical properties of antiretrovirals used to build the PBPK models are represented in Table 1. The models were primarily validated against clinical data at steady state for oral formulations of the antiretrovirals (Table 2). The model was also verified against an existing LA formulation of rilpivirine [6].

2.8 Prediction of Pharmacokinetics Following Intramuscular Injection

In the prediction of intramuscular pharmacokinetics, the dose of the antiretroviral and release rate were optimised such that the mean trough concentration (C_{trough}) was above the 90 % inhibitory concentration (IC_{90}), 95 % inhibitory concentration (IC_{95}) for wild-type HIV virus or protein-binding corrected inhibitory concentration [antiretroviral concentration that can inhibit 95 % of the virus replication in vitro ($PBIC_{95}$)] 7 or 30 days after injection. A dose cut-off of 1,500 mg was considered for intramuscular administration.

2.9 Prediction of Intracellular Concentrations

The active phosphate metabolite of tenofovir, tenofovir diphosphate (TFV-DP) intracellular concentrations were

Table 1 Physicochemical properties, in vitro and population pharmacokinetic data of antiretrovirals

	Atazanavir	Dolutegravir	Efavirenz	Emtricitabine	Etravirine	Raltegravir	Rilpivirine	Tenofovir
Drug properties								
Molecular weight	704	419	316	247	435	444	366	287
log $P_{o:w}$	3.17	2.20	4.60	−0.43	5.20	0.58	4.32	1.25
Protein binding (%)	86.0	99.3	98.0	4.0	99.0	83.0	99.7	0.7
pK_a	7.4	8.3	10.2	2.65	3.75	6.67	3.26	3.75
R	0.75 [13]	0.535 [42]	0.74 [43]	1.0 [33]	0.7 [44]	0.6 [45]	0.67 [27]	1[a]
PSA (Å^2)	–	–	38.33	–	120.64	–	–	–
HBD	–	–	1	–	3	–	–	–
P_{app} (10^{-6} cm/s)	–	–	2.5 [43]	–	–	6.6 [45]	12 [27]	–
k_a (h^{-1})	1.5 [13]	1.63 ± 1.03 [46]	–	1.0 [47]	–	–	–	1.03 [48]
F (%)	68	–	–	93	–	–	–	20
V_d (L/kg)	1.2 [13]	–	3.6	1.4 ± 0.3	6.03[b]	0.6	–	0.813 [38]
Metabolism								
CL/F (L/h)	12.6 (10.5–15.4)[b] [49]	1 (15 %) [31]	–	–	–	4.62[b] [50]	–	–
Renal clearance (L/h)	–	–	–	12.80 ± 5.34 [33]	–	3.6 [45]	–	11.3 ± 6.2 [51]
CYP2A6 CL_{int}	–	–	0.08 [43]	–	–	–	–	–
CYP2B6 CL_{int}	–	–	0.55 [43]	–	–	–	–	–
CYP1A2 CL_{int}	–	–	0.07 [43]	–	–	–	–	–
CYP3A4 CL_{int}	–	3.0[c] [52]	0.007 [43]	–	0.012 [53]	–	2.04 [27]	–
M2 CYP3A4 CL_{int}	–	–	–	–	0.00091 [53]	–	–	–
CYP3A5 CL_{int}	–	–	0.03 [43]	–	–	–	–	–
CYP2C19 CL_{int}	–	–	–	–	0.75 [53]	–	–	–
UGT1A1 CL_{int}	–	3.2[c] [52]	–	–	–	12.4[d] [45]	–	–
CYPs induction								
CYP3A4 Ind_{max}	–	–	6.5 [43]	–	2.5 [53]	–	–	–
CYP2B6 Ind_{max}	–	–	5.7 [43]	–	–	–	–	–
CYP3A4 Ind_{50}	–	–	3.9 [43]	–	–	–	–	–
CYP2B6 Ind_{50}	–	–	0.8 [43]	–	–	–	–	–

Values are expressed as arithmetic mean ± SD, mean (range), Geometric mean (% coefficient of variation) or mean for the presented data

CL/F apparent oral clearance, CL_{int} intrinsic clearance (μL/min/pmol), *CYP* cytochrome P450, *F* absolute bioavailability, *HBD* number of hydrogen bond donors, Ind_{50} 50 % induction expressed as μM, Ind_{max} induction maximum expressed as μM, k_a absorption coefficient, *log* $P_{o:w}$ partition coefficient between octanol and water, *M2* second metabolite, P_{app} drug permeability from apical to basolateral in Caco-2 cell monolayer, *pKa* logarithmic value of the dissociation constant, *PSA* polar surface area, *R* blood-to-plasma drug ratio, *UGT* uridine diphosphate glucuronosyltransferase, V_d volume of distribution, – data not available

[a] Value was an assumption (as the drug is similar to emtricitabine and tenofovir, value was not available in the literature)

[b] An average adult body weight of 70 kg was assumed

[c] The units are in μL/min/mg

[d] The units are in μL/min/10^6 hepatocytes

simulated as previously described [22]. The first-order elimination rate of emtricitabine triphosphate (FTC-TP) from the cellular compartment was calculated considering the elimination half-life of FTC-TP [23]. The maximum uptake velocity (V_{max}) and maximum velocity of anabolism (k_m) were fitted to obtain a zero-order dose input rate (k_{in}) such that the mean intracellular concentration of the active metabolite at steady state ($C_{ss,avg}$) was in accordance with available clinical data following Eq. 9:

$$k_{in} = (V_{max} \times C)/(k_m + C) \quad (9)$$

where C is the concentration in the plasma.

Table 2 Validation of antiretrovirals at steady state: clinical versus predicted AUC, C_{max} and C_{trough}

Drug	Dose (mg)	AUC (ng × h/mL)		C_{max} (ng/mL)		C_{trough} (ng/mL)	
		Clinical	Predicted	Clinical	Predicted	Clinical	Predicted
Efavirenz	600 od	57,150 ± 27,300 [34]	65,006 ± 16,383	4,000 ± 1,710 [34]	3,193 ± 699.7	2,380 ± 1,140 [34]	2,298 ± 671.0
Etravirine	200 bid	3,713 ± 2,069 [35]	3,939 ± 1,249	451.3 ± 232.3 [35]	375.0 ± 106.1	235.9 ± 163.1 [35]	285.4 ± 100.5
Rilpivirine	25 od	2,589 ± 868.8 [27]	3,590 ± 1,256	203.8 ± 75.81 [27]	214.1 ± 53.9	89.85 ± 38.07 [27]	95.7 ± 49.5
Raltegravir	400 bid	7,076 ± 4,071 [36]	9,584 ± 2,160	2,519 ± 1,930 [36]	2,002 ± 378.9	71 ± 50 [36]	54.6 ± 52.1
Atazanavir	400 od	29,303 ± 8,263 [29]	24,354 ± 4,307	5,358 ± 1,371 [29]	2,718 ± 392.2	218 ± 191 [29]	326.9 ± 150.0
Tenofovir	300 od	3,324 (41.2 %) [38]	4,707 ± 1,004	326 (36.6 %) [38]	580.2 ± 80.6	64.4 (39.4 %) [38]	20.7 ± 17.7
Emtricitabine	200 od	10,100 (18 %) [23]	9,747 ± 2,326	1,720 (16 %) [23]	1,136 ± 236.3	73 (28 %) [23]	52.5 ± 32.2
Dolutegravir	50 od	53,600 (27 %) [31]	63,615 ± 8,802	3,670 (20 %) [31]	3,474 ± 519.6	1,110 (46 %) [31]	1,867 ± 309.0

Data are given as arithmetic mean ± SD or geometric mean (% coefficient of variation)

AUC area under the concentration–time curve, *bid* twice daily, C_{max} maximum plasma concentration, C_{trough} trough plasma concentration, *od* once daily

Values of 0.9 ± 0.18 ng × h/mL and 18 ± 3.6 ng/mL for V_{max} and k_m were suitable to obtain $C_{ss,avg}$ values comparable with previously published data for oral administration [23]. The optimisation of the dose and release rates for emtricitabine and tenofovir considered not only plasma concentrations but also the intracellular FTC-TP and TFV-DP over the reported in vitro 50 % inhibitory concentration (IC_{50}) values of 127 and 150 fmol/10^6 cells [24, 25].

3 Results

The validation was conducted by comparing the mean simulated C_{trough}, maximum plasma concentration (C_{max}) and area under the concentration–time curve (AUC) values with the available clinical data for oral administration and also for an existing LA intramuscular formulation of rilpivirine (600 mg; 100 mg/mL) (shown in Table 2 and Fig. 1). For emtricitabine and tenofovir, the $C_{ss,avg}$ of FTC-TP and TFV-DP were compared with clinical data [23]. Although the commonly agreeable limits of variability for validation is twofold from the mean value, we used a more stringent value of 0.5-fold to enhance the accuracy of the predictions [26].

For intramuscular formulations, multiple-dose and release rate combinations were simulated to obtain mean predicted C_{trough} values greater than the defined cut-off. For tenofovir and emtricitabine, the dose and release rates for intramuscular injection were optimised such that the plasma concentrations were over the IC_{90} value and also the $C_{ss,avg}$ values were over the intracellular IC_{50}.

A summary of the predicted AUC, C_{max} and C_{trough} values for eight antiretrovirals following optimal intramuscular injection is shown in Table 3.

3.1 Rilpivirine

The validation of the PBPK model for rilpivirine was performed against available clinical data for the oral formulation. The simulated mean AUC, C_{max} and C_{trough} values deviated +38.7, +5.1 and +6.5 %, respectively, from the clinical data [27]. The PBPK model was additionally validated against an existing LA intramuscular formulation of rilpivirine (600 mg; 100 mg/mL) [6]. The release rate from the intramuscular depot was predicted after matching the model to the clinical pharmacokinetic data. The drug release followed dose-dependent first-order kinetics and the release rate was predicted to be between 0.0009 and 0.0012 h^{-1} (Fig. 1). The mean values for AUC were 84.0 vs. 83.38 ± 33.34 ng × h/mL, C_{max} 96.7 vs. 86.73 ± 30.51 ng/mL and C_{trough} 15.7 vs. 11.81 ± 6.3 ng/mL for clinical versus simulated data. After the validation step with both oral and LA intramuscular formulations, the optimal dose and release rates of both weekly and monthly injection regimens of rilpivirine were identified such that the plasma concentration is over the $PBIC_{95}$ value of 20.3 ng/mL [28]. A weekly dose of 60 mg with a release rate of 0.015 h^{-1} and a monthly dose of 250 mg with a release rate of 0.002 h^{-1} were predicted to maintain the plasma concentrations above the target cut-off.

3.2 Atazanavir

The validation at steady state for the oral administration of unboosted atazanavir resulted in mean predicted AUC, C_{max} and C_{trough} values that were −16.9, −49.3 and +50.0 % of those observed clinically [29]. An intramuscular dose of atazanavir 1,500 mg was predicted to result in plasma concentrations after 30 days below the $PBIC_{95}$ (60 ng/mL) and was therefore deemed unsuitable for monthly injection. An intramuscular weekly dose of 400 mg, however, was predicted to achieve mean

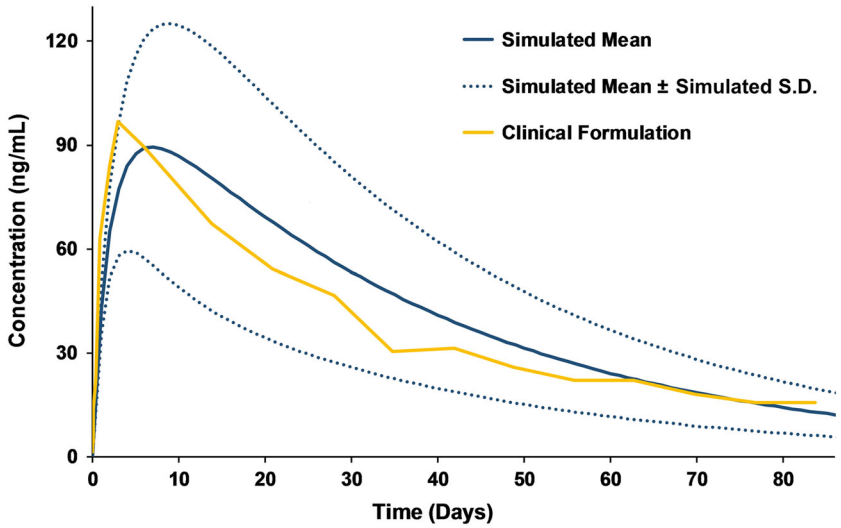

Fig. 1 Validation of the physiologically based pharmacokinetic strategy against clinical data for an existing rilpivirine sustained-release formulation (600 mg; 100 mg/mL) [6]. *S.D.* standard deviation

Table 3 Prediction of the dose and release rate of a single intramuscular injection of antiretrovirals

Drug	Intramuscular dose (mg)	Release rate (h^{-1})	Weekly/monthly	AUC (μg × h/mL)[a]	C_{max} (ng/mL)[a]	C_{trough} (ng/mL)[a]	Cut-off limit (ng/mL)
Nucleoside reverse transcriptase inhibitors							
Emtricitabine	1,500	0.0015	Monthly	43.1 ± 15.4	94.2 ± 32.5	51.2 ± 41.3	50.2 [23] (IC_{90})
	375	0.009	Weekly	20.5 ± 8.9	199.4 ± 76.1	55.6 ± 26.4	
Tenofovir	1,300	0.002	Monthly	52.2 ± 15.4	99.2 ± 28.6	43.8 ± 17.2	18 [2] (IC_{90})
	200	0.008	Weekly	16.6 ± 7.1	155.6 ± 58.5	49.1 ± 23.0	
Non-nucleoside reverse transcriptase inhibitors							
Efavirenz	800	0.007	Monthly	333.9 ± 99.3	850.4 ± 194.6	140.0 ± 50.8	126 [30] ($PBIC_{95}$)
	200	0.015	Weekly	73.2 ± 22.1	546.5 ± 139.5	273.3 ± 81.8	
Etravirine	700	0.015	Weekly	43.4 ± 12.3	359.3 ± 80.7	126.1 ± 60.7	116 [30] ($PBIC_{95}$)
Rilpivirine[b]	250	0.002	Monthly	32.9 ± 14.7	75.4 ± 30.1	22.6 ± 8.2	20.3 [28] ($PBIC_{95}$)
	60	0.015	Weekly	10.8 ± 4.8	97.4 ± 33.3	26.1 ± 18.7	
Integrase inhibitors							
Dolutegravir	105	0.002	Monthly	95.6 ± 8.5	212.1 ± 18.1	65.7 ± 7.1	64 [32] ($PBIC_{95}$)
	20	0.006	Weekly	24.2 ± 2.8	169.5 ± 18.1	101.7 ± 14.8	
Raltegravir	800	0.002	Monthly	26.1 ± 5.2	66.9 ± 13.1	15.7 ± 2.8	15 [37] (IC_{95})
	200	0.005	Weekly	8.23 ± 1.47	69.4 ± 12.1	30.1 ± 5.4	
Protease inhibitors							
Atazanavir	400	0.009	Weekly	38.9 ± 8.3	334.3 ± 70.7	114.8 ± 25.4	60 [30] ($PBIC_{95}$)

AUC area under the concentration–time curve, C_{max} maximum plasma concentration, C_{trough} trough plasma concentration, IC_{90} 90 % inhibitory concentration, $PBIC_{95}$ 95 % protein binding-adjusted inhibitory concentration

[a] Values are shown as arithmetic mean and standard deviation

[b] Note that this dose is not for the existing rilpivirine formulation

concentrations in excess of the $PBIC_{95}$ with a release rate of 0.009 h^{-1} [30].

3.3 Dolutegravir

The predicted variation from the clinical values for mean AUC, C_{max} and C_{trough} in the validation of oral administration of dolutegravir were +18.7, −5.3 and +68.2 %, respectively [31]. Considering the $PBIC_{95}$ value of 64 ng/mL as the target concentration, the simulated intramuscular administration requires an estimated weekly dose of 20 mg with a release rate of 0.006 h^{-1} and a monthly dose of 105 mg with a release rate of 0.002 h^{-1} [32].

Table 4 Validation and prediction of the intracellular concentration of emtricitabine triphosphate and tenofovir diphosphate

Active drug	Intracellular $C_{ss,avg}$ (fmol/10^6 cells)		Weekly/monthly	Intramuscular prediction (fmol/10^6 cells)		In vitro IC_{50} (fmol/10^6 cells)
	Clinical	Simulated		Intracellular C_{trough}	Intracellular C_{max}	
Emtricitabine triphosphate	1124 ± 592 [23]	1,154 ± 456	Monthly	163.0 ± 124.6	228.2 ± 106.1	127 [25]
			Weekly	270.0 ± 130.0	431.8 ± 198.4	
Tenofovir diphosphate	150.7 ± 92.9 [22]	156.5 ± 59.5	Monthly	154.2 ± 46.5	164.6 ± 49.3	150 [24]
			Weekly	163.0 ± 59.0	174.9 ± 62.8	

C_{max} maximum concentration, $C_{ss,avg}$ mean steady-state concentration, C_{trough} trough concentration at the end of the duration, IC_{50} 50 % inhibitory concentration

3.4 Emtricitabine

The predicted variation from the clinical values in mean AUC, C_{max} and C_{trough} for oral emtricitabine were −3.5, −34.0 and −28.1 %, respectively [33], and the simulated mean $C_{ss,avg}$ of intracellular FTC-TP has a deviation of +2.65 % from the clinical value (Table 4). The IC_{90} value of 50.2 ng/mL and intracellular IC_{50} of 127 fmol/10^6 cells were considered as target concentrations [23, 25]. The simulated intramuscular injection required a dose of 375 mg with a release rate of 0.009 h^{-1} for a weekly administration and 1,500 mg with a release rate of 0.0015 h^{-1} for a monthly administration [23].

3.5 Efavirenz

The validated PBPK model yielded AUC, C_{max} and C_{trough} values that were +13.7, −20.2 and −3.4 % compared with the clinical values [34]. In order to obtain plasma concentrations above the $PBIC_{95}$ value of 126 ng/mL, a weekly intramuscular dose of 200 mg (release rate −0.015 h^{-1}) and monthly dose of 800 mg (release rate −0.007 h^{-1}) were required [30].

3.6 Etravirine

The validated PBPK model with oral administration of etravirine resulted in mean AUC, C_{max} and C_{trough} values that were +6.1, −16.9 and +21.0 % compared with the clinical values [35]. The intramuscular predictions indicate that etravirine may be suitable for weekly administration with a dose of 700 mg and release rate of 0.015 h^{-1}, but not for monthly administration as the drug concentrations fall below the $PBIC_{95}$ value even for an intramuscular dose of 1,500 mg [30].

3.7 Raltegravir

The predicted mean AUC, C_{max} and C_{trough} values of orally administered raltegravir were +35.4, −20.5 and −23.1 % compared with the clinical values, respectively [36]. To maintain the plasma concentrations above the IC_{95} value (15 ng/mL), the prediction suggested a dose of 200 mg (release rate −0.005 h^{-1}) and 800 mg (release rate −0.002 h^{-1}) may enable weekly and monthly intramuscular administration of raltegravir, respectively[37].

3.8 Tenofovir Disoproxil Fumarate

The validated PBPK model for tenofovir disoproxil fumarate (TDF) predicted mean AUC, C_{max} and C_{trough} values that were +41.6, +78.0 and −67.9 % compared with the clinical data, respectively [38]. The deviation of the intracellular $C_{ss,avg}$ was +3.85 % compared with the clinical value (Table 4). TDF 200 mg with a release rate of 0.008 h^{-1} was predicted to provide a sufficient exposure following weekly intramuscular injection and TDF 1,300 mg with a release rate of 0.002 h^{-1} was an adequate dose for a monthly intramuscular administration, maintaining mean plasma concentrations above the IC_{90} and intracellular TFV-DP above the IC_{50} [24].

4 Discussion

Imperfect adherence to daily oral antiretroviral formulations continues to hinder the efficacy of HIV therapy and PrEP. Alternative administration strategies are beginning to emerge that provide opportunities for once-monthly or even less frequent administration. A greater understanding of formulation characteristics is required to predict dose and release rates that provide adequate pharmacokinetic exposure relative to potency. This information will facilitate development of newer LA antiretroviral formulations.

In this study, PBPK modelling was used to predict the pharmacokinetics of antiretrovirals following intramuscular injection. The PBPK models were based on mathematical equations describing the human anatomy and physiology and the molecular processes regulating drug

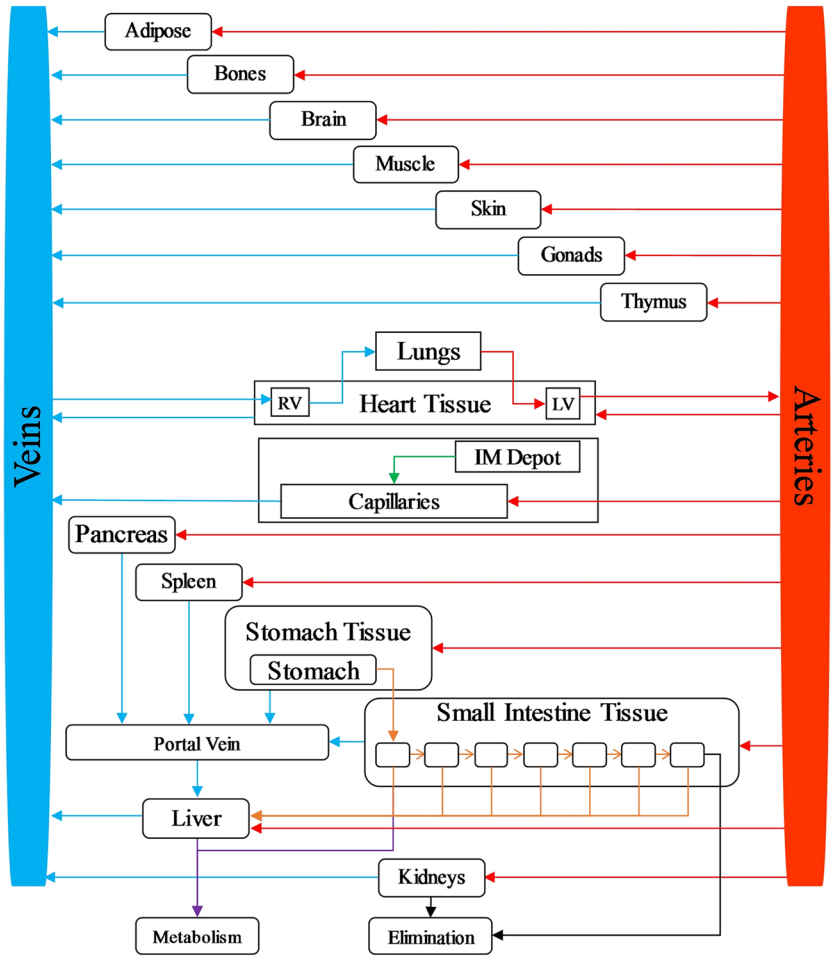

Fig. 2 The whole-body physiologically based pharmacokinetic model with an additional compartment for intramuscular depot and the blood capillaries surrounding the depot. Each of the *arrows* represents the flow kinetics assumed between tissues in the model. The small intestine is divided into seven parts for effective absorption kinetics. Metabolism occurs in the liver and intestine. Unabsorbed drug is excreted through faeces and excretion occurs through the kidneys. The *green line* represents the kinetics of the release from the nanoparticulate intramuscular depot. *IM* intramuscular, *LV* left ventricle, *RV* right ventricle

release and distribution. The PBPK models were initially validated for eight orally administered antiretrovirals against available clinical data for oral formulations. Subsequently, intramuscular administration was simulated by adding an additional compartment to the model to represent the intramuscular depot. For this, a first-order kinetics constant was applied to represent the diffusion of antiretrovirals from the depot to the surrounding blood capillaries (as shown in Fig. 2). This approach was validated comparing simulated pharmacokinetics of rilpivirine following intramuscular injection versus available clinical data for the LA formulation. The primary objective of this study was to identify whether drug-specific doses and release rates from the depot predicted compatibility for intramuscular administration. To obtain the suppression of the viral replication, antiretroviral C_{trough} should reach and be maintained at concentrations above the susceptibility of the virus to that drug. Where possible, dose and release rate were optimised such that the antiretroviral C_{trough} remained above previously identified target values. Target concentrations were identified as equal to the $PBIC_{95}$. Since emtricitabine and TDF are characterised by very low protein binding (<4 and <0.7 %, respectively) IC_{90} values were considered (IC_{95} data not available). The intracellular concentrations of FTC-TP and TFV-DP were also used as target concentrations in the optimisation of intramuscular dose and release rates for emtricitabine and tenofovir. Although the $PBIC_{95}$ represents a rational pharmacodynamic cut-off and has been discussed extensively elsewhere, some antiretrovirals do not exert a good relationship between C_{trough} values and virologic efficacy [30]. In certain cases the clinical data indicate that the minimum effective concentrations for therapy might be considerably higher than the in vitro IC_{95}.

The validation of PBPK models is essential to identify the correct parameters for an accurate simulation of pharmacokinetics for each antiretroviral. Since dissolved molecules rather than nanoparticles are assumed to predominate systemically after intramuscular administration of SDNs, the volume of distribution, metabolism and elimination were fixed irrespective of the route of administration. As such, it should be noted that the presented data are only applicable when this is assumed to be the case, and these models cannot be applied to technologies that change

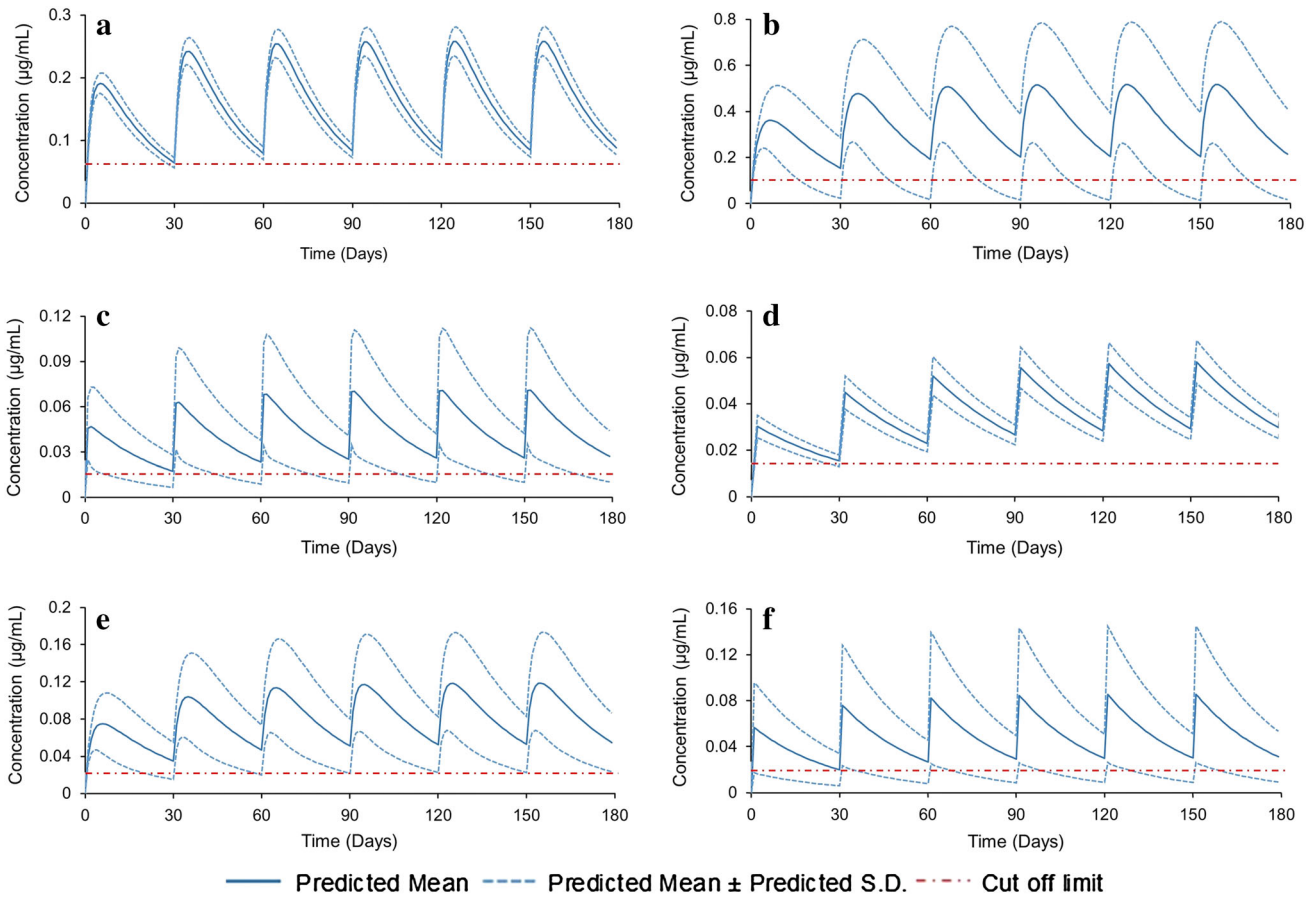

Fig. 3 Pharmacokinetics of monthly administration of antiretrovirals over a period of 6 months: **a** dolutegravir, **b** efavirenz, **c** emtricitabine, **d** raltegravir, **e** rilpivirine and **f** tenofovir. *S.D.* standard deviation

these parameters (e.g. nanocarrier systems such as nanoemulsions or polymeric nanoparticles). Each model prediction was carried out at steady state to better represent the long-term antiretroviral pharmacokinetics and relevant clinical scenarios. The model was considered validated if the mean AUC of the simulation for each antiretroviral was within ±50 % compared with clinically reported values. The simulated values from all of the included PBPK models were in agreement with available clinical data and were therefore used for prediction of pharmacokinetics after intramuscular administration. The simulations were carried out for weekly and monthly administration and the maximum injectable dose was set to 1,500 mg since doses above this have not previously been practical for intramuscular formulations [21]. Table 3 illustrates that the release rate was inversely proportional to the dosage, and a slower release rate was required for monthly administration.

Our PBPK models predicted that two NRTIs, emtricitabine and tenofovir, may be suitable for weekly or monthly administration. Importantly, emtricitabine and tenofovir are widely used in HIV treatment and prevention, and LA formulations of these agents would make possible multidrug regimens that match current clinical paradigms for therapy. These agents also represent good candidates for monthly PrEP due to their excellent safety and efficacy profiles. Tenofovir alone or tenofovir combined with emtricitabine have already been shown to provide good protection against HIV infection in individuals at high risk of infection [3].

Considering NNRTIs, efavirenz and rilpivirine were both predicted to be suitable for monthly administration. Both have long half-lives, which are favourable for development of LA agents. The high systemic clearance of etravirine limits the possibility of a monthly administration format when a dose limit of 1,500 mg is applied. Conversely, both integrase inhibitors, dolutegravir and raltegravir, were predicted to be suitable for monthly administration. A low dose of 105 mg was predicted to be sufficient for monthly administration of dolutegravir if a formulation with an optimised release rate can be developed. It should be noted that cabotegravir is chemically closely related to dolutegravir, and development of a dolutegravir LA formulation may therefore not be

warranted. Our simulation indicated that raltegravir could potentially be developed as a monthly intramuscular injection with a dose of 800 mg. The optimised dose and release rate for unboosted atazanavir predicted feasibility of an injectable weekly option at 600 mg, but doses higher than 1,500 mg would be required for monthly administration.

The optimisation of LA strategies should be based on a rational analysis of several pharmacokinetic factors. For many agents, it is likely that the pharmacokinetic variability after intramuscular administration will be reduced compared to oral formulations, due to the lower complexity of the absorption process and avoidance of intestinal transporters and first-pass metabolism. Therapeutic plasma concentrations were predicted to be achieved in ~8 h for emtricitabine and tenofovir and ~24 h for the remaining antiretrovirals after a single intramuscular injection (as shown in Fig. 3). Since a day is needed to achieve therapeutic plasma concentrations for the other antiretrovirals, a loading oral dose equivalent to the daily regimen may be necessary. This could represent a minor risk for the development of drug resistance and therapeutic failure, but PBPK modelling may help to inform the ongoing debate regarding some aspects of the need for an oral lead-in with the same drugs. The co-administration of multiple injectable antiretrovirals is possible, but a rational selection of concomitant agents is needed to avoid drug–drug interactions. Studies to validate the utility of coadministration of the two existing LA drugs, rilpivirine and cabotegravir, are underway [39].

The PBPK approach is characterised by some intrinsic limitations due to existing knowledge gaps and the complexity of biological processes underpinning drug distribution. For example, muscle tissues are well-connected to the lymphatic circulation, and drugs with high lipophilicity tend to diffuse through lymphatics rather than blood vessels [9]. Consequently, higher accumulation of antiretrovirals in lymph nodes might be possible, but there is a paucity of clinical data in this area for available LA formulations. Similarly, efflux and influx transporters are of clear importance to intestinal absorption, distribution and clearance of drugs, but there are currently very little data on whether these transporters (or metabolic enzymes) are expressed at the depot sites. Gender has been correlated with changes in plasma exposure in a recent clinical study of rilpivirine LA [40]. Although several suggestions have been put forward to explain this, the physiological and anatomical mechanisms characterising the gender differences have not been fully elucidated and consequently have not been included in our simulations.

Although the doses and release rates identified through these simulations set targets for optimal nanoparticle design, it is important to underscore that the technological challenges in achieving these formulation characteristics are not addressed in this work. Long-term stability of the antiretrovirals in the interstitial fluid present in the depot site is another important consideration. Since SDN strategies do not involve the use of a carrier with stable size and dissolution rates over time, the nanoparticle properties are likely to change over the dosing interval. Since this will alter the surface to mass ratio, an effect on antiretroviral release kinetics with downstream effect on the overall pharmacokinetics cannot be ruled out. It may also be important to consider other SDN components, such as polymers and surfactants, which will also be gradually released over time [41]. The use of LA injections of antiretrovirals might determine specific complications at the site of injection as described for other treatments; additionally, given the prolonged systemic exposition of LA antiretrovirals and the inability to interrupt the drug in the event of adverse effects, a period of oral induction might be considered before initiating LA injections. For patients discontinuing the LA antiretroviral therapy, an oral administration strategy should be considered in order to prevent resistance development due to the diminishing plasma concentrations [4].

5 Conclusion

PBPK models for the simulation of pharmacokinetics after LA administration were successfully developed and validated against clinical data for eight approved antiretroviral drugs. The validated models were applied to identify theoretical optimal dose and release rates required to achieve therapeutic concentrations following intramuscular injection. This approach defines a new paradigm for the rational design of LA formulations incorporating pharmacological properties of candidate antiretrovirals. Antiretrovirals with potential for reformulation into intramuscular depots were identified, assuming the technological complexities associated with reformulation can be overcome. Based on their pharmacokinetic properties, dolutegravir, efavirenz, emtricitabine, raltegravir, tenofovir and rilpivirine are potential candidates for once-monthly injection. PBPK modelling represents an innovative pharmacological tool to inform drug and formulation design.

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Merck and ViiV. He also received research funding from Abbvie, Boehringer Ingelheim, BMS, Gilead, Janssen, Merck and ViiV. Charles Flexner received consulting or advisor fees from Abbvie, Boehringer Ingelheim, Bristol Myers-Squibb, Gilead, GlaxoSmithKline, Merck and ViiV. Rajith K.R. Rajoli, Steve Rannard and Caren L. Freel Meyers have no conflicts of interest to declare.

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REVIEW

OPEN

Formulation and pharmacology of long-acting cabotegravir

Christine Trezza[a], Susan L. Ford[a], William Spreen[a], Rennan Pan[b], and Stephen Piscitelli[a]

Purpose of review

Long-acting cabotegravir may provide a novel therapeutic option for both the treatment and prevention of HIV-1 infection that does not necessitate adherence to a daily regimen. The present review will highlight the unique formulation properties and pharmacologic attributes of long-acting cabotegravir nanosuspension.

Recent findings

Cabotegravir is a potent integrase strand transfer inhibitor that has been formulated as an oral tablet for daily administration and as a long-acting injectable nanosuspension. Long-acting cabotegravir is readily absorbed following intramuscular and subcutaneous administration and has an elimination half-life of approximately 40 days, allowing for administration on a monthly or less frequent schedule. Repeat-dose pharmacokinetic studies and population pharmacokinetic modeling indicate monthly and bi-monthly dosing achieves clinically relevant plasma concentrations considered effective for HIV maintenance therapy and that quarterly injections are appropriate for investigation as preexposure prophylaxis. Cabotegravir is primarily metabolized by uridine diphosphate glucuronosyltransferase 1A1 and is unlikely to be impacted by the cytochrome P450 metabolic pathway. *In vitro* and *in vivo* data suggest cabotegravir has a low propensity to cause, or be subject to, significant drug interactions.

Summary

The pharmacologic profile of long-acting cabotegravir supports its continued development for both treatment and prevention of HIV-1 infection.

Keywords

cabotegravir, formulation, long-acting, nanosuspension, pharmacokinetics

INTRODUCTION

The advent of highly active antiretroviral therapy has drastically improved both the length and quality-of-life for HIV-infected patients. There are now over 20 antiretrovirals from six drug classes and multiple effective first-line regimens for HIV-1 treatment [1]. Despite these remarkable advances, strict adherence to daily antiretroviral therapy remains critical to maintaining viral suppression, preventing the emergence of drug resistance, and reducing the risk of HIV transmission [2,3]. Long-acting antiretrovirals capable of achieving prolonged exposures at therapeutic concentrations may enable simplified treatment regimens that do not necessitate daily administration.

Cabotegravir (GSK1265744) is a potent integrase strand transfer inhibitor and a structural analogue of dolutegravir. Cabotegravir's unique physiochemical and pharmacokinetic properties have permitted its formulation and delivery both as an oral tablet for daily administration and as a long-acting nanosuspension for monthly to quarterly intramuscular (i.m.) injection. These attributes have supported the ongoing clinical development of cabotegravir for both the treatment and prevention of HIV-1 infection. For HIV treatment, the novel two-drug regimen of oral cabotegravir and rilpivirine, which has also been formulated as a long-acting injection, has demonstrated potent antiviral activity for the maintenance of viral suppression in

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KEY POINTS

- Intrinsic physiochemical properties have permitted the formulation and delivery of cabotegravir, an investigational integrase strand transfer inhibitor, as a long-acting injectable nanosuspension.
- Cabotegravir's potent antiretroviral activity and less frequent dosing requirements may overcome current limitations associated with daily antiretroviral therapy for HIV-1 treatment and prevention.
- Single-dose intramuscular injections of long-acting cabotegravir achieve and maintain clinically relevant plasma concentrations for approximately 16 weeks after dosing, enabling the design of HIV-1 treatment and PrEP regimens that are administered on a monthly to quarterly basis.
- Cabotegravir is primarily metabolized by UGT1A1 and does not significantly inhibit or induce the CYP450 metabolic pathway.
- In-vitro and in-vivo studies indicate that cabotegravir's propensity to be either a perpetrator or victim of drug–drug interactions is minimal.

antiretroviral-naive patients [4]. The safety and efficacy of this dual–drug regimen administered intramuscularly once monthly or once every 2 months is currently under investigation in a phase 2 clinical trial [5]. In addition, data from nonhuman primate rectal and vaginal simian/human immunodeficiency virus (SHIV) challenge models indicate that cabotegravir long acting is a promising candidate for HIV prevention [6▪,–8▪]. Cabotegravir long acting, dosed once quarterly, circumvents the need for daily or coitally dependent dosing and may provide a useful alternative for HIV preexposure prophylaxis (PrEP). This review will focus on the formulation properties and manufacturing considerations that enable nanosuspension delivery, and highlight the pharmacologic profile of cabotegravir long acting.

FORMULATION AND MANUFACTURING PROCESS

Cabotegravir long acting is a sterile aqueous nanosuspension containing 200 mg/ml cabotegravir free acid as crystalline nano-sized particles with an average particle size of approximately 200 nm. It is manufactured by a wet-bead milling process and terminally sterilized by gamma irradiation [9].

Formulation

Cabotegravir long acting is made with the crystalline-free acid form of cabotegravir, which exhibits low aqueous solubility, a long systemic half-life (approximately 40 h after oral administration), and high antiviral potency. These properties are optimal for a nanosuspension delivery system and permit high drug loading, which in turn minimizes injection volume for a specified dose. Inactive ingredients include polysorbate 20 and polyethylene glycol 3350 as components of a nonionic stabilizing system and mannitol to maintain isotonicity.

Cabotegravir long acting is designed as a dissolution-controlled depot formulation [10]. The rate-limiting step of drug absorption is dissolution of drug particles in interstitial fluid, surrounding the drug depot. In addition to its solubility, the particle size of cabotegravir has an effect on drug dissolution and absorption. Reduction in particle size increases the surface area of drug particles and therefore the dissolution rate. The nanoparticles not only enhance cabotegravir dissolution and absorption, but also improve the syringeability of the formulation.

Manufacturing process

Cabotegravir drug substance is presterilized by gamma irradiation and is manufactured by conventional wet-bead milling. Sterile cabotegravir drug substance undergoes a clean wet-bead milling process with mannitol, polysorbate 20, polyethylene glycol 3350, and water for injection to a target average cabotegravir particle size of 200 nm. The nanosuspension is filled into presterilized depyrogenated glass vials. The vials are stoppered with presterilized stoppers and then sealed. Finally, the sealed vials are sterilized using gamma irradiation at a lethal dose of 25 kGy.

PHARMACOKINETICS

Cabotegravir long-acting pharmacokinetics following intramuscular and subcutaneous administration were first described in a single-dose, dose-ascending study in 72 healthy volunteers [11▪]. In this study, intramuscular gluteal injections were administered at doses of 100, 200, 400, and 800 mg (2 equally split injections of 400 mg), and subcutaneous abdominal injections at doses of 100, 200, and 400 mg (two equally split injections of 200 mg) to cohorts of eight patients (six active/two placebo). Sixteen volunteers received 400 mg i.m. either as a single injection or two equally split injections of 200 mg to assess the differences in cabotegravir disposition following a single versus split dose administration.

The plasma concentration–time profiles were similar between intramuscular and subcutaneous administration, with comparable maximal

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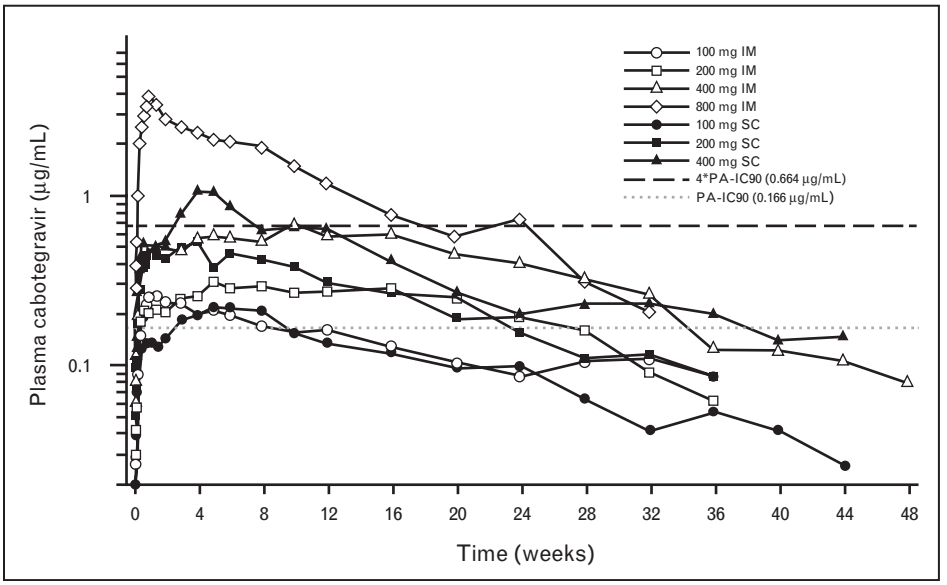

FIGURE 1. Long-acting cabotegravir pharmacokinetics following single-dose administration in healthy adult individuals. Mean cabotegravir plasma concentration–time profiles following a single long-acting intramuscular (gluteal) or subcutaneous (abdominal) injection in healthy volunteers. The dotted line represents the PA-IC90. The black dashed line represents 4∗PA-IC90. 4∗PA-IC90, four times the PA-IC90; PA-IC90, protein-adjusted concentration required for 90% viral inhibition. Adapted with permission from [11▪,12].

concentrations (C_{max}) and total plasma exposures achieved with each route of administration (Fig. 1) [11▪,12]. Rapid absorption from the intramuscular and subcutaneous depot was observed, with detectable plasma cabotegravir concentrations observed 4 h after dosing (first sampling time) in all patients. The median time to C_{max} following a single 400-mg dose was approximately 69 days, although this parameter was highly variable among patients, and ranged from 2 to 213 days. Geometric mean concentrations observed 4 weeks after dosing remained over four times the protein-adjusted 90% inhibitory concentration, 0.66 μg/ml, following intramuscular and subcutaneous split injections of 400 mg (2 × 200 mg) and split intramuscular injections of 800 mg (2 × 400 mg). This concentration is comparable with the mean trough concentration (C_τ) observed in a 10-day monotherapy study of oral cabotegravir 5 mg in HIV-infected antiretroviral-naive adults [13]. In this study, a C_τ of 0.57 μg/ml was associated with a mean −2.2 $\log_{10}$ reduction in HIV RNA, providing a clinically relevant pharmacodynamic target for cabotegravir long acting.

The mean apparent terminal-phase half-life ranged from 25 to 54 days across long-acting dosing cohorts. This is approximately 25-fold longer than the 40-h elimination half-life observed following oral cabotegravir administration [13] and suggests the prolonged concentration–time profiles observed with parenteral administration reflect rate-limiting absorption from the depot rather than elimination from the central compartment. The prolonged apparent plasma half-life noted in this study resulted in detectable cabotegravir concentrations for approximately 52 weeks in some patients.

Splitting the 400-mg dose equally into 2 × 200-mg injections resulted in more than twofold increases in the partial area under the concentration–time curve (AUC) from predose to week 4 (AUC[0–4 wk]), concentration observed 4 weeks after dose (C_{wk4}), and C_{max} relative to unsplit dosing. The total cabotegravir exposures extrapolated to infinity, however, were similar between split and unsplit administration, indicating that dose splitting increases the rate, but not the extent, of cabotegravir absorption. This increase in absorption rate may be because of the increased exposure of nanoparticles to solubilizing biological fluids and subsequent permeation area that results with dose splitting [14].

Pharmacokinetics following repeat doses of cabotegravir long acting have been assessed in a Phase 1, randomized, open-label study [15▪▪]. Healthy volunteers were randomized to receive one of four cabotegravir long-acting dosing regimens: 200 mg subcutaneously monthly × 3 doses, 200 mg i.m. monthly × 3 doses, 400 mg i.m. monthly × 3 doses, or 800 mg i.m. quarterly (every 12 weeks) × 2 doses.

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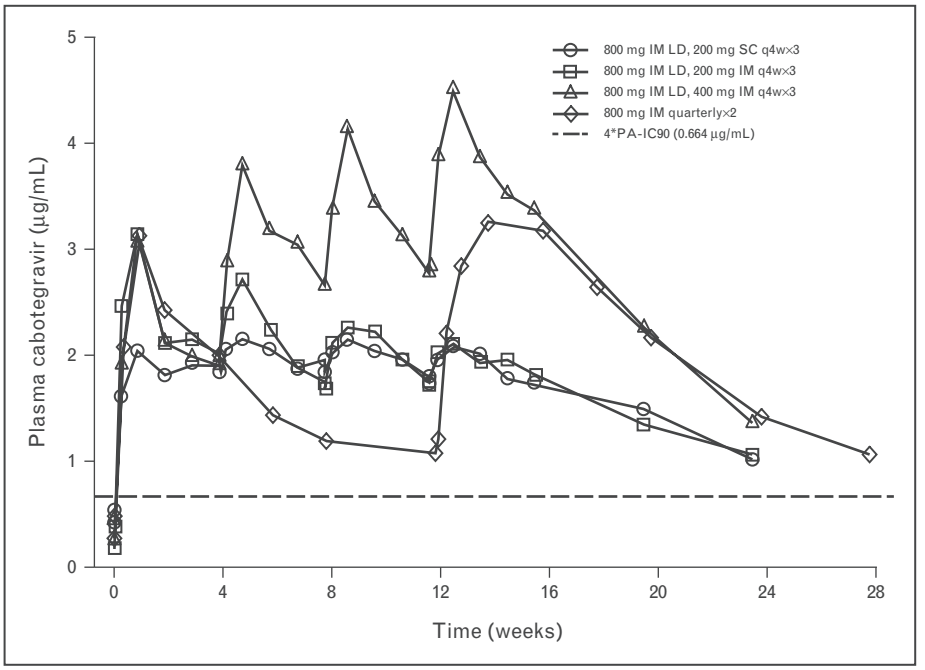

FIGURE 2. Repeat-dose administration of monthly or quarterly injections of cabotegravir long acting. Mean cabotegravir concentration–time curves following monthly (q4w) or quarterly (every 12 week) repeat-dose administration in healthy individuals. Monthly dosing regimens were preceded by an 800 mg (split 2 × 400 mg) intramuscular LD. 4∗PA-IC90, four times the protein-adjusted concentration required for 90% viral inhibition; LD, loading dose; q4w, every 4 weeks. Adapted with permission from [15▪▪].

All monthly regimens were preceded by an 800-mg i.m. loading dose and the cohorts receiving intramuscular cabotegravir (200 and 400 mg) also received long-acting rilpivirine concomitantly with the third and fourth dose. Forty-seven healthy adults were enrolled, with 40 adults receiving at least one cabotegravir injection (37 adults completed all planned injections).

The mean plasma concentration–time profiles of cabotegravir administered subcutaneously or intramuscularly on monthly or quarterly dosing schedules are provided in Fig. 2. By Day 3, all regimens achieved mean plasma concentrations in excess of 0.66 μg/ml (four times the protein-adjusted 90% inhibitory concentration). Cabotegravir concentrations remained above this threshold throughout the monthly or quarterly dosing interval for each of the tested regimens. These data suggest that a two-drug regimen of cabotegravir long acting and rilpivirine long acting, dosed once monthly, is potentially clinically feasible, was generally well tolerated, and achieved plasma concentrations associated with therapeutic efficacy.

Factors affecting long-acting absorption

Wide variability in the apparent absorption rate has been observed following cabotegravir long-acting administration, as evidenced by coefficient of variation of 26.0–96.6% associated with early pharmacokinetic parameter estimates (i.e., partial AUC[0–4wks], C_{wk4}, and C_{max}). The mechanistic explanation and ultimate clinical significance of the variability is currently unknown.

Current phase 1 data suggest concentration–time profiles differ between male and female individuals. Some female individuals appear to have flat concentration–time curves, with blunted C_{max} and a prolonged time to C_{max} relative to male individuals. Despite the difference in the early shape of the concentration–time profile observed in some females, both males and females achieved similar total exposure (AUC[0–∞]), following a single cabotegravir long-acting dose, suggesting the rate, and not the extent of absorption, may be different between males and females. This finding is consistent with a population pharmacokinetic analysis performed using pharmacokinetic data from 346 participants from eight phase 1/2 cabotegravir clinical trials [16]. This analysis showed that both body mass index (BMI) and sex were clinical covariates with significant impact on the rate of absorption following long-acting administration. Slower absorption rates following cabotegravir long-acting administration were associated with increasing BMI [−28% at 90th percentile (30.8 kg/m^2) relative to

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median BMI (25.8 kg/m^2)] and female sex (−35.2% relative to males). The converse was observed with lower BMI, with individuals in the 10th percentile BMI (21.6 kg/m^2) predicted to have 39% faster absorption rate than those in the 50th percentile BMI. A similar effect of sex and BMI was observed on rilpivirine C_{max}, a measure of absorption rate, following long-acting administration [17]. Current hypotheses surrounding the source of variability in cabotegravir long-acting apparent absorption rate include differences in body fat distribution, muscle mass, injection technique, and physical activity. Additional data obtained following cabotegravir long acting in phase 2 studies will be incorporated into the model to better understand pharmacokinetic variability, particularly given the small sample size of patients receiving single or repeat doses of cabotegravir long acting ($n = 121$) in phase 1 studies.

Distribution to tissue

As a potential agent for HIV prevention, there is interest in describing the distribution of cabotegravir into the anatomical tissues associated with sexual HIV transmission. The penetration of cabotegravir long acting to vaginal, cervical, and rectal tissue was assessed following a single dose in healthy adults [11▪]. Males ($n = 8$) and females ($n = 8$) were administered cabotegravir long acting 400 mg i.m. as either a single injection or as 2 × 200-mg split injections and underwent serial and intermittent pharmacokinetic sampling in blood plasma, vaginal tissue, cervical tissue, and rectal tissue. Distribution into these compartments was limited throughout the 12 weeks of sampling, with median tissue-to-plasma ratios ranging from 16 to 28% in the female genital tract and ≤ 8% in rectal tissue. The relatively low penetration into mucosal tissue is likely attributed to extensive plasma protein binding (>99%) and the low volume of distribution associated with oral cabotegravir dosing. Although partitioning into the sampled matrices was low relative to plasma, the tissue-to-plasma ratios noted in humans were comparable with those observed in the nonhuman primate models for PrEP. Despite limited penetration into these tissues in macaques, protection has been demonstrated in rectal and vaginal SHIV challenge experiments, suggesting adequate drug concentrations are achieved and providing proof-of-concept for long-acting cabotegravir as a PrEP development candidate [18].

DRUG INTERACTION ASSESSMENT

The pathways for cabotegravir metabolism, transport, and excretion have been investigated in both in-vitro and in-vivo studies of the oral formulation [19,20]. It is predicted that the results of clinical mass balance and drug-interaction studies using the oral formulation can be extrapolated to the long-acting product, as the pathways of metabolism and route of elimination are believed to be the same among cabotegravir formulations.

Cabotegravir is primarily metabolized by uridine diphosphate glucuronosyltransferase (UGT) 1A1 with a minor contribution from UGT1A9 [20]. In a single-dose mass balance study utilizing a radiolabeled 30-mg oral dose to investigate the recovery, excretion, and pharmacokinetics of cabotegravir, it was found that cabotegravir is primarily eliminated in the feces (58.5% mean recovery) as unchanged drug [19]. Both unchanged cabotegravir and the glucuronide metabolite were excreted in bile, suggesting that enterohepatic recirculation may contribute to the prolonged pharmacokinetic profiles associated with cabotegravir. A small proportion of radioactivity (26.8%) was recovered in the urine, primarily as the glucuronide metabolite. There was no unchanged cabotegravir detected in the urine.

In-vitro studies demonstrate that cabotegravir has a very low potential to be a significant perpetrator or victim of clinically relevant drug interactions [20]. Cabotegravir is a P-glycoprotein and breast cancer resistance protein substrate; however, the impact of these transporters on cabotegravir disposition is predicted to be minimal provided the compound's high passive membrane permeability. Cabotegravir does not induce the activity of cytochrome P450 (CYP) 1A2, 2B6, or 3A4 in human hepatocytes, nor is there evidence that it inhibits CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, or CYP2D6 at clinically relevant concentrations. With the exception of organic anion transporter 1 and 3 (OAT1/3), cabotegravir is not a significant inhibitor of the hepatic, intestinal, or renal drug transporters summarized in Table 1. Although further characterization of the effect of cabotegravir on OAT1/3 substrates, specifically those with narrow therapeutic index (e.g., methotrexate), is needed, it is anticipated that cabotegravir has a low potential for causing, or being subject to, clinically significant drug interactions involving drug transporters.

A clinical study utilizing midazolam as a probe substrate confirmed *in vivo* that cabotegravir does not significantly inhibit or induce the CYP3A metabolic pathway [20]. In this study, 12 healthy individuals received single doses of midazolam 3 mg alone and following 14 days of oral cabotegravir 30 mg/day. The geometric least-squares mean ratios (midazolam and cabotegravir/midazolam alone)

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Table 1. In-vitro assessment of cabotegravir's ability to inhibit hepatic, intestinal, and renal drug transporters

Hepatic and intestinal drug transporters	
Transporter	**Half-maximal inhibitory concentration (μmol/l)**
P-glycoprotein (Pgp)	>30
Breast cancer resistance protein (BCRP)	>30
Bile salt export pump (BSEP)	>30
Multidrug resistance associated protein 2 (MRP2)	>30
Organic cation transporter 1 (OCT1)	>30
Organic anion transporting polypeptide 1B1 (OATP1B1)	>30
Organic anion transporting polypeptide 1B3 (OATP1B3)	>30
Renal drug transporters	
Multidrug and toxin extrusion transporter 1 (MATE1)	18
Multidrug and toxin extrusion transporter 2-K (MATE2-K)	14
Multidrug resistance associated protein 4 (MRP4)	>30
Organic anion transporter 1 (OAT1)	0.81
Organic anion transporter 3 (OAT3)	0.41
Organic cation transporter 2 (OCT2)	>30

In-vitro transporter assays were conducted using cells transfected with Pgp, BCRP, MATE1, MATE2-K, OAT1, OAT3, OATP1B1, OATP1B3, OCT1, or OCT2, and vesicles expressing BSEP, MRP2, or MRP4. Established radiolabeled probe substrates and liquid scintillation counting were utilized to determine transporter inhibition at cabotegravir concentrations up to 30 μmol/l [20].

and the corresponding 90% confidence intervals for midazolam AUC(0–∞) and C_{max} were 1.08 (0.962, 1.22) and 1.09 (0.944, 1.26), respectively, demonstrating that repeat doses of cabotegravir had no significant effect on the CYP3A pathway and midazolam pharmacokinetics.

A clinical drug-interaction study with repeat doses of etravirine was conducted to assess the impact of CYP450 induction on cabotegravir disposition [21]. CYP-mediated metabolism of cabotegravir is expected to be minimal, as evidenced by the lack of effect on cabotegravir pharmacokinetic parameters when co-administered with repeat doses of etravirine, a known CYP3A inducer in 12 healthy volunteers. The C_{τ}, AUC0-τ, and C_{max} geometric least-squares mean ratios (90% confidence intervals) for cabotegravr plus etravirine/cabotegravir were 0.999 (0.942, 1.06), 1.01 (0.956, 1.06), and 1.04 (0.987, 1.09), respectively.

Cabotegravir also has no significant impact on the pharmacokinetics of rilpivirine, a nonnucleoside reverse transcriptase inhibitor [22]. Co-administration of oral rilpivirine 25 mg and cabotegravir 30 mg daily for 12 days had no significant effect on the pharmacokinetic concentration-time profiles of either drug.

CONCLUSION

Cabotegravir long acting is a sterile aqueous nanosuspension containing 200 mg/ml cabotegravir-free acid as crystalline particles. It is manufactured by a wet-bead milling process and terminally sterilized by gamma irradiation. Cabotegravir possesses a number of favorable pharmacokinetic characteristics, including rapid achievement of clinically relevant concentrations, low risk of drug interactions, and potential for monthly or less frequent dosing. Injectable antiretroviral therapies that are less reliant on patient compliance to daily administration are promising, next-generation HIV treatment and prevention strategies.

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Conflicts of interest

C.T. was a postdoctoral fellow on assignment at GlaxoSmithKline at the time this manuscript was written. S.F., W.S., R.P., and S.P. are employees of and own stock in GlaxoSmithKline.

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LEADING ARTICLE

New Approaches to Antiretroviral Drug Delivery: Challenges and Opportunities Associated with the Use of Long-Acting Injectable Agents

Marta Boffito · Akil Jackson · Andrew Owen · Stephen Becker

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Abstract Research on improved treatment of HIV infection and pre-exposure prophylaxis continues. Poor adherence to treatment is the critical risk factor for virological failure and resistance development, and long-acting formulations of anti-HIV medications that need only infrequent dosing may facilitate long-term therapeutic responses. Importantly, long-acting formulations of therapeutic agents have been used to avoid missing doses or treatment fatigue to prescribed lifelong medications in a number of different medical fields, with demonstrable success. However, such formulations are associated with challenges, such as the prolongation of adverse events with the persistence of drug concentrations and concerns over the development of resistance as a result of selective pressure as drug concentrations decline. Furthermore, long-acting injectable formulations of antiretroviral (ARV) agents with infrequent dosing may be advantageous over daily oral drug intake to prevent transmission of HIV. However, the knowledge on protective drug concentrations and frequency of dosing is poor to date and implementation globally is challenging. Importantly, if nanoformulations of ARVs requiring lower drug doses become available globally, the potential for treatment cost reductions is high, as, especially in resource-limited settings, the active pharmaceutical ingredient accounts for the greater proportion of the total cost of the medicine. In conclusion, different long-acting ARVs are being studied in phase I/II for both the treatment and prevention of HIV infection, and research on administering these agents in combination has started.

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1 Introduction

Since the introduction of combination antiretroviral therapy (cART), HIV-infection-related morbidity and mortality have dramatically decreased. However, none of the currently available antiretroviral (ARV) agents provide a cure and, therefore, HIV-infection has become a chronic disease requiring a lifelong commitment to daily oral treatments [1]. Importantly, in patients with chronic diseases, poor adherence to medications has been shown to be common [2]. When adherence to ARV treatment is reduced, drug concentrations are not sufficient to suppress replication and, as a result, fail to control plasma viral load and increase the likelihood for development of resistance. Other factors responsible for incomplete suppression of HIV replication may include drug interactions, altered drug absorption through the gastrointestinal tract, insufficient drug potency, presence of drug resistance, and host factors, such as pharmacogenetic variability [1].

Long-acting formulations of therapeutic agents have been used to improve adherence and prevent issues such as missing doses or treatment fatigue to prescribed medication in a number of different fields such as contraception [3], male hypogonadism [4] and schizophrenia [5], with demonstrable success. Such formulations afford the ability to maintain plasma concentrations at effective concentrations for a sustained duration after dosing, and have been applied during development to new compounds as well as to drugs already used in high doses at frequent intervals.

Reducing the frequency at which a dose is administered (either by the patient or healthcare practitioner) may improve adherence by eliminating the dependence on the user for sustained, reliable daily (or more frequent) drug intake [3]. Furthermore, long-acting contraception, for example, has shown better outcomes than short-acting contraception for the prevention of pregnancy in both resource-rich and resource-poor settings [6, 7].

In current practice, with the exception of the uncommonly prescribed enfuvirtide, all available ARVs are administered orally. However, long-acting formulations are under development for both the prevention and treatment of HIV infection.

The aim of this article is to review the data available on the injectable ARVs under investigation for both the prevention and treatment of HIV infection. Discussion of injection systems is beyond the scope of this review. Furthermore, the humanized monoclonal antibody targeting the CCR5 receptor PRO140 is not discussed, as its development has been stopped.

1.1 Prevention

Pre-exposure prophylaxis (PrEP) is an emerging approach to HIV prevention, and interesting data is being generated for novel interventions. PrEP involves the administration of ARV agents, often the same regimens used in treatment, to HIV uninfected persons who are at high risk of infection because of their pattern of behaviour and/or a high background prevalence of HIV in their social and geographic environment. Several clinical trials with orally administered PrEP have been presented, demonstrating that sustained daily adherence to oral PrEP regimens is vital to ensure efficacy of this prevention strategy [8–12]. Unfortunately, the proportion of trial participants with good adherence was not high, and even in those whose initial adherence was relatively good, there was a tendency for adherence to wane over time [13]. Therefore, parenterally administered long-acting agents are an attractive alternative for use in PrEP because they circumvent the need for high adherence to oral regimens, which is currently a fundamental limitation to the effectiveness of PrEP as a strategy [14].

Applied technologies may complement the numerous important behavioural interventions and augment the prevention strategies. Current investigations are exploring long-acting injectable agents as appropriate options in prevention regimens, with the aim of demonstrating their value in PrEP. However, in addition to carefully conducted clinical trials, it is essential that the overall strategy is evaluated, with careful consideration of the complex issues relating to implementation. These would include individual acceptability of the pharmacological strategy, access to services, compliance to monthly visit schedules, stigma, cost and sustainability of the services, etc. This needs to occur at the earliest stages of development as these will be key determinants of the future efficacy of such agents as a public health intervention.

1.2 Treatment

In the realm of currently established treatment, approved long-acting products in other medical fields have yielded varied beneficial improvements in (i) the management of schizophrenia in refractory and non-compliant patients [5]; (ii) the convenience of chronically administered products, such as insulin [15, 16]; and (iii) the safety profiles of the parent compound, such as the use of pegylated interferon in the treatment of chronic hepatitis C [17]. All of these apply to the clinical difficulties faced in current HIV therapy and thus beneficial advances could be equally useful for lifelong ARV therapy.

Interestingly, a recent report by Williams et al. [18] showed that when HIV-infected individuals on ARV treatment were asked whether they were interested in receiving long-acting injectable nanoformulated cART, 73 % of patients indicated that they would definitely or probably try it.

Nevertheless, advantages and disadvantages of these new medications need to be appropriately analyzed.

2 Overview of Long-Acting Injectable Antiretrovirals (ARVs) Under Investigation

2.1 Rilpivirine Long-Acting

The non-nucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine is now commonly used for the treatment of HIV infection in combination with other ARVs at an oral dose of 25 mg once daily [19]. A parenteral long-acting formulation of rilpivirine is also being developed in order to improve adherence to cART and to investigate its potential use in PrEP [20, 21]. The long-acting formulation consists of a solid drug particle nanosuspension produced by milling of large fragments of drug until particles within the nanometre range are achieved.

A clinical pharmacokinetic study investigating the plasma and genital tract concentrations in 60 women and six men given intramuscular (IM) depot injections of three different doses of rilpivirine long-acting (300, 600, and 1200 mg) showed that rilpivirine concentrations in females were between 26 and 77 % of plasma concentrations in cervico-vaginal fluid and between 45 and 379 % of plasma concentrations in cervico-vaginal tissue, while in males, they were between 33 and >77 % of plasma concentrations

in rectal fluid and tissue [22]. Importantly, the study drug was well tolerated, with no high-grade adverse events or laboratory abnormalities being observed [21]; in particular, there were no hypersensitivity reactions or new electrocardiographic (ECG) abnormalities. Of note, during oral rilpivirine development, doses higher than that approved for treatment, of 75 and 300 mg once daily, were shown to cause QT interval prolongation. However, the drug exposure achieved with the licensed oral dose of 25 mg once daily has been shown to be safe in this respect. To date, rilpivirine exposures achieved following single and multiple IM administration of the long-acting formulation have not been associated with ECG alterations in humans [22, 23]. However, further data on the safety of multiple doses of this agent will be produced in the near future by ongoing phase I/II studies.

Therefore, rilpivirine long-acting appears to be a promising drug for PrEP, and phase II studies are now ongoing to confirm the appropriate IM dose and frequency of dosing. Global phase III studies are being designed and aim to establish the optimal strategy for efficacy and implementation.

Concerns associated with the use of rilpivirine as a PrEP agent centre around the requirement to preserve its use for the treatment of chronic HIV infection. These concerns stem from its low genetic barrier to resistance [24, 25].

2.2 Gsk1265744

GSK1265744 is an investigational integrase inhibitor that is being studied both as oral and parenteral formulations. It is characterized by a long half-life and activity against raltegravir/elvitegravir-resistant HIV clinical isolates [26]. When administered to HIV-infected individuals, GSK1265744 was shown to be efficacious in reducing viral replication and was safe and well tolerated [27]. The long-acting parenteral formulation of GSK1265744 (also a nanosuspension produced by milling) constitutes another long-acting therapy for potential deployment in the treatment of chronic HIV infection and/or PrEP.

Single GSK1265744 injections were generally well tolerated in healthy volunteers, and produced apparent plasma half-lives ranging between 21 and 50 days, supporting once-monthly or even longer dosing intervals [28]. Furthermore, a recent study conducted in rhesus macaques showed that two IM doses 1 month apart protected eight out of eight animals from multiple rectal exposures to simian/human immunodeficiency virus (SHIV), while all eight animals receiving placebo injections acquired infection [29]. These early results are likely to drive further research of this agent for potential as PrEP and possibly lead to studies in humans.

Like rilpivirine, the optimum use of GSK1265744 will likely require its combination with other ARVs or with oral formulations. In this regard, a recent study of the use of repeat-dose co-administration of the two long-acting parenteral nanosuspensions, GSK1265744 and rilpivirine, has provided important data [30]. This study aimed to investigate the safety and pharmacokinetics of the two agents administered to HIV-negative healthy volunteers as a combination. Two out of the four subject cohorts received loading doses of GSK1265744 (800 mg IM) on day 1, followed by a lower dose of 200 or 400 mg IM every 4 weeks (four doses in total). On week 8, rilpivirine long-acting was also administered at a loading dose of 1200 mg IM, followed by a lower dose of 900 or 600 mg IM 4 weeks later. Interestingly, the pharmacokinetic studies of both agents indicated that monthly injections were able to maintain plasma drug concentrations greater than four times the IC_{90} of HIV wild type for GSK1265744 and comparable to the approved oral dose (25 mg once daily) for rilpivirine [30]. The study drugs were safe and well tolerated, with only mild to moderate injection site reactions observed in the majority of study subjects. Further investigations involving both agents both as oral and parenteral formulations are now planned, and will help elucidate the efficacy and durability of this two-drug combination.

2.3 Ibalizumab

Ibalizumab is a non-immunosuppressive investigational monoclonal antibody that binds to the CD4 receptor, the primary receptor for HIV, and inhibits viral entry. It is being investigated as an HIV entry inhibitor with the ability to alter the CD4-gp120 complex and prevent attachment and entry. Ibalizumab is currently undergoing phase III assessment, but results from phase II studies showed that HIV-infected individuals who received the drug in combination with an optimized background regimen, maintained a considerably greater reduction in viral load and experienced a statistically significant increase in CD4+ cells than individuals who were administered the optimized background regimen with placebo [31]. Notably, ibalizumab was well tolerated and safe, and it was administered intravenously and showed prolonged plasma exposure of up to 4 weeks post-dose [32].

A new formulation of ibalizumab (TMB-108) for subcutaneous administration is also currently being studied in HIV-uninfected individuals. Since early data suggest the potential for once-monthly dosing, TMB-108 is being investigated for its utility as a PrEP [33]. Further advantages of ibalizumab stem from its initial favourable safety profile and novel and unique mechanism of action, which would block HIV entry at the earliest stages of the infection

[32]. Data on tissue penetration, efficacy and potential for implementation as a PrEP agent are warranted.

2.4 Other Agents

The pharmacokinetics of other ARV agents (i.e. atazanavir, ritonavir) has also been investigated in pre-clinical studies in rodents and other mammals following the administration of long-acting injectable nanoformulations. The authors concluded that ARV exposure from such formulations was enhanced compared with that of the original drugs and further research in humans was warranted [34–36].

3 Challenges and Opportunities Associated with the Use of Long-Acting Injectable ARVs

Nanotechnology provides previously unobtainable opportunities with the potential to provide substantial benefits for HIV-infected individuals. These advantages include the potential for lower ARV dose without compromising efficacy of the treatment. Solid drug nanosuspensions have also recently been developed using a novel process that does not involve milling for potential dose reduction after oral administration of ARVs [37], and phase I studies are planned. While in their current form these formulations do not offer the sustained-release potential of the IM depot formulations, the underlying technology is more broadly applicable across drugs than milling and may increase the number of ARVs that can be formulated in this way.

Administration of long-acting depot formulations offers the potential to avoid daily oral tablet intake and therefore pill intake exhaustion and need for drug holidays, improved patient-to-patient consistency in drug intake, reduced uncertainty by the physician of patient compliance to cART, avoidance of selective adherence, early detection of non-adherence with lower rates of virological failure, and, importantly, a lower inter-individual variability in drug exposure. Other advantages offered by long-acting injectables when compared with conventional oral ARV formulations include reduced renal, hepatic and other toxicities (i.e. gastrointestinal), as a result of the lower doses achievable through avoidance of first-pass metabolism. However, whether long-acting ARV will reduce chronic toxicity is still a hypothesis and needs to be proven. Moreover, injectable ARV may be associated with injection site reactions, which may limit their use.

Avoidance of first-pass metabolism markedly improves bioavailability and might allow for the administration of drugs that are usually boosted by ritonavir (because of their high susceptibility to first-pass metabolism by the cytochrome P450 [CYP] system) to be given unboosted or with a lower dose of ritonavir. Furthermore, the avoidance of first-pass metabolism in both the gastrointestinal tract and liver might reduce, but not eliminate, the high potential for drug–drug interactions that currently complicates patient management with many ARVs (mainly NNRTIs, boosted protease inhibitors and maraviroc) [38–41]. The more prolonged and consistent drug delivery results in stable plasma exposures over time, which may minimize side effects, especially those that occur within hours of taking medication and are associated with the achievement of high maximum drug concentrations (C_{max}) (e.g. dizziness following efavirenz intake).

If nanoformulations that require lower doses can be effectively developed, validated and translated, they have the potential to reduce treatment costs, which may be particularly important in resource-limited settings where the active pharmaceutical ingredient accounts for the significantly greatest proportion of the total cost of the medicine. Data on the potential reduction in the cost of medical care of HIV-infected individuals should be produced if ARV parenteral formulations become widely available. Ultimately, this is likely to be complex and related to the specific drug, loading and underpinning technology as well as a plethora of process variables, but cost is a key consideration for the ultimate implementation potential of the strategy.

HIV treatment has been shown to be a critical component of HIV prevention (treatment as prevention [TasP]) [42]. Individuals on stable cART should be 100 % adherent to their medication to ensure long-term virological suppression and efficacy of TasP. Therefore, by improving adherence, long-acting injectables may play an important role in large-scale HIV-infected populations where TasP is fundamental to abrogating the spread of HIV infection.

Long-acting injectables may also be a fundamental research innovation to eliminate perinatal transmission (mother to child transmission [MTCT]). In recent times, infants born from HIV-infected mothers receive cART for at least 3 weeks after birth, and this sometimes involves the administration of liquid formulations containing alcohol or tablets crudely broken down to provide an approximately correct dose. A single dose of one or more ARVs administered parentally as a long-acting formulation would clearly improve management in this setting. Even if appropriate long-acting formulations are not available, solid drug nanoparticle suspensions offer the opportunity to deliver oral suspensions that do not contain a solvent to aid dissolution [37].

Moreover, in the context of PrEP, long-acting injectables may be beneficial as they are less behaviourally dependent and they can ensure optimal concentrations at the site of exposure to HIV.

Importantly, data are needed on how to manage toxicity following the administration of long-acting injectables.

Whether using an oral lead-in dose to facilitate the management of adverse effects (in terms of being able to stop drug intake and therefore the cause of reversible drug toxicity) or a system (i.e. an antidote) to enable rapid clearance, strategies are necessary to limit prolonged side effects that would inevitably persist until plasma concentrations fall after the administration of a long-acting formulation.

Prolonged drug persistence may also be undesirable in terms of the development of drug resistance as a result of selective pressure, and an increase in drug resistance would occur in the presence of diminishing and low drug concentrations.

Knowledge of the length of time required to achieve steady-state for parenterally administered drugs is essential since oral drug intake may have to continue until optimal drug exposure is achieved. Existing data on the pharmacokinetics of long-acting compared with oral formulations have shown a longer time to maximum concentration (T_{max} can be several days rather than hours) and a longer time with concentrations close to the C_{max} (days or weeks rather than hours). This may raise different safety concerns than oral dosing but also suggests efficacy targets may relate to C_{max} rather than minimum concentrations (C_{min}).

ARV treatment remains dependent on combination therapy, and individual long-acting drugs will require further development with other drugs to provide long-acting combinations. Ideally, these will be co-formulations providing multiple agents in a single injection of limited volume.

Disadvantages of parenteral administration may include pain, erythema and nodule formation at the site of injection and, even if infrequently, nerves and veins may be damaged. Competent injection technique and dependence on patients attending medical care centres and/or trained healthcare workers for drug administration at defined intervals are other challenges that require consideration in parallel with the development of technology. Furthermore, because of the stigma associated with HIV infection worldwide, the requirement to attend a 'depot clinic', which may be set up in the case of cART or PrEP becoming administered parenterally, may not be ideal for the infected population globally. Therefore, further data on the acceptability of these treatments are needed in order to avoid delayed or missed injections, because if long-acting ARVs are not re-administered on time, there is the potential for long periods of low drug concentrations while the virus starts to replicate and develop resistance.

4 Conclusions

New long-acting formulations involving parenteral administration may find application for the treatment and prevention of HIV infection, as they are characterized by important advantages over oral agents and may lead to improved efficacy and reduced toxicity. However, this approach is not without limitations and there are many potential difficulties for implementing their administration globally that must be taken into consideration. Nonetheless, investigational long-acting parenteral agents are being investigated for the treatment and prevention of HIV infection, and results of phase III clinical trials with one or more long-acting ARVs will be available in the near future. Such trials are being conducted in several high-risk populations (both in the Western world and Africa) and should be able to answer some key questions about the potential of parenteral ARVs in the context of the overall acceptability of such a strategy for HIV treatment and prevention. The latter should also incorporate behavioural and structural interventions to ensure success.

Conflicts of interest Dr. Marta Boffito was principal investigator on the SSAT040 study aimed at investigating the pharmacokinetics of rilpivirine long-acting, funded by the Bill and Melinda Gates Foundation. Dr. Marta Boffito and Prof. Andrew Owen have received funding from the Engineering and Physical Sciences Research Council towards Nanomedicine Interventions for HIV/AIDS.

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The promise and pitfalls of long-acting injectable agents for HIV prevention

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Purpose of review
Preexposure prophylaxis for HIV prevention is highly effective when taken as prescribed. Adherence to required dosing regimens for protection may pose challenges. Long-acting agents for HIV prevention may have the potential to improve adherence via favorable pharmacokinetics supportive of infrequent dosing. This review focuses on the potential benefits and considerations for the study and use of 2 long-acting injectable agents, cabotegravir (GSK1265744LA, CAB LA) and rilpivirine (TMC278LA, RPV LA), for use as chemoprophylaxis for HIV prevention.

Recent findings
Oral RPV is United States Food and Drug Administration approved for HIV treatment (in combination with other antiretrovirals). Both CAB LA and RPV LA are currently in phase 2a safety/tolerability/pharmacokinetic studies in anticipation and support of future efficacy evaluation. Both agents have favorable pharmacokinetics, and use is complicated by injection site reactions.

Summary
Long-acting injectable formulations, if safe and well tolerated, may improve pharmacokinetic coverage of exposures to HIV infection. Complexities around safety, tolerability, and starting/stopping protocols require careful consideration.

Keywords
cabotegravir, HIV-1, long-acting injectable antiretroviral, preexposure prophylaxis, rilpivirine

INTRODUCTION

Chemoprophylaxis for the prevention of HIV infection has been revolutionized by recent studies showing high levels of protection against rectal exposures by daily oral tenofovir/emtricitabine (TDF/FTC) [1,2▪–4▪] and against vaginal exposure in a study of HIV discordant couples [5]. Robust efficacy data led to the approval of daily oral TDF/FTC by the US Food and Drug Administration (FDA) in 2012, for men and women who are at high risk of becoming HIV infected. The registrational studies also indicate a clear dose-response relationship of protection and adherence [6,7]. Although rectal protection afforded by daily oral use of TDF/FTC has been modeled to be 99% when taken 7 days per week as prescribed, modeled data and clinical cohorts suggest some forgiveness of missed doses for protection against rectal exposures – as few as 4 doses per week appears to preserve high levels of protective efficacy [2▪,8]. Protection against vaginal exposures has been less rigorously described, but is modeled to be much less forgiving of missed doses than are rectal exposures [9]. This is believed to be attributable to differential tissue pharmacokinetics of the components of TDF/FTC in rectal and cervicovaginal matrices [10]. The challenges of daily or near-daily oral dosing strategies, and the long-term maintenance of such dosing have driven interest in preexposure prophylaxis (PrEP) agents that have more convenient dosing schedules.

The field of long-acting injectable agents has substantial precedent among antipsychotics (e.g., paliperidone palmitate) and contraception (e.g., medroxyprogesterone acetate). Removable depots of contraceptive agents (Norplant), and transdermal patches for sustained drug delivery are attractive, but are limited by molecular size and chemical properties, including hydrophobicity and charge.

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KEY POINTS

- PrEP effectiveness is compromised by challenges around need for regular adherence.
- Long-acting injectable agents offer pharmacokinetic advantages, but do not entirely solve adherence challenges.
- Use of a long-acting agent that cannot be removed after administration obligates a short-acting lead-in to establish safety and tolerability.
- Prolonged pharmacologic tail makes discontinuation challenging, particularly if exposures/risk are ongoing – with concern for seroconversion with resistant viral quasispecies.
- Injections may be differentially acceptable in diverse populations.

Technologies are evolving rapidly, and a recent presentation of an implantable system appears capable of delivering tenofoviral-afenamide in sustained fashion in a dog model [11].

For HIV, long-acting agents have the potential advantage of requiring less-than-daily dosing intervals, some dosed as infrequently as every 2–3 months. All long-acting HIV antiviral agents currently in development require parenteral injections – via subcutaneous, intramuscular (IM), or intravenous routes of administration. Once administered, a long-acting injectable agent for prevention of HIV would anticipate provision of 'coverage' for HIV exposures during the period at which protective levels of the agent remained in the individual. Although the exact tissue and/or or plasma correlates of protection remain undefined, long-acting injectable agents are intended to provide sustained drug levels in serum, plasma, and relevant mucosal tissues. They additionally have the potential for reduced gastrointestinal toxicity, as well as avoiding some drug–drug interactions. Although long-acting agents would obviate the need for daily pill taking, they raise novel challenges regarding adherence, safety, and optimizing starting and stopping mechanisms. Two chemical entities are in advanced stages of clinical development for long-acting prophylactic use (Table 1).

It will be important to evaluate whether cultural or social norms will make the use of injections for HIV prevention differentially acceptable in diverse populations globally. For this reason, evaluation of such perceptions and acceptability are important to capture as part of early phase development programs.

Cabotegravir

Cabotegravir (CAB, formerly GSK 1265744, ViiVHealthcare) is a novel investigational strand-transfer integrase inhibitor. CAB is a chemical

Table 1. Comparison of agents in advanced clinical development

	Cabotegravir	Rilpivirine
Class of agent	Strand-transfer integrase inhibitor	Nonnucleoside reverse transcriptase inhibitor
Oral half-life (plasma)	40 h	50 h
Injectable half-life (plasma)	≈21–50 days	≈35 days in women
		≈33 days in men
Stage of development	Treatment: phase 2b (oral/LA)	Treatment: approved for treatment of HIV-1 infection, May 2011 (oral); phase 2b (LA)
	Prevention: phase 2a (oral/LA)	Prevention: phase 2 (oral/LA)
Toxicity	Headache	Headache
	Nausea	Nausea
	Diarrhea	Diarrhea
	Insomnia	Nasopharyngitis
	Increased LFTs	Insomnia
		Dizziness
		Increased serum creatinine
		Increased LFTs
		Decreased serum cortisol
To date exposure to LA	≈500	>150
Percentage ISR (IM)	74%	95%

LA, long acting; IM, intramuscular; ISR, injection site reactions; LFT, liver function test.

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congener of dolutegravir, with nanomolar in-vitro activity against HIV-1 clinical isolates. It is available as both a short-acting oral formulation with a half-life of 40 h, and a long-acting injectable formulation comprised solely of active drug nanocrystals and not encased in a polymer, micelle, or lipid matrix. The long-acting formulation has a plasma half-life of 21–50 days. Macaque rectal and vaginal challenge models demonstrate high levels of protection against SHIV acquisition at supraphysiologic challenge doses at drug levels above threefold the PA-IC90 for rectal exposures and fourfold PA-IC90 for vaginal exposures (PA-IC90 is 0.166 μg/ml) [12,13]. Oral CAB 10 mg QD approximates such levels at steady state. CAB LA at a dose of 600–800mg administered every 8–12 weeks provides trough levels approximately six-fold PA-IC90. The 800 mg dose is administered as 2 simultaneous gluteal IM injections of 2 ml each, after which plasma levels are detectable up to 52 weeks after an injection. Because there is no way to remove the drug once injected, a 4-week 'lead-in' strategy using oral CAB has been utilized to establish safety parameters. A 30 mg QD dose has been chosen for the oral lead-in to provide sufficient margin of excess above the 3–4-fold PA-IC90 prophylactic target level, while being a sensitive probe for drug-related adverse events. Ideally, a long-acting injectable agent aimed at those challenged by daily oral pill taking would not require an oral lead-in period; however, this requirement is likely to persist absent extensive safety experience with such agents.

CAB, in combination with other anti-HIV antiretroviral agents, has been investigated for treatment of HIV infection in phase 2a and ongoing 2b studies. In the LATTE trial, oral CAB at doses of 10, 30, and 60 mg daily, in addition to 2 nucleoside-analog reverse-transcriptase inhibitors, were virologically noninferior to a dual-nucleoside plus efavirenz regimen; and virologic noninferiority was maintained after virologically suppressed CAB-treated participants 'simplified' their regimens to CAB plus oral rilpivirine (RPV) at both 48 and 96 weeks of total treatment [14,15]. The ongoing LATTE-2 trial is evaluating oral CAB 30 mg QD with dual nucleosides as a lead-in to virologic suppression prior to transition to IM CAB LA plus IM RPV LA (dosed 400 mg (1 × 2 ml) every 4 or 600 mg (1 × 3 ml) every 8 weeks – each with an 800 mg (2 × 2 ml) loading dose, and the every 8-week regimen with an initial one-time week 600 mg supplement); again, efavirenz with dual nucleosides is the control comparator.

Evaluation of CAB as a potential PrEP agent is currently in phase 2a evaluation. The ÉCLAIR study enrolled 127 US-based HIV-uninfected low-risk men in a safety, tolerability, and pharmacokinetic evaluation that employed a 4-week oral lead-in followed by 3 quarterly IM injection doses, with a 52-week follow-up period after the final injection. ÉCLAIR is randomized 5 : 1 active CAB : placebo, with results expected in early 2016. The ongoing HPTN 077 is enrolling 194 low-risk men and women globally to further evaluate safety, tolerability, and pharmacokinetic in broader populations. A 3 : 1 active CAB : placebo randomization is being used, with data expected in early 2017. It is anticipated that these phase 2a studies will be followed by efficacy studies in populations of men, transgender women, and women at high risk of acquiring HIV infection. Considerations for the design of phase 3 efficacy studies for prevention of rectal exposures are complex and nuanced now that daily oral TDF/FTC has a robust supportive body of evidence for HIV prevention; the results of upcoming dapivirine ring studies, if positive, may additionally complicate these issue for women. A detailed discussion of the ethics of phase 3 efficacy study designs for HIV prevention is provided in an additional article in this issue [28].

Rilpivirine

Rilpivirine (RPV, Edurant, Janssen Scientific, USA) was approved by the FDA in May 2011 for the treatment of HIV-1 infection at an oral dose of 25 mg daily. It is a small-molecule non-nucleoside reverse-transcriptase inhibitor with picomolar activity against HIV-1 primary clinical isolates [16]. Oral RPV is indicated for treatment of HIV-1-infected individuals with viral loads <100 000 cells/ml in combination with 2-NRTI's, and is being developed as a long-acting injectable preparation (also referred to as TMC278LA or RPV LA) [17]. RPV LA has undergone a series of formulation revisions to optimize pharmacokinetics: The particle size and suspension fluid have evolved, with the current formulation (G001) having a particle size of approximately 200 nm in a poloxamer-338 suspension of 300 mg/ml of RPV. Like cabotegravir, RPV comprises the pure parent compound and is not encased in a nanoparticle or micelle. The dose being brought into prevention trials is 1200 mg, requiring 2 × 2 ml IM gluteal injections. For treatment, RPV LA is being evaluated at doses of 600 mg (1 × 2 ml) every 4 weeks, and 900 mg (1 × 3 ml) every 8 weeks.

Humanized bone marrow/liver/thymus mouse studies suggest that RPV LA levels remain above the IC90 level for 4 weeks after a single IM dose, as well as providing protection against vaginal viral

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challenges for 3–4 weeks after dosing [18]. Phase I studies after single doses suggest pharmacokinetics supporting every 8-week dosing for prevention, as well as approximately two-fold excess concentration in rectal tissue over cervico-vaginal tissues [19]. Explant challenge models suggest rectal tissue protection as delayed as 4-months after dosing, but absence of such protection in a comparable cervicovaginal model [20]. One female participant, followed after a single dose of 300 mg IM of RPV LA in a compartment pharmacokinetic study reporting an HIV exposure approximately 40 days after injection, seroconverted approximately 120 days after dosing (80 days after exposure) with K101E virus, conferring resistance to the non-nucleoside reverse-transcriptase inhibitors class [21▪].

RPV LA is now being evaluated in HPTN 076, a phase 2a safety and acceptability study enrolling 132 HIV-uninfected low-risk women in the USA and sub-Saharan Africa in a 2:1 active RPV: placebo randomization. The study also employs a 4-week oral lead-in (uniquely employing direct observed therapy to assure oral drug exposure prior to long-acting dosing), followed by a series of 6 injections of 1200 mg each at 8-week intervals, followed by a 32-week observational period during drug 'washout.' Results are expected in early 2017.

ADHERENCE

Although long-acting agents obviate the need for a daily or peri-coital pill-taking activity, adherence to injections still requires consumers to comply with even infrequent injections. During clinical trials, injections are administered in a clinic-based setting as a gluteal intramuscular injection – essentially a directly observed therapy strategy if the individual presents to the clinical appointment. If a long-acting agent becomes approved for prevention by regulatory agencies, issues regarding administration will require thoughtful consideration, either employing family members/friends to give the injections or potentially allowing self-administration. Although potentially offering the advantage of not requiring clinic visits for administration, towards: homedosing becomes challenging to characterize and track. Decay pharmacokinetics will need to be sufficiently well defined as to allow clear guidance to users how 'late' after the next injection is due protection would be expected to be maintained, and careful guidance developed for late or missed doses. The injectable contraception literature suggests a high rate of nonadherence after initial injectable hormonal contraception use [22,23], and for this reason, even a long-acting injectable PrEP formulation would not be expected to solve adherence challenges for all patients.

SAFETY AND TOLERABILITY

As both cabotegravir and RPV's LA preparations contain nanocrystals of the pure parent compound, there is hope that the long-acting preparation will not have a substantially different safety profile from the short-acting version of each respective agent – save for complications from the injection itself, injection site reactions. A potentially serious safety concern is the nonremovability of the preparation if an idiosyncratic or known adverse event (AE) occurs after the injectable is delivered.

Cabotegravir – clinical experience to date

Approximately 1000 individuals have received the non-FDA approved cabotegravir, divided approximately equally between HIV-infected and HIV-uninfected individuals, and with half receiving the short-acting oral tablet formulation only, and half receiving the long-acting preparation or both the oral tablet and the long-acting preparation. In the LATTE study, doses of 10, 30, and 60 mg daily were administered to HIV-infected treatment naïve participants in combination with 2 nucleoside reverse-transcriptase inhibitors, and compared with 2 nucleoside reverse-transcriptase inhibitors with efavirenz. Treatment emergent AEs included headache, nausea, and diarrhea for cabotegravir-treated participants. Insomnia appeared to be the only AE for cabotegravir with an AE dose–response association [24▪]. Treatment emergent elevations in alanine aminotransferase (ALT) were more common in cabotegravir groups, and were correlated with dose level. Three participants (2 with evidence of steatohepatitis at baseline and 1 HIV/HCV coinfected) developed serious ALT elevations. All were clinically asymptomatic and ALT abnormalities resolved with withdrawal of the study medication. For the long-acting preparation, no ALT abnormalities have been reported requiring discontinuation of study product to date.

In completed studies, ISRs occurred in the majority of participants following IM (74% with any ISR) dosing; however, the reactions were generally mild to moderate (overall ISR Grade 2: 14% in IM without any Grade 3 or 4 ISRs) [25]. The most frequent ISRs for IM dosing were pain (71%), erythema (9%), and nodules (7%). Median IM ISR durations were approximately 5 days for pain and erythema, and approximately 22 days for nodules [26].

Data from the ongoing ÉCLAIR and LATTE-2 studies are preliminary, but has generally been consistent with prior data with few withdrawals

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because of injection-related tolerability (3 discontinuations in ongoing studies). The majority of ISRs has been mild or moderate, with a small percentage of participants reporting more significant (Grade 3) pain/discomfort.

Concern has been raised about the volume of injections and their acceptability. The current preparation of long-acting cabotegravir is 200 mg/ml; doses under evaluation for treatment and prevention therefore would be 3–4 ml. A 3 ml injection could potentially be delivered as a single injection; a 4 ml total injection volume would have to be administered as 2 simultaneous 2 ml injections; one in each buttock. The acceptability of such injections at 4–12 week intervals is currently under evaluation in ongoing phase 2 trials.

Rilpivirine – clinical experience to date

Over 2000 study participants were exposed to oral RPV (TMC278) during its clinical development, and it was approved by the FDA for the treatment of HIV-1 infection in combination with other antiretroviral agents in 2011. In the phase 3 registrational studies, the most commonly reported AEs in the TMC278 group were headache, nausea, diarrhea, nasopharyngitis, insomnia, and dizziness, all with similar incidences in the control group, except for dizziness [27]. The majority of AEs were mild to moderate in severity. Treatment emergent laboratory abnormalities include increases in serum creatinine (usually 0.1 mg/dl), reductions in serum cortisol (and ACTH-stimulated cortisol), and increases in ALT, aspartate aminotransferase, and total bilirubin. Postmarketing experience has found instances of nephrotic syndrome and severe cutaneous reactions including drug reaction with eosinophilia and systemic symptons.

To date, more than 200 individuals have been exposed to TMC278 LA in completed and ongoing studies. Overall, the injections have been well tolerated and safe. ISRs have been the most common adverse events. There have been no safety or tolerability observations that preclude multiple dosing.

STARTING STRATEGIES: THE ORAL LEAD-IN

As mentioned above, both cabotegravir and rilpirivine's developmental programs are employing an oral lead-in period prior to the administration of the long-acting preparation. Although it is somewhat antithetical to the purpose of developing a long-acting preparation to obligate a daily oral run-in, at minimum until the safety profile of the long-acting preparation is informed by a larger number of exposures, an oral phase will likely be part of both product's initial labeling if approved for HIV prevention.

Clearly, a given person's tolerance of an oral lead-in does not obviate concern for an idiosyncratic or late-onset toxicity at a later time-point after injection; however, the intent is to prevent prolonged exposure in individuals who have a fulminant early reaction or issues with tolerability. Adherence to the oral lead-in period may also compromise sensitivity; counseling around the reason for the lead-in will need to be carefully crafted to maximize comprehension of the rationale, whereas the lead-in may otherwise be viewed negatively or as expendable, awaiting the desired injectable preparation. As mentioned, a directly observed therapy strategy in the rilpivirine phase 2a studies attempts to maximize preinjection exposure, however this strategy is unlikely to be tenable in clinical practice.

STOPPING STRATEGIES: THE PROLONGED PHARMACOLOGIC 'TAIL'

In considering discontinuation of long-acting injectable products, the timeline for loss of protective effect will be a critical parameter to determine: How long after the final injection does protection endure? An additional concern then becomes whether as drug levels decline during such a 'wash out,' would exposure to HIV resulting in seroconversion select for resistant virus? As noted above, a single case of seroconversion with nucleoside reverse-transcriptase inhibitors-resistant virus has been documented in a seroconversion event during the pharmacokinetic 'wash out' period of a single dose of RPV LA 300 mg during a phase I tissue pharmacokinetic study [21▪]. This single case suggests that particularly in high-risk populations in whom long-acting injectables are being used for HIV prevention, it may be advisable to 'cover' the pharmacologic tail of the injectable agent with daily oral TDF/FTC. The strength of such a recommendation would likely be governed by the reason for discontinuation and ongoing risk profile of the individual.

OTHER LONG-ACTING CHEMOPROPHYLACTIC AGENTS

Other pharmacologic preparations are in preclinical and various stages of clinical development to provide prolonged drug exposures either as a result of nanosuspensions, novel vehicles or delivery systems, or immunomodulatory effects. These include vaginal rings delivering single or multiple

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ART agents, vaginal and rectal gels, fibers, and pessaries, implantable drug delivery systems, and infused monoclonal antibody preparations (broadly neutralizing antibodies and anti-CD4/antigp120 preparations). Of course, a prophylactic HIV vaccine, particularly one providing high levels of broad protection with finite dosing and good tolerability, is the 'gold standard' from a public health perspective.

CONCLUSION

Until such time as a highly effective preventive vaccine against HIV infection is available, novel HIV prevention strategies are desperately needed to stem an ongoing HIV pandemic. Imperfections in the ability to universalize treatment and suppression of HIV-infected individuals worldwide with ART to minimize their infectivity, as well as the complicated and fluid nature of sexual dyads, leave a prevention need for those who are at risk of acquiring HIV infection. Chemoprophylaxis with HIV antiretroviral agents has been demonstrated with TDF-containing compounds; however, required levels of adher-ence to daily or near-daily oral tablets has proven challenging for some populations. Long-acting preparations promise to offer greater choice for achieving prevention, should their safety, tolerability, and efficacy be confirmed. Such preparations will not solve the need for careful follow-up for repeat dose administration, safety evaluation, and testing for HIV and other sexually transmitted infections, and in fact pose new complications related to safety at initiation, and vulnerability upon withdrawal. Similar to contraception, HIV prevention is unlikely to be a 'one-size fits all' field, and greater variety of options will hopefully provide additional alternatives to at-risk individuals, and ultimately greater numbers of individuals protected against HIV acquisition.

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Conflicts of interest

There are no conflicts of interest.

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Topical Formulations: Options & Challenges

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Formulation and Delivery of Microbicides

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Abstract: The development of pre-exposure prophylactics or microbicide products for the reduction or elimination of the sexual transmission of HIV has numerous challenges or barriers to success. Historically traditional dosage forms such as gels have been developed in the field but more recently controlled release dosage forms such as vaginal rings and novel dosage forms such as polymeric thin films have been studied. Studies have begun to incorporate scientific strategies into the formulation design of microbicide products in order to develop safer and more effective products. In addition advanced drug delivery strategies to overcome barriers to delivery and specific drug targeting methods are being employed. In the present review, a comprehensive discussion of formulation efforts and novel delivery strategies in the field of microbicide product development is presented.

Keywords: Drug Delivery, formulation, HIV Microbicide, pre-exposure prophylaxis.

INTRODUCTION

According to the UNAIDS 2010 update, an estimated 33.3 million people are living with HIV all over the world. In 2009 alone an estimated 2.6 million people became newly infected with HIV [1]. The advents of highly active antiretroviral therapy (HAART) have dramatically improved treatment options for HIV. However, considering the high incidence of new HIV infections each year, prophylactic interventions are warranted. The ideal method would be to prevent or significantly minimize the occurrence of infection, specifically focusing on the prevention of the sexual transmission of HIV.

Microbicides are products intended to prevent or significantly reduce the transmission of sexually transmitted infection, including HIV. Microbicide products are topically (rectal or vaginal) applied. A schematic representation of the various routes used for delivery of microbicides or pre-exposure prophylaxis for HIV prevention is shown in Fig. (**1**). The distribution of specific dosage forms in ongoing microbicide clinical trials is shown in Fig. (**2**). Research in application of nanotechnology for microbicide delivery is also showing an upward trend. Nanocarrier systems like polymeric nanoparticles and liposomes have already been evaluated for microbicide delivery.

This review focuses on microbicides administered/applied *via* topical routes and oral pre-exposure prophylaxis for HIV prevention. For both routes of delivery, the development of a safe and effective formulation remains a challenge. The real challenge is to deliver a sufficient quantity of drug at the local sites (cervicovaginal tissue) to combat viral transmission during sexual intercourse. Historical experience with vaginal microbicides suggests that the dosage form used should not only be well tolerated and accepted by the patients but also should not increase their susceptibility to infection. The formulation should offer a convenient (ideally coitally independent) dosage regimen and should provide ease of application/administration. Data suggests that patient convenience has a profound impact on patient adherence of these products. Other factors that can affect the adherence are the cost of the product and its availability. This review highlights the important role of formulation development and specific drug delivery strategies in the success of a microbicide product. For the convenience of the reader, the text is divided according to routes of administration and various formulations used for each route. Oral tablets containing individual or combination antiretroviral agents are under clinical evaluation as a PrEP therapy. In case of topically applied formulations, gels and vaginal rings are the most advanced in clinical trials. The remaining formulations are still at the stages of early development or preclinical testing.

ROUTE OF ADMINISTRATION

Oral Route

Oral pre-exposure prophylaxis (oral PrEP) is being evaluated as an HIV prevention strategy [2]. To date, two clinical trials evaluating the use of oral PrEP have been completed: a phase II study conducted by the CDC (iPrEx), and MTN-001. Several PrEP clinical trials are in progress: Partners PrEP, VOICE, TDF-2, iPrEx OLE and FEM PrEP studies. Currently all clinical trials either completed or in process for oral PrEP use two antiretrovirals within the reverse transcriptase inhibitor class that are approved for HIV treatment: tenofovir disoproxil fumerate (TDF) also known as Viread® or the combination product of tenofovir and emtricitabine (FTC) also known as Truvada. Both of these products are tablet dosage forms. Oral tablets are the most frequently prescribed commercial dosage form [3]. Oral tablets provide the user with a convenient, stable solid dosage form. The most promising of the completed trials

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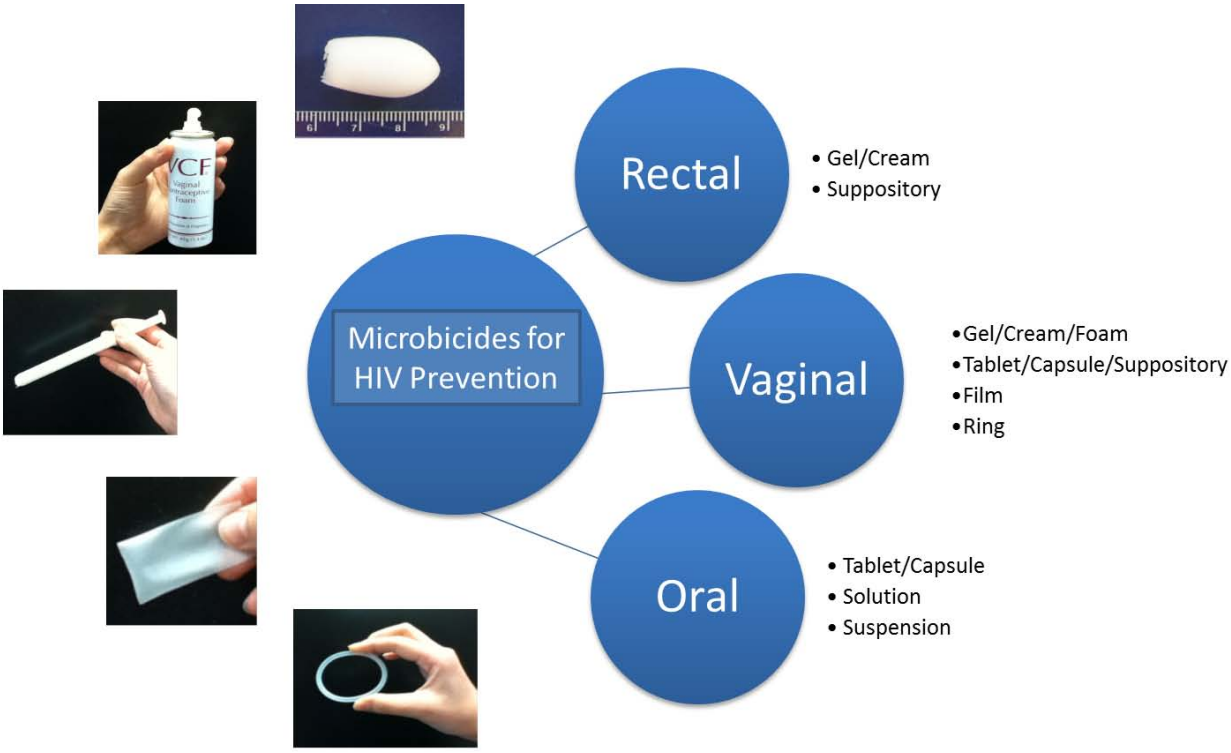

Fig. (1). Schematic description of product dosage forms used.

with oral PrEP is the iPrEx study, which was conducted in 2,500 HIV-negative homosexual men, transgender women, and other men who have sex with men [4]. The study showed the participants who took a daily Truvada had 44 percent fewer infections than participants who received a placebo tablet. This is the first evidence of oral PrEP clinical efficacy.

A second study MTN-001 which evaluated daily oral and vaginal tenofovir use in healthy sexually active women showed interesting results comparing oral and vaginal dosing [5]. Although the preference was for the oral dosage form, women using oral PrEP had greater incidence of GI disturbance as opposed to those using vaginal PrEP. Additionally, tissue levels for the active form of the drug, tenofovir diphosphate, obtained after vaginal administration were greater (1000 fold higher) than those obtained following oral administration [6]. This data suggests a potential advantage for mucosal administration over oral administration for HIV prevention drug candidates. Two mucosal sites have been evaluated for delivery of microbicide products the rectal and the vaginal route.

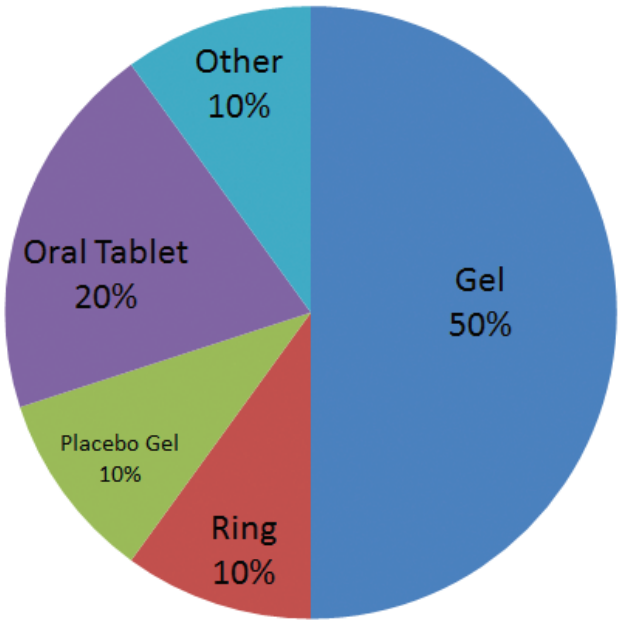

Fig. (2). Distribution of dosage form types in current microbicide clinical trials.

Rectal Route

The initial focus of microbicide researchers has been on vaginal microbicide products. However, the growing number of individuals practicing unprotected receptive anal intercourse (RAI) in both developed and developing countries [7-10] warrants the development and evaluation of rectally applied microbicide products. The risk of acquiring HIV through unprotected anal intercourse is 10 to 20 times greater than that with unprotected vaginal intercourse [11, 12]. This increased risk is likely due to compartmental differences such as the single cell lining of the rectum being a more fragile barrier than the stratified squamous vaginal epithelial and the larger surface area of the rectum available for infection.

Compared to the multiple choices for vaginal delivery of microbicide products (e.g. gels, films, rings, tablets), current rectal microbicides are more likely to take the form of vaginal gels or enema-like products. Historically gel product lubricants have been used in conjunction with anal intercourse [13, 14]. It should be noted that some marketed lubricant products are hyperosmolar due to the presence of a high concentration of glycerin. Hyperosmolar gels used rectally have been associated with rectal epithelial damage due to dehydration of epithelial cells and consequential cell shrinking [15].

Since current antiretroviral drugs being evaluated as microbicide candidates can be either water soluble (e.g. Tenofovir) or water insoluble (e.g. Dapivirine), both aqueous and lipid rectal microbicides formulations may be needed to effectively deliver drug candidates with different chemical properties. Recently, Wang *et al.* developed four rectal placebo formulations [16], which were both aqueous based (Carbopol or poloxamer) and non-aqueous based (Isopropyl Myristate or Caprylic/Capric Triglyceride) (Table **1**). Several key parameters were defined in this work. Iso-osmolality was defined as a target specification. The osmolality of the aqueous gel and aqueous fluid developed were 324±15 and 402±8 (mmol/kg; mean ± standard deviation) respectively. These values are much lower than the commonly used commercial rectal lubricants [16]. Rectal formulations should also be designed with a pH value close to neutral as the pH of the rectum is higher than that of the vagina. Also target viscosities of < 200 cps and >5000 cps for the fluid and gel formulations, respectively were used in this placebo panel. However clinical evaluation of the products is still in progress, and the impact of these viscosity values on patient acceptability has yet to be determined.

Recently, Hendrix *et al.* applied single photon emission computed tomography (SPECT)/computed tomography (CT), magnetic resonance imaging (MRI), and endoscopy to study rectal gel distribution over time and developed predictive models of distribution and clearance based on the physical properties of microbicide gel [17]. To date, rectal microbicide research is in the early phase of clinical development. Aside from a number of studies designed to investigate the behavior of placebo products in the rectum only a few studies have evaluated microbicide products containing antiretrovirals in this compartment. Two Phase I trials (RMP-01 and MTN-006) have been completed, another Phase I trial (MTN-007) is ongoing and one Phase II trial (MTN-017) is in the planning stage. These studies are focused on the safety and acceptability of tenofovir gel for rectal use.

VAGINAL ROUTE

Vaginal Gels

Gels are one of the most extensively studied dosage forms for topical microbicide delivery. Several gel products intended for vaginal application are at various stages of development. Development of first generation topical microbicides in the form of gel was most probably inspired by the history of use of various OTC sexual lubrication products. However, the failed anti-HIV efficacy trials of nonoxynol (N-9) and cellulose sulfate gels highlight serious safety considerations [18]. Since then the field of vaginal microbicide gels has walked a considerable distance. Recently published data from CAPRISA trial is a significant boost for the concept of topical microbicide use as a whole and vaginal gels in particular. Results provided the much needed proof-of-concept that a topical microbicide can be effective. This study involved the administration of 1% tenofovir gel vaginally, with one application of gel no more than 12 hrs before and one application no more than 12 hrs after intercourse (No more than two applications per day). The results showed a statistically significant protection (39%) from HIV infection [19]. Various other trials involving vaginal microbicide gel products are at various stages of clinical development [20].

In general, gels are relatively easy to manufacture, requiring less complicated manufacturing equipment and process. Gels may also contribute to enhance sexual pleasure during intercourse [18]. Vaginal gels most commonly employ water soluble polymers such as gelling agents. Cellulose derivatives like hydroxyl ethyl cellulose (HEC) and poly acrylic acid derivatives like carbopol 974P are the most commonly used polymers due to their GRAS status, cost effectiveness, and safety profile. The universal placebo gel used as a placebo control in a large number of clinical trials consists of 2.7% w/w HEC. The 1% tenofovir gel tested in the CAPRISA trial also contains 2.7% w/w HEC. Another common practice in the formulation of gel products is to include preservatives. A combination of methyl paraben and propyl paraben is one of the most widely utilized

Table 1. Four Rectal Placebo Formulations

		Base Ingredient	Osmolality(mmol/kg)	Rheological Properties	Feature
Aqueous	Fluid	Poloxamer (weight 15%)	402±8	low viscosity initially for rapid distribution upon administration followed by increase in viscosity at body temperature	more localized retention
	Gel	Carbopol (weight 0.5%)	324±15	relatively high viscosity and shear thinning /rapid viscosity recovery	distribution initiated by rectal intercourse/localized retention following distribution
Lipid	Fluid	Isopropyl Myristate (weight 5%)	NR	being relatively thin with little temperature dependence	distribute rapidly and efficiently after rectal administration
	Gel	Caprylic/Capric Triglyceride (weight 74.9%)	NR	high viscosity and shear thinning/slow viscosity recovery	distribution initiated by rectal intercourse/continued distribution

preservative systems in microbicide products currently being evaluated. Many vaginal gels also content cosolvents/humectants like glycerin, polyethylene glycol and propylene glycol. For a more detailed discussion on commonly used pharmaceutical excipients, the reader is referred a review article by Garg *et al.* 2001 [21]. Most microbicide products being studies are aqueous based gels. These gels are generally manufactured by the addition of polymers and other excipients to water with continuous mixing. Normally an overhead mixer with a suitable propeller is used. Complete hydration of the polymers is a very critical requirement and becomes increasingly complex with increased batch size [22].

Preclinical data on vaginal microbicide gel products using polymer bases other than HEC and carbopol is also available. Recently a non-aqueous silicone elastomer based vaginal gel for the delivery of the hydrophobic CCR5 antagonist maraviroc has been reported. The object of this study was to develop a once daily vaginal gel capable of maintaining protective local concentration of microbicide for a prolonged period. *In vitro* release of maraviroc from the gel was studied in vaginal fluid simulant and isopropanol/water system (1:1 and 1:4 ratio) and compared to maraviroc release from aqueous gel consisting of 2.2% w/w HEC. In all three media the rate and extent of drug release from HEC gel was significantly higher than form silicone polymers. However, a pharmacokinetic study in macaque monkeys revealed that the silicone gel was responsible for maintaining a significantly higher maraviroc concentration (up to 24 hrs) in plasma, vaginal fluids, and vaginal tissue. Not much is known about the mucosal toxicity of the silicone polymers. Therefore, further studies are needed to evaluate toxicity and efficacy of this type of gel product [23].

Most of the vaginal gel formulations are developed based on empirical experience of other semisolid products. Recently Mahalingam *et al*. attempted to establish a quantitative relationship between composition of a vaginal gel product and its properties and performance for combination delivery of tenofovir and UC781 using a mixture design of experiment (MDOE) approach. In this study, a series of prototype formulations were manufactured varying the levels of HEC (0-3% w/w) and carbopol 974P (0-2.5% w/w) and characterized for various physical and rheological properties. Computational models were then used to derive a relationship between the composition of the gels and the rheological properties and an effect of variation in levels of gelling agents (individual and in combination) on rheological properties of gels. Further, properties of the gels were correlated to its performance using an optimized fluid flow model. The rheological parameters generated above and other parameters such as the volume of gel, height of the bolus, dimensions of the vagina, and the force exerted by vaginal muscles were used as input functions for the fluid flow model. The response performance in terms of the spread-ability of the gel on the vaginal mucosa was computed. An objective function (called score S) was designed to serve as the DOE response variable. The domain of the function S was defined from zero to 1 in such a way that when score S for a gel equals 1, the gel fully meets performance requirements (in this case spread-ability related coating of vaginal mucosa). Computed scores then were used to generate response surfaces. A typical response surface graphically depicts the relationship between gel composition and the score (which represents the corresponding performance of a prototype gel). Ultimately, it was concluded that a gel containing 3% w/w HEC and 0% carbopol 974 P would completely coat the vaginal surface [24].

An ideal vaginal microbicide gel should have enough spread-ability to coat as much vaginal surface mucosa as possible (ideally 100%). The composition of the gel can affect the spread-ability as discussed by Mahalingam *et al.* [24]. Barnhart *et al.* used MRI imaging to demonstrate that gels varying in their composition exhibited major differences in their coverage of vaginal mucosa. Further, the response of different gels' vaginal coverage after simulated intercourse or dilution was also different from each other. This indicates that the composition of the gel could play an important role in defining how it covers vaginal surface after application (and thereby offers protection against infection). Hence a greater understanding of relation between various formulation components and the spreading properties of gels could be vitally important [25].

Several product parameters can contribute to product safety. The excipients (including the polymers), the pH, and the osmolality of a vaginal microbicide gel could have profound effects on the vaginal environment and epithelium which could result in toxicity or enhanced infection [18]. Clinical trials of a gel containing the surfactant N-9 have pointed out that even a minor perturbation of the vaginal membrane could significantly increase the susceptibility of women towards HIV infection. Further, patient adherence to the product could be compromised if the vaginal gel causes irritation. These issues must be kept in mind while selecting polymers and excipients for development of vaginal gels. The acidic pH of vaginal environment is created by hydrogen peroxide secreting *Lactobacillus*. Studies suggest that the susceptibility of the male-to-female sexual transmission of HIV infection increases with a rise in vaginal pH [26, 27]. Hence the vaginal gel should not have any toxic effects towards innate vaginal lactobacilli. Adriaens *et al.* showed that hyperosmolarity is associated with mucosal irritation and tissue damage in the slug mucosal irritation model [28]. Further it was shown that several commonly used gel excipients like glycerin and disodium EDTA increased the susceptibility of mice towards HSV-2 infection in a dose dependent manner. Propylene glycol and polyethylene glycol-8 also increased the HSV-1 susceptibly by a greater than 10 fold in this mouse model [29]. This paper suggests that selection of excipients and their concentration is crucial as pharmaceutical excipients may compromise safety and contribute to increased susceptibility of infection.

In vivo testing of microbicides is a challenging area. The macaque model has been used to evaluate safety and has been the model of choice for testing microbicide efficacy because of its similarities with female genital tract. However the cost involved and animal availability issues are barriers to the widespread use of this model. Research on the development of alternative animal models is ongoing. Humanized mouse models harboring HIV-1 target cells are reported for the testing microbicide efficacy. The SCID-hu-PBL humanized mice model suffers from the drawback of

variable infection rates, raising concerns of reliability [30, 31]. The BLT mouse model is an improved version of the SCID-hu mouse model [32]. More recently, Neff *et al.* used the RAG-hu humanized mouse model for testing the *in vivo* efficacy of a vaginal gel containing maraviroc [33]. The rabbit model has also been used for safety and pharmacokinetic evaluation of microbicides. This model is cost effective but there are considerable anatomical and physiological differences between the rabbit and human vaginas. The sheep model has also been used for the toxicity evaluation of vaginal microbicide rings [34].

Although CAPRISA 004 has shown that a vaginal gel have the potential as a topical microbicide delivery system, they suffer from some inherent limitations. An applicator is needed for the application of each dose and gels are prone to leakage from the vagina. Vaginal gels are often associated with a messy feeling in women. A recent clinical trial of carrageenan based microbicide gel reveled that only about 42% of participating women adhered to the treatment regimen [35]. Also, most gel products must be used in a coitally dependent manner. There is a consensus amongst the scientific community that a dosage form independent of coital action is more suitable as a microbicide product. Intravaginal rings provide an important option in this regard.

Vaginal Rings

Vaginal rings offer the advantage of sustained or controlled delivery of drug. Vaginal rings are usually positioned in the upper third of vagina and offer advantage of being independent of coital action. Once inserted vaginal rings are capable of releasing drugs for days and often weeks, significantly reducing the frequency of dosing. This result improve adherence to the therapy.

Vaginal rings have historically been fabricated using polydimethylsiloxane, a silicone elastomer, although some thermoplastic elastomers are rapidly becoming popular. The dapivirine ring that is currently being evaluated in clinical trials is made up of silicone elastomer. Poly(ethylene vinyl acetate) is the most commonly used thermoplastic elastomer. The contraceptive intravaginal ring NuvaRing is made up of poly (ethylene vinyl acetate) [36].

Two major types of vaginal rings formulated are matrix or reservoir devices. In a matrix system the drug is distributed throughout the ring polymer and drug release is controlled by a combination of diffusion of drug through the polymer and its ultimate partitioning into surrounding vaginal fluid. In a reservoir system, the ring consists of single or multiple drug core(s) encapsulated in non-medicated polymers. Additionally, vaginal rings can also be developed which consist of a drug loaded polymer layer sandwiched between two non-medicated polymer layers.

The various ring systems described above provide different drug release profiles. For reservoir and sandwich systems, the limiting factor is the thickness of the rate controlling membrane. By adjusting the thickness of the coating membrane it is possible to achieve a near constant daily drug release resulting in zero order release kinetics. The release of drug from a matrix system is governed by the diffusivity of drug through the matrix polymer and the path required for diffusion from the system. Since over time, the drug diffusion path from the center to the surface of polymer matrix increases, the amount of drug released decreases with time. More complex multi-component ring designs have also reported. An excellent review of intravaginal rings was presented by Malcolm *et al.* [36, 37].

A commonly held argument is that rings are more suitable carriers for the delivery of hydrophobic drugs than hydrophilic drugs. However, recently Johnson *et al.* designed a segmented vaginal polyurethane ring for co-delivery of dapivirine and tenofovir. Dapivirine is a hydrophobic molecule while tenofovir is relatively hydrophilic in nature. In this system the two drugs were dispersed in a suitable variety of polyurethane polymers by solvent casting, drug loaded polymers were extruded to form drug containing rods and then the dapivirine and tenofovir containing rods were fused to prepare a segmented ring containing a combination of microbicides [38]. Similarly Morrow *et al.* designed an 'insert vaginal ring' for delivery of proteins [39].

To date the dapivirine ring is the only microbicide vaginal ring tested in clinical trials. Both, reservoir and matrix systems containing dapivirine were found to be well tolerated by women [40]. The rate and extent of drug release from the matrix system was higher than that obtained using the reservoir system. However, the reservoir system was able to release the drug in a more consistent manner over 28 days.

Recently, Gunwardana *et al.* have demonstrated that microbial biofilm develops on the surface of silicon based vaginal rings. It was concluded that development of such a microbial biofilm could be a very common phenomenon, and the implications towards the user are still not known [41].

While microbicide products formulated as gels and rings are currently being investigated for safety and efficacy in clinical trials, research has also been conducted in order to increase the dosage form options available for microbicide delivery. In this respect the potential of vaginal polymeric films, vaginal tablets, foams and sponges as vaginal microbicide products are being considered.

Vaginal Films

The use of polymeric films for topical delivery of microbicide drug candidates is being evaluated. An acceptable vaginal film can be developed using a combination of polymers(s) and plasticizer(s) [42, 43]. This could be a major advantage since use of excipients from only a few categories would enhance the cost effectiveness of this particular dosage form. Vaginal quick dissolve films are primarily developed using water soluble film forming polymers. Use of polymers like hydroxypropyl methylcellulose, hydroxyethyl cellulose, and polyvinyl alcohol for vaginal film development has been reported in the literature [42, 43]. Usually one or more plasticizers are incorporated to obtain acceptable film properties. Commonly used plasticizers include glycerol, propylene glycol, and polyethylene glycol. The type and amount of plasticizer has significant impact on film processing (for example peel ability or manufacturability) and physico-mechanical properties (like brittleness, flexibility, and tensile strength).

Vaginal films are at early state of the development process and the knowledge of the field is expanding slowly.

There is still need to establish a correlation between the composition of the film and its physico-mechanical properties described above. Also like gels, the dissolution of vaginal films inside the vagina, its distribution *in vivo*, and drug release would be affected by the film composition. Some data available shows that selection of film polymers impacts drug release profile. Yoo *et al.* showed that release of sodium dodecyl sulfate (SDS) from a vaginal film was governed by the polymer composition of the film [44]. Films with high HPMC content resulted in burst release of SDS. The prototypes with higher proportions of carbopol (*vs* HPMC) resulted in slower SDS release. No correlation between swelling and SDS release could be established. More systematic studies relating film composition and resulting properties are needed. Studies are also needed which investigate the disintegration and distribution of the film once placed inside vagina.

A vaginal film containing the microbicide drug candidate polystyrene sulfonate has been reported [42]. Another vaginal film containing the microbicide cellulose acetate phthalate was developed using a combination of hydroxyl propyl cellulose and cellulose acetate phthalate [43]. Recently two vaginal film formulations one containing retrocyclin [45] and another containing dapivirine [46] were developed. In these studies vaginal films using water soluble polymers like hydroxypropyl methyl cellulose and polyvinyl alcohol were developed and evaluated. These films were made by solvent casting method using a hand pouring method or automated film applicator. Drug containing films were found to be nontoxic *in vitro* and did not affect the innate microflora, lactobacilli. Both films showed inhibition of HIV replication in cell based assays and were found to be effective in the *ex vivo* cervicovaginal tissue explant model. These studies cumulatively demonstrate the potential for film dosage forms as an option for microbicide drug delivery. Further support is shown in a recently completed acceptability study which indicated women's preference for vaginal films [47].

Vaginal Tablets

Vaginal tablets containing anti-infective agents, hormones, and plant extracts are commercially available. A number of microbicide candidates have been formulated as tablets including cellulose sulfate, dapivirine, and tenofovir. Tablet formulations possess several advantages including portability, precise dosing, ease of storage, handling and administration, feasibility of large scale production, and low cost. In addition to the active ingredient, tablets contain excipients functioning as diluent, binder, disintegrant, lubricant, antiadherent, and glidant. Tablets can be designed with additional characteristics such as bioadhesion, sustained release, and rapid disintegration [48, 49].

Garg *et al.* developed a novel and sustained release bioadhesive vaginal tablet of povidone iodine (PI), which consisted of PI (16%), xanthan gum, chitosan glutamate, polyplasdone, GranuLac, hydroxypropyl cellulose (HPC) and magnesium stearate [50]. Among these polymers, xanthan gum and chitosan glutamate were used for imparting bioadhesiveness. All excipients showed good compatibility with PI, with drug recovery of more than 94% at 50°C for three weeks. The developed tablets showed rapid disintegration (<1min *in vitro*) and sustained release (up to 8 hrs). Bioadhesive strength of the tablets was found to be significantly higher than that of the market vaginal tablets Betadine® and Wokadine®.

More recently, Woolfson *et al.* developed freeze-dried dapivirine tablets (HPMC:PVP (3:1) and Carbopol (3%)), which provide enhanced mucoadhesion, and also increased convenience for the patient. Because the reduced mass of product to be transported (in the absence of water), they improved economy and, also enhanced stability for moisture-sensitive actives [51]. The data presented in their study suggested that freeze-dried systems with a lower HPMC:PVP ratio and higher carbopol concentration represent the optimal candidates for further formulation development.

Tablets provide an additional dosage form option for vaginal delivery of microbicide drug candidates. However, several challenges remain for the effective development of vaginal microbicide tablets [22]. Currently there are no specifications available for microbicide tablets. Uniform drug distribution is difficult to achieve in this dosage form. Additionally, the potential for use of this dosage form for delivery of a combination of microbicide drug candidates remains to be shown.

Several other dosage form options exist for delivery of drug candidates intended for prevention of HIV infection. For vaginal application, combinations of devices with gel products are being evaluated [52]. Dosage forms such as foams and sprays have yet to be explored. Other options may include implants as well as alternative oral delivery strategies.

OTHER DRUG DELIVERY SYSTEMS

Several advanced drug delivery strategies have been evaluated in the field. One strategy which has been explored is the use of nanocarriers either alone or combined with other dosage forms such as films and gels. Nanocarriers which have been explored for delivery of antiretrovirals include both polymeric nanocarriers (nanoparticles) and non-polymeric nanocarriers which include liposomes, solid lipid nanoparticles, ethosomes, nanoemulsions or suspensions, and lipid nanocapsules. Reviews describing the use of nanoparticles for delivery of antiretrovirals were presented by Mallipeddi *et al.* [53], Neves *et al.* [54], Gupta *et al.* [55] and Whaley *et al.* [56].

Humberto *et al.* formulated polyvinylpyrrolidone (PVP)-coated silver (Ag) nanoparticles (NPs) in a non-spermicidal Replens gel (3% glycerin, 0.08% sorbic acid, 1% carbopol 940, 4% liquid paraffin and 16% 1 N NaOH), which was more effective as a virucide compared to the PVP-coated AgNPs dissolved in RPMI (Roswell Park Memorial Institute medium) + 10% Fetal Calf Serum [57]. The mechanism underlying the enhanced activity of the gel was found to be the increased homogeneity of AgNPs diffusion into the cervical tissue. The pH-responsive nanoparticles for vaginal drug delivery have been evaluated. Yoo *et al.* chose Eudragit S-100 (ES) composed of methacrylic acid and methylmethacrylate (1:2, MW= approx. 135,000) as the pH-sensitive polymer and evaluated the ES nanoparticles for mucosal delivery [58]. The model compounds (sodium

fluorescein (FNa) or nile red (NR)) were encapsulated into the ES nanoparticles by using the modified quasi-emulsion solvent diffusion method, and the loading efficiencies were found to be 26% for the hydrophilic compound and 71% for the hydrophobic compound. Both compounds remained incorporated in the nanoparticles at acidic pH but were quickly released when exposed to physiological pH. The confocal study revealed the drug molecules were released from ES nanoparticles in response to pH after entry into the vaginal cells, without obvious cytotoxicity. In another set of studies Zhanga *et al.* developed pH-sensitive nanoparticles containing Tenofovir (TFV) or its prodrug tenofovir disoproxil fumarate (TDF), by blending poly(lactic-co-glycolic acid) (PLGA) and methacrylic acid copolymer (Eudragit® S-100) [59]. The prepared nanoparticles significantly released the encapsulated drugs in a pH-responsive manner without cytotoxicity even when the human semen fluid simulant (SFS) is present. The encapsulation efficiency was 16.1% and 37.2% for TFV and TDF, respectively. The TFV release rate from the 75% S-100 blend was increased by 4-fold in the presence of SFS over 72 hrs. The PLGA/S-100 NPs are noncytotoxic for 48 hrs to vaginal endocervical/epithelial cells and *Lactobacillus crispatus* at concentrations up to 10 mg/ml. The majority of the particles were taken up by these vaginal cell lines through caveolin-mediated pathway (50% in 24 hrs). This data suggest the promise of PLGA/S-100 NPs as a controlled delivery system for the vaginal delivery of anti-HIV microbicides.

Solid lipid nanoparticles (SLN) were evaluated for topical microbicide delivery. Alukda *et al.* presented the first report on engineered functionalized solid lipid nanoparticles for vaginal microbicide delivery [60]. The SLNs can effectively reach the subepithelial layer, thus delivering the microbicide to a wider range of cells. The SLNs were successfully functionalized by using a layer-by-layer technique and did not have any cytotoxic effect on vaginal epithelial cells for 48 hrs. Although the encapsulation efficiency in these studies was relatively low, SLNs appear to be a promising platform for the delivery of hydrophobic microbicides.

Another novel approach is the use of siRNA as an intravaginal viral microbicide. Palliser *et al.* first reported the use of siRNA for intravaginal microbicide-delivered siRNA in a lipid formulation for the protection of mice from HSV 2 infection [61]. *In vivo* studies in mice suggest that knockdown of gene expression is proximal (in the vaginal lumen) and distal (in the uterine horns) to the site of topical delivery, and that nanoparticles also penetrated deep into the epithelial tissue [61]. More recently, Woodrow *et al.* developed an alternative delivery strategy for siRNA based on biodegradable and biocompatible polymers, in which PLGA nanoparticles (<200 nm) were loaded with siRNA (encapsulation efficiency was 40%). This led to efficient and sustained gene silencing for up to 14 days when applied to the vaginal mucosa. In this system, an 8:1 polyamine nitrogen to nucleic acid phosphate ratio produced nanoparticles containing greater than 1,000 siRNA molecules [62].

Although the application of advanced drug delivery strategies in the field is at the early stage, their potential to overcome barriers of drug delivery for HIV prevention has been illustrated. However several challenges remain such as improvement of encapsulation efficiency cost of production, regulatory status, and *in vivo* proof of efficacy.

CONCLUDING REMARKS

Microbicides and pre-exposure oral prophylaxis have been investigated as potential products for prevention/minimization of HIV infection. Several new drug candidates have been identified as having application within potential microbicide products. These drug candidates vary in their hydrophobicity, molecular weight, and stability profile. They also belong to various categories such as small molecules, peptides, and proteins. Obviously, designing an optimum formulation for these microbicides is a challenging task for each candidate demands special formulation considerations based on its physicochemical properties. Currently researched microbicide formulations employ either oral or topical routes. Ongoing research is directed at (a) improving the efficacy and safety of existing formulations and (b) increasing the dosage form options available for microbicide delivery. The first goal can be achieved by directing future research toward designing microbicide products based on more rational formulation development approaches and undertaking mechanistic studies to develop correlations between product formulation and product performance prior to clinical safety and efficacy trials. This work has already been initiated for traditional gel products but needs to be expanded to other dosage form types. With respect to expansion of drug delivery strategies, research is already ongoing for the development of newer dosage forms and drug delivery strategies such as fast dissolving films, nanoparticles and liposomes for microbicide delivery.

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CONFLICT OF INTEREST

Declared none.

ABBREVIATIONS

CDC	=	Center for Disease Control
MTN	=	Microbicide Trials Network
VOICE	=	Vaginal and Oral Intervention to Control the Epidemic
iPrEx	=	Iniciativa Profilaxis Pre Exposicion or Pre-exposure Prophylaxis Initiative
iPrEx OLE	=	iPrEx Open Label Extension
PrEP	=	Pre-Exposure Prophylaxis

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Microbicide vaginal rings: Technological challenges and clinical development☆

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ABSTRACT

Vaginal rings (VRs) are flexible, torus-shaped, polymeric devices designed to sustain delivery of pharmaceutical drugs to the vagina for clinical benefit. Following first report in a 1970 patent application, several steroid-releasing VR products have since been marketed for use in hormone replacement therapy and contraception. Since 2002, there has been growing interest in the use of VR technology for delivery of drugs that can reduce the risk of sexual acquisition of human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS). Although no vaginally-administered product has yet been approved for HIV reduction/prevention, extensive research efforts are continuing and a number of VR devices offering sustained release of so-called 'HIV microbicide' compounds are currently being evaluated in late-stage clinical studies. This review article provides an overview of the published scientific literature within this important field of research, focusing primarily on articles published within peer-reviewed journal publications. Many important aspects of microbicide-releasing VR technology are discussed, with a particular emphasis on the technological, manufacturing and clinical challenges that have emerged in recent years.

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Contents

1. Introduction . . . 0
2. Microbicide-releasing vaginal rings . . . 0
   2.1. Historical overview of vaginal ring technology . . . 0
   2.2. Types of vaginal rings for microbicide delivery . . . 0
   2.3. Material selection . . . 0
      2.3.1. Silicone elastomers . . . 0
      2.3.2. EVAs . . . 0
      2.3.3. Polyurethanes . . . 0
      2.3.4. Other materials . . . 0
   2.4. Mechanisms of microbicide release from vaginal rings . . . 0
      2.4.1. Drug release from matrix-type rings . . . 0
      2.4.2. Drug release from reservoir-type rings . . . 0
      2.4.3. Drug release from pod insert type rings . . . 0

Abbreviations: ACV, acyclovir; AIDS, acquired immunodeficiency syndrome; API, active pharmaceutical ingredient; ARV, antiretroviral; AZT, zidovudine; Boc-LBA, Boc-lysinated betulonic acid; CG, carrageenan; DMPA, depot medroxyprogesterone acetate; DPV, dapivirine; DRV, darunavir; E2, estradiol; EE, ethinylestradiol; ETN, etonogestrel; EVA, ethylene vinyl acetate copolymer; FDA, U.S. Food and Drug Administration; GMP, good manufacturing practice; GRFT, griffithsin; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HRT, hormone replacement therapy; HSV, herpes simplex virus; HPEU, hydrophilic polyether urethane; HPMC, hydroxypropyl methylcellulose; HSV, herpes simplex virus; IgG, immunoglobulin G; IPM, International Partnership For Microbicides; IPA, isopropyl alcohol; IVIVC, in vitro–in vivo correlations; LNG, levonorgestrel; mAb, monoclonal antibody; MIV-150, Medivir-150; MIV-160, Medivir-160; MPT, multipurpose prevention technology; MTN, Microbicide Trials Network; MVC, maraviroc; N9, nonoxynol-9; NES, nestorone; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; PCL, polycaprolactone; PD, pharmacodynamic; PDMS, polydimethylsiloxane; PEU, polyether urethane; PK, pharmacokinetic; PI, protease inhibitor; Pt, platinum; RTV, room-temperature vulcanizing; SE, silicone elastomer; SHIV, simian human immunodeficiency virus; SQV, saquinavir; STI, sexually transmitted infection; SVF, simulated vaginal fluid; TDF, tenofovir disoproxil fumarate; TFV, tenofovir; TPU, thermoplastic polyurethane; USP, United States Pharmacopeia; VR, vaginal ring; ZA, zinc acetate.

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2.4.4. Combination swelling and permeation controlled release systems . . . 0
2.4.5. Osmotically controlled release systems . . . 0
2.5. Manufacturing approaches . . . 0
2.5.1. Thermosetting materials . . . 0
2.5.2. Thermoplastic rings . . . 0
2.6. Microbicide candidates . . . 0
2.6.1. Non-antiretroviral microbicides . . . 0
2.6.2. Antiretroviral microbicides . . . 0
2.7. Combination microbicide and multipurpose prevention technology (MPT) rings . . . 0
3. Challenges moving forward . . . 0
3.1. Formulation and delivery of biomacromolecular microbicides . . . 0
3.2. Manufacturing issues and scale up . . . 0
3.3. Cost . . . 0
3.4. Acceptability and adherence . . . 0
3.5. Correlating in vitro release with in vivo pharmacokinetics . . . 0
4. Conclusions . . . 0
References . . . 0

1. Introduction

In 1983, following two years of increasing number of reported cases in the United States (U.S.) of severe immune deficiency among gay men and infants receiving blood transfusions, scientists first identified the human immunodeficiency virus (HIV) as the retrovirus that causes acquired immune deficiency syndrome (AIDS). By 1987, three biomedical strategies were at the forefront of developments to treat or prevent HIV infection. In March 1987, the U.S. Food and Drug Administration (FDA) approved the first antiretroviral (ARV) drug, zidovudine (AZT), for treatment of HIV by reducing replication of the virus. In August 1987, the FDA sanctioned the first human testing of a candidate vaccine against HIV. Later the same year, the FDA declared HIV prevention as a new indication for male condoms.

Fast-forward three decades and, despite the tremendous advancements in our scientific knowledge and understanding, the HIV/AIDS pandemic remains one of the most serious global public health crises of our time. The latest (2014) global statistics for HIV/AIDS estimate 37 million people living with HIV, 2 million new infections annually, and 1.2 million deaths in 2014 from AIDS-related illnesses [1]. Sub-Saharan Africa remains the hardest hit region, accounting for more than 70% of people presently living with HIV/AIDS.

Development of a safe and effective HIV vaccine has proven very difficult. Ideally, an effective HIV vaccine should induce powerful and durable immunity capable of preventing infection in healthy individuals and/or reducing viral replication and viral load in infected individuals with the aim of slowing or halting disease transmission and progression. To date, more than 250 clinical trials of HIV vaccine candidates have been completed or are presently being conducted; only six of these candidates have reached late-stage clinical testing, and none have demonstrated significant efficacy [2].

With consistent and correct use, male latex condoms can reduce the risk of heterosexual transmission of HIV by more than 70% [3–5]. However, despite widespread and often aggressive promotion, condom use has not reached a sufficiently high level to impact rates of HIV acquisition in Sub-Saharan Africa. One reason lies with gender-power imbalances, resulting in women not always being able to negotiate condom use with male partners. For example, African men are more likely to refuse condom use when there are large differences in age between them and their female partners, if they are married, when they have multiple sexual partners, and where there is no communication about HIV/AIDS between them and their partners [6]. Female condoms, widely promoted as a female-controlled alternative to male condoms, have failed to gain acceptance, despite the introduction of new types [7–10].

On a more positive note, increased access to highly active antiretroviral therapy (HAART) means that an AIDS diagnosis is no longer a death sentence for millions of people. Today, 28 FDA-approved ARV drugs are available for treatment of HIV-1 infections [11]. These drugs are mainly classified into six distinct types based on their mechanism of action: nucleoside-analog reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors (PIs), fusion inhibitors and co-receptor antagonists. As of March 2015, 15 million people living with HIV, including 11 million in Sub-Saharan Africa, were accessing life-saving HAART, up from 13.6 million in June 2014 and only 300,000 in 2002, exceeding the targets set as part of the Millennium Development Goals [1]. Meanwhile, the number of people newly infected with HIV has fallen by 35% since 2000 and global deaths due to AIDS have declined 42% since the peak in 2004. With this halting and reversing the spread of HIV/AIDS, and with continued effort and investment, the world is seemingly on track to end the AIDS pandemic by 2030 [1].

It is widely accepted that ARV treatment alone will not be able to curtail the HIV/AIDS pandemic. In the continued absence of an effective HIV vaccine, there is greater optimism about the clinical potential of HIV microbicides. HIV microbicides are pharmaceutical formulations administered vaginally (or rectally) to reduce sexual transmission of the virus. The concept of an HIV microbicide was first described in a 1990 commentary piece entitled 'HIV Prevention: The Need for Methods Women Can Use' [12]. Recognizing the limitations of behavior-modification strategies and use of condoms in reducing HIV infection rates, Stein strongly advocated research into new methods that women could use to prevent vaginal transmission of HIV. Of course, these 'topical virucides', as they were then called, would have to be acceptable to women in terms of convenience of use, safety and cost, as well as highly effective against the virus. A number of surfactant-type vaginal microbicides were tested in women during the 1990s (Fig. 1), including a compound called nonoxynol-9 (N9). Most of these studies not only failed to protect women against HIV infection, but some actually increased HIV infection rates compared with a placebo product. Surfactant-type microbicides were subsequently abandoned. Next, the focus switched to various polymer molecules (Fig. 1), whose negatively charged functional groups were shown in laboratory experiments to prevent the virus attaching to the immune cells. However, as with the surfactants, these polymer-based microbicides failed to provide protection in clinical studies, and once again, some increased the risk of infection.

The past five years has seen the microbicide field focus almost exclusively on more conventional small molecule ARV drugs, the same or similar drugs to those used since the 1980s for treating people already infected with HIV. A breakthrough came in 2010 when the first results emerged from the CAPRISA 004 trial [13]. For the first time, a vaginally-administered ARV gel product was shown to provide significant protection against HIV infection. A summary timeline describing key moments, and particularly major clinical activities, in the development of HIV microbicides is presented in Fig. 1.

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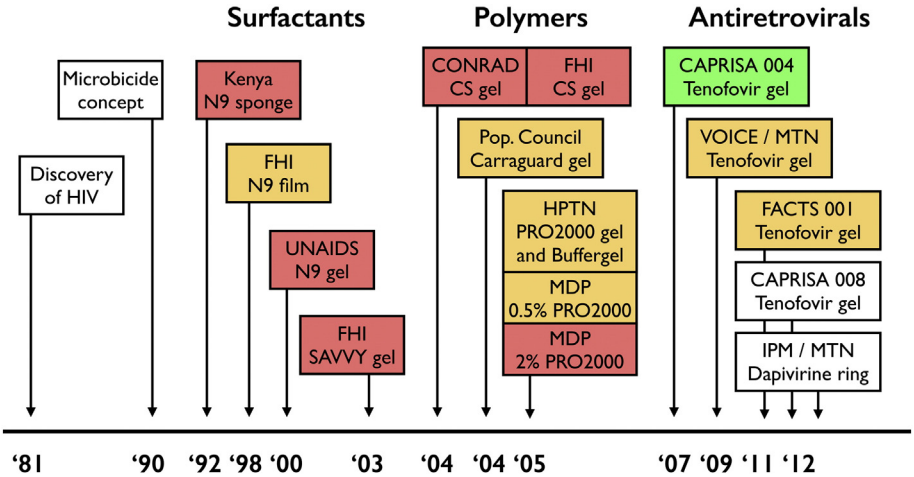

Fig. 1. Summary timeline describing key moments, and particularly major clinical activities (study start dates), in the development of HIV microbicides. Red boxes represent clinical studies that reported an increase in HIV acquisition with use of the microbicide test product. Orange boxes represent trials in which the microbicide test product showed no protective effect. Green box represents a microbicide test product that offered moderate protection. White boxes at the right of the figure represent studies that are ongoing. CS — cellulose sulfate; FHI — Family Health International (now FHI 360); IPM — International Partnership for Microbicides; MDP — Microbicides Development Programme; MTN — Microbicides Trial Network; N9 — nonoxynol-9. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this chapter.)

2. Microbicide-releasing vaginal rings

The application of VR technology, first described and widely reported during the 1970s for vaginal delivery of contraceptive hormones (see Section 2.1), to the formulation of HIV microbicides was first proposed publicly at the 2002 Microbicides Conference in Antwerp, Belgium [14]. At that time, the microbicide field was almost exclusively focused on aqueous vaginal gel formulations containing non-specific microbicide candidates, such as the surfactant N9 and various polymer molecules (see Section 2.6.1), reflecting a lack of engagement and interest in the challenges of HIV prevention by basic scientists with expertise in drug design and formulation development. Today, semi-solid drug products, including gels, are the most common formulation type for vaginal drug administration. For example, of the 24 vaginal products currently marketed in the UK, 16 are either gels or creams, and only two are VR devices (Estring® and NuvaRing®). Since the beginning of the new millennium, a diverse range of formulation strategies for HIV microbicides has been investigated, including films, tablets, diaphragms, capsules, freeze-dried tablets, and nanoparticles [15]. However, ongoing concerns over poor user adherence associated with coitally-dependent gel products and a strong preference for formulations that offer continuous delivery of microbicide(s) over extended time periods has resulted in a very significant shift in focus towards VR-based products that offer sustained/controlled release [15–17].

The first journal article describing a microbicide-releasing VR for HIV prevention was published in 2003 [18]. The matrix-type silicone elastomer ring contained the non-ionic surfactant N9, at that time a lead HIV microbicide candidate although soon to be overshadowed by evidence that in gel format it damaged the vaginal epithelium resulting in increased risk of HIV acquisition [19]. Two years later, the continuous, zero-order release of TMC120 (later renamed dapivirine, DPV) over 71 days from a core-type (also commonly referred to as 'reservoir-type') silicone elastomer VR [20] was described. This reservoir-type VR design was very similar to the marketed VR products Estring® and Femring®.

2.1. Historical overview of vaginal ring technology

VRs are flexible, torus-shaped polymeric devices designed to provide sustained, long-term delivery of pharmaceutical agents to the vagina for clinical benefit. The first VR device for drug delivery was reported in a U.S. patent application filed on 4th January 1968 and subsequently awarded on 8th December 1970 to the UpJohn Company [21]. These polymeric drug delivery ring devices, fabricated using silicone (polydimethylsiloxane, PDMS) elastomers, focused primarily on contraception and hormone replacement therapies (HRTs). Since their inception in the 1960s, numerous ring designs containing various drug combinations have been proposed, tested and documented in the literature [21–26].

The first clinical trials of a VR were performed in the 1970s for the contraceptive progestin medroxyprogesterone acetate [23]. However, this early ring design, which consisted of a metal spring over-molded by a silicone sheath (Fig. 2A) caused numerous problems including erosion and ulceration of the vaginal epithelium [22]. Although first generation VR products were clinically effective, the devices were extremely rigid and inflexible making them prone to expulsion during normal daily activities. An early study of a levonorgestrel (LNG)-releasing ring device reported that 48 out of 139 female participants developed surface lesions/ulcerations, chronic inflammation and thinning of the vaginal epithelium [27]. These adverse effects were most likely the result of a combination of factors including (i) the geometry of the ring, (ii) the inflexible nature of the device causing localized pressure on the vaginal epithelium and (iii) epithelial thinning effects caused by the hormonal contraceptive agent. As a result thinner, more flexible, non-irritating VR designs with optimized geometries (outer diameter ranging from 50 to 58 mm; cross-sectional diameter ranging from 4 to 9.5 mm) were developed [28–32].

Despite initial enthusiasm for these new controlled-release VR devices, formulation issues and concerns regarding the safety of long-acting steroid releasing rings saw many ring development studies discontinued. To date, five VR products have reached marked — Estring® (Pfizer Inc., USA) and Femring® (Actavis, UK) for hormone replacement therapy, NuvaRing® (Merck & Co, USA), Progering® (Laboratorios Silesia, Chile) for contraception, and Fertiring® (Laboratorios Silesia, Chile) for both pregnancy maintenance during in vitro fertilization and hormone replacement therapy in menopausal women. Clinical studies have shown a high degree of acceptability for VR devices over conventional semi-solid vaginal gels and creams [16,33–36].

Historically, silicone elastomers were the polymer of choice for fabrication of VR devices [20–23,37,38] owing to their lightweight, flexible nature and excellent biocompatibility. However, as VR designs have become increasingly more sophisticated a range of polymeric materials including poly(ethylene-co-vinyl acetate) (EVA) [39–41] and more recently thermoplastic polyurethanes (TPUs) have been used for the manufacture of these ring devices [17,26,42–44]. Materials for ring fabrication are discussed in more detail in Section 2.3.

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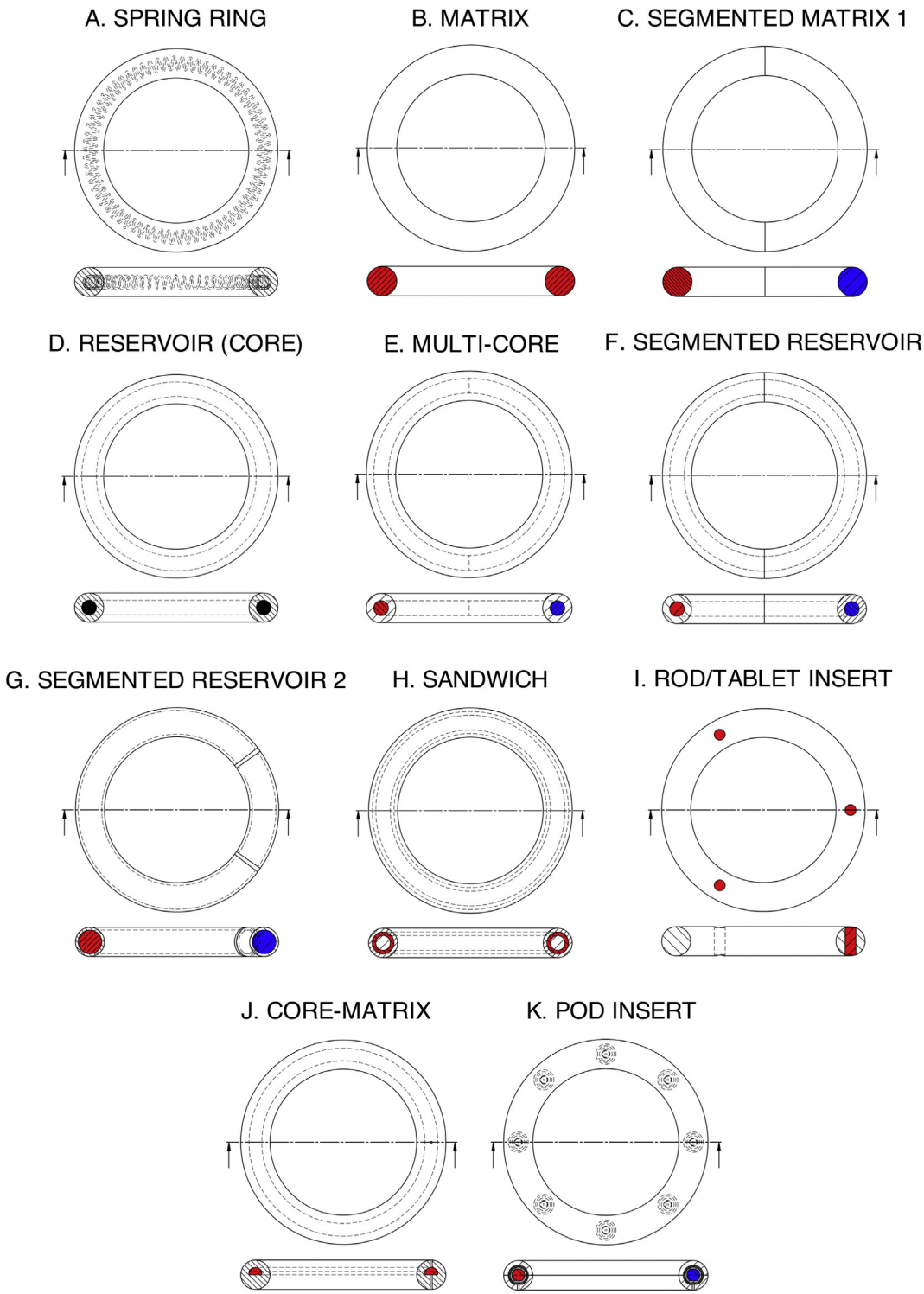

Fig. 2. Full ring (upper) and cross-sectional (lower) views of the various vaginal ring designs reported in the scientific literature for the delivery of HIV microbicides. Dark shading represents the location of the active agent(s).

2.2. Types of vaginal rings for microbicide delivery

The application of VR technology to the delivery of HIV microbicide molecules having a broad range of physicochemical properties has led to very considerable innovation in new ring designs (Fig. 2). Before 2006, VR designs being considered for microbicide delivery were mostly based on conventional matrix and reservoir-type systems, reflecting the design types used in marketed ring devices. However, the highly hydrophobic properties of EVA and silicone elastomer that had thus far proved successful for the formulation of hydrophobic, steroid drugs for estrogen replacement therapy and contraception were often not able to offer suitable permeation characteristics for many lead microbicide candidates. Also, microbicides in general, and ARVs in particular, do not generally possess the same clinical potency as steroid hormones, such that much larger doses (in the form of increased daily release rates) are required for efficacy. For example, while DPV has very similar physicochemical characteristics (e.g. molecular weight, partition coefficient, and water solubility) to many steroid molecules, TFV and TDF are

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significantly more hydrophilic and therefore less capable of achieving clinically significant release rates from conventional VR designs that rely on permeation from hydrophobic elastomers as the primary mechanism of release. As a result, a raft of new ring designs – still largely based around silicone elastomer, EVA and TPU materials (see Section 2.3) – has emerged aimed at overcoming this permeation barrier, including segmented matrix, multi-core, segmented reservoir, rod/tablet insert, core-matrix and pod insert rings (Fig. 2). A major impetus for continued innovation in ring design has been the growing interest in combination microbicide [45] and multipurpose prevention technology products [46–49] (see Section 2.7), for which release characteristics need to be individually tailored for each drug molecule. The release mechanisms that govern these new designs are discussed in Section 2.4.

2.3. Material selection

Drug-releasing VRs, comprising a combination of drug and device, are formally classified as combination products as defined in FDA documentation 21 CFR 3.2(e). Most commonly in VRs, the drug component takes the form of a potent ARV molecule and the device component comprises a polymeric ring device. Because combination products involve components that would normally be regulated under different types of regulatory authorities, and frequently by different FDA Centers, they raise challenging regulatory, policy, and review management challenges. Differences in regulatory pathways for each component can impact the regulatory processes for all aspects of product development and management, including preclinical testing, clinical investigation, marketing applications, manufacturing and quality control, adverse event reporting, promotion and advertising, and post-approval modifications [50].

Biological evaluation of polymeric medical devices that come into direct or indirect contact with the human body are covered by ISO-10993, “Biological Evaluation of Medical Devices Part 1: Evaluation 132 and Testing” [51]. The general principles of this ISO-10993 guidance also apply to combination products, such as drug-releasing VRs, although additional or modified testing may be required. According to the standard, VRs are classified as devices that contact the mucosal tissue, and for which a series of initial tests are mandated to evaluate biological effect. These can include tests of cytotoxicity, sensitization, vaginal irritation, systemic toxicity, subchronic toxicity, genotoxicity, and implantation, depending on the duration of mucosal contact. A supplementary evaluation test for chronic toxicity is also required for drug-releasing VRs that are used for greater than 30-day duration.

To date, all marketed VRs and most prototype microbicide-releasing VRs are fabricated either by high-temperature reaction injection molding of medical grade silicone elastomers (Estring®, Femring®, Progering®, Fertiring®) or high-temperature extrusion of EVA (NuvaRing®). For example, The International Partnership for Microbicides' (IPM) DPV VR 004 is manufactured from Nusil's MED-4870 silicone elastomer. Certain grades of these polymeric materials meet the standards of United States Pharmacopeia (USP) Class VI materials (the most stringent of the six classes of plastic designation). In order to be compliant with USP Class V.e approval, test materials must pass the ‘Systemic Injection Test’ and the ‘Intracutaneous Test’. Here, extracts of the test material in saline, alcohol in saline, polyethylene glycol (PEG 400), and vegetable oil, are injected into mice and rabbits and the animals' response to the sample extracts compared with a blank test. USP Class VI materials must pass both the USP Class V.e test plus an implantation test in which strips of the test material and a negative control are implanted in rabbits for a period of not less than 120 h. Hemorrhage, necrosis, discolorations, and infections are macroscopically observed and degree of encapsulation is scored and compared with a negative control to determine test passage. Although USP Class VI testing is widely used and accepted in the medical products industry, some view it as the minimum requirement a raw material must meet to be considered for use in healthcare applications. USP Class VI testing does not fully meet any category of ISO 10993-1 testing guidelines currently used by the US FDA (General Program/Bluebook Memorandum G95-1) for medical device approval.

2.3.1. Silicone elastomers

Silicone elastomers for use in medical and pharmaceutical applications are prepared through the chemical crosslinking of functionalized, linear, polydimethlysiloxane molecules. The most important chemical crosslinking mechanisms involve condensation-cure and addition-cure chemistries. Condensation-cure systems involve reaction between hydroxy-terminated polydimethylsiloxanes and a tetraalkoxysilane, resulting in the formation of the cured elastomer and an alcohol by-product (Fig. 3) [52–54]. Although the chemistry of this SE crosslinking reaction is generally compatible with a very wide range of chemical functional groups, the alcohol produced can be problematic when an incorporated drug(s) is highly soluble in the alcohol [55,56]. Crosslinking of addition-cure SE systems relies on the platinum-catalyzed hydrosilylation reaction between hydride- and vinyl-functionalized polydimethylsiloxanes (Fig. 4). Usefully, no by-product is formed with this reaction. However, the platinum (Pt) catalyst is particularly sensitive to poisoning by certain chemical functional groups, most notably organotin, organosulfur and certain amine containing compounds. Medical grade silicone elastomers used in the fabrication of VRs are supplied as either restricted (limited to external use or short term implant applications ≤29 days) or unrestricted grades (for any application, including long term implantation >29 days). In general, they are supplied as two-part systems that need to be intimately mixed to initiate cure. Each part includes the silicone base material – a complex mixture of silicone polymers and a reinforcing filler (Table 1) – in addition to platinum catalyst, hydride crosslinker and cure inhibitor components (Table 2).

2.3.2. EVAs

EVAs are copolymers of ethylene and vinyl acetate that have a long history of use in drug delivery applications (Fig. 5A). The vinyl acetate content (typically ranging from approximately 10–40%) and the molecular weight characteristics of the EVA material play a major role in determining the mechanical properties, the ease of processing, and the drug release rates of the finished drug delivery device. The first controlled release drug delivery systems to be commercialized – Alza's Ocusert® (an ophthalmic insert releasing pilocarpine at a constant rate for treatment of glaucoma) and Progestasert® (an intrauterine implant providing constant rate of progesterone delivery) – were fabricated from EVA. More recently, Implanon®/Nexplanon® (a long-acting subdermal contraceptive implant releasing etonogestrel), Virtasert® (a ganciclovir eye implant for treating cytomegalovirus infection) and NuvaRing® (a combination contraceptive VR) are all fabricated from EVA. Despite the success of NuvaRing®, there have been only a small number of reports describing use of EVA for microbicide-releasing VRs. The reasons for this are unclear, although supply of medical grade

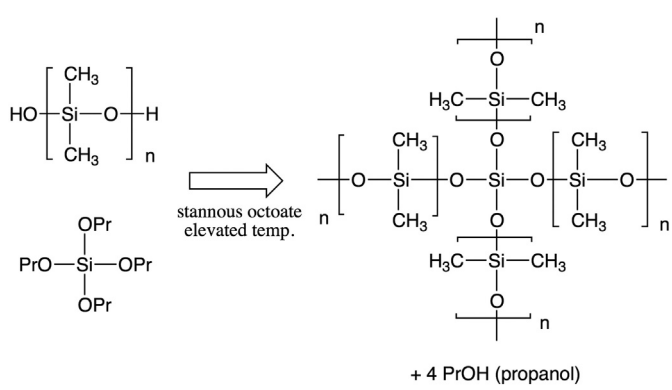

Fig. 3. Curing reaction for condensation-cure silicone elastomer systems.

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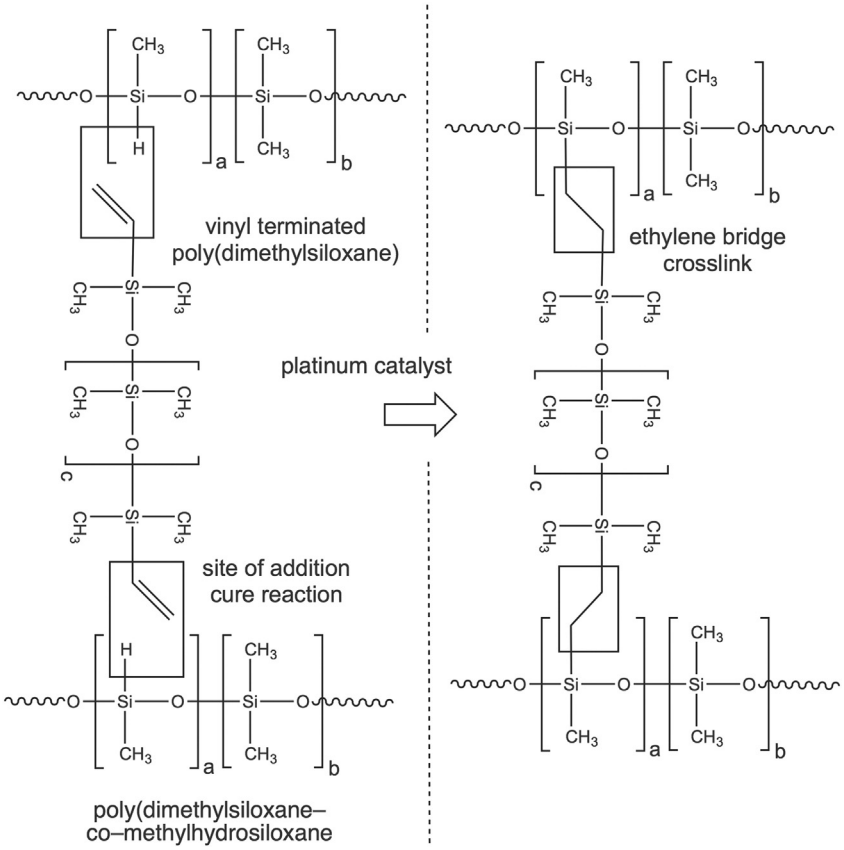


Fig. 4. Representation of the platinum-catalyzed hydrosilylation reaction for cure of addition-cure silicone elastomer systems.

EVA materials is often more constrained than for silicone elastomers (due to limited number of vendors). However, EVA polymers may offer certain advantages over silicone elastomer materials, including lower cost, a wider range of physicochemical and drug permeation properties due to variation in the vinyl acetate ratio, and the potential to produce devices with very thin (less than 100 μm) rate controlling membranes using extrusion processes [57,58]. The experimental NNRTI UC781 showed similar in vivo release rates and kinetics in rabbits following vaginal administration of ring segments fabricated from EVA, polyurethane and silicone elastomer [59]. A combination EVA matrix-type ring providing simultaneous release of the microbicide candidate UC781 and the contraceptive progestin levonorgestrel (LNG) has also been reported [40]. The Population Council are developing an EVA core-matrix ring for simultaneous delivery of Medivir-150 (MIV-150), carrageenan (CG), zinc acetate (ZA) and levonorgestrel (LNG) [60].

2.3.3. Polyurethanes

Alternative biocompatible thermoplastic materials, most notably thermoplastic polyurethanes (TPUs) [42,59,61–64], are also being evaluated for fabrication of microbicide-releasing rings, in order to extend

Table 1
Representative composition of the base material for an addition-cure silicone elastomer system. PDMS — polydimethylsiloxane.

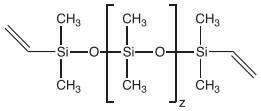
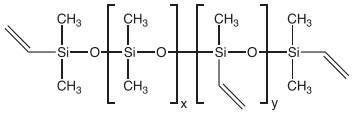
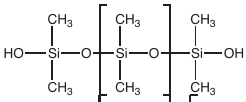
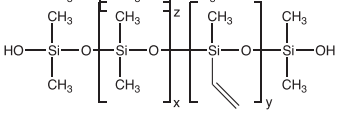
Silicone base component	Chemical structure	Typical conc.
Terminal dimethylvinyl PDMS		35%
Terminal dimethylvinyl + internal vinyl PDMS		35% y = 0.2%
Hydroxy-terminated PDMS oil		5%
Hydroxy-terminated + internal vinyl PDMS oil		5%
Reinforcing fused silica	SiO_2	20%

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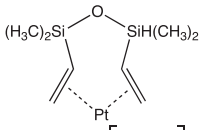
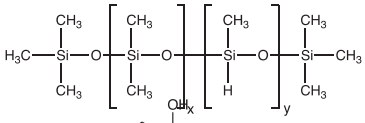
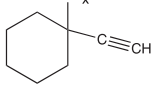
Table 2
Representative part A and B formulation components of addition-cure silicone elastomer system used in the fabrication of vaginal rings.

Component	Representative chemical structure	Part
Silicone elastomer base	(See Table 1)	A and B
Platinum-based hydrosilylation catalyst		A
Hydride crosslinker		B
Inhibitor (used to control work time)		B

material choice beyond the relatively hydrophobic silicone elastomers and EVA polymers. TPUs are multi-phase block copolymers formed by a step-growth polymerization reaction between diisocyanates, a low molecular weight diol and a high molecular weight diol (Fig. 5B). The low molecular weight diol and the diisocyanate combine to form hard segments which contribute to the toughness and physical performance properties; the high molecular weight diol and the diisocyanate combine to form soft segments (responsible for the flexibility and elastomeric character). The TPU polymerization reaction combines the soft segments and hard segments into a linear backbone, giving a copolymer with bi-phasic properties. The hard and soft phases separate as a result of the strong hydrogen bonding between urethane units and/or the hard segment crystallization. By controlling the ratio of hard to soft segments, TPUs offer a wide range of physicochemical and drug release properties useful in developing VR formulations optimized for different types of drug actives. For example, TPUs are available in both hydrophobic and hydrophilic grades, and both have been reported in the literature for VR fabrication. CONRAD's dual-segment, multipurpose prevention technology VR releasing TFV and LNG is fabricated from two different polyurethane materials (Lubrizol's Tecoflex™ and Tecophilic™ polymers; GMP versions are sold under the Pathway™ brand), one for each segment and selected to optimize permeation of the actives [61]. TPU VRs are generally fabricated by blending/compounding the drug with the polymer followed by hot-melt extrusion processes (although injection molding is also possible). Depending upon the properties of the drug in the polymer, the drug may associate mostly with the soft segments or with both the soft and hard segments.

2.3.4. Other materials

Microbicide-release vaginal rings fabricated from hydrogel materials have been reported [65–67]. Han et al. described rings composed of biosoluble acacia gum or nonbiodegradable hydrogel of 2-hydroxyethyl methacrylate (HEMA) and sodium methacrylate (SMA) [P(HEMA-co-SMA)] for the in vitro release of AZT and various non-hormonal contraceptives [65]. In a follow-up paper, the same technology was extended to release of dapivirine (TMC120), PMPA and Boc-lysinated betulonic acid (Boc-LBA) [66]. For each drug, in vitro release was maintained for no less than 15 and 28 days from the acacia gum and 2-hydroxyethyl methacrylate and sodium methacrylate rings, respectively, at concentrations higher than the minimum effective dose for HIV inhibition. The same group of researchers have more recently reported a biocompatible VR composed of a nanoporous poly(diol citrate) elastomer hydrogel for the delivery of non-hormonal contraceptives and anti-HIV agents [67]. Following synthesis of the prepolymer (by mixing and heating a mixture of 1,8-octanediol and citric acid) and subsequent mixing/processing with the active agents, the viscous polymer mixture was poured into a ring mold and heated at 80 °C for 4 days to form the final ring device. Use of organic solvents and a complex and protracted manufacturing method are likely to restrict the scope and practicality of this VR technology.

Biodegradable polycaprolactones (PCLs) are commonly used for production of biomedical implants and drug delivery devices in the form of films, micro- and nanoparticles. Pertinent to this review, microporous PCL vaginal insets offering 30-day controlled release of TFV have recently been reported [68]. Unlike more conventional polymer materials used to construct ring devices, preparation of these prototype rings involves dissolving/dispersing the polymer/drug in acetone followed by a methanol extraction process, resulting in a relatively flexible porous insert. Such a solvent method is not safe, practical or scalable for commercial ring manufacture. However, PCL is amenable to hot-melt injection molding and extrusion. The porous nature of these materials, which facilitates fluid uptake, offers an alternative mechanism of release for those microbicidal drugs that are not amenable to permeation control polymer systems.

2.4. Mechanisms of microbicide release from vaginal rings

All of the VR products currently marketed (Estring®, Femring®, NuvaRing®, Fertiring® and Progering®) and the DPV Ring-004 in

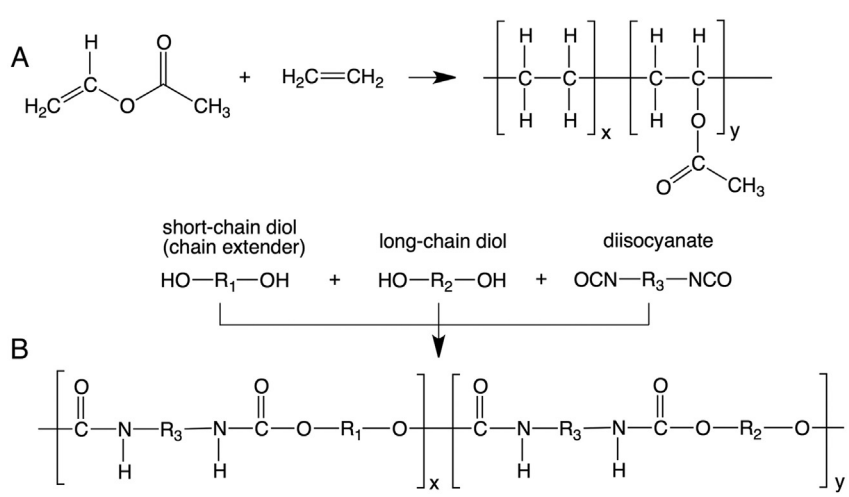


Fig. 5. General synthetic reactions and representative chemical structures for (A) poly(ethylene)-co-vinyl acetate (EVA) polymers and (B) polyurethanes used in the fabrication of thermoplastic vaginal rings.

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Phase III clinical testing rely on release of the active from the device via a permeation-controlled mechanism [20]. This permeation process can be considered as three discrete and consecutive steps — drug solvation in the surrounding polymer, molecular diffusion of the solvated drug molecules within the polymer, and partition of the drug from the surface of the ring into the surrounding release medium [25]. The driving force behind the permeation mechanism of drug release from VRs is passive diffusion down concentration gradients that exist from within the device to the fluid in the vaginal vault. The nature of the release profile observed in vitro depends upon the ring design and allows differentiation of ring type based on characteristic release profiles e.g. matrix and reservoir-type rings. Recently other mechanisms of drug release beyond permeation control have been investigated including combination swelling and permeation controlled systems, pod-insert type systems and osmotically controlled systems.

2.4.1. Drug release from matrix-type rings

In the simplest matrix-type ring design, drug is homogeneously dispersed (at least, at a macroscopic level) throughout the entire ring body (Fig. 2B). For most drug compounds, solubility in the polymer is lower than the amount of drug present, such that drug is present in both the solvated and solid (usually crystalline) states. Upon placement of the ring into a release medium, solvated and solid drug initially present at the outer surface of the ring will diffuse/dissolve into the surrounding fluid, giving rise to the so-called 'burst effect' [25,55]. Once this very outermost layer of drug has ben released, other solvated drug molecules from within the bulk of the VR will diffuse to the ring surface and partition into the surrounding fluid. The solubility sites within the polymer that have been depleted due to drug release are then replenished by dissolving of further drug molecules from the solid drug particles dispersed within the matrix. As a result of these processes, a series of equilibria are established – solid drug in polymer ⇌ dissolved drug in polymer ⇌ drug in release medium/vaginal fluid – which are maintained so long as excess solid drug is present within the device. Given sufficient release time, an advancing drug-depletion zone forms with matrix-type VRs [69].

Usually, the rate-limiting step in release of drugs from matrix-type VRs is molecular diffusion of the drug through the polymer, which, under sink condition, is commonly modeled by the Higuchi equation [70,71]. Given that the Higuchi model was originally derived for planar matrices rather than a torus-shaped system [72], Helbling et al. present an alternative and more accurate mathematical model for controlled release of drugs from torus-shaped matrix-type devices [72]. Representative daily and cumulative release profiles for matrix-type VRs are presented in Fig. 6. There is characteristic reduction in the daily release as the depletion zone boundary recedes into the ring thereby increasing the diffusional path of dissolved drug to the surface of the ring. The release rate is dependent upon the drug solubility in the polymer, the diffusion coefficient of the drug in the polymeric material of the ring, the drug loading and the ring surface area.

Through judicious choice of release medium, matrix-type rings can give rise to both diffusion-controlled and partition-controlled release mechanisms [73]. Diffusion-controlled release predominates when the drug solubility in the release medium is sufficiently high [74]. In this scenario, the shape of the release profile is relatively insensitive to the partition coefficient and solubility of the drug considered. However, as the solubility of the drug in the release medium falls the mechanism of release will shift to a partition-controlled one [74]. Here the cumulative drug release profile is linear with time, corresponding to zero-order release. This can be thought of as having a constant supply of dissolved drug in the polymer waiting to be released into the surrounding fluid with the rate-limiting step being the partitioning of the drug into vaginal fluid/release medium rather than diffusion through the polymer matrix. The drug release process is then a function of the partition coefficient of the drug between the polymer and the fluid surrounding the ring.

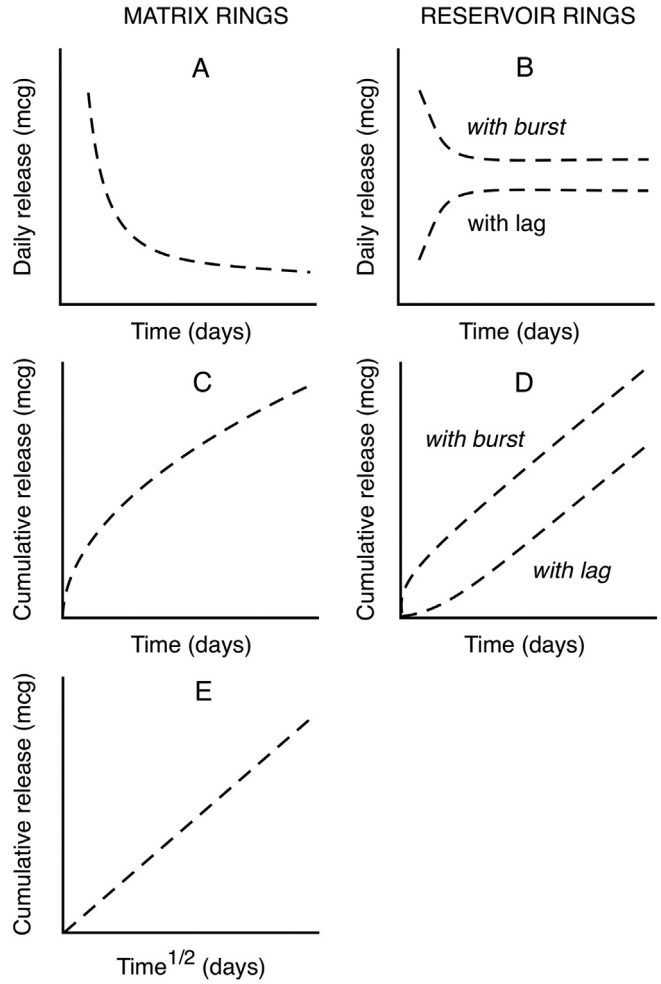

Fig. 6. Representative daily and cumulative drug release vs. time profiles for non-degradable, non-swelling matrix-type and reservoir-type vaginal rings. Matrix-type rings contain crystalline drug distributed throughout the entire ring body and exhibit root time kinetics. Here, reservoir rings can refer to either a conventional reservoir-type ring comprising one or more drug cores encapsulated by a non-medicated membrane (Fig. 2D–G), a core-matrix ring (Fig. 2J) or a pod insert type ring (Fig. 2K), all of which display (pseudo) zero-order drug release kinetics.

The 25 mg DPV Ring-004 – the most advanced microbicide ring, currently in Phase III clinical trials – has a matrix design [75,76]. Other examples include the EVA rings containing either MIV-150 [77] or MIV-160 developed by the Population Council [78], both of which exhibit partition-controlled release into an acetate buffer with surfactant system and the hot melt extruded polyether urethane ring containing UC781 [79]. Combination matrix-type rings containing more than one microbicide have also been investigated [80,81].

2.4.2. Drug release from reservoir-type rings

In simplest form, reservoir-type VR designs comprise a central drug-loaded polymer core (again, drug is generally present in both the dissolved and solid state) surrounded by a non-medicated polymeric rate-controlling membrane (Fig. 2E–G). For example, each of the marketed VRs NuvaRing®, Estring® and Femring® is a reservoir design, although the length of the drug-loaded core varies between the devices. Most commonly, release is governed by a permeation mechanism involving dissolution and diffusion of drug molecules in the polymer materials from which the ring is fabricated. Drug release rates from reservoir VRs are typically constant with time, consistent with zero-order kinetics. Sometimes, depending on the drug/polymer combination, the manufacturing conditions and the stability conditions, a lag

or burst effect can be observed during the initial release period (Fig. 6). As only dissolved drug can migrate through the ring structure from the core to the periphery, the rate of release is controlled by the fixed thickness of the membrane layer. Constant rate of drug release will continue until the solid drug within the core becomes depleted. A fixed diffusional path length gives rise to a fixed release rate dependent upon the rate of partitioning into the surrounding fluid and the size of the sheath layer. Drug release kinetics in this case are controlled by the thickness of the rate-controlling membrane and the relative partition coefficient of the drug between the polymer and the release medium [82]. Owing to their design, reservoir-type devices offer lower release rates compared with matrix-type rings. Examples of reservoir-type microbicide VRs include the 90-day TFV ring [83], the tenofovir disoproxil fumarate (TDF) reservoir ring [63,84] and the DPV reservoir rings tested by IPM [56,85].

2.4.3. Drug release from pod insert type rings

Pod insert VRs comprise compacted drug powder inserts coated with a semipermeable polymer and embedded in a polymeric (often silicone elastomer) VR body [86–89]. This design offers pseudo zero-order release profiles and can be used to deliver a broad range of compounds including hydrophilic and macromolecular actives. Drug release, which occurs by permeation through a delivery window in the ring body, can be readily altered by changing the window diameter, altering the amount and composition of the core polymer coating, or by increasing or decreasing the number of pods per ring [86]. The pod ring has been investigated for the delivery of the hydrophilic drugs TFV and ACV [89, 90], and simultaneous delivery of five different drugs – TFV, nevirapine (NVP), saquinavir (SQV) and the hormonal contraceptive combination etonogestrel (ETN) and ethinyl estradiol (EE) [91]. The release of antibodies has also been investigated (see Section 3.1) [92].

2.4.4. Combination swelling and permeation controlled release systems

TPUs have been used in the manufacture of various ring types, including matrix [42,44,79], reservoir [44,63] and segmented designs [43,61] (Fig. 2). Segmented polyurethane rings, comprising a water swellable polyurethane segment and non-water swellable polyurethane segment within the same ring device, are useful for release of compounds with very different hydrophilicities. For example, a segmented ring has been reported for simultaneous delivery of TFV and DPV [43]. Depending upon microbicide solubility in the release media, the release mechanism can be diffusion controlled or partition controlled [42,79]. The use of a water swellable polymer requires that polymer swelling be taken into account for the release mechanism [44]. Variations on the segmented ring design have also been described, including the use of hollow tube like cores with osmotically active excipients present to encourage water ingress and drug release [83] and segmented dual reservoir-type designs (Fig. 2G) [61]. A dual-segment version of this latter ring type has been developed as an MPT device offering release of TFV from one segment and LNG from the other [61].

2.4.5. Osmotically controlled release systems

A new core-matrix MPT ring containing four different APIs has recently been reported by the Population Council (Fig. 2J) [60]. This VR comprises a compressed core containing the solid hydrophilic agents ZA (targeting HIV-1 and HSV-2) and CG (targeting HPV and HSV-2). The core is embedded within a hot-melt extruded EVA ring body containing the hydrophobic antiretroviral MIV-150 and the hydrophobic progestin LNG. Pores drilled in the EVA ring allow fluid ingress to dissolve and release the ZA + CG from within the core. In this manner, different mechanisms control release of the various actives. The concept of using rings to hold inserts containing highly hydrophilic excipients to promote release of macromolecular drugs has also previously been reported [93]. Recently, a device utilizing polymer swelling for the controlled release of macromolecules has been reported [94].

2.5. Manufacturing approaches

VR manufacturing techniques are dependent on the design, APIs, materials and production volume requirements of the device. For most microbicide-releasing VRs, the polymeric excipients used in their manufacture play a major role in controlling the release of drug(s), usually by limiting the rate of drug diffusion through the matrix body or a non-medicated layer. Thermosetting and thermoplastic polymers are the most commonly used materials used in the manufacture of microbicide-releasing VRs; thermosetting polymers cure irreversibly, while thermoplastic polymers can be thermally cycled. Summaries of the manufacturing approaches used for the different material categories are found in Figs. 7 and 8.

2.5.1. Thermosetting materials

Silicone elastomers are the main thermosetting polymer used in the manufacture of VRs. They are available in a variety of curing chemistries including condensation-cure (Fig. 3), addition-cure (Fig. 4), room-temperature vulcanizing (RTV) and ultra-violet (UV). The chemistries of condensation-cure and addition cure systems are described in Section 2.3.1.

The basic manufacturing principles for condensation-cure silicone elastomer rings are the homogenous distribution of n-propylorthosilicate into a silicone elastomer base followed by addition of the active ingredient and thorough mixing (Fig. 7). A tin catalyst is dispersed into the formulation and final forming operations are performed at temperatures typically above 100 °C.

For drug molecules with non-reactive functional groups, addition-cure systems are the preferred option. Addition-cure silicone elastomers are two component systems (part A and part B) which are typically combined in a 1:1 ratio [20,38,56,69,80,95–97]. Active ingredients are usually dispersed in equal amounts into each elastomer component in separate batch mixing operations, and then these active mixtures are combined in appropriate ratios using an additional mixing operation before final forming, heating and curing of the ring.

At a laboratory scale, mixing of components and API into the elastomer base has been performed by hand, overhead paddle, planetary [80] and double-asymmetric centrifugal (DAC) mixing [80,96]. The selection of a suitable mixing method depends on the viscosity of the silicone component, the amount of excipients and/or API being added, the sensitivity of the components to processing conditions, batch size requirements and also the degree of scalability necessary for a given stage of product development. DAC mixing is capable of dispersing multiple APIs in silicone elastomer materials to allow the production of combination microbicide matrix-type VR formulations [80,81,96].

Given the relatively rapid kinetics of the crosslinking reaction for both condensation and addition cured silicone elastomer systems, final downstream mixing is ideally performed immediately before the product forming operation occurs. Working with small batches of premixed material can mitigate the problem of the elastomer curing prior to completion of final product forming but accepted best practice is the use of static mixing equipment to combine the incoming streams of components; just subsequent to forming. This contrast is highlighted again by Fetherston et al. [80] in their different approaches to producing R&D scale batches using a DAC mixer working with mixed batches of part A and part B API loaded elastomer compared to the method used during large scale manufacturing runs for stability trial samples where the two separately pumped A and B streams were combined using a static mixer, prior to being fed into an injection molding machine.

Silicone elastomer VRs are usually fabricated using injection molding processes. After the final combination of all liquid components, material is transferred into an injection vessel that pressurizes and provides a mechanism of control over the 'shot', specifically the volume of material that flows into a mold tool containing a negative ring cavity. The mold tool is temperature controlled and heated to a set point that provides crosslinking of the silicone elastomer in the ring as rapidly as

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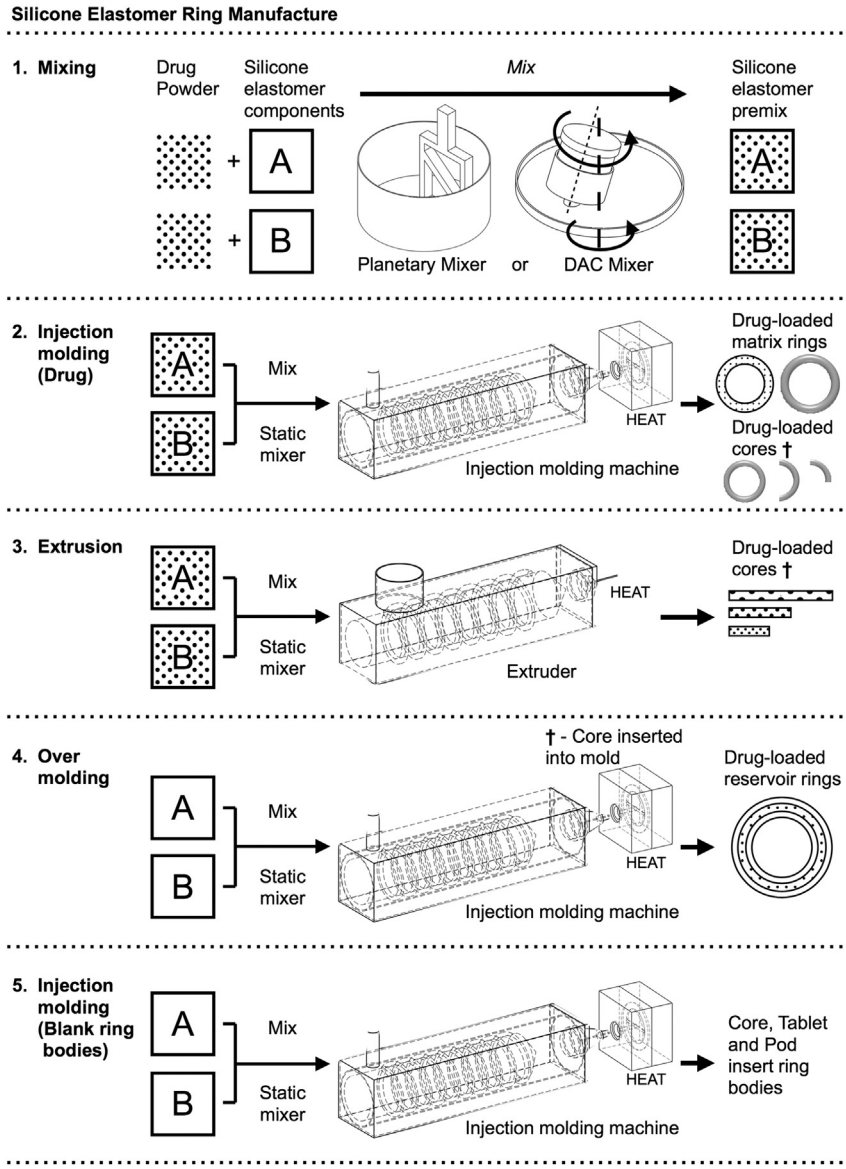

Fig. 7. Representative steps in the manufacturing process for fabrication of silicone elastomer vaginal rings.

possible without causing detrimental effects to the initial injection — linked to ring quality, or degradation of API contained therein. The processing parameters that can be controlled during injection molding operations of liquid silicone elastomers and their potential effect on product quality have not been widely reported. Evidence from studies of Pt-catalyzed silicone elastomer maxillofacial prostheses suggests that low temperature-long duration vs high temperature-short duration curing conditions produce no appreciable differences in material hardness but mold material could have an effect [98].

For silicone elastomer reservoir-type VRs, the drug-loaded core component is formed as for a matrix-type ring. This core is then placed into a mold that allows half of the core cross-sectional diameter to be covered in a non-medicated membrane of chemically-compatible silicone elastomer. When cured, the half-sheathed ring is removed and placed into a third mold assembly that allows the final part of the sheath layer to overmolded around the core, forming a full rate controlling membrane around the API loaded core [20]. There is increased complexity in the manufacture of silicone reservoir rings compared to their matrix counterparts due to the importance of centrally locating the core within the membrane to ensure that consistent drug release rates are obtained. In addition a two or three step injection molding process is required that has implications for manufacturing costs.

Reservoir-type microbicide rings can also be manufactured by injection molding to contain partial-length cores (unpublished). Here, full cores are molded and the required segment size is cut from the full core, e.g. half and quarter. The overmolding process is then performed in the same manner as for full core, injection molded reservoir rings. A non-microbicide example of a silicone multi-core reservoir VR containing oxybutynin examined different fractional segment cores, using this approach to reduce the day one 'burst effect' observed in full-length cores [55].

An additional forming operation for silicone elastomer products is the extrusion of rod geometries. This approach is particularly useful when working with high drug loadings (>40% *w*/w) in silicone elastomer systems, where viscosity of the silicone mixes exceeds the capability of the injection molding process. For silicone elastomer extrusion, drug-loaded material is conveyed using an Archimedean screw inside a temperature-controlled barrel. A circular die placed at the output from the extruder forms a rod of defined diameter and this is passed through a line of convection ovens or a static oven to cure the elastomer.

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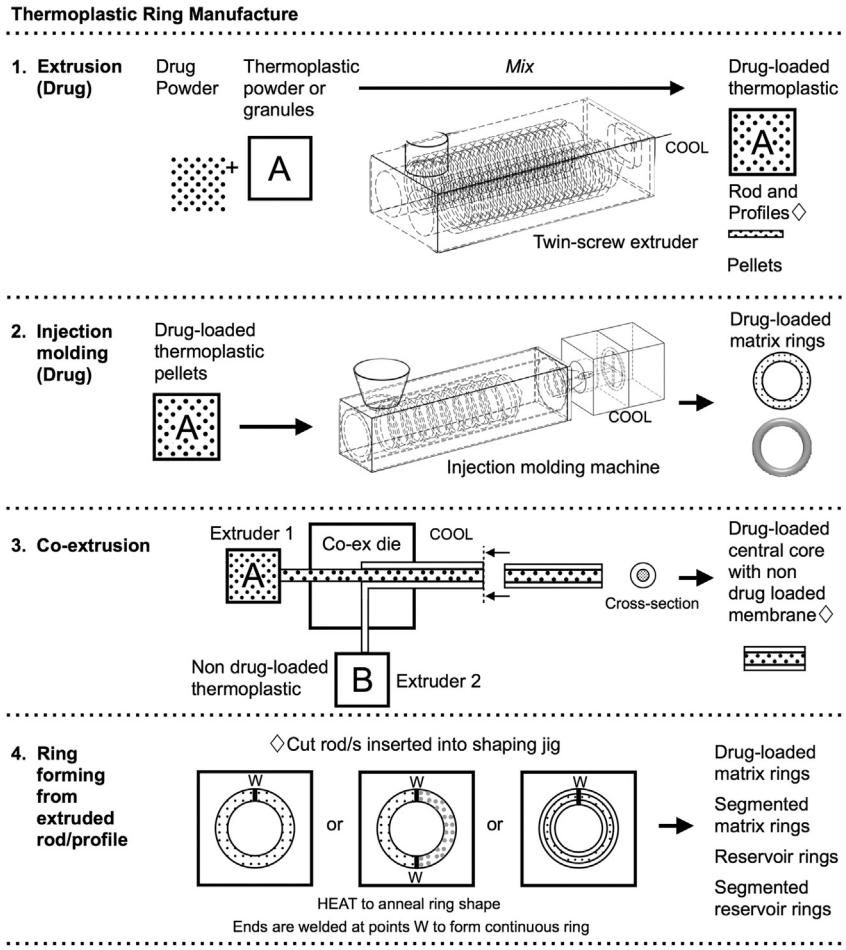

Fig. 8. Representative steps in the manufacturing process for fabrication of thermoplastic vaginal rings.

One example of the use of API-loaded silicone rods is the Population Council's nestorone (NES)/EE ring [99] that has two separate rods inserted into a single ring device. Once active silicone rods have been extruded, they are cut to length and either overmolded with a compatible silicone or, as in the case of the NES/EE ring, inserted into a separately produced, non-medicated silicone ring body. While this example is for a contraceptive ring application, the manufacturing techniques could equally apply for microbicide releasing VRs delivering two or more actives.

A subset of reservoir-type silicone VRs exists where the ring body acts as a non-medicated holder for active silicone cores. Examples include the Population Council's NES/EE VR, where the ring body is manufactured separately and the cores are added in a separate operation [99], and a tablet insert ring in which API-loaded capsules are inserted into an injection molded ring body manufactured using mold tooling that forms defined hole diameters traversing the cross section of the ring [93,100]. One advantage of this manufacturing approach is that thermally-sensitive actives, such as proteins or peptides, can be readily incorporated into ring devices without exposure to the elevated temperatures required to cure the silicone elastomers. A different type of pod ring has been developed using cropped, spherical pods of solid API coated with permeable and semi-permeable polymers. Early prototypes of the device were manufactured with a recess for pod insertion that was backfilled with silicone [86]. Delivery channels were mechanically punched through the base of the ring body. The pods were manufactured separately giving the capacity to deliver multiple APIs [91]. In recent advances to the manufacturing process, the delivery channels were molded directly into the silicone sheath layer [101]. First, a ring body is injection molded with a recess for each pod that includes a cylindrical orifice or 'delivery channel' at the base, formed during the molding process — designed to control release rate of the individual API loaded pods. The pods are inserted into their recess and fresh silicone is used to backfill the recess, locking the pods in position and completing the ring profile.

2.5.2. Thermoplastic rings

Thermoplastic rings differ from thermosetting VRs in their manufacturing approach, specifically in the steps required to create homogenous dispersions of API in the viscous thermoplastic melts. For matrix-type VRs, the API is first dispersed in the selected matrix polymer (powder or pellet form) before final forming operations occur (Fig. 8). The types of equipment used to disperse API throughout the base polymer are Banbury type mixers and single/twin screw compounders suitable for melting the polymer and high shear/torque mixing. Another method used to disperse API into polymer prior to extrusion is solvent casting [42,43] with drug and polymer dissolved in suitable solvent then evaporated to form films that are subsequently chopped up and fed into the extruder. Extruders have the capability to provide a continuous output of API loaded rod, sized according to a mold or 'die' fitted to the output of the extrusion barrel. This rod can be used directly to create rings if it is cut, shaped into a torus and butt welded to form seam joint [42]. Also, with the advantage of simple thermo-mechanical jointing methods, it has been possible to combine segments of compatible API matrix rods to form segmented matrix rings containing multiple API [43]. Alternatively, as for DPV loaded EVA matrix rings, the rod can be cut into granules via pelletization and

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used in injection molding operations to form the final ring. Injection molded thermoplastic matrix rings have also been adopted for combinations of API for HIV and contraceptive function [40].

Co-extrusion is a widely-adopted method for manufacture thermoplastic reservoir VRs. Here, API is compounded in an extruder to provide a homogenous output of API + polymer, while an additional extruder provides a secondary stream of non-medicated polymer that is compatible with the polymer used in the active stream. The API loaded stream forms a rod that is coated with the non-medicated stream forming a core/sheath configuration with core diameter and sheath thickness dictated by the geometry of the die. The rod is cut into defined lengths, placed into a jig that bends it into a toroid and butt-welded to form a seam. A non-microbicide example of a commercial thermoplastic reservoir ring manufacture is NuvaRing [58,102]. Powdered EVA is mixed with the contraceptive hormones using a twin screw extruder and fed through a co-extrusion die, while a second extruder feeds non-medicated EVA to provide a rate controlling sheath layer thickness of approximately 100 μm.

Reservoir-type VR design principles have also been used to create microbicide loaded thermoplastic devices using polyurethane sheathes [83]. Hydrophilic polyurethane was extruded into a hollow tube configuration and the lumen filled with microbicide powder only or powder and glycerol/water combinations, followed by end-sealing using an induction weld. This straight piece of sealed tubing was then shaped in a ring die, annealed then the two ends of the ring were joined using a final induction weld. Different formulations of a combination microbicide/contraceptive hormone VR were fabricated from conventionally co-extruded LNG reservoir strands and hollow core design TFV strands [61]. To reduce leakage of LNG into the TFV reservoir strand, low permeability polymer caps were placed between the different reservoir segments, and all of the various joints were induction welded.

The Population Council has recently reported a multipurpose prevention ring containing API to prevent HIV, HSV, and HPV in combination with a contraceptive hormone [60]. The device is a combination of matrix and core technologies in order to release both hydrophobic and hydrophilic actives. The outer body of the ring was formed in two halves using separate hot melt extrusion stages combining EVA, microbicide and contraceptive hormone. The first extrusion stage produced a semi-circular; half ring with a channel into which an inner core formulation using hydrophilic actives was formed. The second hot melt extrusion step produced the upper half of the matrix ring minus the channel and completed the ring profile. Orifices of defined diameter were drilled through the matrix outer body to facilitate release of the hydrophilic core.

2.6. Microbicide candidates

It is not the remit of this article to provide a comprehensive review of the dozens of HIV microbicide candidates that have been evaluated over the years. Instead, the focus here is on those molecules that either have previously been tested or are being actively developed in VR formulations. A short overview of non-specific microbicides is presented first, before a more comprehensive review of ARV-based microbicides, and particularly the lead candidate ARVs DPV and TFV.

2.6.1. Non-antiretroviral microbicides

First generation microbicide candidates tended to be non-antiretroviral compounds that had broad-spectrum activity against HIV and other sexually transmitted infections (STIs) (Table 3). These non-specific microbicides covered several modes of action, often not specific to the HIV life cycle and including surface active detergents and surfactants that could destroy or disrupt the viral membrane, pH acidifiers that could maintain the protective pH of the vagina and long chain polyanionic compounds that could non-specifically inhibit viral fusion, attachment and entry into the host cells [103–107].

Although the majority of broad-spectrum microbicide candidates (e.g. Savvy, BufferGel, cellulose sulfate) were formulated in vaginal gels and creams, several VR formulations have also been evaluated (Table 3). In 2003, a silicone elastomer matrix-type VR releasing nonoxynol-9 was reported [18]. Although N-9 had shown promising antiviral activity against HIV-1 and other sexually transmitted infections in vitro [107–109], development of N9 microbicide products was halted following the discovery that it failed to provide protection against HIV-1 during clinical trials and that repeated exposure to the spermicidal compound actually damaged the vaginal epithelium and increased the risk of HIV-1 acquisition [19,110–112]. ZA has shown early promise as a broad-spectrum microbicide against HIV-1 and herpes simplex virus 2 (HSV-2) [113–115], despite an unknown mechanism of action.

Another non-specific microbicide being developed as a ring formulation is Boc-LBA, a betulonic acid derivative with anti-HIV activity in vitro [66]. This multi-step entry and fusion inhibitor was first formulated in a bio-soluble acacia gum reservoir-type VR (Table 3) [26,66]. More recently a nanoporous hydrophilic hydrogel-based combination ring product (Bioring™) containing Boc-LBA in combination with ferrous gluconate (a non-hormonal contraceptive), ascorbic acid, polyamino-polycarboxylic acid mixtures and the nucleotide reverse transcriptase inhibitor (NRTI) TFV is undergoing early preclinical development by Bioring LLC [67].

Griffithsin (GRFT), a naturally occurring lectin found in red algae (Griffithsia sp.) is also undergoing early preclinical evaluation in a VR device. GRFT has shown potent, broad-spectrum antiviral activity as a non-specific entry inhibitor against HIV-1, HIV-2 and other STIs including HSV-2 in vitro warranting further investigation as a topical microbicide [116–119]. Studies have shown that GRFT, a carbohydrate binding agent, binds to mannose rich glycans on viral envelope glycoproteins of HIV and HSV-2, coating the virus surface and thereby preventing/inhibiting penetration of host cells [117,120].

The high profile clinical failures of many non-ARV microbicides [121–123] have directed the microbicide field, and VR development

Table 3
Summary of non-ARV HIV microbicide candidates that have been formulated in vaginal ring devices.

Microbicide candidates/APIs	Ring type/polymer	Clinical indications				Organization	Development stage
		HIV	HSV-2	HPV	Pregnancy		
Nonoxynol-9 (N-9)	Matrix/silicone elastomer	✔				Queen's University Belfast	Halted
Zinc acetate, carrageenan (ZC)	Core/EVA	✔	✔	✔		Population Council	Advanced preclinical
Zinc acetate, carrageenan, MIV-150 (MZC)	Core/EVA	✔	✔	✔		Population Council	Advanced preclinical
Zinc acetate, MIV-150, LNG (MZL)	Core/EVA	✔			✔	Population Council & ProMed Pharma	Early preclinical
Zinc acetate, carrageenan, MIV-150, LNG (MZCL)	Core/EVA	✔	✔	✔	✔		Early preclinical
Boc-lysinated betulonic acid (Boc-LBA)	Reservoir/bio-soluble acacia gum	✔				Weill-Cornell Medical College & Bioring LLC	n/a
Bioring™; Boc-lysinated betulonic acid, ferrous gluconate, ascorbic acid, polyamino-polycarboxylic acid, TFV	Nanoporous elastomer (hydrophilic) hydrogel	✔			✔	Bioring LLC	Early preclinical
Griffithsin	n/a	✔	✔	✔		Population Council	Early preclinical

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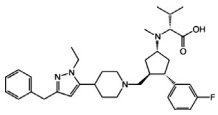
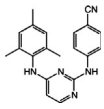
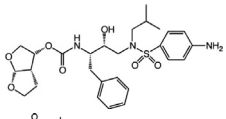
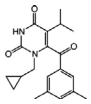
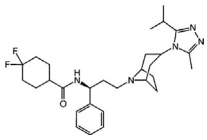
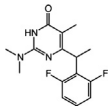
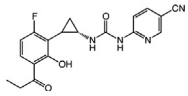
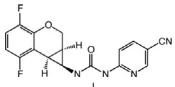
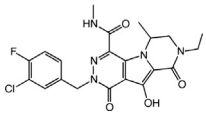
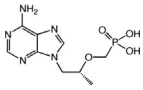
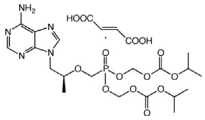
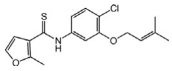
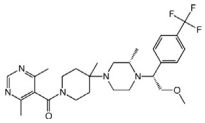
specifically, towards use of potent ARVs that act specifically against the HIV life cycle. However, concerns over the development of ARV resistant strains mean that there may still be a place for non-specific, broad-spectrum microbicidal agents that are safe and effective against HIV and a range of sexually transmitted infections.

2.6.2. Antiretroviral microbicides

A large number of both approved and experimental ARV drugs have been evaluated for formulation in VR devices (Table 4). The two lead candidate microbicides – TFV and DPV – are now discussed in detail.

Tenofovir (({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)phosphonic acid, also known as PMPA, Table 4) and its oral prodrug form TDF (marketed as Viread, Table 4) are nucleotide analog antiviral drugs made by Gilead Sciences Inc. and commonly used in the treatment of HIV infection. Consistent with other hydrophilic, negatively charged, acyclic phosphonate nucleotide analogs, TFV suffers poor oral bioavailability. TDF, however, in which the negative charges of its phosphonic acid groups are masked by phosphodiester modification, is significantly more lipophilic resulting in greatly enhanced oral bioavailability [124–127]. Following absorption, TDF rapidly undergoes esterase hydrolysis to TFV, which is then metabolized intracellularly to its active anabolite tenofovir diphosphate, a competitive inhibitor of HIV-1 reverse transcriptase that interferes with and terminates DNA replication.

Table 4
Summary of antiretroviral drugs that have been formulated in vaginal rings as HIV microbicides. ENT — entry inhibitor; INT — integrase inhibitor; NNRTI — non-nucleoside reverse transcriptase inhibitor; PI — protease inhibitor; NRTI — nucleoside reverse transcriptase inhibitor.

Antiretroviral	Mechanism of action	Chemical structure	Vaginal ring types	Reference(s)
CMPD167	ENT		Matrix; silicone elastomer	[96,158]
Dapivirine	NNRTI		Matrix and core; silicone elastomer	[20,38,56,75,76,80,81,153,195]
DRV	PI		Matrix; silicone elastomer	[81]
IQP-0528	NNRTI		Matrix; polyurethane	[64,232]
Maraviroc	ENT		Matrix; silicone elastomer	[80,96]
MC1220	NNRTI		Matrix; silicone elastomer	[69]
MIV-150	NNRTI		Matrix; silicone elastomer and EVA	[77,233,234]
MIV-160	NNRTI		Matrix (solvent cast)	[78]
MK-2048	INT		Reservoir; also being evaluated in combination with Vicriviroc	n/a
Tenofovir	NRTI		Pod; reservoir	[61,83,86,87,89,91]
Tenofovir disoproxil fumarate	NRTI		Matrix, reservoir and pod; TPU, EVA and silicone elastomer	[62,63,84,88,152]
UC781	NNRTI		Matrix; EVA, TPU and silicone elastomer	[41,79,229]
Vicriviroc	ENT		Reservoir; also being evaluated in combination with MK-2048	n/a

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In 2006, Gilead Sciences granted a co-exclusive, royalty-free license to CONRAD and the IPM to develop TFV vaginal formulations for use by women in developing countries to prevent HIV infection. Since then, a large number of journal papers have been published describing either TFV or TDF formulated for vaginal application, including gels [128–144], tablets [145–147], nanoparticles [148–150] and VRs [43, 61–63,68,83,86–89,91,151,152].

The relatively hydrophilic nature of both TFV and TDF results in poor release characteristics from traditional permeation-controlled VR formulations fabricated from silicone elastomer or EVA, a consequence of the limited solubility of the TFV and TDF in these polymeric materials. Therefore, many of the VR strategies pursued for these drugs have involved use of novel ring designs and/or alternative polymer systems that overcome this permeation obstacle. The various ring types reported for delivery of TFV and TDF are summarized in Table 5. 'Pod VRs' comprise one or more drug-loaded pods embedded within a non-medicated silicone elastomer ring body containing delivery channels sited adjacent to each pod (Fig. 2). The pods themselves are effectively small, compressed, solid tablets coated with a semi-permeable polymer (such as polylactic acid) to offer osmotic control [86]. By adjusting the number of pods and the width of the delivery channels, VRs can be fabricated containing multiple drug compounds with independent control of release rate. These pod rings have been widely studied for controlled release of TFV and TDF, either alone [88,89] or in combination with other ARV [91], antiviral [86, 89] and contraceptive drugs [91]. Typically, pod rings offer near constant drug release rates in vitro and maintain constant levels in the relevant biological compartments (cervicovaginal fluid, vaginal tissue and blood plasma) during the ring use period. In a 28-day comparative pharmacokinetic study in sheep, pod rings containing TDF produced drug tissue levels 86-fold higher than similar ring containing TFV, despite similar concentrations of each drug reported in cervicovaginal lavage [88]. A similar pharmacokinetic study in pig-tailed macaques demonstrated that TFV concentrations in vaginal lavage and tissue could be modulated by modification of the pod ring design by adjusting the size of the TFV reservoir and/or the width of the delivery channel [87]. The ability of the pod VR design to simultaneously deliver multiple drug compounds makes it an interesting platform for development of a multipurpose technology (MPT) VR, as exemplified by a pharmacokinetic study in sheep demonstrating steady state release of five – TFV, NVP, SQV, ETN and E2 – from a single pod-type ring device [91].

An alternative approach that has been used successfully to provide sustained vaginal delivery of TFV or TDF involves VR devices manufactured, at least in part, using hydrophilic thermoplastic polyurethanes (TPUs). The ability of these polymers to swell in the presence of

Table 5
Summary of vaginal rings reported for the delivery of tenofovir (TFV) or tenofovir disoproxil fumarate (TDF). Abbreviations: ACV — acyclovir; E2 — estradiol; ETN — etonogestrel; LNG — levonorgestrel; NVP — nevirapine; PK — pharmacokinetic; SQV — saquinavir; TPU — polyurethane.

Drug(s)	Vaginal ring type	Materials	Image	Study details	Reference
TFV + DPV	Segmented matrix	Hydrophilic and hydrophobic TPUs		In vitro characterization of dual segment polyurethane VRs; 30 day release of TFV and DPV achieved	[43]
TFV	Reservoir (tubing) filled with either solid TFV or TFV + glycerol + water paste	Hydrophilic TPUs		In vitro characterization; PK testing in sheep; 90 day of TFV achieved	[83]
TFV + ACV	Pod (multiple)	Silicone elastomer ring body; polylactic acid-coated pellets		In vitro characterization; 28 day of both TFV and ACV achieved	[86]
TFV and TDF	Pod (×2)	Silicone elastomer ring body; polylactic acid-coated pellets		28-Day PK study in sheep; tissue levels of TDF were 86-fold higher than TFV	[88]
TFV + ACV	Pod (×4)	Silicone elastomer ring body; polylactic acid-coated pellets		In vitro characterization; 28-day PK evaluation in rabbits and sheep	[89]
TFV	Pod (×4)	Silicone elastomer ring body; polylactic acid-coated pellets		In vitro characterization; safety and 28-day PK evaluation in pig-tailed macaques	[87]
TDF	Matrix	Hydrophilic TPU; EVA; silicone elastomer		In vitro characterization, including testing in cell and explant models	[62]
TFV	Matrix	PLA and EVA blends		In vitro characterization	[151]
TFV + NVP + SQV + E2 + ETG	Pod (×10)	Silicone elastomer ring body; polylactic acid-coated pellets		28-Day PK study in sheep; demonstration that five different drugs can be administered simultaneously	[91]
TFV	Matrix	PCL		In vitro characterization	[68]
TDF	Matrix; reservoir (tubing) with solid TDF ± solid excipients	Hydrophilic TPU		In vitro characterization; multiple low-dose SHIV challenge study in macaques; 100% protection achieved	[63]
TDF	Reservoir	NA		6-Month safety and PK study in pig-tailed macaques	[152]
TFV + LNG	Dual-segment reservoir	Hydrophilic TPU		In silico, in vitro and in vivo (rabbit) evaluation	[61]

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aqueous liquids (including in vitro release media and presumably vaginal fluid) offers an alternative release mechanism to the permeation control offered by rings fabricated from hydrophobic silicone elastomer and EVA materials. Equilibrium water absorption values for TPUs can range from 20 to 900% depending on selection of the polymer grade. For VR fabrication, water absorption capacities at the lower end of the range are used [43,83], since excessive swelling in vivo would likely be problematic from the perspectives of both ring expansion/mechanical pressure and vaginal fluid uptake. Rings containing two different matrix-type TPU segments, one hydrophilic and the other hydrophobic, have been reported for co-delivery of TFV and DPV [43]. As expected with matrix-type configurations, the amount of drug released from these rings decreased with time. By incorporating TFV powder or a TFV + glycerol + water mixture into the lumen of extruded TPU tubing and then joining the ends of the tubing to form a reservoir-type ring device, zero-order TFV release kinetics were achieved [83]. Release rates were greater for rings comprising the TFV + glycerol + water mixture, and the TFV release rate increased with equilibrium swelling value of the hydrophilic TPU. In a sheep PK study, rings fabricated from the 35% *w*/w swelling TPU and containing the TFV + glycerol + water mixture provided maintenance of TFV vaginal fluid concentrations close to 10^6 ng/g over the 90-day study period [83]. By comparison, the control 1% w/w TFV gel administered once daily showed TFV vaginal fluid levels steadily declining from 10^6 to 10^4 ng/g over a 28-day period. A similar ring design containing TDF (rather than TFV) in combination with sodium chloride as an osmotic agent offered protection against repeated vaginal challenge with simian human immunodeficiency virus 162p3 (SHIV162p3) in pig-tailed macaques over 28 days [63]. TFV levels in vaginal secretions and tissue remained consistent for 6 months with no adverse safety concerns [152].

TFV vaginal gel has previously shown a 39% reduction in HIV and an unanticipated 51% reduction in HSV-2 acquisition when used by women [13]. This HSV activity has also been demonstrated in in vitro cell and explant models for a TPU VR containing TDF [62], further supporting the concept of a MPT ring. TFV, in the form of a glycerol paste, has also been successfully combined with the contraceptive progestin LNG in a segmented dual-reservoir TPU VR offering continuous release of both drugs over 90 days [61].

Dapivirine (DPV), also known as 4-[[4-[(2,4,6-trimethylphenyl)amino]-2-pyrimidinyl]amino]benzonitrile and referred to in the early literature as TMC120, is an experimental ARV drug that acts against HIV by inhibiting the reverse-transcriptase enzyme. Like many ARV HIV microbicides, DPV was originally developed – by Janssen Research and Development (formerly Tibotec Pharmaceuticals Ltd.), a subsidiary of Johnson & Johnson – as an oral ARV compound for treatment of HIV/AIDS. However, DPV showed such poor oral bioavailability in early stage clinical studies (due to its extremely low aqueous solubility) that this treatment option was abandoned. The compound was subsequently repurposed for vaginal application in 2004 when Tibotec granted IPM a non-exclusive, royalty-free license to develop DPV as a microbicide for use in low/middle income countries. In 2014, Janssen granted IPM exclusive worldwide rights to DPV. During this time, DPV has been extensively tested in a wide range of vaginal formulations, including gels, films, rings, freeze-dried matrices, nanoparticles, capsules, tablets and rings. IPM has completed numerous Phase I/II clinical trials of the compound in Africa, Europe and the United States (Table 6), all of which have demonstrated good safety, tolerance, user acceptability and pharmacokinetic profile. In response to concerns over poor user adherence to gel products and the preference for a single device offering sustained vaginal delivery of ARV compounds over extended time periods, IPM has now prioritized development of their DPV-releasing VR [153].

Proof-of-concept for a DPV-releasing VR was first demonstrated in in vitro studies that reported continuous, zero-order release from core-type (also known as reservoir-type, Fig. 2), silicone elastomer, VRs over 71 days [20]. Based on upper limits for the volumes of cervicovaginal fluid and semen, and assuming that in vivo release rate matched in vitro release, the 136 μg/day release rate was calculated to be capable of maintaining vaginal concentrations of DPV several orders of magnitude in excess of reported HIV inhibitory concentrations. Subsequent Phase I clinical studies conducted in Belgium assessed the safety and PK of a matrix-type (25 mg DPV loading) and two different core-type (25 mg and 200 mg DPV loadings) silicone elastomer VRs [56,85]. The 25 mg matrix-type ring, in which the solid crystalline drug is dispersed throughout the entire volume of the device, produced higher concentrations of DPV in vaginal fluid, vaginal tissue and blood plasma compared with the core-type rings, reflecting the ready availability of drug at the ring surface. Both the core-type and matrix rings were safe and well tolerated and delivered DPV to the vaginal region for 28 days at concentrations over 4 logs greater than the EC_{50} for wild-type HIV-1 (LAI) in MT4 cells (0.3 ng/mL) [154,155]. Importantly, systemic exposure of DPV with all ring formulations was deemed sufficiently low to alleviate concerns concerning the emergence of resistance strains of HIV.

Early DPV ring prototypes were fabricated using condensation-cure silicone elastomer systems [20,26,56,85,153] (Fig. 3). The curing reaction associated with these materials produces a volatile alcohol by-product that detrimentally affected DPV distribution within the ring and its release after storage. As a result, the current version of the DPV-releasing VR, a 28-day matrix-type device containing 25 mg micronized DPV (Ring-004), is fabricated using an addition-cure silicone elastomer that produces no cure by-product (Fig. 4). Compared with core-type rings (Fig. 2), the simplicity of the matrix design of Ring-004 ensures ease of manufacture, low cost of manufacture, and higher pharmacokinetic exposure. A recent safety and pharmacokinetic study in women testing consecutive use of multiple 004 rings for up to 57 days reported detectable DPV concentrations in vaginal fluid and plasma within 4 h after ring insertion, indicating rapid release and absorption of DPV [76].

With a view to expanding options for testing human-sized VR formulations in animal models, Holt et al. recently reported safety and pharmacokinetic evaluation of the DPV Ring-004 in Suffolk cross sheep [156]. DPV plasma and vaginal fluid levels were lower than those measured in previous ring clinical studies [56,76,85,157]. DPV was also detected remotely in the neighboring rectal compartment, as reported previously with vaginal administration of the experimental entry inhibitor CMPD167 using aqueous gels and VRs [158].

IPM and clinical trial partner the Microbicide Trials Network (MTN) are currently conducting two Phase III long-term safety and efficacy studies of the monthly DPV ring as part of IPM DPV Ring Licensure Program, with efficacy results expected as soon as early 2016 (Table 6). The Ring Study, started in April 2012 and conducted by IPM, enrolled 1959 HIV-negative women aged 18 to 45 across seven research centers in South Africa and Uganda. The ASPIRE study, started in August 2012 and conducted by MTN, enrolled 2629 HIV-negative women aged 18 to 45 across 15 sites in Malawi, Uganda, South Africa and Zimbabwe. In both trials, women were randomly assigned to use the monthly Ring-004 or placebo rings for at least one year. Results of both studies will be reported early 2016.

2.7. Combination microbicide and multipurpose prevention technology (MPT) rings

HAART for treatment of HIV/AIDS involves the use of ARV combinations. By using drugs from different therapeutic classes and with different mechanisms of action, the virus is targeted at multiple stages of the infection/replication cycle, which can increase the breadth of activity and reduce the propensity for emergence of resistant viral strains [159–162]. It is rational to extend this combination strategy to ARV-based vaginal microbicides [163,164]. A combination of emtricitabine (a nucleoside reverse transcriptase inhibitor) and TDF (a nucleotide analog reverse transcriptase inhibitor) administered orally has already been shown to confer HIV protection in men who have sex with men

Table 6
Completed, ongoing and planned microbicide vaginal ring clinical trials. Abbreviations used in table: DPV — dapivirine; IPM — International Partnership for Microbicides; FTC — emtricitabine; PK — pharmacokinetics; LNG — levonorgestrel; MTN — Microbicide Trials Network; MPT — multipurpose prevention technology; MVC — maraviroc; TDF — Tenofovir disoproxil fumarate; TFV — tenofovir.

Trial	Description	Phase	Countries	No. women	Status
IPM 001	DPV ring safety (Ring-001)	1	Belgium	12	Completed
IPM 008	DPV ring safety (Ring-002)	1	Belgium	13	Completed
IPM 011	Placebo ring safety & acceptability	n/a	South Africa/Tanzania	170	Completed
IPM 013	DPV ring PK (Ring-004)	1	Belgium	48	Completed
IPM 015	DPV ring safety (Ring-004)	1/2	Kenya, Malawi, South Africa, Tanzania	280	Completed
IPM 018	DPV ring PK (Ring-002 & Ring-003)	1	Belgium	24	Completed
IPM 024	DPV ring PK (Ring-004)	1	Belgium	16	Completed
IPM 026/MTN 013	MVC, DPV, and DPV-MVC combination rings	1	USA	48	Completed
IPM 027	'The Ring Study' — DPV ring long-term safety and efficacy	3	South Africa, Uganda	1959	Ongoing
IPM 028	DPV ring drug–drug interaction (Ring-004)	1	Belgium	36	Completed
IPM 029	DPV ring & male condom functionality (Ring-004)	n/a	USA	70 couples	Completed
IPM 030/MTN 023	DPV ring safety (Ring-004)	2a	USA	96	Ongoing
IPM 031/MTN 024	DPV ring safety and acceptability (Ring-004)	2a	USA	96	Ongoing
IPM 033	DPV ring and female condom functionality (Ring-004)	n/a	USA	80 couples	Study report in progress
IPM 034	DPV ring PK (Ring-004)	n/a	Belgium	40	Completed
IPM 035	DPV ring & menses and tampon use (Ring-004)	n/a	Belgium	32	Ongoing
IPM 036	DPV ring drug–drug interaction (Ring-004)	1	Belgium	36	Ongoing
MTN 020	ASPIRE — DPV ring efficacy & safety (Ring-004)	3	Malawi, South Africa, Uganda, Zambia, Zimbabwe	3475	Completed; awaiting results
IPM	Combination MPT ring containing DPV + LNG	1	n/a	n/a	Planned (2016)
CONRAD	TDF ring/safety and PK (IVR-001)	1	USA	30	Completed
CONRAD-128	TFV-only ring and TFV + LNG ring/Safety, PK & PD	1	USA, Dominican Republic	50	Completed, awaiting results
Auritec	TDF-only, TDF + FTC and TDF + FTC + MRV rings/safety and PK	0	USA	6	Ongoing
MTN 027/NIAID	MPT rings containing Vicriviroc and MK-2048A/Safety and PK	1	USA	48	Recruiting
MTN 028/NIAID	MPT rings containing Vicriviroc and MK-2048A/PK	1	USA	18	Recruiting
Population Council	MPT ring containing griffithsin	1	n/a	n/a	Planned (2017/18)

[165] and the same combination was investigated as part of the VOICE trial [166].

A number of combination microbicide VRs are in the early stages of preclinical/clinical development. Following on the heels of the DPV Ring-004 are second-generation formulations containing DPV in combination with maraviroc (MVC) [80,157], darunavir (DRV) [81] and TFV [43]. MVC is an entry inhibitor ARV that binds the CCR5 co-receptor and prevents the cell entry of the most frequently transmitted HIV-1 strains [167–170]. It is considered a highly promising microbicide candidate because of its activity against HIV strains resistant to other ARVs and its use as a component of current HAART regimes. Aqueous vaginal gel formulations of MVC have previously been shown to prevent the vaginal transmission of SHIV-162P3 to macaques [171,172], and subsequent PK testing of the gels in macaques has helped define the local concentrations required for protection [158,173]. The first report of a MVC-only VR formulation demonstrated that pretreatment of macaques with Depo-Provera (a subdermal injectable contraceptive) significantly modified biodistribution of the drug [96]. Following a 2008 licensing agreement with Pfizer (now ViiV Healthcare), IPM is developing MVC as a microbicide, initially as a combination with DPV in a matrix-type ring device. Results from the MTN-013/IPM026 clinical study (a multisite PK/pharmacodynamics (PD) study among 48 women of silicone elastomer VR containing 25 mg DPV, 100 mg MVC, both DPV and maraviroc, or placebo; Table 6) showed that (i) MVC vaginal fluid concentrations in both the maraviroc-only arm and the combination arm were 2–10 times lower than DPV levels (despite the higher initial drug loading), (ii) cervical tissue levels of MVC were mostly below the limit of quantification, and (iii) no in vitro HIV inhibitory activity was observed with the maraviroc cervical tissue samples [157]. It was concluded that MVC was not released from the rings in sufficient quantities to provide cervicovaginal concentrations capable of providing protection against HIV transmission. Previous in vitro testing of the same combination ring device showed that the quantities of DPV and MVC released from a 25 mg DPV + 100 mg MVC combination matrix-type silicone elastomer ring were similar [80]. Therefore, the poor release of MVC from the same ring formulation in the clinical setting is most likely due to physiological constraints placed upon MVC in the in vivo environment. These could include poor solubility in vaginal fluid, poor stability in vaginal fluid, poor tissue absorption, and/or rapid elimination from the tissue.

DRV is a second-generation PI used in combination with other ARVs in the treatment of HIV infection. PIs inhibit the HIV protease enzyme required to produce mature infectious virus particles by cleaving structural proteins and enzymes from their precursors. Their high potency within HAART regimens and the relatively high genetic barrier to the emergence of resistant HIV strains (compared with other ARVs) suggest that they have good potential as microbicides, administered alone or in combination with other ARVs [174,175]. Preclinical development, including testing of pharmacokinetics in macaques, has recently been reported for matrix-type silicone elastomer VRs containing various loadings of DPV and DRV [81]. Serum and vaginal concentrations of both DPV and DRV in macaques during 28-day ring placement were measured within the same general range to those reported previously for DPV-only rings in women [56,76,85,157]. Based on the results of this study, the potential of PIs as vaginal microbicides, either alone or in combination, warrants further investigation.

VRs composed of biosoluble acacia gum or a non-biodegradable hydrogel of 2-hydroxyethyl methacrylate and sodium methacrylate have previously been assessed for formulation of microbicide combinations, selected from DPV, TFV, AZT and Boc-LBA [66]. A potential issue with these gum and hydrogel matrices for ring fabrication is their propensity to absorb aqueous fluids and swell, which could be problematic in vivo. These ring formulations are not being actively developed.

The combination microbicide VRs discussed so far have been limited to two ARV drugs incorporated into the same compartment within the ring device, a strategy previously exploited with the contraceptive ring NuvaRing in which etonogestrel and ethinyl estradiol are located within the same core. However, this simple and relatively inexpensive approach to incorporating multiple drug compounds within a ring also introduces challenges, including increased potential for drug–

drug interactions and reduced ability to independently control the release of each drug. A formulation strategy to overcome these challenges involves the fabrication of ring devices having multiple separate compartments, each compartment containing a single drug active. Several variations on this formulation approach have been reported. Dual-segment rings comprising DPV incorporated into a hydrophobic polyurethane segment and TFV incorporated into a hydrophilic polyurethane segment (Fig. 5) showed good drug stability and in vitro release properties [43]. This approach is particularly useful for microbicide molecules having contrasting hydrophilic/hydrophobic character.

Despite the fact that a safe and effective vaginal microbicide product has yet to reach market, there is already considerable interest and early-stage development activity around next-generation products that combine HIV prevention with contraception and/or prevention/treatment of other sexually transmitted infections (STIs) and reproductive tract infections. Formulation strategies for multipurpose prevention technologies (MPTs) are generally based upon the extensive range of product types available within both the mature contraceptive market and the emerging HIV microbicide pipeline [48]. Many of the MPT products currently undergoing development have prioritized use of LNG as the contraceptive hormone component, based on its historical record of safety and effectiveness [46,176,177]. Both DPV and TFV are being developed as MPT rings in combination with LNG [61,153]. Clark et al., describe a segmented dual-reservoir polyurethane VR (Fig. 2G) that delivered the TFV and LNG continuously for 90 days in a rabbit pharmacokinetic model [61]. TFV was incorporated into a hydrophilic polyether urethane reservoir segment in the form of a glycerol paste, while the levonorgestrel was located in a separate polyether urethane reservoir segment. A DPV + LNG VR is also in development, based on a similar silicone elastomer matrix-type design to that of the dapivirine-only VR [153].

A number of MPT VR prototypes containing ZA in combination with the ARV agent MIV-150, the linear sulfated polysaccharide CG and the contraceptive steroid LNG are being actively developed by The Population Council (Table 3). A 2014 study by Ugaonkar et al. reported that sustained in vitro release of ZA from MIV-150/ZA/CG and MIV-150/ZA/CG/LNG combination core-type EVA VRs could be achieved for up to 90 days thus offering the potential for protection from HIV-1, HSV-2 and unwanted pregnancy from a single ring device [60]. Results from macaque efficacy and pharmacokinetic (PK) studies have also been promising, indicating that the ZA combination VR devices are capable of providing protection from SHIV-RT and reducing viral shedding of HSV-2 [60].

3. Challenges moving forward

3.1. Formulation and delivery of biomacromolecular microbicides

Small-molecule ARVs are currently the major focus of the microbicide field. However, there is some interest in the use of biomacromolecular compounds as vaginal microbicides, including proteins (cyanovirin-N, GRFT, 5P12-RANTES), peptides (T-1249, PIE12 trimer, retrocyclin RC-101), monoclonal antibodies (mAbs) (b12, 2F5, 4E10, 2G12, VRCO1) and nucleic acids (DNA, short interfering RNA (siRNA)). Many of these biopharmaceuticals agents can inhibit transmission of HIV and other STIs by either directly targeting the free virus or by blocking the host cell receptors [178–180].

Broadly neutralizing mAbs such as b12, 2F5, 4E10 and 2G12 [93, 181–185] have shown promise due to their high potency, excellent safety profile and their unique ability to be both specific while having a broad spectrum of action when combined in a multi-antibody formulation [178,180]. MABGEL1, a monoclonal antibody gel containing 2F5, 4E10 and 2G12 developed by the European Microbicides Program (EMPRO) was the first reported mAb vaginal product to undergo Phase I pharmacokinetic and safety testing. The study demonstrated that daily application of up to 50 mg of each Ab was safe over a 12 day period and was able to achieve concentrations with the potential to block HIV transmission. However, stability of the mAbs was a significant issue for these gel formulations [185]. Until recently challenges regarding production costs, production capacity, quality control and safety of biological therapeutics have prevented large-scale development and evaluation of mAbs in a microbicidal formulation [178]. However, recent advances in the production of mAbs in plants such as Nicotiana benthamiana has provided the potential for safe, rapid, cost effective production of N-produced recombinant human monoclonal antibodies (N-mAbs) [186].

The first antibody-releasing VR was reported in 1992 [187]. These proof-of-concept VR devices formulated using lyophilized antibody particles of bovine serum albumin (BSA) and anti-human chorionic gonadotropin (anti-hCG) in thermoplastic EVA demonstrated sustained Ab release for up to 30 days and prevented HSV-2 transmission in mice, thus paving the way for future Ab-releasing ring studies [188,189]. In 2011 Morrow et al., reported that a rod-insert VR device was capable of releasing the mAb 2F5 [93]. The ring comprised a silicone elastomer ring body into which multiple 2F5-loaded lyophilized hydroxypropyl methylcellulose (HPMC) gel inserts could be placed. In vitro release testing demonstrated that the rod-insert device was capable of delivering over 1 mg of 2F5 for a period of up to 100 h dependent on the lyophilized gel insert formulation. These VR devices provided the capability to deliver temperature-sensitive biologically-based microbicides as production of the lyophilized gel inserts did not involve the use of high temperatures normally associated with polymer ring manufacture [16].

Currently, several antibody-containing VRs are undergoing early preclinical development. In a recently reported study by Gunawardana et al., a novel pod-type platinum-catalyzed silicone elastomer VR demonstrated sustained in vitro delivery of ovine IgG (ov-IgG), a model IgG human antibody, over a period of 14 days further confirming that a VR device has the potential to provide sustained effective release of antibody-based microbicides [86,92]. Mapp66 is a novel multi-antibody microbicide currently under investigation by Integrated Preclinical/Clinical Program for HIV Topical Microbicides (IPCP-HTM) in conjunction with Mapp Biopharmaceutical Inc. [190]. Mapp66 contains a triple combination of N-mAbs (4E10-N, VRCO1-N and HSV8-N) that have the potential to neutralize a range of HIV isolates and prevent sexually transmitted HSV-2 infection. Early studies suggest that a mapp66 VR device utilizing the Versaring pod-insert technology developed by Auritec Pharmaceutical and Oak Crest Institute of Science [86] is capable of efficient intravaginal release of N-mAbs [191].

In addition to monoclonal antibodies, sustained delivery of Llama heavy-chain antibody fragments (VHH) have been reported from a rod-type ring device [192]. Similar to the rod-insert rings reported by Morrow et al. [93], these VR devices manufactured using silicone elastomers were capable of holding multiple HPMC compressed or lyophilized gel antibody tablets. In vitro release testing demonstrated effective release of the highly potent HIV-1 entry inhibitor (VHH A12) over a 7-day period in concentrations sufficient to offer protection in the vaginal environment [192].

As discussed earlier in this review (see Section 2.6.1) GRFT, a naturally occurring algal protein is also undergoing early preclinical evaluation as a potential virus entry inhibitor against multiple STIs including HIV-1, HIV-2 and HSV-2. VR devices containing GRFT in combination with CG or MIV-150 are currently under evaluation [119,193].

While these biopharmaceuticals have shown early promise as microbicidal candidates their high production costs, stability and formulation issues remain major obstacles for their successful development as effective microbicidal products.

3.2. Manufacturing issues and scale up

A potential disadvantage of silicone elastomer VRs is the increased complexity in scaling the elastomer/API mixing processes. Most silicone elastomer VR projects use DAC mixers to disperse API into the elastomer base. However, scalability of this equipment limits batch sizes to 5 kg,

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thereby requiring totally new classes of mixers to be trialed and validated for anything other than early clinical testing. Thermoplastic extrusion processes on the other hand are generally scalable provided that the screw geometry and length to diameter ratio of the extruder are appropriately matched. Also, given the high output capabilities of hot melt extrusion equipment, it is feasible to use the same equipment for early clinical trial product manufacture through to commercial scale production by simply increasing manufacturing duration. A particular disadvantage associated with thermoplastic extrusion as a manufacturing technique to produce VRs is the requirement to cut, bend and weld the extrudate ends to form a full ring; this process is complex to automate and ultimately the rate limiting step in the production output. These issues are not present for thermoplastic or silicone elastomer rings manufactured via injection molding.

Suppliers of injection molding and extrusion equipment often have limited experience of the pharmaceutical industry and hence the requirements of cGMP and stringent quality systems that must be employed. Significant investment in partnerships between original equipment manufacturers and pharmaceutical stakeholders has been necessary to commission suitable equipment. As these manufacturing techniques become more widely adopted by the pharmaceutical industry, equipment that is capable of fulfilling cGMP requirements should be more readily available across the injection molding and extrusion equipment supply sectors.

Multi-cavity injection mold tooling for production of high volumes of rings per cycle requires significant detail and technical expertise to ensure that conditions such as pressure and temperature are uniformly experienced for each ring cavity. In particular, silicone elastomer mold tooling is highly specialized with only a handful of companies worldwide with the expertise to machine tools with the significantly higher tolerances required compared to thermoplastic tools.

Limited choice of contract manufacturing organizations (CMO) with the expertise and capability to manufacture either thermoplastic or thermosetting-based VRs has also slowed the progress of development of microbicide VR products.

Methods for determining the assay value (drug content) of VRs can be time consuming and costly. A method for Process Analytical Testing (PAT) has been proposed using Raman spectroscopy for the 25 mg DPV ring currently under development [194,195]. This method rotates a manufactured ring while the Raman spot is focused on a fixed point providing wide area illumination and the results were correlated to provide a prediction of content assay values for the ring with good levels of accuracy.

3.3. Cost

Since the inception of the vaginal microbicide concept in 1990 [12], the cost factor has been uppermost in the minds of developers. The impact of the HIV pandemic is greatest in Sub-Saharan Africa and Asia/Pacific region, where 24.7 million and 4.8 million respectively are currently living with HIV/AIDS; Sub-Saharan Africa alone accounts for almost 70% of the global total of new HIV infections. Many of the countries within these regions have gross domestic product per capita values significantly less that $1000, with major implications for the availability and quality of healthcare provision. In order to gain widespread use, HIV microbicide products must be affordable to at-risk populations. As with all pharmaceutical products, manufacturing costs will comprise a very substantial part of the total cost structure of a microbicide product, including the costs of the active pharmaceutical ingredients, formulation excipients and product packaging. For the 1% TFV gel tested in the CAPRISA 004 trial, manufacturing costs were reported as US$ 0.50 per dose, a significant proportion of which was for provision of the plastic applicator [196]. By comparison, microbicidal VRs will be much more costly to manufacture, due to increased drug loadings, complexity of product design, advanced manufacturing processes and the use of relatively expensive excipients. However, unlike gels, for which a new dose needs to be applied either daily or before every act of intercourse (depending on the prescribed regimen), microbicide-releasing VRs currently in development are intended to be worn continuously for at least 28 days. This longer duration of use compared with gels will compensate to some degree for the increased costs of ring manufacture. Assuming a fixed manufacturing cost per ring device, extending the duration of ring use will result in a proportional lowering of the daily cost of use. IPM is developing 60 and 90-day versions of their 28-day DPV-releasing ring [153]. Advocates are working with researchers and policy makers to make sure that any approved microbicide will be as affordable and accessible as possible. For example, efforts are already underway to ensure that manufacturing costs of the DPV ring are kept as low as possible.

3.4. Acceptability and adherence

Numerous studies have reported high user acceptability of VRs for contraception and estrogen replacement therapy [29,30,33–35,39,197–202]. Of particular significance is the strong preference for rings over semi-solid systems [34,197] which should hopefully extend to vaginal microbicide products, since high levels of user acceptability/satisfaction generally correlate with user adherence.

Medication adherence is defined as the extent to which users/patients take their medications as prescribed. An estimated 20% to 50% of patients do not take their medications as prescribed and are said to be non-adherent or non-compliant with therapy [203,204]. Medication non-adherence is a major and growing concern for many current drug therapies, including HAART for the treatment of HIV infection [205] and statin medication for chronic coronary artery disease [206]. User adherence to vaginal microbicide products in late-stage clinical studies has proved problematic, particularly for regimens that require regular daily application (i.e. once-daily products) or require timing of application close to coitus (i.e. coitally-dependent products) [207–209]. The most widely cited example is that for the Phase IIb CAPRISA trial of vaginal TFV gel, in which HIV acquisition was reduced by an overall estimated 39% [13,210–212]. However, adherence estimates based on vaginal applicator returns indicated that HIV incidence was 54, 38 and 28% lower in the TFV gel arm for high, intermediate and low adherers, respectively, demonstrating unequivocally that high adherence is key to microbicide effectiveness. In fact, it has primarily been the growing concern over lack of user adherence to gel-based microbicides in clinical studies that has driven the prioritization of ARV-releasing VR products [213,166,214].

It has long been assumed that use of sustained or controlled release delivery systems for vaginal administration of microbicides to prevent infection with HIV will lead to increased microbicide product adherence, acceptability and efficacy compared with more conventional, coitally-dependent, vaginal formulations by simplifying use instructions and requiring less user behavior [15,16,56,215]. Indeed, based on adherence data from other clinical indications [204,216,217], including hormonal contraception for which long-acting depot injections, subdermal implants, transdermal patches and VRs are available [218,219], the case for sustained/controlled release of HIV microbicides is generally well made and widely accepted. Previous studies have reported high levels of user adherence to VRs for non-microbicide clinical indications. For example, in a 3-month study comparing adherence to the contraceptive VR NuvaRing and a daily low-dose oral contraceptive pill, ring users were more likely to report perfect use [220]. Surprisingly, given the acknowledged importance of adherence, only a very limited number of studies have directly addressed the topic of adherence to microbicide VRs [221–223].

One of the major challenges for the HIV microbicide field is the accurate (and preferably quantitative) measurement of adherence in late stage clinical trials [207,215,224]. Generally, methods for measuring adherence can be divided into two distinct categories. Direct measures of adherence, also referred to as “biomarkers”, are substances or effects

whose presence or absence indicates that a biological or pharmacological process has occurred in response to a drug [207]. Indirect measures of adherence comprise two major sub-categories: "objective measures" and "self-report measures", both reliant on the observations or reports of clinicians, trial participants, or others [207,225]. Self-reporting tends to overestimate adherence behavior compared with other assessment methods and generally has high specificity but low sensitivity [225]. Some of the methods previously reported for assessing adherence to microbicides are specific to a particular product type. For example, several advanced vaginal gel applicators have been developed, either containing a dye that changes color upon exposure to mucin or that record the date and time that the syringe piston is depressed into the applicator barrel [226]. Both Phase III clinical studies for the DPV ring – The Ring Study and APSIRE – will attempt to measure women's adherence to the ring by measuring concentrations of DPV in blood and vaginal fluid and testing the residual DPV content in rings after 28-day use.

The recording of vaginal temperature offers an alternative and interesting biomarker option for monitoring adherence to microbicide-releasing VRs. Boyd et al. recently reported the testing in macaques of a vaginally-administered silicone elastomer device fitted with a miniature, battery-operated, temperature logger [97]. The device responded quickly and accurately to vaginal removal and insertion, and produced a regular diurnal temperature pattern comprising higher temperatures during daytime activity and lower temperatures during nighttime inactivity (matching the diurnal cycle observed in a woman's basal body temperature). Ring devices fitted with temperature loggers could be used to directly monitor user adherence as part of late-stage clinical testing.

3.5. Correlating in vitro release with in vivo pharmacokinetics

Development of in vitro-in vivo correlations (IVIVC) for complex, non-oral, extended release products is a long-term aim of many pharmaceutical development programs [227]. The overall aim is to reduce the regulatory burden associated with certain pre- and post-approval changes. For example, manufacturing process, equipment and site changes can be reduced in the presence of a Level A or point-to-point IVIVC. However, developing IVIVC for non-oral extended release products is extremely challenging due to the complex nature of the formulations and the difficulty in accurately mimicking the in vivo release process with an in vitro method [227]. These problems are magnified in the microbicide ring field due to the number of variables about which we have limited information and the fact that many of the biological factors will vary throughout the hormonal cycle. There is also an awareness of the need to define PK-PD relationships for microbicides. This is also beset by challenges due to the unique nature of the products [176].

Completed, current and pending clinical studies involving microbicide VRs are presented in Table 6. Relatively few candidate microbicide compounds have proceeded to clinical testing in a ring device. The current Phase III clinical trials of the DPV 25 mg VR should provide key values for the vaginal fluid, tissue and plasma concentrations seen on repeated use in a much larger sample of women than has been reported to date [56,75,76]. This information, coupled with knowledge of the seroconversion status of trial participants, will help establish the vaginal fluid and tissue concentrations necessary for protection with this microbicide in a clinical setting. More generally, how well these data relate to in vitro IC_{50} values may also prove informative, giving an indication of how close in vitro estimates of activity are to the clinical scenario. However, because of the large differences between candidate microbicides physicochemical properties and mechanisms of action, other microbicide compounds will need to be assessed individually [176].

The PK of DPV released from IPM reservoir-type Ring-002 and matrix-type Ring-003 has been compared [56,153]. (The ring designs and silicone elastomer type used in these rings are not the same as for the Ring-004 design currently being tested in Phase III [153].) The matrix ring led to increased vaginal fluid and plasma levels compared with reservoir ring, although inter-subject variability was significantly lower for the reservoir ring. Interestingly, the vaginal fluid concentration profiles measured did not reflect the differences typically observed in vitro with these rings. The other microbicide tested clinically from a ring device is ACV. A ring containing 64 mg ACV was found to provide comparable cervicovaginal lavage concentrations over 7 or 14 days use, to samples provided 2 h after oral valaciclovir ingestion [90].

Several attempts have been made to correlate in vitro release with that observed in different animal models. A selection of published non-human studies involving microbicide releasing VRs is presented in Table 7. Overall, these studies have provided some evidence for the development of IVIVC in animal species but whether and how this will translate to humans is unclear. First attempts at IVIVCs have been published for TFV and ACV release from pod-insert rings into rabbits and sheep [89], and for double and triple combination microbicide release (TDF, emtricitabine and MVC) in macaques [228].

Several articles have reported a lack of correlation between the in vitro and in vivo release rates. For example, in vitro release rate of UC781 was much higher than that observed in vivo, presumably attributed to the exceptionally poor water solubility of UC781 [79,229]. It has been reported that non-sink in vitro conditions exhibiting partition-controlled release better predicted the total amount of experimental pyrimidinedione microbicides released from polyurethane VRs in pigtail macaques, whereas sink in vitro release conditions, exhibiting typical matrix-type kinetics, over predicted release [83]. In vitro release of MVC and CMPD167 from silicone elastomer rings into simulated vaginal fluid (SVF) was a relatively good predictor of the amount released in rhesus macaques in vivo [96]. Notably, this work also highlighted the differences observed in vivo with the use of depot medroxyprogesterone acetate (DMPA) pre-treatment and the impact this can have on measured absorption. Release of the more hydrophobic MC1220 from matrix-type silicone elastomer rings in macaques was somewhere between that measured in vitro into a mixture of equal parts of propan-2-ol and water or SVF [69]. Other researchers have found conflicting results between in vitro and in vivo testing in animal models. For example, the in vivo concentrations of MIV-150 in vaginal fluids were similar when the microbicide was released from both silicone and EVA rings, despite the EVA rings having a higher drug loading and showing higher in vitro release rates [77].

Recently deterministic models of vaginal distribution of drugs delivered from both gels and rings have been presented [230]. Methods used to determine vaginal drug permeation have also recently been reviewed [231]. Given the highly complex and variable nature of the vaginal environment and the relative simplicity of the currently used in vitro release rate tests, it may prove difficult to effectively correlate values from one

Table 7
List of published articles describing animal testing of microbicide vaginal rings.

Animal species	Compound(s) tested	Reference
Macaque (cynomolgus)	DPV + DRV	[81]
	IQP-0528; IQP-0532	[232]
Macaque (pigtail)	TDF	[63,84,152]
	TDF + FTC + MVC	[228]
	UC781	[229]
	CMPD167	[96,158]
	MC1220	[69]
Macaque (rhesus)	MIV-150	[77,233,234]
	MIV-150 + ZA + CG + LNG	[60]
	MIV-160	[78]
	MRV	[96]
Rabbits	TFV + ACV	[89]
	TFV + LNG	[61]
	UC781	[79]
Sheep	DPV	[156]
	TFV; TDF	[83,88]
	TFV + ACV	[89]
	TFV + NVR + SQN + ETN + EST	[91]

Table 8
Values for the vaginal fluid and plasma/serum concentration of DPV at various time points after initial ring insertion, compared with daily release values measured in vitro (into IPA:H_2O) at equivalent times.

Compartment	Species	Time (days)			
		4	8	15	22
Plasma/serum (pg/mL)	Human[a]	299	357	357	327
	Sheep[b]	58	59	37	32
	Macaque[c,d]	164	94	78	110
Vaginal fluid (μg/g)	Human[a]	44	45	44	37
	Sheep[b]	4.2	2.6	1.7	1
	Macaque[c,d]	3.7	6.1	6	4.9
In vitro daily release (μg/day)[e]	Human ring	684	425	273	212
	Macaque ring	416	278	201	156

[a] Values estimated from published graphs of plasma and vaginal fluid concentration against time; weighted by the number of participants [75,76].
[b] Values estimated from published graphs of plasma and vaginal fluid concentration against time [156].
[c] Values interpolated from concentrations measured at time points either side of the time in question [81].
[d] Macaque sized rings (25 × 6 mm) utilizing an alternative platinum catalyzed silicone were used.
[e] In vitro release was measured into IPA:H_2O, 100 mL for human sized rings, 50 mL for macaque rings.

to another. However, it may be possible to draw some broad inferences from a given release rate test in relation to available clinically tested products, as attempted in Table 8 for vaginal fluid concentrations, plasma concentrations and in vitro release data of DPV during use of the 25 mg VR. Available data for the same ring tested in sheep are presented as are data for a smaller macaque sized ring with the same 25 mg loading, composed of similar but not identical type of silicone. This table is informative if merely to show the large range of values that may be seen between in vitro release rates and those measured in vaginal fluid at any time.

In the first instance, the primary aim of such correlations should be to link previously established in vitro release rates with consistently achieved protective vaginal fluid and tissue levels in the compartments of interest. It might then be possible to tie together in vitro release rate testing, clinical PK profiles and ex vivo assays including challenge assays to provide a more holistic picture of drug loadings and release profiles necessary to afford protection. However, all of the above in vitro tests will need to be benchmarked against clinical concentration and effectiveness data. The ultimate usefulness of IVIVC may only be seen when sufficient clinical data is available to allow such comparisons to be drawn.

4. Conclusions

The past ten years has witnessed unprecedented advances in vaginal ring technology for the delivery of drugs, driven almost exclusively by the development of practical, long-acting and user-friendly vaginal microbicide products for prevention of sexual transmission of HIV. Considerable innovation in the development of novel ring designs has emerged in attempts to achieve clinically effective release rates for microbicide candidates that often possess very different physicochemical properties from the small molecular weight hydrophobic steroid molecules for which the original vaginal ring devices were first described back in the 1970s. The future of vaginal microbicide VRs will likely depend on the outcome of ongoing clinical studies testing dapivirine and tenofovir-releasing rings. In particular, success of the monthly dapivirine ring in two recently completed Phase III studies ('The Aspire Study' and 'The Ring Study') is likely a prerequisite for the future viability of not only vaginal ring strategies for HIV prevention, but for vaginal microbicides in general. If the key indicators for success are met – at least moderate protection against HIV infection; long-term safety; ease of use; user acceptability; good user adherence; and global access – microbicide-releasing VRs are positioned to make a valuable contribution in the fight against one of the greatest threats to women's health globally. Success should also stimulate priority development of next-generation combination microbicide and MPT VR products aimed at further enhancing protection, minimizing development of resistant HIV strains, and additionally offering contraception and protection against other STIs.

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Nano-Formulations for Local and Injectable Use

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Nanoparticle-Based ARV Drug Combinations for Synergistic Inhibition of Cell-Free and Cell−Cell HIV Transmission

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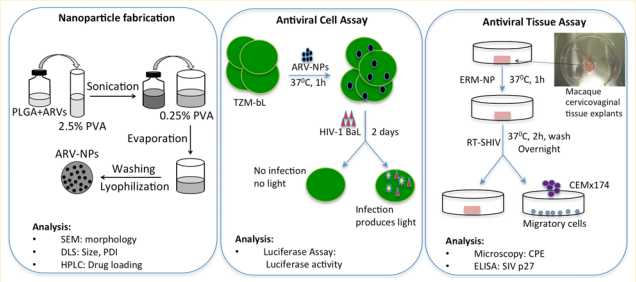
Supporting Information

ABSTRACT: Nanocarrier-based drug delivery systems are playing an emerging role in human immunodeficiency virus (HIV) chemoprophylaxis and treatment due to their ability to alter the pharmacokinetics and improve the therapeutic index of various antiretroviral (ARV) drug compounds used alone and in combination. Although several nanocarriers have been described for combination delivery of ARV drugs, measurement of drug−drug activities facilitated by the use of these nanotechnology platforms has not been fully investigated for topical prevention. Here, we show that physicochemically diverse ARV drugs can be encapsulated within polymeric nanoparticles to deliver multidrug combinations that provide potent HIV chemoprophylaxis in relevant models of cell-free, cell−cell, and mucosal tissue infection. In contrast to existing approaches that coformulate ARV drug combinations together in a single nanocarrier, we prepared single-drug-loaded nanoparticles that were subsequently combined upon administration. ARV drug-nanoparticles were prepared using emulsion-solvent evaporation techniques to incorporate maraviroc (MVC), etravirine (ETR), and raltegravir (RAL) into poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles. We compared the antiviral potency of the free and formulated drug combinations for all pairwise and triple drug combinations against both cell-free and cell-associated HIV-1 infection in vitro. The efficacy of ARV-drug nanoparticle combinations was also assessed in a macaque cervicovaginal explant model using a chimeric simian-human immunodeficiency virus (SHIV) containing the reverse transcriptase (RT) of HIV-1. We observed that our ARV-NPs maintained potent HIV inhibition and were more effective when used in combinations. In particular, ARV-NP combinations involving ETR-NP exhibited significantly higher antiviral potency and dose-reduction against both cell-free and cell-associated HIV-1 BaL infection in vitro. Furthermore, ARV-NP combinations that showed large dose-reduction were identified to be synergistic, whereas the equivalent free-drug combinations were observed to be strictly additive. Higher intracellular drug concentration was measured for cells dosed with the triple ARV-NP combination compared to the equivalent unformulated drugs. Finally, as a first step toward evaluating challenge studies in animal models, we also show that our ARV-NP combinations inhibit RT-SHIV virus propagation in macaque cervicovaginal tissue and block virus transmission by migratory cells emigrating from the tissue. Our results demonstrate that ARV-NP combinations control HIV-1 transmission more efficiently than free-drug combinations. These studies provide a rationale to better understand the role of nanocarrier systems in facilitating multidrug effects in relevant cells and tissues associated with HIV infection.



KEYWORDS: *antiretroviral therapy and prophylaxis, nanoparticles, drug delivery, ARV combination, HIV*

1. INTRODUCTION

The delivery of drug combinations is a paradigm for treatment of cancer and infectious diseases, including HIV/AIDS. Although the use of drug combinations is standard for the treatment of HIV, topical delivery of combination ARV drugs for pre-exposure prophylaxis has only been recently investigated. However, the physicochemical diversity of ARV drugs may prohibit their coformulation in conventional vaginal dosage forms.[1] Clinical implementation of combination microbicides requires coformulating drugs with different physicochemical properties and engineering the delivery of these drug combinations to have optimal inhibitory activity against both cell-free and cell−cell virus transmission. Therefore, the development of drug delivery systems that formulate multiple ARVs into a single platform has the potential to enhance the efficacy of current ARV-based prevention strategies. The design of these systems should be flexible enough to harness drug combinations with demonstrated synergistic activity against

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various modes of HIV infection associated with sexual transmission.[2,3]

A number of nanotechnology-based systems have been developed to deliver incompatible drug combinations for the purpose of enhancing potency, reducing toxicity and prolonging drug residence time to reduce dosing frequency.[4,5] For example, a lipid−drug nanoparticle formulation containing lopinavir/ritonavir and tenofovir produced intracellular drug concentrations in lymph nodes that were 50-fold higher than those obtained with the unformulated drugs.[6] In a related study, HIV-1-infected cells treated with biodegradable nanoparticles containing efavirenz combined with lopinavir/ritonavir produced significantly higher nuclear, cytoskeleton, and membrane antiretroviral drug levels compared to cells treated with unformulated drugs.[7] These examples demonstrate that nanocarriers can overcome challenges with coformulating drugs. However, in these cases and others, drug combinations are typically coformulated, which makes it challenging to control the drug ratios and bioavailability needed to achieve optimal antiviral potency. For example, lipid−drug nanoparticles entrapped 10-fold higher amounts of lopinovir/ritonavir compared to tenofovir even though a different ratio was identified as having the greatest potency.[6] Formulating drugs separately and combining them upon administration is an alternative approach that is both modular and may provide better control over bioavailability of individual drugs.

Although several antiretroviral agents have been successfully incorporated in diverse nanocarriers,[8,9] the use of these systems for identifying unique drug−drug activities for topical prevention has not been fully investigated. To fully understand the potential of these platforms to address critical gaps for HIV prevention, a comparison of safety and efficacy between the unformulated and nanoparticle formulated drug combinations is needed in relevant models of cell-free, cell−cell, and mucosal tissue infection. In the current study, we measured the antiviral potency emerging from nanoparticle codelivery of etravirine (ETR), maraviroc (MVC), and raltegravir (RAL) in these various models of HIV infection. ETR is a non-nucleoside reverse transcriptase inhibitor (NNRTI) with high intrinsic antiviral potency against wild-type as well as various resistant HIV variants.[10] ETR exhibits interesting nonlinear dose−effect responses at high concentrations, which may reflect a particular form of set-point intermolecular cooperativity controlling virus replication.[11,12] However, it is unclear what contribution these dose−effect properties contribute to its synergistic activity when combined with other ARV drugs. MVC is an entry inhibitor that blocks binding of HIV gp120 to CCR5, an essential coreceptor for HIV to enter host cells during infection.[13,14] RAL is an integrase inhibitor that inhibits insertion of the proviral DNA into host cell genome.[15−17] MVC and RAL have been highly effective in combination therapy although both have significantly lower intrinsic antiviral potency compared to ETR. Despite the fact that MVC acts on an extracellular receptor, we formulated all of the ARV drugs to compare with the delivery of unformulated compounds. Nanoparticle formulation of drugs with extracellular targets may also be advantageous for modulating bioavailability, targeting, and preventing drug degradation or metabolism. The purpose of this study was to demonstrate that physicochemically diverse APIs, which may be difficult to coformulate in conventional vaginal dosage forms, could be individually formulated in nanoparticles and codelivered to study their combination effects.

Here, we present data on poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles individually encapsulating ETR, MVC, and RAL. All pairwise combinations of the unformulated and NP-formulated ARVs were tested for antiviral potency against both cell-free and cell-associated HIV-1 infection in vitro. Our results show that unformulated and ARV-loaded nanoparticles (ARV-NP) have similar inhibitory activity but that the drug-loaded nanoparticle combinations exhibited greater antiviral potency against both cell-free and cell-associated HIV-1 BaL infection in vitro. We observed a significant dose-reduction of up to 10-fold when using ARV-NP combinations compared to the free-drug combinations or individual ARV-NP. The dose-reduction appeared to arise from synergistic activity from drug combinations involving ARV-NP but not the unformulated drugs. ARV-NP combinations also resulted in higher intracellular drug concentrations in specific subcellular fractions. The antiviral potency of combined nanoparticle ARV drugs was also measured in a macaque cervicovaginal explant model against challenge with RT-SHIV. This is the first demonstration of dose-reduction and synergy using combination ARV-NPs of ETR, MVC, and RAL across relevant models for mucosal HIV transmission. Our findings support a rationale and need for understanding drug−drug interactions mediated by polymeric nanomaterials and may support a new paradigm for topical delivery of combination ARVs to enhance potency and long-acting efficacy of current microbicides.

2. MATERIALS AND METHODS

2.1. Chemicals and ARV Drugs. Reagents for nanoparticle fabrication and characterization were purchased from Sigma-Aldrich and Fisher Scientific unless otherwise specified. PLGA (50:50 ester terminated, MW 52−54 kDa, inherent viscosity 0.55−0.75dL/g) was purchased from Lactel. ETR, MVC, and RAL were isolated from their pharmaceutical formulations using standard techniques consisting of (i) solubilization and extraction of ARVs from their commercial formulations, (ii) isolation of crude ARVs from excipients, and (iii) large-scale secondary purifications. Secondary purifications consisted of acid−base extractions, silica gel chromatography, and recrystallization and were adapted from the published syntheses.[18−20] Purified ARVs were characterized by ^{1}H NMR (400 MHz),^{13}C NMR (100 MHz), LC-MS, and UV-HPLC (Supporting Information Figures SI-1−5). The spectroscopic and spectrometric data for ETR and MVC were identical to those obtained with standards provided by the NIH AIDS Research and Reference Reagent Program. Both RAL and MVC were isolated as the neutral compounds, which are highly soluble in dichloromethane. Because the neutral form of RAL was unavailable through the NIH AIDS Research and Reference Reagent Program the spectroscopic and spectrometric data for RAL were compared to literature values and were identical to those reported by Summa et. al.[20] All purified ARVs produced inhibition curves identical to those obtained with reagent standards using the cell-free HIV infection assay described below.

2.2. Fabrication of ARV-Loaded Nanoparticles. ARV-loaded nanoparticles and blank nanoparticles (vehicle control) were formulated individually such that only a single drug was encapsulated in an individual nanoparticle formulation. Nanoparticles were fabricated using a single emulsion/solvent evaporation method. A theoretical drug loading of 10 wt % ETR, MVC, or RAL (w/w, weight of ARV relative to weight of PLGA polymer) was combined with PLGA (5% w/v) in a

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Mol. Pharmaceutics 2015, 12, 4363−4374

volatile organic solvent to generate the "oil"-phase. MVC and RAL were dissolved together with PLGA in DCM, whereas ETR was dissolved together with PLGA in ethyl acetate.[21] This mixture was added dropwise into 2.5% w/v aqueous poly(vinyl alcohol) (PVA) solution while vortexing. The solution was emulsified using a probe sonicator (500W, Ultrasonic Processor GEX 500) with a 3 mm diameter microtip probe for intervals of 20 s at 50% amplitude for three rounds with vortexing between intervals. The sonicated emulsion was transferred to a continuously stirred aqueous solution of PVA (0.25% w/v) for 3−5 h to evaporate all residual organic solvent. Nanoparticles were washed with Milli-Q water three times by centrifugation at 14 000 rpm for 10 min at 4 °C in order to remove any residual PVA surfactant and unencapsulated drug. Following these wash steps, the nanoparticles were suspended in water, frozen at −80 °C and lyophilized (24 h, 0.133 mbarr at −87 °C). Lyophilized nanoparticles were stored at −80 °C until use.

2.3. Characterizations of ARV-Loaded Nanoparticles (ARV-NPs). ARV-NP size, polydispersity (PDI), and zeta (ζ)-potential were measured by dynamic light scattering (DLS) in 10 mM NaCl (pH 5) using a Zetasizer Nano ZS90 (Malvern Instruments). HPLC was used to measure drug loading, which is defined as the measured mass of ARV drug per mass of PLGA-NP, and encapsulation efficiency, which is defined as the ratio of the drug loaded to the total drug used for fabricating the nanoparticles (see equations, Table 1). A direct measure of drug loading was determined by dissolving a known mass of lyophilized ARV drug-NP in DMSO and analyzing the samples using an integrated reversed-phase high-pressure liquid chromatography (HPLC) system. In brief, separations were made on a C18 column (5 μm, 250 × 4.6 mm, Phenomenex) at either 25 °C (MVC) or 30 °C (RAL and ETR) and based on methods previously established for detecting ETR, RAL, and MVC.[22,23] Detections were performed at wavelengths of 300 nm (RAL), 193 nm (MVC), and 234 nm (ETR). Sample retention and run-times for RAL, MVC and ETR were: 4/10 min, 9/15 min, and 5/22 min, respectively. Long-term storage stability of blank and ARV-NPs was assessed after lyophilization in the presence or absence of trehalose (1:1 by mass). Stability was monitored by measuring the ARV-NP size, PDI and ζ-potential at day 0, 1, 3, 5, 7, 14, and 21 during incubation at 25 °C, 39 and 60 °C. Transmission electron microscopy (TEM) images were obtained on a FEI Tecnai F20 transmission electron microscope operating at 200 kV at Molecular Analysis Facility (MAF), University of Washington.

2.4. In Vitro Drug Release from ARV-NP Formulations. ARV drug release kinetics from PLGA nanoparticle formulations were measured under sink conditions. Sink conditions are defined as concentrations ≤10-fold the drug solubility, which was achieved for MVC and RAL by using PBS and for ETR by using 2 wt % solutol (Sigma-Aldrich, Kolliphor HS 15, cat. no. 42966-1KG) in PBS. Lyopholized ARV-NPs were individually resuspended in their respective release media at sink conditions, aliquoted into individual tubes for each time point to quantify drug release, and incubated at room temperature. At various times up to 215 h, the aliquots were centrifuged to remove nanoparticles, and the supernatant was analyzed by HPLC to quantify released drug. Percent release was measured in triplicate from three independent samples and was based on loading data obtained for the identical nanoparticle formulations.

Table 1. Properties of ARV Drug-Loaded PLGA Nanoparticles

measured properties[a]	Etravirine NNRTI	Raltegravir integrase	Maraviroc CCR5 inhibitor
molecular weight	435.3	444.5	513.7
size (diameter, nm)	371.4 ± 71.6	311.2 ± 9.3	331.6 ± 27.4
polydispersity index	0.36 ± 0.11	0.09 ± 0.03	0.2 ± 0.1
zeta-potential (mV, pH 6)	−21.0 ± 1.8	−22.5 ± 3.0	−26.5 ± 2.5
loading (wt %)[b]	8.1 ± 0.8	2.4 ± 0.2	1.2 ± 0.1
encapsulation efficiency (%)[c]	91.0 ± 9.9	16.8 ± 2.6	12.0 ± 0.6
polymer yield (%)[d]	66.7 ± 9.3	64.2 ± 7.8	91.7 ± 2.9
IC50 (nM) [95% CI][e]	1.4 [1.2, 1.6]	6.9 [5.2, 9.2]	17.4 [9.2, 32.7]

[a]Measured properties are reported as the mean ± SD from $n \geq 3$ independent nanoparticle fabrications. [b]Mathematical relationship to determine the amount of drug loaded:

$$\text{drug loading\%} = \frac{\text{mass of drug in nanoparticles}}{\text{mass of nanoparticles recovered}} * 100$$

[c]Mathematical relationship to determine the effectiveness of the encapsulation:

$$\text{encapsulation efficiency\%} = \frac{\text{mass of drug in nanoparticles}}{\text{mass of drugs used in formulation}} * 100$$

[d]Mathematical relationship to determine the yield of the polymer:

$$\text{polymer yield\%} = \frac{\text{mass of nanoparticles recovered}}{\text{mass of polymer used in formulation}} * 100$$

[e]Antiviral activity is reported for HIV-1 BaL infection of TZM-bL cells. The value represents the mean of triplicate measurements obtained from ≥3 independent nanoparticle fabrications for each ARV drug.

2.5. Cell Lines and Viruses. TZM-bL (JC53-BL), PM-1, and CEMx174 cell lines and HIV-1 BaL virus were obtained from the NIH AIDS Research and Reference Reagent Program. TZM-bL cells are a genetically engineered HeLa cell clone that expresses CD4, CXCR4, and CCR5 and contains Tat-responsive reporter genes for firefly luciferase (Luc) and *Escherichia coli* β-galactosidase under regulatory control of an HIV-1 long terminal repeat, which permits sensitive and accurate measurements of HIV infection. TZM-bL cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, and 25 mM HEPES. PM-1 is a human T cell line while CEMx174 cell line is a human T/B-lymphocyte hybrid. Both cell lines were grown in RPMI1640 medium supplemented as above. All cell lines were incubated at 37 °C in a humidified 5% CO_2 air environment.

HIV-1 BaL is a CCR5 tropic virus, which uses CCR5 as a coreceptor to enter target cells and is dominant in HIV mucosal transmission.[24] HIV-1 BaL stock was propagated in PM-1 cells and titrated in both PM-1 and TZM-bL cell lines. RT-SHIV, a chimeric SHIV where the reverse transcriptase (RT) of the backbone SIV_{mac239} is replaced with that of HIV-1HXB2 was originally provided by L. Alexander at Yale University and was propagated in CEMx174 cells.

2.6. Antiviral Activity Assay. *2.6.1. Evaluation of ARV Particle Drugs against Cell-Free HIV Infection.* Antiviral

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activity was measured as a reduction in luciferase reporter gene expression after infection of TZM-bL cells with HIV-1 BaL. All single ARV drugs and ARV-NPs were dosed based on molar concentrations of the drug. Drug combinations were dosed to achieve a total drug concentration that combined the drugs at an equimolar ratio. Briefly, TZM-bL cells were seeded at a concentration of 1×10^4 per well in 96-well microplates (~30 000 cells/cm^2). After a 24 h culture, TZM-bL cells were incubated with various concentrations of either free drugs or NP-formulated drugs at 37 °C for 1 h prior to virus exposure. At this time, cell free HIV-1 BaL at 200 TCID50 (50% tissue culture infective dose) was added to the cultures and incubated for an additional 48 h. Untreated cells (100% infected) were used as a control. Antiviral activity was measured by luciferase expression using the Promega Luciferase Assay System. Antiviral activity was plotted as a percent inhibition relative to virus control (no drug treatment, 100% infected) and cell control (no virus exposure, 0% infected). Nonlinear regression was used to fit the data to an inhibition model with a constant Hill coefficient to determine the slope parameter and IC50 value, which is the sample concentration giving 50% of relative luminescence units (RLUs) compared to the virus control after subtraction of background RLUs.

For drug combinations that showed significant dose-reduction, we used the model of Bliss independence with variable Hill coefficients to quantitatively analyze the combination effect as synergistic, additive, or antagonistic. For this analysis, dose−response data for the combination and single ARV assays were plotted as median-effect plots with the response transformed to $\log(f_a/f_u)$, where f_a is the fraction of infection events affected by drug and f_u is the fraction of infection events unaffected by drug ($f_u = 1 - f_a$). To calculate the Bliss independence prediction for combinations of ARVs exhibiting variable Hill slopes, we followed the method of Jilek et al.,[11] restricting the analysis to combination data within the dynamic range of our assay ($-2 < \log(f_a/f_u) < 3$). Bliss independence predictions were calculated using eq 1, where D is the concentration of each drug present in the combination and m is the slope of the median-effect plot for each drug at a given concentration (local Hill coefficient). Variable Hill coefficients and IC_{50} values were determined via linear regression centered at the concentration of interest. The magnitude of the combined effect was quantified by measuring the difference between the observed and predicted fraction affected following the approach of Laird et al.[25] Synergy, additivity, and antagonism as defined by the Bliss model occurs for values of $0<\Delta f_{axy} \leq 1$, $\Delta f_{axy} = 0$, and $-1 \leq \Delta f_{axy} < 0$, respectively (see Figure 3e).

$$f_{u1+2} = \frac{1}{1 + \left(\frac{D_1}{IC_{50_1}}\right)^{m_1}} \times \frac{1}{1 + \left(\frac{D_2}{IC_{50_2}}\right)^{m_2}} \quad (1)$$

2.6.2. Cell-to-Cell Transmission Assay. For cell−cell transmission assays, TZM-bL cells were seeded at a concentration of 1×10^4 per well in 96-well microplates (~30 000 cells/cm^2). The following day, TZM-bL cells were incubated with various concentrations of either free drugs or ARV-loaded nanoparticles in combination at an equimolar ratio at 37 °C for 1 h prior to exposure to chronically infected PM-1 cells. Infected PM-1 cells were produced by infecting with HIV-1 BaL and culturing these cells for 3 days. These cells were washed twice with phosphate-buffered saline (PBS) to remove cell free virus before they were added to TZM-bL cell culture (5×10^3 cells/well). After 1 h of incubation, PM-1 cells were removed by washing wells twice with PBS. TZM-bL cells were then incubated in the presence of drug for an additional 48 h. Cells were lysed, and the luciferase activity of the cell lysate was measured using the luciferase assay system (Promega). Antiviral activity was expressed as described above.

2.6.3. Culture of Macaque Cervicovaginal Tissue Explants and RT-SHIV Infection. Macaque reproductive tracts were purchased from the tissue distribution program at Washington National Primate Research Center (WaNPRC), University of Washington. All macaques were confirmed to be serologically negative for simian type D retrovirus, SIV, and simian T-cell lymphotropic virus (STLV) prior to sample collection. Cervical and vaginal explants, comprising both epithelial and stromal tissue, were obtained using a 3 mm-diameter biopsy punch (Miltex Medical Instruments). Intact explants (3 mm) were pretreated with combined ARV-NPs containing ETR, MVC, and RAL with equimolar ratios of the three drugs (EMR-NP, 1:1:1 molar ratio) at 37 °C for 1 h prior to exposure to RT-SHIV (10^5 TCID50) for 2 h at 37 °C. Explants were then washed three times with PBS and were cultured individually in 300 μL of complete RPMI1640 in a 96-well plate in a humidified atmosphere containing 5% CO_2. Following overnight culture, explants were transferred to fresh culture plates and cultured for an additional 14 days, with two-thirds of the medium exchanged every 2 to 3 days. Cells that spontaneously migrated out of the tissue explants during overnight culture were washed twice with PBS, transferred to fresh plates, and cocultured with 5×10^3 CEMx174 cells/well to assess the blockade of virus transfer by migrating cells. Cocultures were examined daily for cytopathic effect (CPE). RT-SHIV infection was determined by the measurement of SIV p27 in culture supernatants by ELISA (ZeptoMetrix Corporation RETROtek SIV p27 Antigen ELISA Kit).

2.7. Quantification of ARV Drug Uptake and Subcellular Distribution. Intracellular uptake was investigated in TZM-bL cells for the triple ARV-NP combination and the equivalent free-drug combination. Cells were cultured at 37 °C, 5% CO_2 for 48 h in the presence of 1 μM total drug at equimolar ratio for each drug. After removing culture medium, cells were detached with 1 mL ice-cold PBS and collected by centrifugation at 500*g* for 10 min at 4 °C. Cells were processed using the Subcellular Protein Fraction Kit (Thermo Scientific, cat. no. 78840) following the manufacture's guidelines to isolate cytoplasmic, membrane, and nuclear/chromatin-bound and cytoskeletal subcellular fractions.

Drug concentration in the different subcellular compartments was analyzed by LC-MS/MS at the University of Washington Mass Spectrometry Center. A Chromolith Performance RP-18e 100-3 mm column was used for LC analysis, with a gradient method of 10 mM formic acid in H_2O and 10 mM formic acid in 80:20 ACN:MeOH. Etravirine-$^{13}C_6$, maraviroc-d_6, and raltegravir-d_6 (Alsachim, Strasbourg, France) were used as internal standards and combined prior to spiking into samples. All subcellular fraction samples were aliquoted at a volume of 50 μL and spiked with 5 μL of a 10 ng mL^{-1} solution of the combined internal standard mix (50 pg per sample). A standard curve ranging from 0.01 to 100 ng mL^{-1} was prepared in MeOH and run before and after unknown sample sets. Samples were analyzed using a single LC-MS/MS method for detection of ETR, MVC, and RAL on a Waters Xevo TQ-S couple with an I-Class Acquity UPLC (Waters Corporation, Milford, MA,

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Mol. Pharmaceutics 2015, 12, 4363−4374

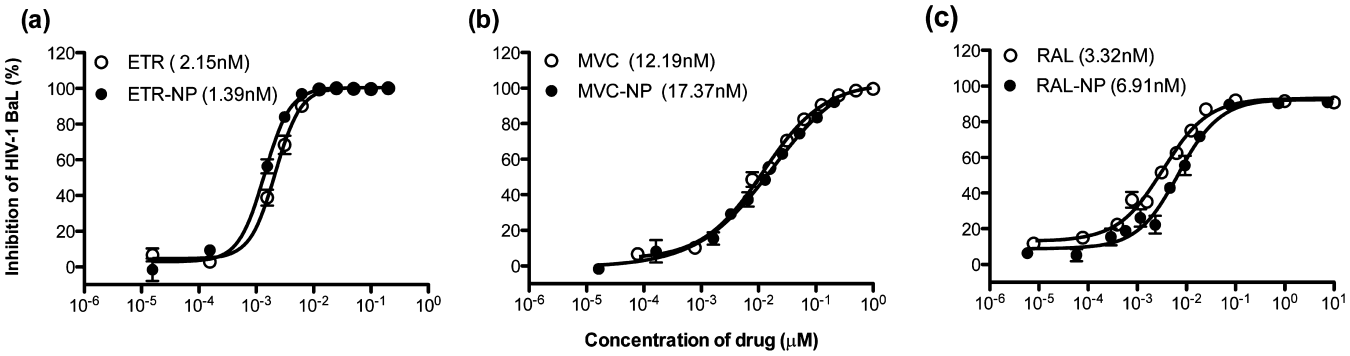

Figure 1. Antiviral activity of single ARV and ARV-NP drug against cell free HIV-1 BaL in TZM-bL cells. TZM-bL cells were pretreated with ARV and ARV-NP drugs individually for 1 h prior to HIV-1 BaL exposure (200TCID50). Luciferase activity was measured 48 h after virus infection. Dose−response curves show the mean and standard deviation of the percent inhibition of cell-free HIV-1 BaL virus for free drug (o) and nanoparticle drug (•) of (a) ETR-NP, (b) MVC-NP, and (c) RAL-NP. The numbers in parentheses indicate the 50% inhibitory concentration (IC50). Data from three independent experiments measured in duplicate were analyzed by GraphPad Prism 5.0.

U.S.A.). All analytes were acquired in electrospray positive mode, and the drugs and internal standards were analyzed at the following m/z transitions: 435.22 to 162.98 (ETR), 441.20 to 162.98 (ETR-$^{13}C_6$), 445.35 to 109.10 (RAL), 451.35 to 115.10 (RAL-d_6), 514.48 to 290.22 (MVC), and 520.48 to 280.22 (MVC-d_6). All species were analyzed at a cone voltage of 50 kV and collision energy of 28 V (ETR and MVC) or 30 V (RAL).

2.8. Cytotoxicity Assay. TZM-bL cells were cultured in 96-well plates in the absence or presence of various concentrations of ARV-loaded nanoparticles. After 3 days in culture, cell viability was assessed using CellTiter-Blue Cell Viability Assay (Promega) following the manufacturer's recommended procedures. Cells were incubated for 4 h with 20 μL/well of CellTiter-BlueReagent, and fluorescence was recorded at 560/590 nm.

3. RESULTS

3.1. PLGA Nanoparticles Successfully Formulate Mechanistically and Physicochemically Distinct ARV Drugs and Are Noncytotoxic. ETR, RAL, and MVC were purified by standard techniques (Supporting Information Figures SI-1−5) and used to prepare ARV-loaded nanoparticles based on PLGA by emulsion−solvent evaporation techniques. Irrespective of the specific drugs, all of our ARV-NPs showed an average diameter of ∼340 nm, PDI of ∼0.2, and an average negative ζ-potential of −23 mV (Table 1). Size estimates obtained from TEM images are consistent with the size of the ARV-NPs reported by DLS measurements (Supporting Information, Figure SI-6). Nanoparticles of this size range (200−500 nm) have been reported by others to transport through mucus and penetrate the vaginal epithelium after topical delivery to the mucosa.[26−28] We used a nominal initial drug loading of 10 wt %, which resulted in formulated ARV-NPs with measured drug loadings of 8.1 ± 0.8 wt %, 2.4 ± 0.2 wt %, and 1.2 ± 0.1 wt % for ETR-NP, RAL-NP, and MVC-NP, respectively (Table 1). These respective drug loadings translated to encapsulation efficiencies of 91%, 17%, and 12%. As expected, encapsulation efficiency and drug loading were much greater for the hydrophobic ETR compared to the ionizable and more water-soluble RAL and MVC.

We observed that our ARV-NPs were stable during storage at room temperature for 21 days and showed minimal change to their originally measured value of size, PDI, and ζ-potential (Supporting Information, Figure SI-7). However, addition of trehalose during lyophilization was required to maintain the stability of PLGA NPs at elevated temperatures of 39 and 60 °C (Supporting Information, Figure SI-8). We also measured ARV drug release kinetics for each of the NP-formulated drugs under sink conditions in vitro. We observe that ETR, RAL, and MVC all exhibit a burst-phase of release that is likely due to surface associated drug (Supporting Information, Figure SI-9). In particular, MVC, which acts extracellularly with the host CCR5 cell-surface receptor, shows complete drug release in <15 min under sink conditions. In contrast, ETR and RAL exhibit a burst phase of release within the first 15−60 min followed by a slower pseudolinear release up to 150 h (∼6 days) of the remaining 20% and 40% of the entrapped drugs, respectively. In summary, we have successfully fabricated three physicochemically and mechanistically distinct ARV drugs into PLGA nanoparticles that allow their modular combination.

The ARV-NPs were tested for cytotoxicity in the cell line used to measure HIV infection. No significant reduction in cell viability was observed in TZM-bL cells treated with ARV-NPs compared to blank NPs or untreated control groups (Supporting Information, Figure SI-10). The data indicates that blank PLGA nanoparticles cause no reduction of cell viability at concentrations below 2.5 mg/mL. At this maximum concentration of ARV-NPs, the corresponding drug concentrations would be 6.6×10^5nM ETR, 1.2×10^5 nM RAL and 5.8×10^4 nM MVC. All of the antiviral assays involving ARV-NPs in this study were conducted at concentrations well below these limits. For this reason, cytotoxicity from the nanoparticles themselves is unlikely to confound the antiviral activity results.

3.2. ARV-NP Combinations That Include ETR Exhibit Significant Antiviral Potency and Dose-Reduction against HIV-1 BaL Infection in Vitro. The ARV drug loading for all of our ARV-NP formulations were sufficient to measure antiviral potency against HIV-1 BaL infection in vitro. We dosed the ARV-NPs based on their total drug loading and observed a dose-dependent inhibition of TZM-bL infection by HIV-1 BaL (Figure 1). The measured IC50 of the individual ARV-NPs was in the low nanomolar range and similar to the anti-HIV activity measured for the equivalent unformulated drugs. We also performed the antiviral assay with blank NPs (vehicle control) dosed at the same polymer concentrations as the ARV-NPs to control for the possibility that NP sedimentation resulting from aggregation could cause non-specific antiviral activity from steric hindrance (data not

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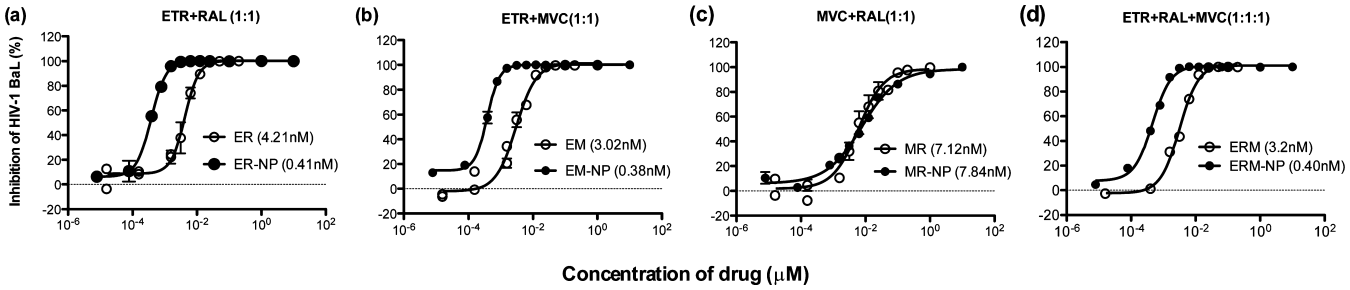

Figure 2. ARV-NP combinations show potent antiviral activity and dose-reduction compared to the analogous free-drug combinations against HIV-1 BaL transmission. TZM-bL cells were pretreated with free ARV drug and ARV-NP drug combinations at equimolar ratio for 1h prior to HIV-1 BaL exposure (200TCID50). Luciferase activity was measured 48 h after virus infection. Dose−response curves show the mean and standard deviation of the percent inhibition of cell-free HIV-1 BaL virus for free drug (o) and nanoparticle drug (•) combinations of (a) ETR-NP and MVC-NP, (b) ETR-NP and RAL-NP, (c) MVC-NP and RAL-NP, and (d) ETR-NP, RAL-NP and MVC-NP. The numbers in parentheses indicate the 50% inhibitory concentration (IC50). Data was analyzed by GraphPad Prism 5.0. Graphs show values of the means ± standard deviations from three separate experiments.

shown). No HIV inhibition was observed, which indicates that PLGA NPs alone do not contribute to any measurable antiviral efficacy seen with the ARV-NPs. Based on these viral inhibition results, the individual ARV-NPs were evaluated in all three pairwise combinations using the same assay for HIV-1 BaL virus transmission. We first measured the antiviral potency of different molar ratios of the three ARV drugs and identified that equimolar concentrations of ETR, RAL, and MVC resulted in the greatest antiviral potency (Supporting Information, Table SI-1). Therefore, we used this ratio to compare the antiviral potency of the free and ARV-NP drug combinations. We observed that combinations involving ETR-NP exhibited significant dose-reduction compared to the equivalent free-drug combinations (Figure 2a,b) and compared to the antiviral potency of the single ARV drug-NP (Figure 1). In contrast, drug combinations of free and NP-formulated MVC and RAL (Figure 2c) showed similar IC50s and no dose-reduction compared to the potency of only the single ARV drugs (Figure 1b,c). For example, ETR-NP combined with MVC-NP or RAL-NP showed an approximately 10-fold dose reduction in the IC50 compared to the same combinations using unformulated drugs (Figure 2a,b). The drug combinations that included ETR-NPs also showed a 2−40 fold dose-reduction compared to the IC50 measured for the single ARV drug-NP (Figure 1 and Table 1). The unformulated drug combinations of free ETR combined with either free RAL or MVC did not show a dose-reduction relative to the most active component present. Instead, these free-drug combinations had a measured IC50 that was of median value between the IC50 for the single ARV drugs. The triple drug combination of either the free or NP-formulated drugs also did not show an enhanced potency compared to the two-drug combinations (Figure 2d). In summary, we observed that ARV-NP combinations including ETR-NP show significant dose reductions relative to all unformulated drug combinations tested against HIV infection in vitro.

For the ARV-NP combinations that showed significant dose-reduction compared to the equivalent unformulated drug combinations, we used the model of Bliss independence to identify if the observed combination effects were synergistic. The Bliss independence model can be used to predict the additive effect arising from combinations of multiple drugs that have independent mechanisms of action, as in the case with ETR, MVC, and RAL. We observed steep upward inflections in the median-effect plots of ETR (free and nanoparticle-loaded), which is consistent with previous findings[11] (Figure 3). Because we combined ETR in an equimolar ratio with MVC or RAL (1:1), the Bliss independence prediction is expected to be dominated by the response observed for ETR alone due to the large Hill coefficients observed for ETR at high concentration.[11] Drug combinations involving ETR-NP with MVC-NP or RAL-NP resulted in higher antiviral activity than would be predicted by the Bliss model if the combination effect were strictly additive (Figure 3b,d). The combination effect was dose-dependent such that the greatest deviation from the Bliss prediction was observed at concentrations much higher than the IC50 (Figure 3e). In contrast to the two drug combinations that included ETR-NP, the antiviral effect measured for the equivalent unformulated drug combinations was within error of the Bliss model for additivity. Therefore, the combined effects from these ARV-NP combinations but not the free-drug combinations appear to be acting synergistically.

3.3. ARV-NP Combinations Show Enhanced Cell-Associated Drug Content. We used LC-MS/MS to measure the concentration of ETR, RAL, and MVC in subcellular fractions after dosing to TZM-bL cells. Since ETR-NP showed a dose-reduction with both MVC-NP and RAL-NP, we hypothesized that the triple ARV-NP combination would allow us to capture the intracellular drug concentration of both binary combinations in a single study. TZM-bL cells were cultured in similar conditions used in the antiviral assay, and the cells were dosed with 1 μM of the triple ARV drug combination either as NP-formulated or free-drug combinations. We selected a dose where the difference between the triple ARV-NP and free-drug combinations was the largest on the median-effect plot but still within the dynamic range of our antiviral assay. A commercial kit with specific extraction buffers was used to isolate cytoplasmic, membrane, and nuclear/chromatin-bound and cytoskeletal cell fractions. LC-MS/MS was used to quantify the concentration of each drug in these separate fractions. In general, we observed higher cell-associated drug concentration for ETR, RAL, and MVC when dosed as the triple drug ARV-NP formulations compared to the equivalent free-drug combination (Figure 4). The triple ARV-NP combinations resulted in the highest measured cell-associated total drug content for ETR when dosed as a NP combination (~350 ng per 10^6 cells) compared to the free-drug combination (~94 ng per 10^6 cells), of which there was almost a 10-fold difference in the measured cytoplasmic drug content (ETR-NP = 20 ng versus ETR-free = 2.8 ng per 10^6 cells). The data

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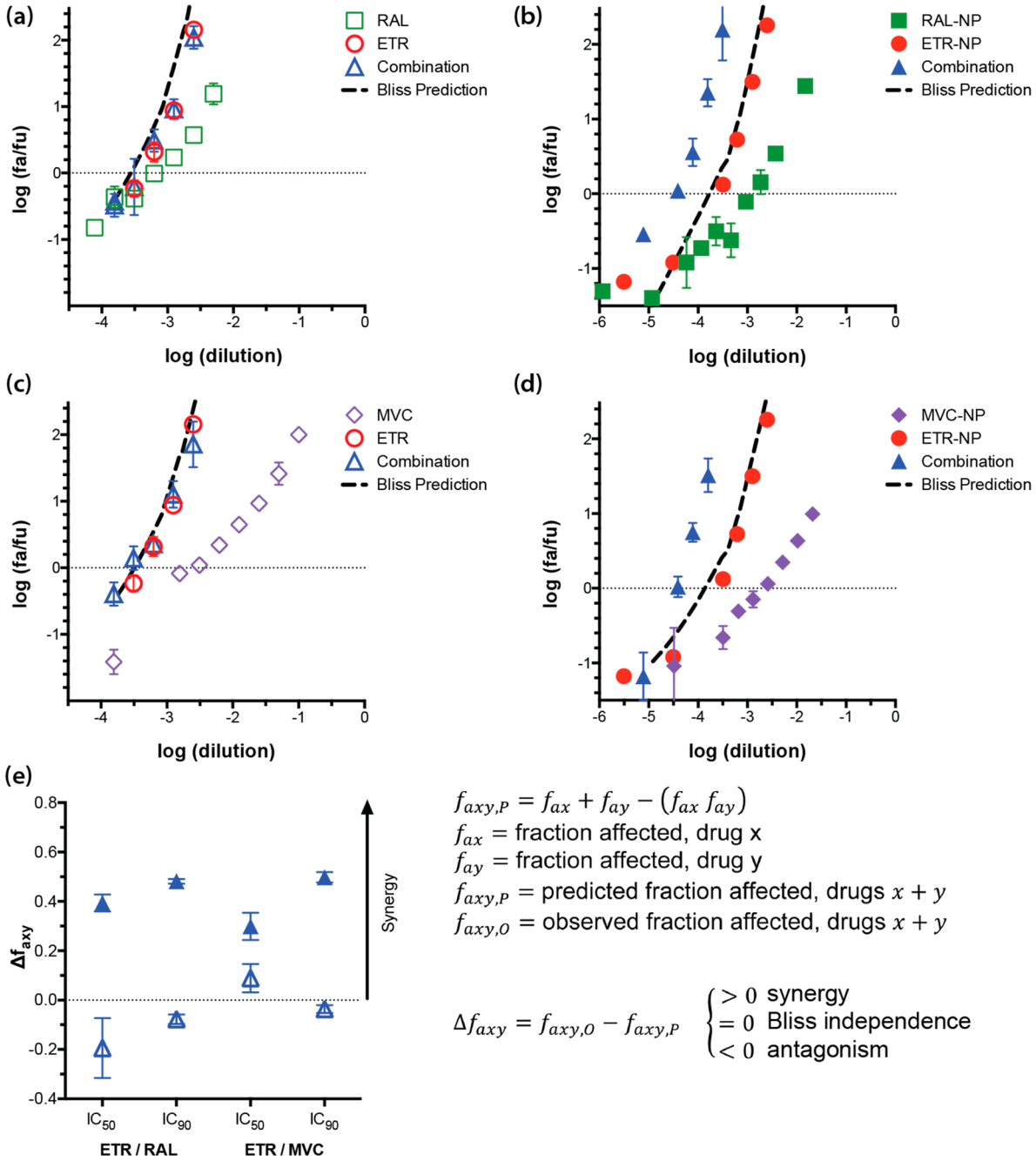
Mol. Pharmaceutics 2015, 12, 4363−4374



$$f_{axy,P} = f_{ax} + f_{ay} - (f_{ax}\, f_{ay})$$

f_{ax} = fraction affected, drug x

f_{ay} = fraction affected, drug y

$f_{axy,P}$ = predicted fraction affected, drugs $x + y$

$f_{axy,O}$ = observed fraction affected, drugs $x + y$

$$\Delta f_{axy} = f_{axy,O} - f_{axy,P} \quad \begin{cases} > 0 & \text{synergy} \\ = 0 & \text{Bliss independence} \\ < 0 & \text{antagonism} \end{cases}$$

Figure 3. Median-effect plots for ARV drug combinations compared to the Bliss independence prediction. Median-effect plots for (a) RAL/ETR, (b) RAL-NP/ETR-NP, (c) MVC/ETR, and (d) MVC-NP/ETR-NP. The combination and single ARV concentrations have been adjusted to a log dilution scale to allow a comparison to the activity observed for single ARVs at the concentrations present in the combination experiment. The activity predicted by the Bliss independence model is shown (dashed line). This prediction incorporates the variable Hill slopes observed in the single ARV median-effect plots. The Bliss independence prediction tracks closely with the effect observed with ETR alone in all combination experiments because the median-effect plot for ETR exhibits large Hill slopes at high concentrations. Combination experiments using ARV-loaded nanoparticles containing ETR exhibit drug synergy with the observed response exceeding that predicted by the Bliss independence model. Free drugs and their combinations (a, c) are shown as open shapes whereas ARV-NP and their combinations (b, d) are shown as filled shapes. The Bliss prediction (dashed line) is compared to the observed data for the different combinations (triangles). (e) Data are plotted as the difference between the observed fraction affected ($f_{axy,o}$) and predicted fraction affected ($f_{axy,p}$) at concentration values near the IC50 and IC90 for the ARV-NP combinations (closed triangle) or free-drug combinations (open triangle). Drug combinations that exhibit synergistic activity as defined by the Bliss model have values of $0 < \Delta f_{axy} \leq 1$.

indicate that a greater fraction of RAL partitioned to the cytoplasm (80%) compared to the membrane (10%), whereas MVC showed almost equivalent partitioning into these same cellular fractions (44% versus 42%, respectively). Despite being dosed at equimolar ratios in both the triple ARV-NP combinations and free drug, we measured ~100-fold less MVC and RAL associated with the cells compared to ETR. This may be a result of variable solubility of the drugs within the different buffers leading to different extraction efficiencies, which were not accounted for in our measurements. In

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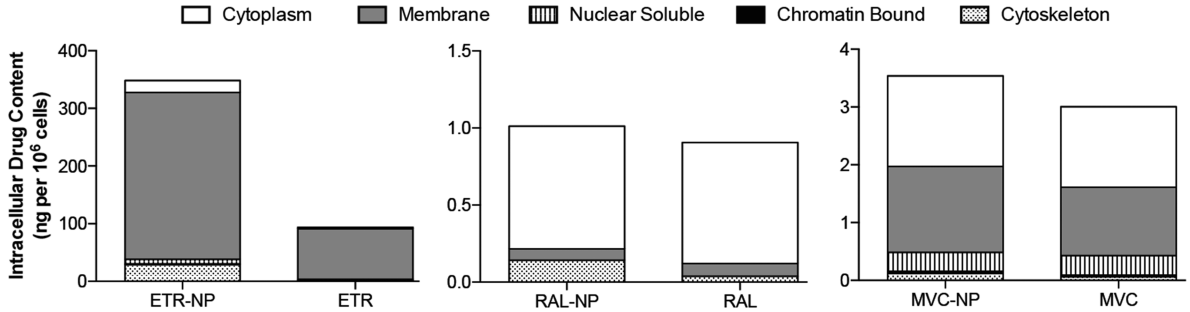

Figure 4. Subcellular distribution of ARV drug combinations delivered by nanoparticles. TZM-bL cells were dosed with the triple drug combinations formulated in NPs or delivered as the free-drugs. The amount of cell-associated ETR, RAL, and MVC content (ng per 10^6 cell) resulting from the drug combinations were measured in subcellular fractions collected for the cytoplasm, membrane, nuclear/chromatin-bound, and cystokeleton. Data are plotted for the individual ARV drugs and show the cumulative drug amount that is cell-associated from the different subcellular fractions.

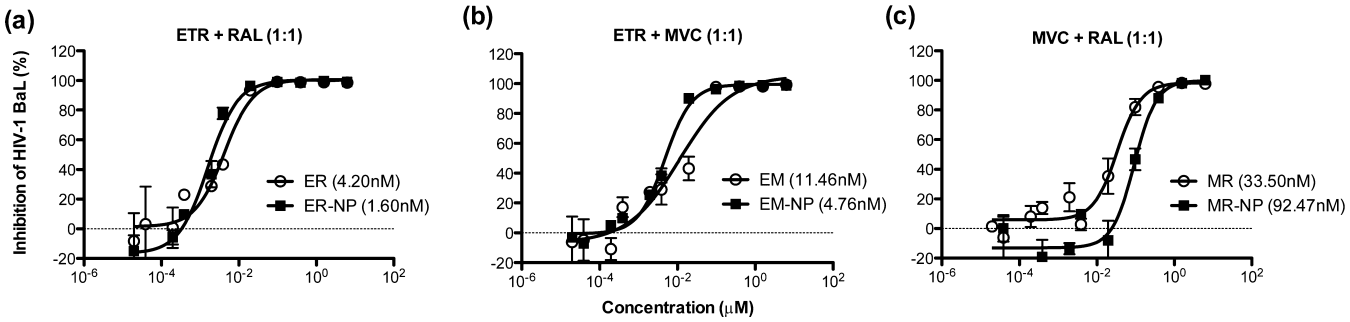

Figure 5. ARV-NP combinations show potent antiviral activity and dose-reduction compared to the analogous free-drug combinations against cell−cell HIV-1 BaL transmission. TZM-bL cells were exposed to different free ARV drug and ARV-NP drug combinations in the presence of PM-1 cells infected with HIV-1 BaL. Dose−response curves show the mean and standard deviation of the percent inhibition of cell−cell HIV-1 BaL transmission from infected PM-1 cells to the TZM-bL reporter cells for free drug (o) and nanoparticle drug (■) combinations of (a) ETR and RAL, (b) ETR and MVC, and (c) MVC and RAL. The numbers in parentheses indicate the 50% inhibitory concentration (IC50). Data was analyzed by GraphPad Prism 5.0 and presented as the means ± standard deviations from three separate experiments; each condition was tested in duplicate.

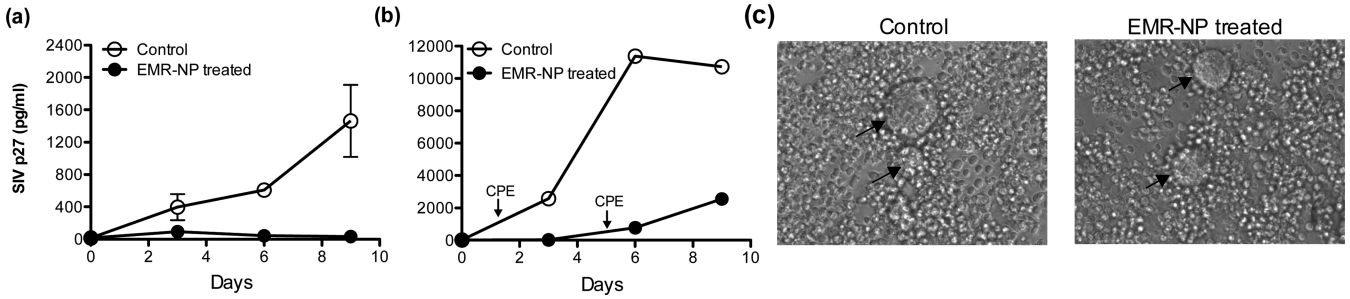

Figure 6. ARV drug-NP combinations inhibit RT-SHIV infection of cervicovaginal tissue explants. Pigtailed macaque cervicovaginal tissues were pretreated with 5 μM etravirine/maraviroc/raltegravir nanoparticle combinations (EMR-NP) at equimolar ratio prior to exposure with 10^5 TCID50 RT-SHIV. (a) Infection of tissue explants was monitored by detection of SIV p27 antigen in the tissue culture supernatants. Untreated tissue biopsy controls (o) showed a trend toward increasing p27 concentration in culture over the course of 9 d. In contrast, tissue biopsies treated with ARV drug-NP combinations (•) showed no significant change in p27 concentration during the same time course. (b) Migratory cells were cocultured with CEMx174 cells, and infection was monitored by p27 ELISA at the same time points. Virus dissemination by migratory cells from cervicovaginal tissues was also blocked by ARV drug-NP combinations treatment (•) compared to untreated tissues (o). (c) Migratory cells cocultured with CEMx174 cells were examined by light microcopy for CPE. Virus-induced giant cells (indicated with arrows) were observed in the coculture of the untreated control group at day 1. Whereas, CPE was first observed at day 5 for the EMR-NP treatment group. Each condition was tested in duplicate or triplicate. Data represent the mean ± SD.

addition, for the cells treated with ARV-NPs, we are unable to distinguish between released drug and drug that is still entrapped in the nanoparticles. These results suggest that ARV-NPs achieve better intracellular uptake over free-drug combinations.

3.4. ARV-NP Combinations Are Effective against Cell-Associated HIV-1 Infection in Vitro. We tested the efficacy of our ARV drug-loaded nanoparticles to inhibit cell-to-cell HIV-1 transmission between HIV-1 BaL infected PM-1 cells and TZM-bL cells. The target TZM-bL cells were pretreated with all three pairwise combinations of unformulated and NP-formulated ETR, RAL, and MVC delivered at equimolar ratios and then exposed to infected PM-1 cells but not free virus. We observed that higher ARV drug dosing is required to prevent the direct infection of TZM-bL cells by PM-1 cells for all combinations tested (Figure 5). This observation is consistent

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Mol. Pharmaceutics 2015, 12, 4363−4374

with previous findings that show cell−cell virus transmission is much more efficient than infection from cell free virus.[3,29] Drug combinations that included ETR-NP (Figure 5a,b) resulted in significantly higher inhibition of cell−cell virus transmission compared to two drug combinations of MVC-NP and RAL-NP (Figure 5c). ETR-NP combined with MVC-NP or RAL-NP also showed an approximately 3-fold higher potency compared to the unformulated combinations of the same ARV drugs (Figure 5a,b). In contrast, the free combinations of MVC and RAL showed an approximately 2-fold higher potency compared to the equivalent ARV-NP combinations, a decrease in potency not observed in the cell-free assay (Figure 2c). In summary, combinations of ARV-NPs that include ETR show improved inhibition of cell−cell transmission compared to the equivalent free-drug combinations.

3.5. ARV-NP Combination Inhibits RT-SHIV Transmission in Nonhuman Cervicovaginal Explants and Blocks Virus Propagation by Migratory Cells. Motivated by the antiviral activity of ARV-NP in our cell culture infection assays, we investigated a triple combination of ARV-NP to prevent RT-SHIV infection of nonhuman primate cervicovaginal explant tissue. RT-SHIV virus replication in infected tissue was measured to evaluate the pre-exposure prophylaxis of EMR-NP. The EMR-NP combinations were dosed at 5 μM in culture with the explant biopsy (∼25 mg), and the biopsies were challenged with 10^5 TCID50 RT-SHIV. The tissue explants were cultured for 9 days, and culture supernatant was removed every 3 days to monitor for SIV p27 concentration by ELISA. Compared to tissue biopsy controls that were untreated, ARV-NP combinations show protection of the explant tissue from RT-SHIV infection (Figure 6a). In particular, after 9 days in culture, the untreated tissue biopsy showed measurable SIV p27 of ∼1200 pg/mL in the supernatant whereas the combination ARV-NP treatment groups were similar to the media control measured at the start of the study (day 0) and had no detectable virus replication.

We also assessed the same EMR-NP combinations for its ability to inhibit virus dissemination by infected migratory cells emigrating out of the cervicovaginal tissue explants. Cells that had migrated from the tissue explants after overnight culture were collected and cocultured with permissive CEMx174 cells. At day 6, CEMx174 cells cocultured with migratory cells from untreated explants showed SIV p27 levels of 11 600 pg/mL. In contrast, migratory cells from EMR-NP treated explants cocultured with CEMx174 cells showed SIV p27 levels of 700 pg/mL (Figure 6b). Infection in migratory cells was also confirmed by coculture with CEMx174 cells and monitoring for CPE, which refers to morphological changes in the host cells that are caused by viral infection including rounding of the infected cell, fusion with adjacent cells to form multinucleated cells. For the EMR-NP treated coculture, CPE was not observed until day 5 whereas the untreated coculture showed CPE at day 1 (Figure 6c). Together, these results indicate that EMR-NPs also delay infection against viral dissemination by migratory cells.

4. DISCUSSION

Here, we investigated the role of nanoparticles to enhance the antiviral potency of ARV drug combinations in models of in vitro cell-free and cell−cell HIV infection as well as in an ex vivo tissue organ system. We focused on delivering nanoparticles containing MVC, ETR, and RAL because the inhibitory potential of this triple combination is predicted to be sufficiently large to halt viral replication.[10,11] In addition, this triple drug combination is one of many promising ARV drug combinations that would be difficult to formulate in a single topical microbicide due to low intrinsic aqueous solubility and incompatible drug physicochemical properties.[30] The combination of MVC, ETR, and RAL is also promising due to its documented antiviral activity against drug-resistant HIV viral isolates, its demonstrated efficacy when used in salvage therapy, and the absence of identified adverse drug−drug interactions.[30−32] In addition, clinical studies of highly treated/experienced patients show that this combination is associated with good long-term efficacy and safety profiles.[33]

In contrast to existing approaches that coformulate ARV drug combinations together in a single liposome or nanoparticle,[6,7] we chose to prepare single-drug-loaded nanoparticles that were subsequently combined upon administration. Our data for drug loading in PLGA nanoparticles indicate that ETR, a hydrophobic drug, shows greater loading and encapsulation efficiency than either MVC or RAL, which are both ionizable at neutral pH. As such, these particular drugs are interesting not only for their mechanism of action and potency when used alone and in combination but also because they represent a challenge for codelivery in current vaginal dosage forms. If coformulated together in a single NP, these drugs would be likely to combine at a ratio near 8:2:1, which is the ratio observed for the individual loading of ETR, RAL, and MVC, respectively (Table 1). However, there are many applications where combining drugs at ratios that differ from their loading capacity may be beneficial. The molar ratio of drug combinations is known to be a significant factor in determining if the combined effect will be additive, synergistic, or antagonistic.[34−37] While formulating drugs together in the same carrier may ensure that they will be in fixed ratios when delivered intracellularly, most clinical applications require high inhibition ($f_a \gg 90\%$) and, in this limit, the dosing of multiple drugs either separately or combined in a single carrier may be similar. Our data provide a rationale for formulating the drugs separately in order to understand and maximize beneficial drug ratios for increased antiviral potency.

We observed that the ARV-NPs used in this study maintained potent HIV inhibition and were more effective when used in combinations. In particular, ARV-NP combinations with ETR exhibited significantly higher antiviral potency and dose-reduction against cell-free HIV-1 BaL infection despite no enhanced potency observed for the single ARV-NP. Based on the in vitro releases kinetics, we expect that MVC instantaneously releases from the NP once dosed to the cells and would behave similarly to free MVC by interacting with its extracellular receptor. This may explain the similar dose−effect response observed for free MVC and MVC-NP. ETR and RAL exhibited both a burst and significant sustained release phase. We anticipate that under physiological conditions, in the absence of a solubilizing agents such as solutol, ETR could show significantly slower release kinetics. Coupling the observed release kinetics with knowledge of the cellular uptake kinetics of nanoparticles should help clarify the mechanisms responsible for observed dose-reductions. Although NP internalization by cells can occur within minutes to hours, the kinetics of internalization is likely to depend on cell type/cycle as well as nanoparticle size, shape, and colloidal stability.[38−41]

ARV-NP combinations that showed large dose-reduction compared to their single drug components and the equivalent

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Mol. Pharmaceutics 2015, 12, 4363−4374

unformulated drug combinations were identified as synergistic based on the model of Bliss independence. For these ARV-NP combinations, deviation from the modified Bliss prediction for additivity was dose-dependent and showed the greatest difference at effect levels that were much higher than the IC50 ($f_a \gg 90\%$). In contrast, the combination effect of free ETR and RAL appeared to be strictly additive. A previous study with similar two-drug combination but using a single-round infection assay with PBMCs showed that free ETR and RAL are additive in combination, which is consistent with our observed data, whereas free ETR and MVC were strongly synergistic.[11] It is possible that the lack of synergy observed for the unformulated ETR/MVC combination in our study originates from differences in the assay employed, including sensitivity and the choice of viral isolate. We used the model of Bliss independence with a variable slope to predict the additive effect from our drug combinations, which provides a more stringent criterion for synergy than existing approaches.[42] An alternative approach that is more widely used to describe the effect of multidrug combinations is the Loew additivity model, which is based on the widely used isobolograms to describe an interaction index.[42−44] However, based on binding mechanism alone, the Loew additivity model is only applicable to drugs that have the same mechanism of action and therefore compete for the same binding site. In addition, existing models are only valid when the Hill coefficient is constant and not applicable for drugs that exhibit nonlinear dose−effects as is seen with ETR and many other ARV drugs.[10] It is interesting that the combination effect we observed from the free drugs differed from the ARV-NP combinations, but our result is consistent with other studies showing unique combination effects from nanoparticles used for combination chemotherapy.[45]

Although most studies on HIV transmission have focused on cell-free viral infection, an effective prophylaxis strategy including microbicides must also block the direct transmission of virus from cell−cell contact with infected cells such as seminal macrophages and T cells. It is now well established that both cell free-virus and cell-associated virus play important roles in initiating mucosal infection by HIV-1.[46−48] In general, cell−cell HIV transmission is more difficult to inhibit than cell-free virus infection.[2,3,29,49,50] We observed that cell−cell HIV-1 BaL transmission was inhibited at least 10-fold more effectively with drug combinations that included ETR-NP. Nanoparticle combinations of ETR and MVC showed the greatest dose-reduction in cell−cell HIV transmission compared to all other free- or formulated-drug combinations that were tested. Our observation that ARV-loaded nanoparticle combinations effectively inhibit cell-to-cell HIV transmission demonstrates their potential in this important aspect of HIV prophylaxis and should be further investigated. Finally, as a first step toward evaluating challenge studies in animal models, we also measured the activity of ARV-loaded nanoparticles in a virus infection assay using a macaque cervicovaginal explant model. We observed that ARV-NP combinations inhibit RT-SHIV virus propagation in tissue and also block virus transmission by migratory cells emigrating out of the tissue. The value of the explant infection data is to demonstrate that the ARV-NP combinations could be dosed and inhibit RT-SHIV infection in a relevant model. Unlike the cell culture assays, the tissue explant measures the activity of ARV-NP combinations against both cell-free virus and cell-to-cell transmission modes. Together, these results indicate that ARV-loaded nanoparticles can inhibit both cell-free and cell-associated HIV transmission in relevant model systems. Our results also demonstrate that ARV-NP combinations control HIV-1 transmission more efficiently than free-drug combinations in both cell-free and cell-associated HIV infection assays.

The underlying mechanism behind the dose-reduction observed with ARV-NP combinations involving ETR-NP is unclear but appears to be dependent on the formulation of the drugs in nanoparticles. Drug-loaded nanoparticles can improve intracellular drug uptake and biodistribution in tissues and cells due to a combination of tissue penetration and trafficking properties.[5] The bioavailability of ARV drugs delivered in NPs is associated with both the intracellular uptake of the nanocarriers as well as the dissolution of the drug from these polymeric solid dispersions. Given the impact on viral dynamics caused by the temporal uptake and release of these drugs, it would be valuable to correlate the time-dependent intracellular bioavailability with measured antiviral activity. Although we observe higher intracellular drug concentration in cells dosed with the triple ARV-NP combination compared to the equivalent unformulated drugs, this does not prove that the higher intracellular concentration underlies the mechanism that drives synergy. That is, higher intracellular drug concentrations are sufficient but not necessary to explain the dose-reduction from synergistic combinations. Although some synergistic combinations can be correlated to changes in intracellular drug concentrations (e.g., ritonavir boosted saquinavir), dose reduction due to synergy can also arise without changes in intracellular drug concentration (e.g., allosteric effects). Numerous ARV drug combinations exhibit synergy when dosed clinically but the underlying mechanism for enhanced potency is not known for the vast majority of these combinations.[11] Although measuring the intracellular PK of drugs dosed in combinations is a first step, alternative approaches are likely needed to fully understand the complex mechanisms underlying synergy and other combination effects.[51]

We envision that these combination NP drug cocktails can be administered topically to the vaginal mucosa using a conventional dosage form such as a gel where they can be incorporated as a suspension. In this case, nanoparticles may benefit the formulation of hydrophobic drugs into these water-based gels. Nanoparticles may also be incorporated in quick-dissolving films and intravaginal rings. However, these solid dosage forms have processing requirements such as hot-melt extrusion or solvent-cast evaporation that are likely to alter the integrity of the nanoparticles. Electrospun fibers, which are an emerging solid dosage form for vaginal delivery,[52] could also be used for nanoparticle delivery. While the current nanoparticle drug delivery system is being considered for use as a topical microbicide, we expect it will have other applications including parenteral administration. Although the PLGA nanocarriers used in our studies were effective at inhibiting HIV transmission across three different models of virus infection (cell-free, cell−cell, and tissue), the low loading observed for ionizable and water-soluble drugs may limit this platform's utility to study highly soluble drugs at high doses in other interesting combinations. Evaluating other nanocarrier systems that may be more amenable to loading highly soluble drugs, or developing pro-drugs that allow better loading into PLGA nanoparticles, could overcome these limitations. These and other strategies are needed to fully understand the role of nanocarrier systems in facilitating multidrug effects in relevant cells and tissue associated with HIV infection.

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5. CONCLUSION

We have developed ARV-loaded nanoparticles for ETR, RAL, and MVC and tested them in combination for prophylaxis against both cell-free and cell-associated HIV-1 BaL infection. We have also evaluated the protective efficacy of ARV-NP combinations in a nonhuman primate cervicovaginal tissue explant model against RT-SHIV infection. We observe that the ARV-NP combinations used in this study exhibit nonlinear and steep dose−effect responses at lower concentrations than the equivalent free-drug combinations. Combined with the ability to extend the half-life of the drugs by providing sustained release, this increased potency could enable an extended dosing interval. Identifying drug combination regimens that exceed the antiviral potency needed to achieve complete virus replication may allow for less frequent dosing as well as decreased dosing, which may depend on the modular ability to deliver and tune the release of different drug combinations. To move toward these applications, it will be important to determine if the synergistic activity measured for some of our ARV-NP combinations is also observed across diverse models of HIV infection and to investigate the underlying mechanism of these combination drug effects in vivo. We expect that the versatility of nanoparticle delivery platforms, particularly their ability to deliver high concentrations of physicochemical diverse agents, will result in broad applications for HIV chemoprophylaxis and treatment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00544.

Characterization of purified ARV drugs (^{1}H NMR,^{13}C NMR, LC-MS and UV-HPLC); additional characterization data of ARV-NP including transmission electron microscopy (TEM) images, stability studies, and drug-release kinetics; additional biological characterization including cytotoxicity of ARV-NP and antiviral activity of free-drug combinations at different ratios (PDF)

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Notes

The authors declare no competing financial interest.

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Review

A review of nanotechnological approaches for the prophylaxis of HIV/AIDS

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ABSTRACT

Successful treatment and control of HIV/AIDS is one of the biggest challenges of 21st century. More than 33 million individuals are infected with HIV worldwide and more than 2 million new cases of HIV infection have been reported. The situation demands development of effective prevention strategies to control the pandemic of AIDS. Due to lack of availability of an effective HIV vaccine, antiretroviral drugs and nucleic acid therapeutics like siRNA have been explored for HIV prophylaxis. Clinical trials shave shown that antiretroviral drugs, tenofovir and emtricitabine can offer some degree of HIV prevention. However, complete prevention of HIV infection has not been achieved yet. Nanotechnology has brought a paradigm shift in the diagnosis, treatment and prevention of many diseases. The current review discusses potential of various nanocarriers such as dendrimers, polymeric nanoparticles, liposomes, lipid nanocarriers, drug nanocrystals, inorganic nanocarriers and nanofibers in improving efficacy of various modalities available for HIV prophylaxis.

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1. Introduction

Approximately three decades ago, human immunodeficiency virus (HIV) was found to be the cause of acquired immunodeficiency syndrome (AIDS) [1]. Since its discovery, HIV is responsible for more than 25 million deaths worldwide [1–7]. At the moment, more than 33 million individuals are infected with HIV across the globe. During last 5 years, more than 2 million new HIV infections have been reported every year and around same number of individuals have died because of HIV [7]. The scenario is projected to get worse in the next decade mainly in Asia, Africa and Eastern Europe [2]. Thus, HIV represents one of the insurmountable problems of the 21st century. HIV/AIDS has caused significant socioeconomic damage worldwide. With the advent of highly active antiretroviral therapy (HAART) that uses a cocktail of antiretroviral drugs, improvements have been achieved. HAART has been responsible for significant improvement in life expectancy and quality [1–6]. However, HAART is associated with disadvantages such as emergence of drug resistant viral strains, inconvenient dosage regimen (daily administration of one or more pills for lifetime), serious adverse effects, and inability to eradicate HIV from reservoirs [1–6]. In view of this, significant attempts have been focused on prevention of HIV infection.

The majority of HIV infections are transmitted through sexual contact. Hence, early efforts were focused on advocating the use of physical barriers such as condoms and behavioral modifications (ABC, abstinence, be faithful, and correct consistent use of condom). However, these methods have not been very successful [7]. Studies have shown that male circumcision can considerably reduce chances of contracting HIV but this approach has its own limitations and is not a female controlled method of HIV prophylaxis [7]. Thus, it is important to develop more effective modalities for prevention of HIV. This review focuses on the applications of nanotechnology for HIV/AIDS prevention.

2. Modalities for prophylaxis of HIV

The process of development of prophylactic modality is always focused on making it widely available for disease prevention. Over the years, various modalities have been and are being developed for prophylaxis of HIV. The desired properties of an ideal modality for HIV prophylaxis are shown in Fig. 1 [8–10]. Broadly, prophylactic modalities can be divided into four categories viz. vaccines, macromolecular HIV entry inhibitors, antiretroviral drugs, and nucleic acid-based therapeutics (Fig. 2). There are numerous reviews that discuss each of these modalities in detail [2,7,8–10]. In this review, we intend to give an overview of various modalities available for HIV prophylaxis.

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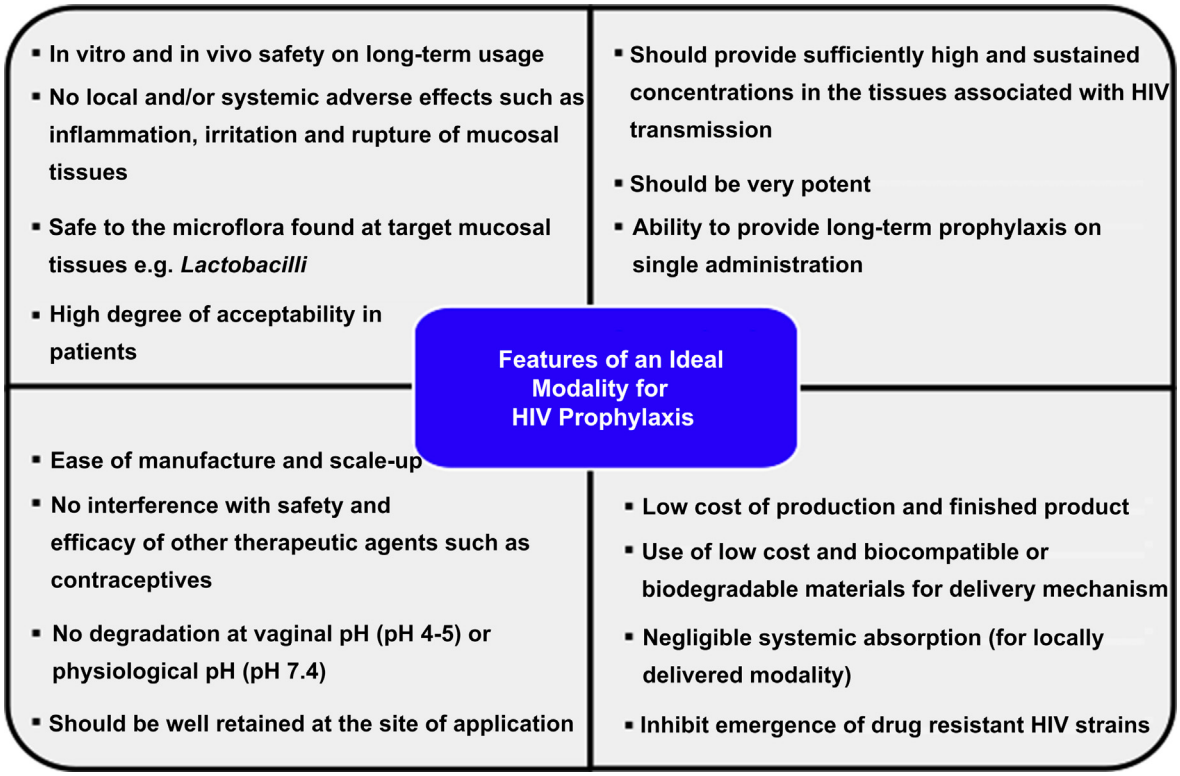

Fig. 1. Desired properties of an ideal prophylactic modality.

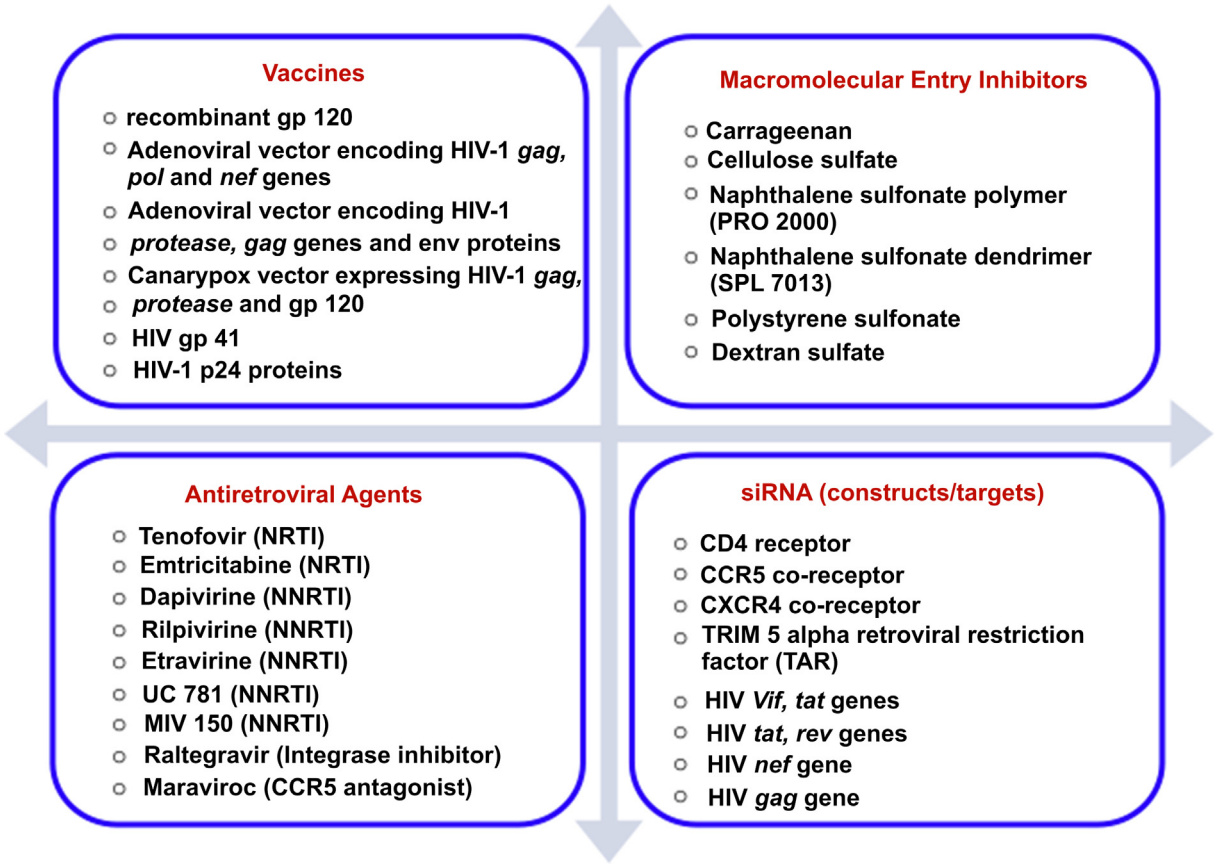

Fig. 2. List of various modalities available for HIV prophylaxis.

2.1. Vaccines

Development of vaccines has been the most prominent prophylactic strategy for a variety of viral infectious diseases. However, developing safe and effective vaccines against HIV is a very challenging task. The vast genetic diversity and high mutation rate are the major hurdles in the development of a HIV vaccine [11–14]. Moreover, structural characteristics of HIV envelope glycoprotein (gp120) such as variable loops, glycosylated N-terminus and flexible conformation are responsible for evasion of host immune response by virus [11–13]. Despite a plethora of challenges, scientists are continuously exploring various strategies to develop an effective vaccine. Until today, few clinical trials have been carried out to evaluate efficiency of vaccines in HIV prevention (Table 1). It is noteworthy that the first three clinical trials failed to show any prophylactic effect against HIV infection. RV144 was the first trial that demonstrated 31% protection from HIV infection in the Phase III [11–13]. Although some progress has been made, complete prophylaxis with a HIV vaccine still remains out of reach.

2.2. Macromolecular entry inhibitors

Several anionic macromolecules were found to inhibit binding of HIV-1 to CD4 cells by interacting with envelope glycoproteins [3]. Hence, their potential as prophylactic modality has been evaluated in many clinical trials (Table 1). Although macromolecular entry inhibitors showed a great promise in animal models, clinical trials in humans failed to show any significant protection as compared to placebo [3,9,10]. In fact, certain entry inhibitors like cellulose sulfate showed an increased risk of HIV acquisition due to destruction of vaginal epithelium [10]. At the moment, nanotechnology based macromolecular entry inhibitor (VivaGel®) is being evaluated in clinical trials [14–17]. Further details are discussed in a later section.

2.3. Antiretroviral agents

Due to lack of efficacy of vaccines and macromolecular entry inhibitors, there is a growing consensus on the use of drugs with proven antiretroviral activity for prophylaxis of HIV. It is anticipated that the presence of sufficient concentrations of antiretroviral drugs at the site would help to prevent HIV infection. It should be noted that drugs which act before integration of HIV with the human DNA are deemed to be useful for HIV prophylaxis [18]. Hence, drugs which act on HIV entry, HIV fusion, HIV reverse transcriptase and HIV integrase are being explored for the HIV prophylaxis (Fig. 2). For HIV prophylaxis, antiretroviral agents have been delivered either orally or locally. At the moment, nucleoside (or nucleotide) reverse transcriptase inhibitors (NRTIs) such as tenofovir and emtricitabine have been widely explored for HIV prophylaxis in various clinical trials (Table 1) [4,15,19–22]. Although results of these clinical trials are promising, none of the trials showed complete protection. In fact, some trials were discontinued due to lack of efficacy [19–22]. At the moment, several trials are ongoing that evaluate potential of non-nucleoside reverse transcriptase inhibitor (NNRTI) such as dapivirine and UC 781 (vaginal gel and/or ring) [23,24]. Use of antiretroviral drug combination is a commonly employed strategy for HIV therapy to increase efficacy and reduce resistance and side effects. In view of this, clinical trial using combination of antiretroviral drugs (dapivirine and maraviroc) has also been initiatedfor HIV prophylaxis[23,25].

2.4. Nucleic acid therapeutics (siRNA)

Discovery of RNA interference (RNAi) mechanism has brought a revolution in many fields including medicine. RNAi employs short RNA constructs to induce degradation of mRNA machinery in a sequence specific manner [26–29]. RNAi can be accomplished by using a short double stranded RNA (21–25 nucleotides in length; siRNA) or a short hairpin RNA (shRNA), a stably expressed hairpin like precursor. As siRNAs can achieve sequence specific gene silencing at a very small concentration, they are being actively pursued as therapeutic agents for a variety of indications [26–29]. In the last few years, considerable efforts have been made to develop RNAi constructs for prophylaxis of HIV by systemic and/or local delivery. siRNA targeting various HIV-1 encoded genes like *tat*, *rev*, *pol*, *nef*, *gag*, *vif*, *env*, *vpr* and LTR have shown potential to inhibit HIV-1 infection or suppression of HIV-1 infection in the cells. Transmission of HIV occurs through utilization of various cellular receptors from the host (CD4, CCR5, CXCR4) [27–29]. Hence, siRNAs targeting expression of these receptors have also shown potential in HIV prophylaxis. Although RNAi therapeutics has potential for HIV prophylaxis, there are several challenges associated with the delivery of RNAi therapeutics. Extreme hydrophilicity and anionic charge of siRNAs significantly hamper their cellular uptake [28–30]. Moreover, siRNAs should be released in the cytoplasm of the cells in order to achieve silencing. Hence, it is important that the delivery mechanism of siRNA should prevent lysosomal degradation of siRNA. siRNAs are also susceptible to nuclease mediated degradation in the body [28–30]. All these challenges pose a great difficulty in successful delivery of siRNAs. Until today, siRNA mediated HIV prophylaxis has been explored only in animals.

Various routes (oral, nasal, intramuscular, subcutaneous, intravenous, vaginal and rectal) have been employed to administer modalities for HIV prophylaxis. Prophylactic modalities (mainly chemical products) applied locally to either vagina or rectum are termed'microbicides'. Importance of routes of administration on the efficacy of prophylactic modalities will be discussed later.

3. Factors affecting efficacy of prophylactic modalities

For effective prophylaxis, it is important to attain sufficient concentrations of prophylactic modality at the potential site of infection. Various physicochemical properties such as aqueous solubility, permeability and log *P* value have considerable influence on the local and/or systemic distribution of the prophylactic modality. According to biopharmaceutics classification system (BCS), therapeutic agents are classified as Class I (high solubility, high permeability), Class II (low solubility and high permeability), Class III (high solubility and low permeability) and Class IV (low solubility and low permeability). Table 2 enlists physicochemical properties and BCS Classification of the antiretroviral drugs that are being considered for prophylaxis [31–39]. Prophylactic modalities such as vaccines and siRNA should be considered as BCS Class III drugs.

Tenofovir has been extensively evaluated as a prophylactic modality. Tenofovir is a BCS Class III drug with high solubility but low permeability [40]. In order to improve permeability of tenofovir, a prodrug (tenofovir disoproxilfumarate) was synthesized for oral administration. Studies indicate that tenofovir prodrug yields 1000-fold higher intracellular concentration of tenofovir diphosphate as compared to tenofovir base [40]. Moreover, tenofovir prodrug has 100-fold lower IC_{50} as compared to tenofovir base which clearly indicates the importance of permeability [41]. Interestingly, tenofovir gel used in clinical trials have employed tenofovir base instead of tenofovir prodrug. CAPRISA004 trial which employed pre-and post-coital administration of 1% tenofovir gel showed 39% protection from HIV in women [21]. However, the VOICE trial that employed coitus-independent once daily application of 1% tenofovir gel was stopped due to fulity. One of the reasons for this failure could be low permeability of tenofovir base. It is

Table 1
Details of completed and ongoing human clinical trials of various modalities for HIV prophylaxis [9–25].

Trial name	Prophylactic modality	Study size	Regimen	Clinical phase	Results
VAX003 (AIDSVAX B/E)	Recombinant gp120 + alum	2527	7 i.m. Injections over 30 months (dose: 300 μg)	Phase III	No efficacy
VAX004 (AIDSVAX B/B)	Recombinant gp120 + alum	5403	7 i.m. Injections over 30 months (dose: 300 μg)	Phase III	No efficacy
STEP	Ad5 vector encoding HIV-1 *gag*, *pol* and *nef* genes	3000	3 i.m. Injections (day 0, week 4 and week 26) containing 1.5×10^{10} adenovirus genomes	Phase III	No efficacy
RV144	ALVAC-HIV (canarypox vector expressing HIV-1 *gag*, *protease* and gp120) + AIDSVAX (B/E)	16,395	4 i.m. Injections of ALVAC-HIV (day 0, week 4 12 and 24) at the dose of $10^{6.5}$ $TCID_{50}$ + 2 i.m. injections of 300 μg AIDSVAX B/E on week 12 and 24	Phase III	31.2% protection
HVTN 505	Ad5 vector encoding HIV-1 *gag*, *pol*genes and env A, B, C + DNA vaccine encoding *nef*	2200	3 i.m. Injections of DNA vaccine over 8 weeks + single injection of Ad5 vector on week 24	Phase II	Results awaited
Carraguard® (PC 515)	3% Carrageenan	6202	Vaginal application of 4 ml of 3% Carrageenan gel 1 h before intercourse	Phase III	No efficacy; Carraguard was found to be safe
Ushercell	6% Cellulose sulfate	1398	Vaginal application of 3.5 ml of 6% cellulose sulfate gel 1 h before intercourse	Phase III	No efficacy, increased risk of HIV acquisition
MDP 301	0.5% and 2% PRO 2000 (naphthalene sulfonate polymer)	9385	Vaginal application of 0.5% or 2% gel before intercourse	Phase III	No efficacy
CAP	13% cellulose acetate phthalate gel	6	Vaginal application of gel	Phase I	Mucosal irritation due to hyperosmolarity
MTN 004	VivaGel (3% SPL7013, dendrimers containing naphthalene sulfonate) end groups	61	Twice daily application of 3.5 g VivaGel for 14 days	Phase I	VivaGel was well tolerated although higher incidences of low grade genitourinary adverse effects were observed
VivaGel	VivaGel (3% SPL7013)	11	One time application of 3.5 g VivaGel	Phase I	Cervicovaginal fluid collected at 3 h after application of VivaGel showed complete inhibition of HIV-1 and cervicovaginal fluid collected at 24 h after application showed 88% protection
VOICE 004	1% Tenofovir gel	889	Vaginal application of 4 ml gel up to 12 h before and after sex	Phase III	39% Protection; 54% protection in women with high adherence
iPrEx	Tenofovir (300 mg) + Emtricitabine (200 mg) (Truvada®)	2499 (MSM)	Daily oral Truvada®	Phase III	44% Protection
TDF2	Tenofovir (300 mg) + Emtricitabine (200 mg) (Truvada®)	1200	Daily oral Truvada®	Phase III	63% Protection
PIP	Tenofovir (300 mg) + Emtricitabine (200 mg) (Truvada®) or Tenofovir (300 mg)	4747	Daily oral Tenofovir or Truvada®	Phase III	62% Protection for tenofovir group and 73% protection with Truvada®
FEM-PrEP	Tenofovir (300 mg) + Emtricitabine (200 mg) (Truvada®)	1951	Daily oral Truvada®	Phase III	Trial stopped due to lack of efficacy
VOICE	Tenofovir (300 mg) + Emtricitabine (200 mg) (Truvada®) or Tenofovir (300 mg) or 1% tenofovir gel	5029	Daily oral Tenofovir or Truvada® or once daily application of 1% tenofovir gel	Phase III	Oral tenofovir and 1% tenofovir gel did not show efficacy
IPM 012	Dapivirine gel	36	Once daily vaginal application of two different 0.05% dapivirine gels (2.5 g) for a period of 11 days	Phase I	Dapivirine concentration in cervicovaginal fluid was five logs higher than *in vitro* IC_{50}
IPM 014A	Dapivirine gel	280	Once daily vaginal application of 0.05% dapivirine gel (2.5 g) for 6 weeks	Phase I/II	Ongoing
IPM 014B	Dapivirine gel	100	Once daily vaginal application of 0.05% dapivirine gel for 6 weeks	Phase I/II	Results awaited
IPM 020	Dapivirine gel	128	Once daily vaginal application of two different 0.05% dapivirine gels for a period of 12 weeks	Phase I/II	

(*continued on next page*)

Table 1 (*continued*)

Trial name	Prophylactic modality	Study size	Regimen	Clinical phase	Results
IPM 013	Dapivirine (25 mg) vaginal ring	48	Group A: dapivirine ring inserted on day 0 and 31 Group B: dapivirine ring inserted on day 0, 38, 59	Phase I	Peak dapivirine concentrations reached in a day and dapivirine released at a concentration above IC_{50} for up to 35 days
IPM 015	Dapivirine (25 mg) vaginal ring	280	Dapivirine ring inserted once in 28 days over a period of 12 weeks	Phase I/II	Results awaited
IPM 027	Dapivirine (25 mg) vaginal ring	1650	N.A.	Phase II	Ongoing
MTN 013/ IPM 026	Maraviroc (100 mg), dapivirine (25 mg) and maraviroc (100 mg) + dapivirine (25 mg) vaginal rings	48	Insertion of vaginal ring and checking of drug levels and safety for 28 days	Phase I	Results awaited
UC 781	UC 781 gel	25	Twice daily application of 3.5 ml 0.1% or 0.25% UC 781 gel for 14 days	Phase I	Cervicovaginal lavage collected from 13 (out of 15) women treated with 0.25% UC 781 gel showed inhibition of HIV

possible that due to low permeability, once daily application of tenofovir base may not yield sufficient concentration of tenofovir diphosphate in various tissues of reproductive tract. A recent study showed that less than 5% of the tenofovir base permeated through HEC-1A cells (endometrial adenocarcinoma cells) placed on a transwell membrane [33], which may corroborate this hypothesis. Thus, strategies that could improve permeability and local distribution of tenofovir would be very advantageous. At the moment, dapivirine (NNRTI) is also being evaluated as a microbicide in various clinical trials [25]. Due to high permeability and intracellular half-life [33], dapivirine has high potency. However, dapivirine is a hydrophobic drug. For solubilization of dapivirine, a considerable amount of cosolvents like glycerine and ethanol are required [42]. Studies indicate that use of high concentrations of cosolvents can increase osmolarity of gels which can affect integrity of vaginal epithelium and lead to increased susceptibility to HIV infection [43]. Furthermore, dapivirine tends to form aggregates in solution and/or at acidic pH [44]. No studies have been carried out to study formation of dapivirine aggregates in the vaginal milieu. Higher generation hydrophobic NNRTIs like etravirine and rilpivirine also share similar problem which may pose difficulties in their use as a microbicide. UC 781 is a hydrophobic NNRTI that is being evaluated as a microbicide. UC 781 was found to undergo degradation in aqueous solution [45]. UC 781 is also light sensitive and undergoes metal catalyzed oxidation [45]. Thus, developing suitable and stable formulation of microbicides such as UC 781 is a challenging task. Raltegravir is an integrase inhibitor that belongs to BCS Class II. However, a recent study has shown the permeability of raltegravir is significantly dependent on the pH, which would have an impact on its use as a microbicide [46]. Various studies have shown that drugs like tenofovir, raltegravir and maraviroc are substrates for drug efflux transporters [47–49]. Until today, there are no *in vitro* studies on the expression of drug efflux transporters on the cells relevant to vaginal and/or rectal delivery of microbicides. It could be interesting to study the use of P-glycoprotein modulators on the *in vitro* and *in vivo* distribution of microbicides.

Vaccines, peptides and siRNA-based modalities are continuously being explored for HIV prophylaxis. However, these modalities have poor tissue permeability due to their extreme hydrophilicity and/or anionic charge [26–30]. Furthermore, these modalities are quite susceptible to chemical and metabolic instability. Acidic environment of vagina and cervicovaginal mucus are also major barriers for effectiveness of peptides, vaccines and siRNA. It is important to identify strategies that would increase permeability, chemical and metabolic stability and immunogenicity of vaccines and/or siRNA therapeutics.

High osmolarity of delivery vehicle (gel) is another factor that hinders/compromises efficacy of prophylactic modalities. Tenofovir gel used for CAPRISA004 trial contained 20% w/v glycerine. Usually, 2.25% w/v glycerine is sufficient to attain anosmolarity similar to cervicovaginal fluids. The tenofovir gel used in the CAPRISA004 trial had osmolarity of 3111 mmol/kg and *in vitro* studies showed that it caused epithelial stripping of polarized explant [50]. Recently, a Phase 1 trial of an HIV-1 entry inhibitor (cellulose acetate phthalate) was halted due to significant mucosal irritation caused by hyperosmolarity of the gel [51]. In order to avoid mucosal irritation and damage of vaginal epithelium, microbicide gels should have osmolarity less than 1000 mmol/kg [43]. Thus, suitable strategies

Table 2
Physicochemical properties and pharmacokinetic parameters of selected antiretroviral drugs [31–39].

Drug	Solubility	pK_a	log *P*	BCS classification	Half-life	% Bioavailability
Tenofovir	13.4 mg/ml	1.3; 7.9; 3.0; 5.3	−1.1	Class III	17	25–39
Emtricitabine	112 mg/ml	2.63	−0.43	Class I	10	93
Efavirenz	<10 μg/ml	10.2	3.68	Class II	40–50	42–80
Nevirapine	100 μg/ml	2.8	2.05	Class II	25–30	>90
Dapivirine	<10 μg/ml	5.8	5.27	Class II	N.A.	N.A.
Etravirine	<10 μg/ml	3.5	5.2	Class IV	30–40	N.A.
Rilpivirine	<10 μg/ml	5.6	4.86	Class II	34–55	24 (monkeys)
Raltegravir	<1 mg/ml	~1.25	1.06	Class II	9	~65%
Maraviroc	~1 mg/ml	3.3, 7.9	4.37	Class III	14–18	23–33

N.A., not available.

need to be designed to tackle the problem of hyperosmolarity without compromising efficacy of microbicides.

4. Nanotechnology for HIV prophylaxis

According to the National Nanotechnology Initiative, nanotechnology involves study of materials/architectures of size 1–100 nm in at least one dimension [1,5,52–54]. However, materials with size up to several hundred nanometers are also included under nanotechnology. At the moment, several nanotechnology based products are being used and evaluated in clinical practice. Since last decade, efforts are being made to improve diagnosis, therapy and prophylaxis of HIV/AIDS with the help of nanotechnology. In last few years, several reviews have been published which mainly focus on application of nanotechnology for improving therapeutic effect and targeting of antiretroviral modalities [1,5,32,52–56]. In the present review, we have mainly focused on application of nanotechnology pertaining to prophylaxis of HIV. Until now, various types of nanocarriers have been developed for improving HIV prophylaxis (Fig. 3). Nanocarriers offer various advantages which would be useful to overcome challenges/problems associated with current prophylactic modalities. Various types of nano-archi tectures developed for HIV prophylaxis are discussed in the following sections and their salient features are shown in Fig. 4.

5. Nano-architectures for HIV prophylaxis

5.1. Dendrimers

Dendrimers are a versatile class of polymeric nano-structures with unique architectural and topological features. Unlike conventional linear polymers, dendrimers exhibit three-dimensional tree-like structures, narrow polydispersity index and precise number of terminal groups [52,55,57]. Interestingly, it is possible to synthesize dendrimers with precise physicochemical and desired biological properties by manipulating the structure of central core, structure and number of branching units and composition and number of surface functional groups [52,55,57].

Due to presence of numerous surface functional groups, it is possible to conjugate multiple drug or targeting ligand molecules to a single dendrimer. Dendrimers also have the ability to encapsulate hydrophobic drugs in their interior cavity[52,55,57]. However, due to limitations on the cavity size, drug payload obtained after encapsulation is significantly lower than that obtained with conjugation [52,55,57]. Dendrimers with the right composition and number of surface functional groups can form complexes with cell or viral receptors through multivalent interactions resulting in inhibition of viral fusion to target cells [52,58]. In fact, the potential of dendrimers to prevent viral infection was demonstrated back in 1996 [59]. Thus far, dendrimers based on diverse building blocks such as polyamidoamine (PAMAM), poly-amino acids, polypropylene imine, polyethers and carbosilane have been evaluated.

5.1.1. Dendrimers as topical microbicides

Due to a lack of efficacy of polyanionic natural, semi-synthetic or synthetic linear polymers in the clinical trials (Table 1), dendrimers were actively pursued as topical microbicides. Until today, dendrimers is the only nanotechnology that has advanced to human clinical trials for HIV prophylaxis[52]. Extensive studies on the linear polyanionic inhibitors provided a basis for the design of suitable dendrimers. It is well known that the anionic moieties of the linear polyanionic inhibitors inhibit viral fusion to cell membrane by binding to viral glycoproteins such as gp120 and/or gp41 [3]. Hence, dendrimers with anionic surface functional groups have been designed and explored to maximum extent. Although most of the linear polyanionic entry inhibitors exhibit sulfated end groups, dendrimers with various other anionic end groups have also been designed [58, 60]. Dendrimers that are being evaluated as HIV entry inhibitors can be broadly classified into (1) classical dendrimers with anionic end groups; (2) carbohydrate terminated dendrimers (glycodendrimers); and (3) dendrimers based on inorganic–organic backbones.

5.1.1.1. Classical dendrimers with anionic end groups. Dendrimers with classical branching units such as polyamidoamine (PAMAM) and poly-L-lysine, but different anionic end groups have been extensively investigated by researchers to identify optimal design parameters for dendrimers with maximum antiviral activity and minimal toxicity [58,60,61]. These systematic investigations have resulted in development of first topical nanomicrobicide, SPL7013 (also referred as VivaGel®). SPL7013 is a g-4 poly-L-lysine dendrimers synthesized from divalent benzhydrylamine amide of L-lysine and it contains 32 sodium 1-(carboxymethoxy) naphthalene-3,6-disulfonate as terminal anionic functional groups [58,62]. The precise chemistry and process design have ensured large-scale synthesis of SPL7013 as single molecular entity with molecular weight of 16,581 Da. Various *in vitro* studies have established broad spectrum antiviral activity of SPL7013 against HIV and HSV (EC_{50}

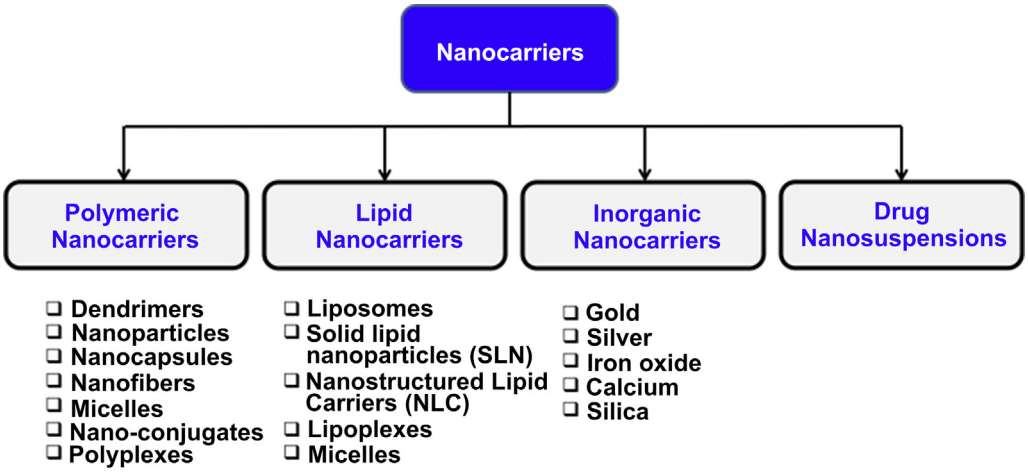

Fig. 3. Various types of nano-architectures available for HIV prophylaxis.

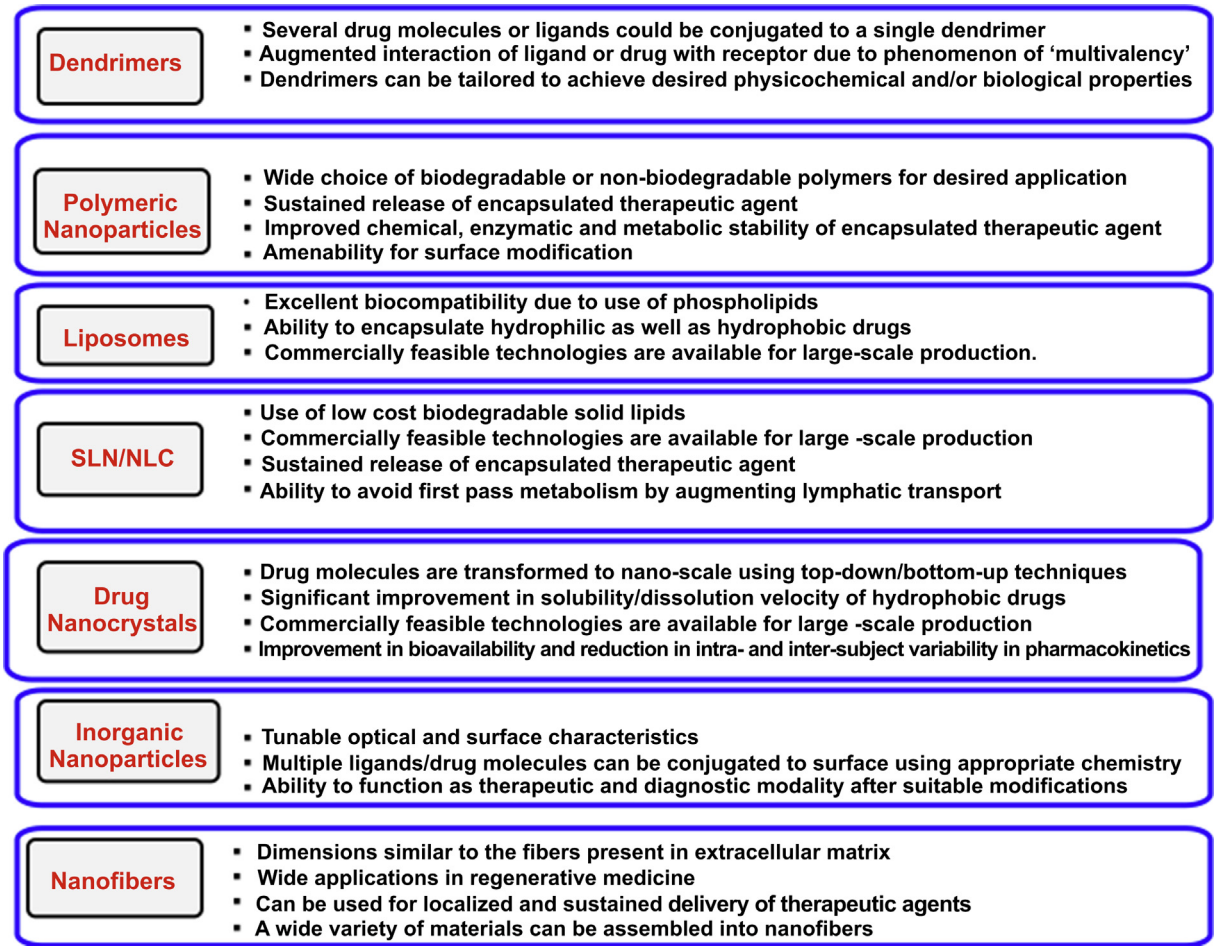

Fig. 4. Salient features of various nano-architectures.

ranging from 0.5 to 3 μg/mL) whereas SPL7013 was not cytotoxic to Vero cells at a concentration as high as 10,000 μg/mL [62]. Thus, SPL7013 showed excellent selectivity index. Unlike dextran sulfate and other linear polyanionic entry inhibitors, SPL7013 showed similar potency against entry of CXCR4-(X4) and CCR5-using (R5) HIV-1 strains [60]. Telwatte et al. have demonstrated that SPL7013 has potent virucidal activity against the CXCR4-tropic HIV-1 strains [63]. SPL7013 (3% w/w) was formulated into an aqueous mucoadhesive Carbopol® gel buffered to aphysiologically acceptable pH (VivaGel®). Single intravaginal application of VivaGel to pigtailed macaques completely prevented simian-human immunodeficiency virus infection. Moreover, VivaGel® was found to be safe in pigtailed macaques after vaginal as well as rectal administration [64].

VivaGel® is the first nanotechnology based microbicide to enter human clinical trials. During the Phase I clinical trial, VivaGel was found to be safe and well tolerated in women after once daily intravaginal application for 7 days. Moreover, no systemic absorption of SPL7013 was observed indicating good vaginal retention and absence of systemic toxicity [65]. An additional Phase I randomized placebo controlled trial was carried out to evaluate safety of VivaGel® in healthy young women after twice daily application for 14 days. Women in VivaGel® arm showed higher incidences of grade 1 or 2 genitourinary adverse events as compared to control but no serious adverse events were observed in both the arms [15]. Price et al. evaluated antiviral efficacy of SPL7013 present in the cervicovaginal fluids of the women treated with VivaGel®. VivaGel demonstrated activity against HIV and HSV for at least 3 h post-dose. This is the first clinical evidence of the efficacy of the VivaGel® and it may be possible to administer VivaGel® 3 h before coitus [16]. In a recent Phase I trial, Mosicicki et al., evaluated the effect of VivaGel treatment on various mucosal biomarkers associated with epithelial damage [17]. It was observed that VivaGel treated women showed higher levels of IFN-γ, IL-2, IL-5, IL-6 and IL-10 after 7-day and/or 14-day administration. Incidentally, the VivaGel treated group also showed higher population of $CD8^+$/$CD69^+$ T cells, $CD4^+$/$CD69^+$ cells and $CD4^+$/$CCR5^+$ T cells. However, all these changes abolished 7 days after discontinuation of VivaGel treatment. Thus, changes observed after VivaGel treatment appeared to be reversible. However, this trial indicated that subsequent VivaGel cytotoxicity should be monitored meticulously.

5.1.1.2. Glycodendrimers. Carbohydrates are an integral part of several complex biological processes such as cell differentiation, metastasis and infection. Carbohydrate receptors are expressed on variety of immune cells such as macrophages as well as epithelial cells such as vaginal epithelial cells [66–69]. Carbohydrate receptors such as C-type lectin receptors present on the antigen presenting cells (dendritic cells) and mannose receptors present on the vaginal epithelial cells and human spermatozoa have important role in the HIV infection [66–69]. Cell surface glycosphingolipids such as galactosylceramide can act as a receptor for the HIV gp120 and facilitate HIV entry in the absence of CD4 receptors [70]. On the other hand, a variety of linear sulfated polysaccharides have shown the ability to inhibit HIV entry *in vitro* although they were unsuccessful in clinical trials [3]. In view of this, it was hypothesized that

multivalent presentation of carbohydrates or sulfated carbohydrates on the dendrimeric scaffold could be useful for inhibition of HIV entry. Tabarani et al. demonstrated that mannose hyperbranched dendritic polymers can inhibit interaction between HIV gp120 and DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin), a C-type lectin receptor present on dendritic cells [71].

Kensinger et al. designed various generations of polypropylenimine (PPI) glycodendrimers with galactose or sulfated galactose as terminal groups and evaluated their ability to prevent HIV infection [70,72]. It was observed that dendrimers terminated with sulfated galactose were more active as compared to galactose terminated dendrimers. In fact, sulfated galactose generation 5 (G-5) dendrimers were found to be more potent than linear polysaccharide dextran sulfate in certain cases [72]. This indicates the potential of glycodendrimers. In a series of investigations, Han et al. have evaluated potential of various types of sulfated cellobiose terminated poly-L-lysine dendrimers to inhibit HIV entry [73,74]. Generation 3 (G-3) sulfated cellobiose terminated poly-L-lysine dendrimers showed similar potency to the NRTI (nucleosidereverse transcriptase inhibitor) ddC (2′–3′-dideoxycytidine) [73]. The same group evaluated effect of derivatization of lysine core with stearylamide on the anti-HIV effect of sulfated cellobiose terminated dendrimers. The fatty acid derivatization reduced the potency of the dendrimers [74]. Hence, fatty acid derivatization was not found to be useful for increasing potency. Clayton et al. synthesized sialic acid and sulfated sialic acid terminated PAMAM dendrimers and evaluated their anti-HIV activity in comparison to dextran sulfate. The sialic acid dendrimers were much less potent as compared to dextran sulfate [75]. Schengrund et al. designed G-5 PPI dendrimers terminated with either 3′-sialyllactose (GM3) or globotriose (Gb3). Depending on the type of sugar, cell and virus, the IC_{50} value of dendrimers ranged from 0.1 to 15 μg/mL [76]. Sattin et al., demonstrated that tetravalent Boltron-type dendrimers terminated with linear trimannoside mimics (termed as Dendron-12) can inhibit infection of lymphocytes (up to 90%) by CXCR4-(X4) and CCR5-using (R5) HIV-1 strains at 50 μM concentrations [77]. Moreover, the dendrimers did not show any signs of toxicity at 4-fold higher concentrations. The authors also carried out *in vitro* studies that involved pre-treatment of cells with Dendron-12 for 30 min or 2 h, thorough washing to remove Dendron-12 followed by HIV infection at 0, 6, or 12 h. In all cases, Dendron-12 significantly reduced HIV infection. Thus, glycodendrimers could have potential for long-term prophylaxis [77]. The same group evaluated the potential of Dendron-12 in the cervical explants. Dendron-12 could successfully prevent infection of cervical tissue by various clades of R5- and X4-tropic viruses and was found to be well tolerated by cervical explants at tested concentrations [78].

Mannose and lactose terminated dendrimers based on gallic acid core have been evaluated for ability to inhibit dimerization of HIV-1 capsid protein [79]. Unfortunately, none of the glycodendrimers showed inhibition of capsid protein dimerization whereas benzoic acid sodium terminated gallic acid dendrimers showed considerable inhibition of capsid protein dimerization [79]. From various studies on the glycodendrimers, it can be inferred that the anti-HIV activity considerably depends on the nature of the terminal carbohydrate group and its subsequent derivatization. The influence of the type of core and branching units on the activity of glycodendrimers has not been explored yet.

5.1.1.3. Dendrimers based on inorganic-organic backbones. In recent years, dendrimers based on inorganic compounds such as phosphorus and silicon have been designed as HIV entry inhibitors. Blanzat et al. have evaluated anti-HIV potential of phosphorus dendrimers based on hexachlorocyclotriphosphazene or thiophosphoryltrichloride core in a series of investigations [80–82]. Phosphorous dendrimers with either cinnamic acid or phosphonic acid end groups were prepared. These anionic dendrimers interacted with cationic galactosylceramide analogue (amino lactitol) to yield self-assembled catanionic dendrimers [80,81]. The anti-HIV activity of these dendrimers was evaluated. Interestingly, all the dendrimers demonstrated the ability to inhibit HIV infection at nM or μM concentrations. However, all the dendrimers yielded considerably low selectivity index (less than 100) [80,81]. Thus, this series of dendrimers was deemed to be unsuitable for further development. The same group designed phosphonate terminated poly-(phosphorhydrazone) dendrimers with different pendant alkyl chains [82]. Unlike earlier investigation, no attempts were made to fabricate cataninonic dendrimers. Interestingly, these dendrimers did not show any cytotoxicity up to 10 μM concentration whereas IC_{50} against HIV was in the nM range [82]. This study clearly indicated the influence of dendrimers architecture and pendant alkyl groups on the anti-HIV activity of dendrimers. Munoz-Fernandez et al. have designed a series of dendrimers containing carbosilane (Si–C) or carbosiloxane (Si–O) core and various cationic or anionic end functional groups for various applications [83–85]. Recently, the same group designed second generation carbosilane dendrimers with anionic sulfonate end groups and evaluated their ability to inhibit HIV [90]. The dendrimers were non-toxic to various epithelial cell lines and PBMC at concentrations ranging from 20–100 μM. Moreover, carbosilane dendrimers were able to prevent HIV-1 and HIV-2 infection in activated PBMC. Carbosilane dendrimers also partially inhibited translocation of HIV through trans-epithelial monolayer *in vitro*. Carbosilane dendrimers were also safe to rabbits after 2 weeks of intravaginal application [85]. Thus, dendrimers based on inorganic–organic core do have potential to become topical microbicides. However, there have been no studies to establish their safety on chronic administration.

5.1.2. Dendrimers as carriers

A small number of studies have been reported in the literature that explores the potential of dendrimers as a carrier for antiretroviral modalities. Jain et al. have designed poly(propylene imine; PPI) dendrimers terminated with tuftsin (a macrophage activating tetrapeptide) or mannose (ligand for lectin receptors present on dendritic cells and macrophages) as targeting ligands [86,87]. Antiretroviral drugs such as efavirenz and lamivudine were incorporated in the dendrimers and the efficacy and safety of these dendritic nanocarriers was evaluated. The PPI dendrimers functionalized with targeting ligands resulted in greater *in vitro* anti-HIV activity and reduced cytotoxicity as compared to PPI dendrimers without targeting ligands. Functionalization of PPI dendrimers with targeting ligands masked their cationic charge leading to reduced cytotoxicty and greater efficacy.

Munoz-Fernandez et al. evaluated the potential of water-soluble amine terminated cationic carbosilane dendrimers to deliver various siRNAs to PBMC and lymphocytic SupT1 cells [84]. The carbosilane dendrimers prevented siRNAs from RNAse mediated degradation and dendrimers–siRNA comeplexes were non-toxic to cells up to 30 μg/mL. The dendrimers could successfully transfect PBMC (which are usually hard to transfect) and SupT1 cells with siRNA and prevented HIV infection [84]. In an interesting investigation, Navath et al., fabricated in-situ forming hydrogel based on crosslinking of thiopyridine terminated G-4 PAMAM dendrimers and thiol terminated 8-arm PEG [88]. Vaginal delivery of dendrimeric hydrogels to guinea pigs revealed that hydrogels were retained in the vaginal cavity for at least 72 h and did not cause any alteration in the vaginal pH. The hydrogels did not show any signs of local toxicity and started biodegradation after 72 h. Thus, dendrimeric hydrogels could be used for sustained delivery of water-

soluble antiretroviral drugs such as tenofovir or emtricitabine into vaginal cavity. In short, dendrimers can be successfully used as carriers for antiretroviral agents.

5.2. Polymeric nanoparticles

Polymeric nanoparticles (or nanospheres) are solid colloidal nano-scale particles composed of macromolecular substances of natural or synthetic origin and have size range between 10 and 1000 nm [89]. The therapeutic agent(s) can be dissolved, encapsulated, adsorbed or conjugated to polymeric nanoparticles by means of various methods [89]. Over the years, various methods have been developed to engineer polymeric nanoparticles that can carry a variety of hydrophobic or hydrophilic drugs as well as biomolecules like proteins and siRNA [90]. A variety of natural or synthetic biodegradable polymers as well as non-biodegradable polymers have been employed for fabrication of polymeric nanoparticles. However synthetic polymers such as poly-lactic-co-glycolic acid (PLGA), poly-caprolactone (PCL), polyalkylcyanoacrylates, polymethylmethacrylates (Eudragits) and natural polymers like chitosan are most widely used for fabrication of polymeric nanoparticles. Although there are several literature reports and reviews about delivery of antiretroviral drugs [32,53,54], we will discuss reports relevant to prophylaxis of HIV. We have also included some recent reports on potential of polymeric nanoparticles for HSV prophylaxis as they have implications in vaignal delivery of microbicides using polymeric nanoparticles.

5.2.1. Polymeric nanoparticles for delivery of antiretroviral drugs and siRNA for HIV prophylaxis

Polymeric nanoparticles received great attention for (vaginal) delivery of microbicides after investigations by Ham et al., and Woodrow et al., were published in 2009 [91,92]. Until today, most of the investigations focusing on HIV prophylaxis have employed PLGA nanoparticles to deliver microbicides. PLGA is a US FDA approved biodegradable polymer and is acceptable for delivering drugs via all major routes of administration. PLGA nanoparticles can undergo endolysosomal escape and deliver encapsulated cargo into cytoplasm [93]. This aspect is very important for successful delivery of antiretroviral drugs as well as RNAi therapeutics. There are numerous examples in the literature that demonstrate sustained release potential of PLGA nanoparticles. In view of this, PLGA nanoparticles were thought to have potential in achieving long-term HIV prophylaxis. Ham et al., engineered PLGA nanoparticles containing PSC-RANTES, a CCR5 chemokine receptor inhibitor [91]. Encapsulation of the PSC-RANTES in the PLGA nanoparticles did not affect its anti-HIV activity as compared to PSC-RANTES solution. Interestingly, ex-vivo permeation studies in human ectocervical tissue demonstrated that PSC-RANTES-PLGA nanoparticles have significantly higher (4.8 times) uptake as compared to non-encapsulated PSC-RANTES within a period of 4 h. Furthermore, PSC-RANTES-PLGA nanoparticles could reach the basal layer of the cervical epithelium, which is critical component of the HIV infection process [91]. The augmented delivery of PSC-RANTES from the nanoparticles could be due to greater cellular uptake of nanoparticles as well as protection of PSC-RANTES from the acidic/enzymatic degradation in cellular milieu due to its encapsulation into nanoparticles. Saltzman et al. have carried out various investigations on the vaginal delivery of PLGA nanoparticles. In the first investigation, Saltzman et al. fabricated PLGA nanoparticles (<200 nm) containing siRNA–spermidine complex in their core. *In vitro* studies confirmed ability of PLGA nanoparticle encapsulated siRNA to silence target genes in a cell- and dose-dependent manner [92]. *In vivo* efficacy of the PLGA nanoparticles carrying siRNA (that targets enhanced green fluorescent protein expression; eGFP) was evaluated in female mice expressing eGFP in their reproductive tract. Interestingly, single intravaginal application of siRNA carrying PLGA nanoparticles induced sustained eGFP silencing in the entire reproductive tract for the period of 14 days [92]. PLGA-siRNA nanoparticles achieved deep penetration into the epithelial tissue and were better tolerated than sirna-lipoplexes. In the second investigation, the authors evaluated distribution of various types of fluorescent nanoparticles in the reproductive tract of female mice after single intravaginal administration [94]. It was observed that surface characteristics of the PLGA nanoparticles significantly governed their intravaginal distribution and retention. PLGA nanoparticles without any surface modification and PLGA nanoparticles bearing avidin on their surface showed significantly lower retention in the reproductive tract as compared to PLGA nanoparticles bearing PEG chains on their surface [94]. This study clearly indicated need for surface PEGylation of PLGA nanoparticles to achieve optimal intravaginal delivery. Surface PEGylated PLGA nanoparticles were recovered from cervicovaginal lavage even after 24 h. However, the study was not carried beyond 1 day to study further fate of the PLGA nanoparticles. The same group has developed PLGA nanoparticles containing siRNA that can target genes (UL29.2 or nectin-1) relevant to HSV-2 infection [95]. The authors evaluated *in vitro* and *in vivo* efficacy of siRNA containing PLGA nanoparticles and siRNA-lipoplexes. PLGA nanoparticles containing siRNA showed significantly higher gene silencing (*in vitro*) as compared to siRNA-lipoplexes. The PLGA nanoparticles and lipoplexes containing siRNA were intravaginally administered to mice before and after lethal HSV-2 challenge and disease progression and mortality in mice was observed for 28 days. Interestingly, PLGA nanoparticles treated mice showed higher survival as compared to lipoplexes [95]. Moreover, histopathological studies indicated that siRNA-lipoplexes treatment caused a greater accumulation of polymorphonuclear neutrophils in the vaginal mucosa whereas no gross inflammation and epithelial damage was observed after delivery of PLGA-siRNA nanoparticles. The *in vivo* silencing of target genes by PLGA-siRNA nanoparticles was confirmed 7 days after the treatment. The highlight of the study is that for the first time, siRNA carrying nanosystems were able to increase the survival of HSV infected mice for 28 days [95]. This investigation clearly demonstrates the ability of PLGA nanoparticles to become a carrier for variety of microbicides.

Hanes et al. have recently evaluated potential of acyclovir containing PLGA nanoparticles to prevent HSV infection [96]. The authors engineered acyclovir loaded conventional PLGA nanoparticles as well as PLGA nanoparticles with ability to rapidly penetrate vaginal mucus. The authors demonstrated that simple coating of PLGA nanoparticles with Pluronic F127 (a US FDA approved polymeric emulsifier) imparts rapid mucus penetrating ability to PLGA nanoparticles. Interestingly, a significant amount (more than 60%) of mucus penetrating PLGA nanoparticles was retained in the reproductive tract of female mice as compared to the conventional PLGA nanoparticles. Moreover, conventional PLGA nanoparticles caused acute inflammatory events like Nonxynol 9 after administration whereas mucus penetrating nanoparticles did not show any such events. Intravaginal administration of acyclovir containing mucus penetrating nanoparticles was found to protect significantly higher number of mice (53%) as compared to acyclovir solution (16%). Furthermore, administration of acyclovir solution containing 10 times higher concentration of acyclovir than acyclovir nanoparticles offered only 30% protection [96]. This clearly establishes utility of mucus penetrating nanoparticles in vaginal delivery.

Youan et al. focused on developing polymeric nanoparticles for delivering tenofovir. In view of the lack of success with VOICE trial, developing smart carrier for delivery of tenofovir is an urgent need.

Youan et al. developed pH-sensitive PLGA nanoparticles as well as mucoadhesive chitosan nanoparticles for vaginal delivery of tenofovir [97,98]. Tenofovir loaded pH-sensitive PLGA nanoparticles were developed by blending different ratios of PLGA with Eudragit S100, a pH-sensitive polymer that dissolves at pH 7.4. The developed nanoparticles were well tolerated by vaginal epithelial cells and *Lactobacillus* sp. Interestingly, due to presence of Eudragit S100, the NPs released minimal amount of drug in simulated vaginal fluids (pH 4.5) and significantly higher release of drug was observed in the simulated seminal fluid (pH 7.6). The form of tenofovir (tenofovir base or tenofovir disoproxilfumarate) affected the rate of drug release. Although these nanoparticles released higher amount of drug at pH 7.6, the drug release was sustained for 24 h due to presence of PLGA. The S100-PLGA nanoparticles demonstrated 50% uptake in the vaginal epithelial cells over a period of 24 h [97]. Tenofovir loaded chitosan nanoparticles were also evaluated for drug release, cytotoxicity and mucoadhesivity. Size of the chitosan nanoparticles was found to have considerable impact on the encapsulation efficiency and release rate of tenofovir. Chitosan nanoparticles with larger size yielded greater encapsulation efficiency and slower release rate. The chitosan nanoparticles were tolerated by vaginal epithelial cells and *Lactobacillus* sp. The mucoadhesive nature of chitosan nanoparticles was confirmed using porcine vaginal mucosa [98]. Recent evidence suggests that nanoparticles should preferably have mucus penetrating ability to reach to vaginal epithelial cells and deliver the drug. Although chitosan is a mucoadhesive polymer, it also imparts positive surface charge to nanoparticles due to free primary amine groups. It has been demonstrated that positively charged nanoparticles with certain features can penetrate the mucus. Thus, whether chitosan nanoparticles have mucoadhesive and/or mucus penetrating ability still remains to be clearly elucidated.

Beletti et al. fabricated tenofovir loaded hybrid nanoparticles containing chitosan and PLGA [99]. The nanoparticles were prepared by multiple emulsion method and chitosan was added in the inner phase of the primary emulsion. The presence of chitosan helped in increasing the encapsulation efficiency of tenofovir in the nanoparticles as compared nanoparticles prepared without chitosan. Although the nanoparticles showed a tendency to sustain release of tenofovir in the pH 7.4 media, the release was not studied for more than 9 h [99]. Although smart delivery strategy for tenofovir is warranted, the extreme hydrophilicity of the tenofovir poses a major problem for its nanoencapsulation. None of the reported investigations on tenofovir polymeric nanoparticles have been able to achieve encapsulation efficiency greater than 40%. This is a major challenge that still needs to be overcome.

dasNeves et al. developed poly-caprolactone (PCL) nanoparticles containing dapivirine, a hydrophobic NNRTI under development as a vaginal microbicide [100]. PCL nanoparticles with anionic, cationic and neutral surface charge were developed. It was observed that cationic nanoparticles had greater cell uptake but they were also more cytotoxic. Poloxamer 338 containing neutral PCL nanoparticles were found to be well tolerated by cells. PCL nanoparticles significantly improved intracellular delivery of dapivirine as compared to dapivirine solution in a variety of cells. However, no appreciable difference in IC_{50} values has been reported. Moreover, no attempts were made to test the intracellular concentrations after 8 h [100]. Yoo et al. fabricated nanoparticles of Eudragit S100 due to its pH-sensitive nature [106]. The Eudragit S100 nanoparticles were non-toxic to vaginal epithelial cells even at higher concentrations (1 mg/mL). Due to their pH-sensitive behavior, nanoparticles showed very less drug release at vaginal pH whereas immediate release was observed at physiological pH. The nanoparticles were abundantly taken up by the vaginal epithelial cells [101]. Thus, S100 nanoparticles could be pursued further as carriers for antiretroviral drugs.

Use of antiretroviral drug combination is gaining popularity in the field of HIV prophylaxis mainly to maximize success of prophylaxis and to obviate possibilities of resistance. We have earlier demonstrated that it is possible to fabricate PLGA nanoparticles containing a combination of three antiretroviral drugs, viz. lopinavir, ritonavir and efavirenz [102,103]. Moreover, these combination nanoparticles demonstrated sustained release of antiretroviral drugs *in vitro* as well as *in vivo*. However for prophylaxis, it is important to develop formulations of drugs that act before integration of HIV to host DNA. Thus, we focused our investigation on developing PLGA nanoparticles containing different antiretroviral modalities. Until today, there are no reports on use of HIV integrase inhibitors for vaginal prophylaxis of HIV infection. Raltegravir (RAL) is the only HIV-1 integrase inhibitor approved by US FDA. There were two recent reports demonstrating potential of RAL in oral pre-exposure prophylaxis [104,105]. Moreover, Koh et al. demonstrated that RAL pre-treated HeLa-T4 cell and primary human cells can resist HIV-1 infection even after washout of RAL from culture medium [106]. Thus, RAL was thought to have potential for vaginal pre-exposure prophylaxis of HIV infection. We aimed at developing nanoparticles containing a combination of RAL, an integrase inhibitor and efavirenz, a NNRTI. Although there are concerns about the use of efavirenz as a microbicide due to resistance issues and teratogenicity [107,108], we used it as a model NNRTI to establish a proof-of-concept. We fabricated PLGA nanoparticles containing a combination of raltegravir and efavirenz (RAL-EFV-NPs) using simple and scalable emulsion-solvent evaporation technique [109]. Furthermore, nanoparticles were fabricated using Pluronic F127 as a stabilizer, which is already known to impart mucus-penetrating properties to nanoparticles [96]. It was observed that RAL and EFV exhibited different encapsulation efficiency due to differences in their physicochemical properties. The RAL-EFV-NPs demonstrated significantly less cytotoxicity as compared to RAL + EFV solution at the same concentration. Thus, PLGA nanoparticles can improve selectivity index of antiretroviral drugs. RAL-EFV-NPs (at RAL and EFV concentration of 5 μg/mL) did not show any signs of toxicity to HeLa cells over a period of 14 days. The ability of RAL-EFV-NPs in the prophylaxis was evaluated *in vitro* using TZM-bl HIV indicator cells. The cells were pre-treated with RAL-EFV-NPs or RAL-EFV solution for 1 day and cells were infected with HIV after complete removal of treatments. Interestingly, RAL-EFV-NPs demonstrated lower EC_{90} value as compared to RAL + EFV solution [109]. This clearly indicated the ability of PLGA nanoparticles to augment delivery of drugs to the cells. The intracellular concentrations of RAL and EFV in RAL-EFV-NPs treated HeLa cells were observed over a period of 14 days. RAL intracellular concentration was maintained for a period of 6 days whereas EFV was detected in the cells (concentration 100 times greater than reported IC_{50} of EFV) even at the end of 14 days [22]. The intracellular concentrations of the drugs seemed to be dependent on the metabolic enzymes expressed in the HeLa cells. In summary, PLGA nanoparticles can offer sustained intracellular delivery of encapsulated drugs and can be useful for long-term prophylaxis.

It is also important to develop a suitable vehicle for the delivery of PLGA nanoparticles. Unfortunately, none of the literature reports describe development of suitable vaginal delivery vehicle for the polymeric nanoparticles. The gelling agent can considerably affect the size and colloidal stability of the nanocarriers as observed by Patravale et al. [110]. Instead of developing a conventional gel, we aimed at developing a thermosensitive gel containing RAL-EFV-NPs. Thermosensitive gels are liquid at room temperature and form a highly viscous gel at 37 °C once delivered inside the body [111]. Thermosensitive gels are easy to handle and deliver as

compared to conventional gels due to their liquid nature, can spread evenly on gelation whereas their high viscosity at body temperature can minimize chances of gel leakage [112]. We successfully developed thermosensitive gel based on combination of Pluronic F127 and Pluronic F68. Incorporation of nanoparticles in the gel did not affect their size or other physicochemical characteristics. We carried out transwell experiments on the thermosensitive gel containing Rhodamine 6G labeled fluorescent PLGA nanoparticles to check whether incorporation of PLGA nanoparticles in the thermosensitive gel has any effect on their release or uptake profile. Interestingly, fluorescent PLGA nanoparticles traversed through transwell membrane and were taken up by the HeLa cells within 30 min [109]. This clearly indicated that thermosensitive gel enabled quick release of the PLGA nanoparticles from the matrix. Thermosensitive gel containing fluorescent PLGA nanoparticles were administered to mice by intravaginal route and various tissues were excised after 24 h to observe presence of fluorescence in the tissues. Interestingly, tissues such as vaginal epithelium showed considerable fluorescence even after 24 h (unpublished data). Thus, RAL-EFV-NPs carrying thermosensitive gel showed promise for further development.

5.2.2. Polymeric nanoparticles for delivery of vaccines for HIV prophylaxis

In view of the failures observed with most of the HIV vaccine clinical trials, development of smart vaccine delivery system is highly warranted. Polymeric nanoparticles (especially biodegradable nanoparticles) have shown a great potential to become the next generation vaccine adjuvants. Moreover, biodegradable polymeric nanoparticles offer a safer alternative to conventional adjuvants such as alum salts. Polymeric nanoparticles can (1) protect encapsulated antigen from proteolytic degradation; (2) yield sustained and enhanced cross-presentation of antigen to immune cells; (3) undergo endolysosomal escape after uptake by immune cells; and (4) be tailored by various methods to elicit a desired immune response [113–115]. There are several reviews in the literature discussing advantages of nano-scale carriers as vaccine adjuvants for a variety of vaccines [113–115]. Readers are requested to refer to these excellent reviews for further information. Although various polymers are available for nanoparticle fabrication, polystyrene, PLA, poly-glutamic acid (PGA) and polymethylmethacrylate (PMMA) have been mainly used for fabrication of the nanoparticles. In most of the studies, electrostatic interactions between anionic nanoparticles and cationic HIV vaccines have been utilized for enabling delivery of vaccines to target cells/organs. Various routes of administration (oral, nasal, dermal and vaginal) have been employed for achieving mucosal immunization.

Ataman and colleagues fabricated surfactant-free anionic PLA nanoparticles by a simple diafiltration technique [116]. HIV p24 protein was electrostatically adsorbed onto nanoparticles and the process did not result in loss of its antigenicity and immunogenicity. The antigen loaded nanoparticles were subcutaneously injected to mice, rabbits and macaques. Interestingly, p24-PLA nanoparticles elicited significantly higher antibody titers including strong cytotoxic T-lymphocyte (CTL) responses in mice as compared to soluble antigen or alum/Freund adjuvanted antigen. The similar observations were noted in rabbits and macaques. Moreover, the PLA nanoparticles induced high levels of IFN-γ-producing T cell responses. These T cell responses were similar to that observed with viral vectors such as Modified Vaccinia virus Ankara against the p24 antigen in macaques [116]. In another study, the authors explored feasibility of developing divalent HIV vaccines based on PLA nanoparticles. Interestingly, authors succeeded in co-adsorbing p24 antigen and gp120 envelope glycoprotein onto PLA nanoparticles [117]. Both the antigens were found to have similar affinity for PLA nanoparticles and their structural and functional integrity was preserved even after adsorption onto PLA nanoparticles. Furthermore, subcutaneous administration of this divalent nanovaccine yielded strong antibody responses against both the antigens [117]. This clearly indicates the feasibility of designing smart vaccine delivery systems with the use of polymeric nanoparticles. The immunogenic ability of antigen carrying PLA nanoparticles has also been compared to MF59, a nanoemulsion based adjuvant. Various HIV antigens (p24*gag*, wild-type Tat and a mutated and detoxified version of Tat) were delivered to rabbits with either PLA nanoparticles or MF59 and immune responses were monitored [118]. It was observed that the nature of adjuvant as well as type of antigen influenced the type or extent of immune responses. In case of the p24 antigen, the immune response induced by PLA nanoparticles was focused on immunodominant domain as compared to MF59. No such differences in immune responses were observed in the case of wild-type Tat. For mutated detoxified Tat, PLA nanoparticles augmented the number of epitopes recognized by serum IgG as compared to MF59 [118]. Thus, efficacy of vaccine adjuvants would have to be evaluated on a case-by-case basis and no generalization can be made about superiority of particular type of adjuvant.

Aline et al. investigated the ability of p24-PLA nanoparticles to boost adjuvant capacity of dendritic cells. Dendritic cells were incubated (pulsed) with p24 antigen, blank PLA nanoparticles and p24 antigen adsorbed PLA nanoparticles [119]. Only p24-PLA nanoparticles were capable of inducing maturation of dendritic cells leading to enhance expression of cell surface markers such as MHC classes I and II, CD40, CD80 and CD86 [119]. Moreover, p24-PLA nanoparticles treated dendritic cells were capable of releasing cytokines such as IL-4 and IL-7. Dendritic cells treated with p24-PLA nanoparticles were capable of inducing high antibody titers in the blood and intestine (mucosal immunity) of mice. Dendritic cells treated with p24 antigen or blank PLA nanoparticles alone were incapable of inducing mucosal immune response. Thus, PLA nanoparticles can also be used to boost capacity of adjuvants [119]. Liard et al. evaluated systemic and mucosal immune responses after administration of p24-PLA nanoparticles in mice at different sites of the skin viz. subcutaneous, intradermal and transcutaneous route [120]. Subcutaneous delivery of p24-PLA nanoparticles was associated with generation of HIV-1 p24-specific IgG in the absence of antigen-specific CD8 T cells whereas intradermal administration yielded cellular and humoral responses. Administration of p24-PLA nanoparticles by transcutaneous route (hair follicle mediated transport of nanoparticles) induced major CD8 effector cells in the absence of IgG. Moreover, transcutaneous delivery also yielded generation of IgA in the stratified epithelium of the vagina (mucosal immunity). Thus, site of administration in skin seemed to be of significant importance for nano-particulate vaccine delivery.

Poly-amino acids such as Poly-γ-glutamic acid (PGA) have been actively pursued as an adjuvant for HIV vaccines. PGA is a highly anionic biodegradable polymer. HIV antigens can be adsorbed onto PGA by electrostatic interaction. Wang et al. evaluated immune responses elicited after intranasal administration of HIV gp120, HIV gp120 + cholera toxin B subunit and HIV gp120 adsorbed onto PGA nanoparticles. Interestingly, only HIV gp120 adsorbed onto PGA nanoparticles induced significant antigen-specific lymphocyte proliferation [121]. The PGA nanoparticles also induced long-lived memory $CD8^+$ T cells. Moreover, after single intranasal immunization with PGA nanoparticles, the central memory T cells remained relatively constant from day 30 to day 238 although decay in the effector memory T cells was observed. In another study, PGA nanoparticles were evaluated for *in vitro* and *in vivo* immunostimulation and for ability to deliver ovalbumin to dendritic cells (DC) [122]. PGA nanoparticles yielded significant increase in uptake of ovalbumin by dendritic cells and also offered

sustain release of ovalbumin in the cells. Moreover, PGA nanoparticles were capable of inducing the maturation of DCs which is indicative of their adjuvant potential. PGA nanoparticles containing HIV p24 were subcutaneously administered to mice. PGA nanoparticles induced antigen-specific IFN-γ-producing T cells in spleen cells and p24-specific serum antibodies. The levels of p24-specific serum antibodies induced by PGA nanoparticles were comparable to complete Freund's adjuvant. However, PGA nanoparticles predominantly activated p24-specific IFN-γ-producing T cells which were not seen in case of complete Freund's adjuvant.

Uto et al. compared adjuvant effect of PGA nanoparticles with alum salt [123]. Ovalbumin containing PGA nanoparticles were more efficiently taken up by dendritic cells as compared to alum adjuvanted ovalbumin. Mice immunized with PGA nanoparticles induced significantly higher antigen-specific $CD8^+$ T cells as compared to alum and monophosphoryl lipid A (MPLA) adjuvanted ovalbumin [123]. This indicates potential of PGA nanoparticles in vaccine delivery. Himeno et al., compared immune responses after administration of HIV gp120 and PGA nanoparticles containing HIV gp120 to rhesus macaques [124]. PGA nanoparticles elicited stronger gp120-specific cellular and humoral immune responses than gp120 alone. However, PGA nanoparticles could not offer protection against challenge by simian/human immunodeficiency chimeric virus (SHIV) in the macaques. Further studies would be required to clarify these results.

Surface modified polystyrene nanoparticles (PS-NP) have also been evaluated as vaccine adjuvants. Baba et al. designed polymethacrylic acid decorated polystyrene nanoparticles. The nanoparticles were coated with concanavalin A (a lectin with high affinity for HIV gp120). These surface modified nanoparticles were capable of capturing infectious HIV-1 (irrespective of cell tropism) as well as heat inactivated HIV-1 [125,126]. In the first study, the authors compared immunogenic potential of heat inactivated HIV-1, concanavalin A decorated PS-NP and heat inactivated HIV-1 capturing concanavalin A PS-NP (HIV-PS-NP) after intravaginal administration to mice [126]. Vaginal fluids of immunized mice were evaluated for presence of anti-HIV-1 IgG and IgA. No detectable levels of anti-HIV-1 IgG were observed in all treatment groups. However, HIV-PS-NP showed significantly higher anti-HIV-1 IgA levels as compared to other groups. Moreover, vaginal fluids obtained from HIV-PS-NP immunized mice were able to neutralize immunizing HIV-1 strain. HIV-PS-NP showed different intravaginal distribution as compared to other treatments [126]. In another study, the authors compared the effect of route of administration on the immunogenicity of HIV-PS-NP. HIV-PS-NPs were administered to mice by oral, nasal, vaginal and intraperitoneal route and HIV-1 specific IgA levels in the vaginal tissues were evaluated [127]. It was observed that intranasal administration of HIV-PS-NP yielded highest levels HIV-1 specific IgA in vagina and vaginal fluids of these mice were able to neutralize $HIV\text{-}1_{IIIB}$. Furthermore, intranasal immunization of mice with HIV-PS-NP also showed presence of HIV-1 specific cytotoxic T-cells in the spleen [128]. Finally, immunization potential of SHIV capturing PS-NP was studied in macaques. Intranasal administration of SHIV-PS-NP resulted in detectable levels of vaginal anti-HIV-1 gp120 IgA and IgG antibodies in all the macaques [129]. Although these studies are promising, PS-NPs are non-biodegradable in nature. Moreover, PS-NPs were found to be less effective in inducing antigen-specific $CD8^+$T-cell responses as compared to biodegradable PGA nanoparticles [121].

Delair et al. developed nanoparticles based on ionic interaction of two natural polymers viz. chitosan and dextran sulfate. The authors developed positively as well as negatively charged polysaccharide nanoparticles by altering the ratio of chitosan to dextran sulfate and studied adsorption of HIV-1 p24 antigen on these nanocarriers. Interestingly, negatively charged nanoparticles yielded higher binding and stability of the adsorbed p24 [130]. The nanocarriers were efficiently internalized by dendritic cells. Subcutaneous administration of polysaccharide nanoparticles yielded significant p24-specific cellular and humoral immune response in mice [131].

Researchers have evaluated polymethylmethacrylate (PMMA) based nanocarriers for delivering HIV antigens or HIV DNA vaccines. PEGylatedpolymethylmethacrylates have been evaluated for delivery of HIV *gag* and *tat*DNA vaccines [132,133]. Intramuscular immunization with PMMA nanoparticles carrying HIV *tat* gene DNA vaccine yielded significantly higher Th-1 type T-cell response and HIV-1 Tat-specific cytotoxic T cell response [132]. Intranasal administration of PMMA nanoparticles carrying HIV *gag* DNA vaccine resulted in significantly higher number of Gag-specific IFN-γ secreting cells as well as Gag-specific IgG as compared naked DNA vaccine [133]. Nanoparticles with cationic PMMA core and anionic Eudragit L 100-55 corona have been evaluated as vaccine adjuvants [134,135]. The nanoparticles were able to adsorb significant amount of HIV-1 Tat (antigen) and also increased stability of Tat. HIV-1 Tat adsorbed nanoparticles were administered by intramuscular, subcutaneous or intranasal route and immune response was monitored. Nanoparticles were able to induce potent and long-lasting immune response (Tat-specific cytotoxic T lymphocytes as well as IgG) and were well tolerated by animals.

Zhu et al. evaluated immunization potential of Eudragitmicroparticles encapsulating HIV vaccine carrying PLGA nanoparticles on oral delivery [136]. The authors developed PLGA nanoparticles encapsulating PCLUS3−18IIIB (a CD4+ T cell helper epitope fused with an HIV Env CD8+ cytotoxic T lymphocyte epitope) and TLR ligands (MALP2 + poly(I:C) + CpG) and these nanoparticles were encapsulated in Eudragit L 100-55 or Eudragit FS-30D microspeheres. Eudragit L 100-55 and Eudragit FS-30D microparticles were orally administered to mice and immune responses were compared with intracolorectal administration of HIV antigens. Interestingly, Eudragit FS-30D microparticles induced significant colorectal immunity after oral administration and protected animals against rectal and vaginal viral challenge. Thus, it is possible to develop smart carriers that can induce mucosal immunity and can protect peptide antigens from harsh environment of gastrointestinal tract. In short, polymeric nanoparticles have a great potential to be successful in delivery of HIV vaccines.

5.3. Liposomes

Liposomes were the first to be developed as well as commercialized among all the nanocarriers and have a longest history of research and development. Liposomes are vesicular carriers composed of phosholipid bilayers and aqueous core. The size of the liposomes can range between 80 nm to 10 μm depending upon preparation method and composition. There are numerous reviews that provide detailed description of liposome components, fabrication methods, types of liposome, biophysical properties, characterization and their applications [137–141]. The tendency of liposomes to get rapidly recognized by phagocytic cells of liver and spleen after intravenous administration (passive targeting) and at least partly localize in lysosomes has been utilized for augmenting prophylaxis and/or therapy of various infectious diseases [138,142]. Moreover, the surface of the liposomes can be easily engineered with various moieties to enhance their recognition as well as uptake by macrophages or other components of immune system. Liposomes have mainly been explored for delivering HIV vaccines although few studies have focused on delivering antiretroviral agents or siRNA.

5.3.1. Liposomes for delivery of HIV vaccines and siRNA

The first attempt to deliver HIV antigens using liposomes was reported almost two decades ago [143]. Since then, influence of several aspects such as liposome components, fabrication methods, HIV antigen, route of administration and type of adjuvant has been evaluated to develop liposomeal HIV vaccines and the research is still in progress. Philips et al., evaluated the influence of liposome components on the immune responses to HIV gp120 after subcutaneous administration [144]. Liposomes were fabricated with various phospholipids such as dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidylethanolamine (DPPE) and phosphatidylserine (PS) and their effect on immunization was monitored. It was observed that liposomes composed of DPPC/DMPG were able to elicit the highest levels of HIV gp120-specific IgG as compared to the other liposomes and the IgG levels were significantly higher than HIV gp120 adjuvanted with alum. It was also observed that liposomes composed of DPPC/PS yielded IgG levels considerably lower than HIV gp120 adjuvanted alum [144]. This indicated the influence of liposome composition on the immune response. The potential of cationic liposomes for delivery of HIV-1 DNA vaccine was evaluated by Okuda et al. [145]. Intranasal administration of HIV DNA vaccine (encoding *env* gp160 and *rev* genes) via cationic liposomes resulted in significantly higher levels of mucosal IgA in feces and vaginal fluids and antibodies against HIV-1 were detected for at least 10 months. Moreover, co-administration of HIV DNA vaccine with plasmid encoding for interleukin-12 (IL-12) and granulocyte/macrophage-CSF through cationic liposomes induced high levels of HIV-1 specific cytotoxic T-lymphocytes (CTL) [145].

It is well known that antigen presenting cells such as dendritic cells and macrophages express receptors that can bind to mannose and mannose containing polysaccharides. Hence, liposomes coated with mannose containing moieties (mannan) were evaluated for targeted delivery of HIV vaccines. Toda et al., evaluated immunization potential of DNA vaccine (encoding HIV-IIIB *env* and *rev* genes) loaded onto cationic liposomes with or without mannan coating [146]. The immune responses obtained after intramuscular and intranasal administration of different liposomal vaccines were compared. Interestingly, mannan coated liposomes showed significantly higher serum IgG, fecal IgA and IFN-γ levels in mice as compared to liposomes without mannan coating. This clearly indicated the importance of targeting of liposomal vaccines [146]. Mannosylated glycolipids have also been used for coating liposomal HIV vaccines. Liposomes containing either peptide sequence from HIV gp120 or SIV GST-Nef protein were functionalized with mannosylated glycolipids and their immunization potential was evaluated [147,148]. Once again, targeted liposomes were associated with better immune response as compared to other controls.

Usually, exogenous antigen is presented to MHC Class II molecules through endosomes which leads to $CD4^+$ T-cell response. However, for efficacy of HIV vaccines, strong CTL response is required. Hence, utility of the pH-sensitive liposomes was explored for delivering HIV vaccines. It is known that liposomes containing pH-sensitive lipids like dioleoylphosphatidylethanolamine (DOPE) can fuse with endosomal membrane and release the antigens into the cytosol [149]. The endosomal escape of antigen facilitates its presentation to MHC Class-I that can lead to CTL response [150]. Superiority of pH-sensitive liposomes (containing ovalbumin as a model antigen) over pH-insensitive liposomes has been established by *in vitro* and *in vivo* studies [150,151]. Chang et al. prepared various pH-sensitive liposomes by using either DOPE or 1-Palmitoyl-2-Oleoyl-Phosphatidylethanolamine (POPE) as a pH-sensitive lipid and incorporated peptide sequences from V3 loop of HIV-1 gp120 into these liposomes [152]. It was observed that POPE containing liposomes elicited significantly higher immune response as compared to DOPE containing liposomes indicating importance of liposome composition.

Liposomes containing microbial adjuvants (Cholera toxin B subunit) or inactivated virus (hemagglutinating virus of Japan; HVJ) have been explored for potentiating mucosal immune response to HIV antigens [150–155]. Cholera toxin B subunit exerts adjuvant effect by increasing localization of antigen to the galglioside GM1 present on surface of the antigen presenting cells [153]. Lian et al. formulated liposomes containing GM1 and incorporated CTB as well as recombinant HIV envelope protein into the liposomes [153]. It was hypothesized that CTB bound to surface of GM1 containing liposomes would also be able to bind to GM1 present on the mucosal cells; thereby increasing presentation of HIV antigen to mucosal cells. Intranasal administration of CTB decorated GM1 liposomes was associated with significantly higher HIV envelope protein-specific mucosal IgA and IgG as compared to liposomes devoid of GM1 or antigen adjuvanted with alum [153]. Similarly, HIV gp160 loaded liposomes decorated with inactivated HVJ and HIV gp41 peptide loaded liposomes containing adjuvant MA729 (analogue of muramyl dipeptide) elicited significantly higher antigen-specific antibody titers as compared to antigen adjuvanted with alum [154,155].

Dendritic cells express several microbial pattern recognition receptors such as Toll-like receptors (TLR). Hence, adjuvants that can bind to TLR were incorporated in liposomes for increasing immunogenicity [156]. Rao et al. used this approach for vaccine delivery. Liposomes containing monophosphoryl lipid A (MPLA; binds to TLR4) and oligodeoxynucleotides containing cytosine phosphate guanosine (CpG ODN; bind to TLR9) were fabricated. The ability of these liposomes to elicit immune response to HIV-1_{IIIB} gp140 was evaluated [156]. It was observed that MPLA and CpG ODN containing liposomes yielded highest level of immunization as compared to the treatment groups that contained individual adjuvants. The study also concluded that incorporation of MPLA in the liposomes is important for maintaining high level of immunization [156]. Singh and Bisen reported the importance of surface characteristics of liposomes on the immune response to HIV antigen [157]. The authors fabricated pH-sensitive liposomes with or without surface PEGylation and conjugated HIV gp41 epitopes to the liposomes. Interestingly, surface PEGylation of liposomes was found to have considerable influence on the immunization. Surface PEGylated pH-sensitive liposomes elicited significantly higher anti-gp41 antibodies as compared to the pH-sensitive liposomes without surface PEGylation [157].

Watson et al. synthesized various lipid derivatives of peptides from membrane proximal region (MPR) of HIV gp41 and incorporated them into MPLA containing liposomes [158]. The effect of type of lipid anchor on the immunogenicity of the antigen was evaluated by immunizing animals with the different liposomes. Liposomes containing lipid conjugated antigen elicited significantly higher antibody titer as compared to non-conjugated antigen. The type of the lipid anchor had significant effect on the secondary structure of the antigen as well as extent of immune response. Antigen conjugated to a pH-sensitive lipid cholesterylhemisuccinate (CHEMS) was found to yield highest immune response [158]. The authors also studied effect of type of antigen association to liposomes on the immune response [159]. For this purpose, two strategies were employed. In the first strategy, ability of chelated divalent metal ions such as nickel to form coordinate bonds with short histidine residues was utilized. Liposomes containing nickel chelating lipids (Ni-Lipo) were developed and HIV antigen (MPR peptides) with short sequence of histidine residues (his-tag) was employed to augment interaction of HIV antigen with liposomes. In the second strategy, liposomes containing CHEMS conjugated antigen were fabricated. It was observed that Ni-Lipo elicited significantly higher immune

response in comparison to liposomes devoid of nickel chelating lipids [159]. This indicated effect of type of antigen association to liposomes on immune response.

Fairman et al. designed complex of cationic liposomes and DNA containing CpG motifs (CLDC). Two SIV antigens viz. simian immunodeficiency virus (SIV) gag protein and SIVmac239 were delivered using the CLDC and immune responses were evaluated in macaques [160]. Intramuscular administration of CLDC yielded stronger SIV-specific T-cell and B-cell responses as compared to antigens delivered without CLDC. Moreover, CLDC treated macaques showed better memory responses several months later following boosting by SIVmac239 [160]. Recently, application of liposomes in delivering self-amplifying RNA vaccines has been reported [161]. The liposomes employed for this purpose had important features such as presence of cationic lipid (for condensing RNA vaccine), ionizable lipid (for pH triggered release) and PEGylated lipids [161]. As described earlier, all these features were shown to improve immune responses. The authors used 9-kb self-amplifying RNA derived from an alphavirus for designing vaccines against various viruses including HIV and the vaccines were incorporated into liposomes with a very high encapsulation efficiency of 85%. Self-amplifying RNA encoding HIV envelope protein gene (Env, SF162 gp140) was delivered using liposomes by intramuscular, intradermal and subcutaneous routes. Interestingly, liposomal vaccine delivered through intramuscular route showed significantly higher antigen-specific immune response as compared to the other routes. The authors also established utility of liposomal vaccines against various other viruses [161].

Liposomes have also been used for systemic or intravaginal delivery of siRNA targeting HIV-1 or HSV-2 [162–164]. Intravaginally applied liposomes carrying siRNA targeting HSV-2 UL27 gene (encode an envelope glycoprotein) and UL29 gene (encode DNA binding protein) were efficiently taken up by epithelial and lamina propria cells and also showed sustained gene silencing in vagina and ectocervix of mice for at least nine days [162]. The liposomal siRNA formulation did not cause induction of interferon-responsive genes or inflammation in the reproductive tract. Moreover, the liposomal siRNA protected mice from lethal HSV-2 challenge [26]. Wu et al. developed alginate based scaffolds for delivery of muco-inert PEGylated cationic liposomes containing fluorescently labeled siRNA [163]. It was observed that liposomal siRNA could reach to vaginal epithelium and silence gene expression [163]. Kim et al. developed neutral liposomes decorated with hyaluronic acid on the surface. Monoclonal antibodies targeting human integrin LFA-1 were conjugated to the liposomes through hyaluronic acid [164]. An siRNA with ability to silence leukocyte-specific HIV co-receptor CCR5 expression was incorporated into liposomes. Due to presence of anti-integrin antibody, systemically administered liposomal siRNA was selectively taken up by integrin receptors present on T-cells and macrophages and showed *in vivo* gene silencing for as long as 10 days. Interestingly, humanized BLT mice pre-treated with liposomal anti-CCR5 siRNA showed enhanced resistance to infection after HIV challenge [164]. Thus, liposomal siRNA formulations have a great potential in HIV prophylaxis.

5.3.2. Liposomes for delivery of antiretroviral agents

Phospholipids such as cardiolipin have shown ability to inhibit HIV-1 *in vitro*. However, cardiolipid has relatively low selectivity index [165]. Malavia et al. formulated various liposomes containing cardiolipin and synthetic phospholipids by simple ethanol injection method. The composition of liposomes exhibited considerable effect on the anti-HIV activity as well as selectivity index of cardiolipin [170]. Fluorescently labeled cardiolipin liposomes were found to be retained in the vaginal cavity of mice for approximately 24 h after intravaginal administration and liposomal formulation did not cause any adverse effects [165]. MC-1220, a hydrophobic non-nucleoside reverse trascriptase inhibitor was encapsulated in liposomes and liposomes were encapsulated in Carbopol® gel [171]. Pre-treatment of macaques with liposomal gel showed 50–60% protection after SHIV challenge whereas control group showed 100% infection. The RNA viral load at necropsy was significantly lower in the macaques treated with liposomal gels as compared to control group [166]. In summary, liposomes can be used for augmenting efficacy of prophylactic modalities of HIV.

5.4. Lipid nanocarriers

Lipid nanocarriers such as solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) are extensively being explored for a variety of applications for the last two decades. SLN are composed of low cost and biodegradable solid lipid(s) which are nanosized with the help of a suitable stabilizer and emulsification technique [142,167,168]. Lipids that are solid at body temperature (fatty acids and their mono-, di- or tri-glycerides, monoglycerides, hard fat or even waxes) are employed for fabrication of SLN. NLC are next generation lipid nanocarriers that contain a mixture of solid lipids and liquid lipids (oil) in appropriate proportions as a matrix. Various liquid lipids such as medium chain tri-glycerides, mono- and di-glycerides of short chain fatty acids and propylene glycol esters of short chain fatty acids are employed for fabrication of NLC [142,167,168]. The presence of liquid lipid in the NLC confers long-term colloidal stability and greater drug encapsulation and loading unlike SLN [142,167,168]. Although several fabrication techniques have been developed for fabrication of SLN/NLC; melt-emulsification followed by high-pressure homogenization is most commonly used. SLN/NLC have already been used in few commercial topical preparations [142]. Despite these advantages, SLN/NLC have not been explored to their full potential for HIV treatment and prophylaxis. Most of the investigations reported hitherto, focus on development of SLN of protease inhibitors for improving their permeability and *in vitro* transport through blood-brain barrier and these investigations are out of scope of this review. Alukda et al. have reported development of SLN of tenofovir for vaginal prophylaxis [169]. The SLN were fabricated by a phase inversion method and surface of the SLN was functionalized with multiple layers of poly-L-lysine and heparin. Poly-L-lysine coat was introduced to augment cellular uptake of SLN whereas functionalization with heparin was carried out for targeting of SLN to natural cytotoxic receptors of natural killer cells. However, the authors did not establish any proof for this hypothesis. The functionalized SLN were well tolerated by vaginal epithelial cells even at a high concentration (900 μg/mL) [169].

In past few years, researchers have evaluated potential of SLN as an adjuvant for HIV vaccines. Mumper et al. have carried out several investigations to establish potential of lipid nanocarriers as HIV vaccine carriers [170–174]. These investigators have developed a 'microemulsion template' method to fabricate emulsifying wax based anionic SLN. The anionic SLN were coated with HIV-1 Tat (1–72) protein [175]. The HIV-1 Tat (1–72) coated SLN were injected twice to mice at an interval of 2 weeks by subcutaneous route and immune responses were compared to Tat adjuvanted with alum or Lipid A. On day 28, Tat-specific IgG and IgM levels observed with SLN were comparable to that of Tat adjuvanted with alum. However, splenocytes isolated from Tat coated SLN immunized mice showed 3-fold higher release of IFN-γ as compared to mice immunized with Tat adjuvanted with alum [170]. This indicates potential of Tat coated SLN in eliciting cellular and humoral

responses for HIV vaccine. Studies were also carried out to evaluate immune responses of Tat coated SLN and Tat adjuvanted with alum at various doses of Tat [171]. Tat coated SLN carrying lower amount of Tat (1 μg) showed similar Tat-specific total IgG titers as compared to Tat coated SLN carrying higher amount of Tat (5 μg). However, Tat-specific total IgG titers obtained with SLN carrying lower amount of Tat were significantly higher than Tat adjuvanted with alum. This indicated potential of SLN over conventional vaccine adjuvants. The study also established that all Tat (1–72) immunized groups elicited Tat-neutralizing antibodies capable of inhibiting Tat-initiated long terminal repeat (LTR) and that anti-sera from Tat-SLN immunized mice showed higher reactivity towards N-terminal and basic regions of Tat as compared to that of Tat adjuvanted with alum [171].

Mumper et al. also devised strategies to increase interaction between HIV antigen and nanoparticles and studied the effect on the immune responses. The authors explored two strategies for increasing interaction of HIV antigens with the nanoparticles viz. (1) chelated divalent metal based coordination of HIV antigen to SLN and (2) conjugation of HIV antigen to SLN. In the first strategy, the ability of chelated divalent metal ions such as nickel to form coordinate bonds with short histidine residues was utilized. SLN containing nickel chelating lipid (Ni-SLN) were developed and HIV antigen (HIV-1 Gag p24) with short sequence of histidine residues (his-tag) were employed to augment interaction of HIV antigen with SLN [172]. Immune responses to his-tag HIV-1 Gag p24 bound Ni-SLN were compared to HIV-1 Gag p24 adjuvanted with alum and HIV-1 Gag p24 adsorbed onto anionic SLN. Interestingly, Ni-NP yielded significantly higher Gag p24-specific serum IgG and IgG2a levels in mice as compared to alum as well as anionic SLN. These results clearly demonstrated effect of increasing antigen-nanoparticle interaction on the immunization [172]. The ability of Ni-SLN to co-deliver two HIV-1 antigens (his-p24 and his-Nef) was also evaluated in comparison to his-p24 and his-Nefadjuvanted alum [173]. Interestingly, co-delivery of his-p24 and his-Nef through Ni-SLN resulted in significantly higher p24-specific and Nef-specific serum IgG as compared to alum. Moreover, co-delivery of his-p24 and his-Nef through Ni-SLN elicited significantly higher IgG levels as compared to delivery of individual antigen through Ni-SLN [178]. This shows advantage associated with co-delivery of HIV antigens. Conjugation of HIV antigen to SLN was employed as a second strategy to augment immune responses. In this study, HIV-1 p24 was conjugated to Brij 700 (a stabilizer for SLN) by means of a facile tresyl conjugation [174]. The immune response to Brij 700-p24 conjugate and Brij 700-p24 conjugate incorporated in SLN was studied. SLN containing conjugated p24 showed significantly higher p24-specific IgG levels as compared Brij 700-p24 conjugate alone and p24 adjuvanted with alum [174]. This demonstrates advantage associated with the SLN. However, whether antigen conjugation is more immunogenic as compared to nickel mediated antigen coordination still remains to be evaluated.

Arias et al. have reported potential of carnauba wax based SLN as an adjuvant for HIV antigens [175]. The authors developed carnauba wax SLN with different surface characteristics and excellent long-term colloidal stability. HIV gp140 antigen and TLR-9 (Toll-like receptor-9) ligands such as CpGB or PolyI:C were adsorbed on SLN. Intradermal administration of these SLN in mice yielded significantly higher levels of HIV gp140-specific IgG as compared to antigen alone. Moreover, intranasal administration of these SLN in mice resulted in significantly higher serum and vaginal IgG and IgA levels as compared to antigen alone [175]. Carnauba wax SLN could induce systemic as well as mucosal immunity to HIV antigens. Thus, lipid nanocarriers may emerge as low cost and high efficacy adjuvants for HIV vaccines.

5.5. Drug nanocrystals or nanosuspensions

As the name suggests, drug nanocrystals are essentially nano-scale drug particles dispersed in an aqueous (water or buffers) or non-aqueous (polyhydric alcohols or oils) medium. Drug nanocrystals also contain a suitable stabilizer or mixture of stabilizers to maintain long-term colloidal stability [55,176]. Drug nanocrystals can be generated using methods based on top–down approach or bottom-up approach. Top–down approaches such as media milling and high-pressure homogenization are the most preferred methods for generation of nanocrystals due to their amenability for large-scale production. Various platform technologies such as Nano-Crystal®, Nanopure® and NANOEDGE® have been developed for fabrication of drug nanocrystals [55,176]. Various products based on drug nanocrystals are currently available in the pharmaceutical market. Thus, 'drug nanocrystals' is an industrially feasible nano-technology. Conversion of pure drug to nano-scale particles leads to dramatic increase in the surface area and dissolution velocity leading to increase in bioavailability and reduction in pharmacokinetic variability [55,176]. Drug nanocrystals have mainly been developed for (1) extremely hydrophobic drugs that are difficult to deliver as a solution by intravenous route and (2) low dose hydrophobic drugs exhibiting dissolution rate limited oral bioavailability.

Baret et al. developed nanocrystals of rilpivirine (a non-nucleoside reverse transcriptase inhibitor) with the help of media milling technique [177]. Rilpivirine nanocrystals of 200, 400 and 800 nm were injected in mice and dogs by intramuscular and subcutaneous routes and their pharmacokinetic behavior was monitored. Interestingly, all the treatments showed detectable rilpivirine levels up to 90 days in dogs and 3 weeks in mice indicating their utility in long-term prophylaxis of HIV. The pharmacokinetic behavior of rilpivirine was influenced by the size of the nanocrystals as well as route of administration. It was observed that 200 nm nanocrystals yielded higher and less variable rilpivirine plasma concentration as compared to 400 and 800 nm nancocrystals [177]. In another study, the authors compared 200 nm rilpivirine nanocrystals delivered to rats and dogs at 5 mg/kg dose by IM and SC routes [178]. The IM administration resulted in higher initial peak plasma concentration as well as higher clearance as compared to SC administration of rilpivirine nanocrystals. The SC administration yielded stable plasma concentration for at least 6 weeks in dogs. Rilpivirine concentrations were also detected in lymphoid tissues throughout the treatment, indicating uptake of nanocrystals by macrophages. However, rilpivirine levels in lymphoid tissues were considerably higher for 1 month for IM route as compared to SC route in dogs[178]. Thus, drug nanocrystals can serve as a long-term prophylactic modality for HIV infection.

5.6. Inorganic nanoparticles

Inorganic metals such as antimony, iron, platinum, calcium, gold and silver have long history as therapeutic agents. Since last decade, nano-structures based on various inorganic metals are actively being pursued as therapeutic and imaging modalities. Moreover, inorganic nanomaterials have also been tailored to act as carriers for drugs and RNAi therapeutics.

Elicheguerra et al. were the first to demonstrate the effect of silver nanoparticles on HIV-1 [179]. Polyvinyl pyrrolidone coated silver nanoparticles were found to be effective against an array of HIV-1 strains including M tropic strains, T tropic strains and drug resistant strains [179,180]. The silver nanoparticles exhibited significantly higher selectivity index as compared to silver salts (silver nitrate and silver sulfadiazine) indicating the importance of the nanosizing of the particles. Mechanistic studies indicated that silver nanoparticles bind to disulfide bonds in the CD4 binding domain of gp120 and inhibit CD4 mediated viral fusion to host cells [180]. Interestingly, silver

nanoparticles were also found to interfere with other stages of viral replication cycle, which may minimize emergence of resistant viral strains. Lara et al. evaluated HIV inhibitory potential and tolerability of silver nanoparticles in cervical explants [181]. Silver nanoparticles were well tolerated by cervical explants at the concentration of 0.15 mg/mL. Pre-treatment of cervical explant with silver nanoparticles prevented its infection by cell free as well as cell-associated virus. The anti-HIV activity of silver nanoparticles was evident within 1 min after topical treatment of the cervical explant. Moreover, 20-min pre-treatment of cervical explants with silver nanoparticles gel (followed by thorough rinsing to remove gel) was able to prevent HIV infection for 48 h [181]. This clearly indicates the potential of silver nanoparticles in the long-term prophylaxis of HIV. Silver nanoparticles have also shown to have an additive effect with neutralizing antibodies developed against HIV-1 envelope proteins [182]. Thus, silver nanoparticles can also be used for combination therapy. Recently, the ability of silver nanoparticles coated polyurethane condoms were evaluated for prevention of HIV and HSV infection [183]. Interestingly, condoms impregnated with silver nanoparticles did not show cytotoxicity to HeLa cells and T cells after 3 h. Furthermore, silver nanoparticles impregnated condoms were able to inhibit macrophage (M)- and T lymphocyte (T)-tropic HIV as well as HSV. This study opens a new door to male controlled microbicides.

Gold nanoparticles have been extensively explored for an array of biomedical applications [184]. The advancement in fabrication methodologies has enabled generation of gold nanoparticles with varying sizes and tunable optical and surface properties. It is well known that appropriately designed gold nanoparticles can enable multivalent presentation of the materials present on their surface; thus, enabling maximal interaction with the target receptors. It is possible to functionalize surface of gold nanoparticles with a variety of biomolecules such enzymes, antibodies, carbohydrates, siRNAs and even drugs without loss of their inherent activity [184]. In fact, gold nanoparticles carrying TNF-α (Aurimune™, CytImmune Sci. Inc.) are currently being evaluated in clinical trials for cancer therapy. Paradoxically, very few studies have focused on applications of gold nanoparticles for the HIV treatment or prophylaxis. Melander et al. observed that conjugation of SDC-1721 (a derivative of HIV fusion inhibitor TAK-779) to gold nanoparticles exhibited IC_{50} value against HIV-1 at concentration as low as 10 nM. Interestingly, neither gold nanoparticles nor SDC-1721 alone were able to demonstrate HIV-1 inhibitory potential [185]. This study clearly indicates that multivalent presentation of biologically inactive modality with the help of gold nanoparticles can transform them into biologically active therapeutic agents.

Penades et al. have carried out a series of experiments to explore the effect of multivalent presentation of various oligomannosides on the gold nanoparticles (Manno-Glyconanoparticles) for the inhibition of DC-SIGN mediated HIV trans-infection [186–188]. Te-50, a manno-glyconanoparticles containing 56 units of linear tetrasaccharide were found to be active against infection by both R5 and X4 tropic HIV at nM concentrations [187]. The experiment clearly showed that manno-glyconanoparticles prevented attachment of gp120 to DC-SIGN resulting in HIV inhibition. Moreover, these nanoparticles were found to mimic carbohydrate epitope of the 2G12, a broadly neutralizing monoclonal antibody against HIV [188]. The same group evaluated anti-HIV potential of gold nanoparticles bearing anionic sulfate or phosphate end functional groups [189]. Gold nanoparticles with phosphate end groups did not show any anti-HIV activity whereas gold nanoparticles decorated with sulfate groups and inert monosaccharide (5-(thio)pentyl D-glucopyranoside) on the surface inhibited HIV at nM concentrations [189]. A recent study explored cytotoxicity and antiviral activity of PEG coated gold nanoparticles [190]. Gold nanoparticles were well tolerated by cells and they were able to inhibit M-tropic, T-tropic, dual-tropic and resistant strains of HIV-1. The authors observed that gold nanoparticles prevented HIV-1 entry by binding to gp120. Shiang et al. fabricated gold nanoparticles functionalized with apatamers that can recognize polymerase and RNAse H region of HIV-1 reverse transcriptase [191]. Interestingly, aptamer functionalized gold nanoparticles were stable to nuclease mediated degradation and also inhibited HIV-1 infection by blocking reverse transcriptase. Thus, suitably functionalized gold nanoparticles could have potential as topical microbicides, vaccines, vaccine adjuvants and delivery systems for vaccine and nucleic acid.

Magnetic nanoparticles (Resovist®) are currently being used as MRI contrast agents. However, several studies have established that magnetic nanoparticles can also be functionalized to deliver drugs and biomolecules to target cells or organs [192]. Saiyad et al. studied the ability of magnetic nanoparticles to deliver azidothymidine triphosphate (AZTTP) to PBMC [193]. AZTTP was bound to magnetic nanoparticles by simple ionic interaction. *In vitro* studies indicated that the AZTTP bound nanoparticles did not offer any significant advantage over free AZTTP. Extensive studies need to be carried out to realize full potential of magnetic nanoparticles in the HIV prophylaxis.

Silica nanoparticles are being explored for a variety of biomedical applications such as diagnostics and delivery of therapeutic agents [194]. Recently, Vasilyeva et al. have fabricated 2′-deoxyribonucleoside triphosphate (dNTP) functionalized silica nanoparticles using click chemistry [195]. Interestingly, dNTP functionalized silica nanoparticles were able to get integrated into growing DNA chain. Furthermore, these nanoparticles were capable of reaching to nuclei after incubation with cells [195]. Thus, NRTI conjugated silica nanoparticles could be an interesting approach for prophylaxis of HIV.

Calcium nanoparticles have attracted great attention as a delivery system for oligonycleotides and siRNAs [196]. The excellent biocompatibility of calcium nanoparticles is of particular interest to researchers. He et al. carried out studies to check the ability of calcium phosphate nanoparticles to function as mucosal adjuvants [197]. Mice were treated with calcium phosphate nanoparticles and HSV-2 antigen by intravaginal and intranasal route and generation of mucosal immunity was evaluated. Interestingly, nanoparticle + HSV-2 antigen combination generated HSV-specific mucosal IgA and IgG with concomitant increase in systemic IgG responses. Moreover, intravaginal administration of nanoparticle + HSV-2 antigen combination yielded higher antibody titers at mucosal surfaces as compared to intransal administration [197]. Thus, calcium nanoparticles could have utility as vaccine adjuvants for HIV vaccines.

5.7. Nanofibers

As the name suggests, nanofibers are the sphagetti-like like masses (fibers) with diameters ranging from 1 to 1000 nm [198–200]. Various materials such as polymers, peptides, polysaccharides can be tailored to form nanofibers. Usually, nanofibers are fabricated using electrospinning process. It is possible to vary diameter, length and pore size of nanofibers by controlling parameters of the electrospinning process. The nano-scale dimensions of nanofibers are quite close to that of extracellular matrix (ECM) fibers [198–200]. Hence, nanofibers have been extensively explored in the field of regenerative medicine. Applications of nanofibers as tissue engineering scaffolds, wound dressings and vascular grafts have been widely investigated [198–200]. Furthermore, nanofibers are also being explored for the localized and controlled delivery of drugs and biotechnology based therapeutics (peptides, nucleic acids) [198–200].

Recently, nanofibers have been explored for delivering various microbicides for HIV prophylaxis [201]. Huang et al., have

fabricated nanofibers of cellulose acetate phthalate (CAP), a macromolecular HIV-1 entry inhibitor. CAP nanofibers were well tolerated by vaginal epithelial cells and vaginal microflora. Due to pH-sensitive nature of CAP, nanofibers maintained integrity in acidic pH (vaginal environment). However, addition of semen to nanofibers led to immediate dissolution of CAP. CAP nanofibers retained ability of CAP to prevent HIV-1 entry. Moreover, incorporation of tenofovir in CAP nanofibers significantly improved its antiviral activity [201]. CAP nanofibers can release antiretroviral drugs on coitus which would help in preventing sexual transmission of HIV. Low cost of CAP and ease of scale-up of electrospinning process indicate that drug loaded CAP nanofibers have good potential in HIV prophylaxis.

Woodrow et al. have fabricated nanofibers of various biodegradable polymers such as PLA and PCL [202]. Nanofibers of various dimensions were easily formulated by using blends of polyethyleneoxide (PEO) and biodegradable polymers. Nanofibers did not show any toxicity to various cells and explants. Several microbicides such as maraviroc, azadothymidine, acyclovir, glycerylmonolaurate were successfully incorporated into the nanofibers. Nanofibers sustained release of the microbicides and showed similar antiviral activity as compared to free drugs. Intravaginal administration of fluorescent nanofibers showed that they were very well retained in the reproductive tract. This study also established spermicidal activity of GML loaded nanofibers [202]. Thus, polymeric nanofibers could be used for delivering combination of microbicides and contraceptives.

6. Factors governing *in vitro* and *in vivo* fate of nano-architectures

There are several factors that influence *in vitro* and *in vivo* fate of nano-architectures. Several studies have shown that factors such as material properties, size, shape, surface charge and surface chemistry can affect cellular uptake, intracellular distribution, toxicity, immunogenicity and biodistribution of nano-architectures. Hence, the process of design and fabrication of nano-architectures is very crucial.

6.1. Properties of material used for fabrication

The nano-architectures can be fabricated using various materials such as polymers, lipids and inorganic metals. Inherent properties of these materials can significantly influence cellular and/or tissue uptake and immunological properties of nano-architectures. For example, due to the intrinsic properties of metals like gold and silica, fluorescent nano-architectures can be generated without using any fluorescent dye whereas nano-architectures based on polymers and lipids do not have such capability [184,203]. It has been observed that phospholipid coated polymeric nanoparticles and gold nanoparticles showed significantly higher intracellular uptake as compared to nanoparticles without phospholipid coating [204,205]. Similar results were found for albumin coated polymeric nanoparticles [206]. Studies have also shown that micelles based on polyoxyethylene oxide (PEO)-polypropylene oxide (PPO) copolymers are capable of complement activation whereas PEG-phospholipid micelles do not exhibit this property [207]. These investigations clearly indicate importance of inherent properties of materials. Furthermore, composition of materials belonging to same class (polymer or lipid or metal) can influence the *in vitro* and *in vivo* performance. Studies have shown that the chemical structure of cationic lipids used can dramatically impact efficacy, cellular uptake, transfection and toxicity of cationic nano-architectures. Nano-architectures composed of cationic lipids with single alkyl chain were more toxic and incapable of transport through cervicovaginal mucus and transfection [208,209]. On the contrary, nano-architectures composed of cationic lipids with two alkyl chains showed enhanced transport through mucus and cells with concomitant reduction in cytotoxicity [208–210]. Similarly, Sunshine et al., have shown that the end groups of the polymer can substantially impact cellular uptake and extent of transfection [211]. Molecular weight is another important property that can govern performance and/or fate of nano-architectures. The process of glomerular filtration (renal clearance) has a molecular weight cutoff of ~48 kDa [212–214]. Thus, non-biodegradable polymers with molecular weight higher than the cutoff would not be eliminated by kidneys and should be avoided for fabrication. Hanes et al. have shown that molecular weight of PEO-PPO block copolymers (Pluronics) and/or length of PPO chain can have significant impact on the transport of nano-architectures through cervicovaginal mucus [215]. The authors report that coating of nanocarriers with Pluronic F127 (high molecular weight and PPO chain length > 3000 Da) yielded quick transport through cervicovaginal mucus as compared to Pluronic F68 (low molecular weight and PPO chain length < 3000 Da) coated nanocarriers. Polymer molecular weight can influence particle size, rate of *in vitro* and *in vivo* drug release which in turn affects efficacy of nano-architectures [216,217]. In case of polyethylenimines (PEI), polymer molecular weight has a dramatic impact on their transfection capability, antiviral activity and cytotoxicity [218,219]. Thus, properties of material used for fabrication of nano-architectures are of great importance to obtain an optimal effect.

6.2. Size

The size of nanoparticles is a very important feature that governs their biodistribution and uptake in tissues and cells. Physiological barriers such as mucus, blood capillaries, endothelium, renal vasculature and fenestrations in the liver and spleen have different size cut-offs and have a key role in determining *in vivo* biodistribution and clearance of nanocarriers [214]. Understanding the size limits of various physical barriers is an important aspect of the nanocarrier designing process. The size of the nanocarriers has also been found to influence their transport through mucosal barriers like cervicovaginal mucus [220]. Cervicovaginal mucus is one of the key components that determine the success of vaginal prophylaxis of HIV. Cervicovaginal mucus has a nanoscopic mesh of mucin fibers and various other components. The mesh size of the ovulatory cervical mucus is anticipated to be ~100 nm [53,220]. In view of this, the effect of size of the nanocarriers on their transport through cervicovaginal mucus has been studied. Initial studies indicated that viruses with size less than 100 nm (polio, rotaviruses) easily diffuse through cervicovaginal mucus whereas viruses like herpes simplex virus (HSV, particle size ~ 180 nm) get trapped into the mucus [221]. Hence, it was believed that nanoparticles with size ≤ 100 nm would show rapid mucosal transport. However, Lai et al. observed that particles with size between 200 and 500 nm were able to quickly diffuse through cervicovaginal mucus whereas particles with average size of 100 nm were trapped in the mucus [222]. However, a recent study from the same group showed that nanoparticles with size less than 100 nm can also rapidly diffuse through mucus after suitable surface engineering [96].

While designing nanocarriers for prophylaxis of HIV through systemic routes, one should be aware that cutoff size for renal clearance is ~10 nm and 48 kDa [212–214]. It is well known that the width of the sinusoidal space in liver and spleen is about 50–100 nm and the size limit for blood–brain barrier is considered to be ~12 nm [212,214]. Studies have shown that particles less than 10 nm undergoes rapid renal clearance and particles higher than 1000 nm are rapidly opsonized by reticuloendothelial system [212–214,223,224]. Size of the nanoparticles also influences plasma

protein adsorption pattern. PEGylated polyhexadecyl cyanoacrylate (PHDCA) nanoparticles of different size showed differential adsorption of serum proteins. Smaller PHDCA nanoparticles (80 nm) showed very less protein adsorption (6%) whereas larger nanoparticles (243 nm) showed higher protein adsorption (34%) [212]. Studies have shown that smaller nanoparticles (<100 nm) have higher residence time in blood than larger nanoparticles [212–214,223,224].

The size of nanocarriers also governs intracellular uptake, receptor interaction, immune response and toxicity. Size dependent uptake of PLGA nanoparticles has been studied in Caco-2 and HT-29 cells. Interestingly, particles in the size range of 100–150 nm showed highest cellular uptake in the cells whereas particles with size ≤50 nm and ≥200 nm showed lesser cellular uptake [225,226]. Similar results have been observed for mesoporous silica nanoparticles [227]. The relation between nanocarrier size and cellular uptake is considered to be dependent on the competition between the bending energy and the stretching energy of cell membrane [226]. Huang et al. showed that polyvinyl pyrrolidone coated iron oxide nanoparticles of size 100 nm exhibited highest MRI contrast in liver [228]. In case of HIV prophylaxis, uptake of nanocarriers by macrophages is an important criterion for the successful results. Yu et al. observed that Pluronic coated iron oxide nanoparticles of size 100 nm had higher uptake in THP-1 human leukemic monocytes as compared to nanoparticles of size 40 nm [229]. Similar results have been observed for uptake of gold nanoparticles (90 nm) in mouse macrophages [230]. In short, studies indicate that nanocarriers of size ~100 nm would have higher propensity for cellular uptake. Binding affinity of a ligand to receptor was found be dependent on the particle size. Binding affinity of Herceptin to the ErbB2 receptor was higher for 70 nm particles as compared to 10 nm particles [224].

Various *in vitro* and/or *in vivo* studies have been carried out to evaluate the impact of nanocarrier size on immune responses. This is of particular interest in development of HIV nano-vaccines. Gutierro et al. evaluated PLGA nanoparticles of size 200, 500 and 1000 nm for their ability to elicit immune response [231]. It was observed that 1000 nm particles yielded higher total serum IgG levels as compared to smaller particles. Interestingly, there was no significant difference in the serum IgG2a/IgG1 ratio for particles of all size. Hence, it was concluded that antigen processing and presentation was similar in case of all the particles [231]. Cohen et al. observed that acid sensitive hydrogel particles of size 35 nm and 35 μm show similar extent of T-cell activation *in vitro* as well as *in vivo* [232]. On the contrary, polystyrene nanoparticles of different size showed size dependent immune responses. Polystyrene particles with size <200 nm were found to be more efficient in generating cytotoxic T lymphocytes as compared to 2 μm particles [233]. Recently, Wang et al. noticed that mesoporous silica nanoparticles of size 430 nm elicited higher serum IgG and IgA levels than 130 nm and 1–2 μm particles [234]. Studies have shown that 250 nm dextran coated iron oxide nanoparticles showed significant complement activation whereas 600 nm particles did not activate complement [223]. In an interesting study, Xiong et al. observed that macrophages treated with PLGA nanoparticles of size 60 nm and 100 nm show significantly higher TNF-α release (indicator of inflammatory response) as compared to 200 nm particles [235]. However, these observations were also dependent on the concentration of the nanoparticles. Size dependent toxicity and free radical generation has been observed for silver nanoparticles. Silver nanoparticles of size 15 nm were significantly toxic to macrophages as compared to 30 and 55 nm silver nanoparticles [236]. On the contrary, PLGA nanoparticles did not show size dependent toxicity in macrophages [235]. All these studies indicate that other factors such as material type are also crucial in size dependent responses.

6.3. Shape

In last 10 years, extensive studies have been carried out to understand the effect of nanocarrier shape on cellular uptake, intracellular trafficking, *in vivo* circulation and/or biodistribution. There are many methods available to generate nanocarriers with different geometry and aspect ratio [237,238]. Various *in vitro* studies have been carried out to study the effect of nanocarrier shape on uptake in different cells. For the process of cell uptake, attachment of nanocarrier to cell surface is the first step. Internalization of nanocarriers can occur by various mechanisms such as phagocytosis, macropinocytosis, clathrin or caveolae-mediated endocytosis and clathrin/caveolae-independent endocytosis [237,238]. The mechanism of cellular internalization is governed by size and nature of the extracellular object. Hence, morphology of the nanocarrier has significance in the internalization process [237,238]. Phagocytosis is a process of internalization of foreign particles by macrophages through rearrangement of the actin skeleton. Uptake of nanocarriers by macrophages is an important aspect in drug delivery. For effective treatment of bacterial and viral infections, nanocarriers should be effectively taken up by macrophages. Studies indicate that particles with oblate ellipsoid shape exhibit higher macrophage uptake as compared to the spherical and prolate ellipsoid shape particles [239–241]. Lin et al. observed that sphere and hexagon shaped polymeric nanoparticles of 70 nm do not show a difference in the macrophage uptake. However, hexagonal shape particles of 120 nm showed considerably lower uptake [242]. Nowacek et al. studied uptake of antiretroviral drug nanocrystals (nanoART) of different shapes in macrophages. It was observed that nanocrystals with rounded particles and irregular edges showed quite low macrophage uptake as compared to rods with smooth and regular edges [243]. Between rods, longer rods were more efficiently taken up as compared to shorter rods. Similar results have been found for silica nanorods [244]. On the contrary, gold nanospheres were found to have much greater uptake in macrophages as compared to gold nanorods [245]. Champion and Mitragotri showed that polystyrene nanospheres exhibit higher macrophage uptake than worm-like polystyrene particles of equal volume [240]. It should be also noted that nonphagocytic cells might show different preference towards nanocarrier shape. Studies have also shown importance of aspect ratio (ratio of width to height) of particles with similar shape on the cellular uptake. Monodisperse hydrogel nanoparticles of different aspect ratios were fabricated using PRINT technology. It was observed that particles with higher aspect ratio were internalized much quicker than particles with lower aspect ratio. Also, cylinders with larger diameter were taken up faster than cylinders with smaller diameter although their aspect ratios were same [237,238]. Thus, one should keep in mind that factors other than size are also important in the cellular uptake.

In vivo studies have shown that gold nanorods exhibit longer circulation in the blood as compared to gold nanospheres [245]. Furthermore, gold nanospheres showed significantly higher accumulation in the liver as compared to gold nanorods. Similar results have been observed for the polymeric nanospheres and hexagonal shaped particles [242]. In an interesting work, *in vivo* circulation of biodegradable filamentous micelles was compared to stealth liposomes. Interestingly, filamentous micelles resided in circulation for a week whereas liposomes were cleared within 2 days [237,238]. Intravascular behavior of spherical, quasi-hemispherical, cylindrical and discoidal silica particles was examined in various studies [246–248]. It was observed that discoidal particles were preferentially taken up by lungs, spleen and heart but not by liver [248]. Devrajan et al. showed that irregular shape polymer–lipid hybrid nanoparticles (LIPOMERs) undergo selective splenic uptake (in

mice, rabbit and dog) as compared to spherical particles [249]. Szoka et al. have shown that PEGylated cyclic polymers have significantly higher residence time as compared to the linear polymers of the same molecular weight [38]. Thus, it may be possible to modulate biodistribution of particles by manipulating shape. These studies might help in designing nanocarriers for HIV prophylaxis through systemic route but until today, distribution of nanocarriers with different shape on local (vaginal/rectal) delivery has not been studied.

6.4. Surface characteristics

Surface characteristics such as surface charge, surface coating, surface functional groups and surface hydrophilicity (PEGylation) have significant impact on *in vitro* as well as in *in vivo* behavior of nanocarriers [213,214]. The surface charge of nanocarriers has a great impact on their colloidal stability, interaction with blood components, cellular uptake, toxicity and mucosal transport [213,214]. Generally, a minimum zeta potential (surface charge) of ±30 mV is required for good colloidal stability of nanocarriers prepared via electrostatic interactions whereas in the case of nanocarriers fabricated via combination of electrostatic and steric interactions, a minimum zeta potential of ±20 mV is desirable [176]. Studies have shown that nanocarriers with high negative surface charge (liposomes; zeta potential of ~−40 mV) are rapidly cleared from blood as compared to the neutral liposomes. Moreover, negatively charged liposomes showed increased mononuclear phagocytic system (MPS) uptake in the liver as compared to neutral liposomes [213]. Hanes et al. have demonstrated that highly anionic polymeric nanoparticles (zeta potential of ~−40 mV or higher) have very slow mucosal transport as compared to particles with zeta potential close to neutral charge [222]. The presence of anionic proteoglycans in the mucus is believed to be responsible for repulsion of anionic nanocarriers leading to reduce mucosal transport. Generally, positively charged nanocarriers are known to have significantly higher cellular uptake as compared to negatively charged nanocarriers [213,214]. This is mainly due to their increased electrostatic interaction with the anionic cell membrane. Furthermore, cationic nanocarriers may also exhibit different mechanism of cell uptake as compared to anionic nanocarriers. Kannan et al. observed that anionic dendrimers exhibit caveolae-mediated endocytosis whereas cationic dendrimers show non-clathrin, non-caveolae dependent endocytosis [250]. Until now, cationic nanocarriers are widely used for delivering nucleic acid therapeutics *in vitro* and *in vivo* [212,214]. Studies also indicate that cationic nanocarriers show selective uptake in the angiogenic endothelium of tumors, which led to clinical development of paclitaxel containing cationic liposomes (EndoTAG-1®) [251]. Positively charged liposomes have been shown to have activity against parasites like leishmania and viruses like HSV [142,228]. Positively charged nanocarriers containing appropriate cationic components (like dicotadecyldimethyl ammonium bromide and PEI) are also known to act as vaccine adjuvants [252,253]. However, positive charge is also associated with increased cytotoxicity. Many studies indicate that positively charged nanocarriers have higher cytotoxicity as compared to anionic and neutral nanocarriers [213,214]. It has been observed that positively charged nanocarriers form aggregates with serum proteins after i.v. administration, which may increase the risk of transient embolism in lung capillaries in certain cases [212,214]. Furthermore, positively charged nanocarriers are rapidly cleared from blood stream and accumulate in liver and lung to a greater extent. Recently, oral administration of positively charged dendrimers to mice was found to cause adverse effects such as hemobilia and splenomegaly [254]. On the contrary, negatively charged dendrimers did not show such adverse effects and were well tolerated even at 10-fold higher dose [254]. Recent study indicates that polymeric nanoparticles containing cationic surfactant cetyltrimethylammonium bromide (CTAB) have significantly lower diffusivity through cervicovaginal mucus as compared to anionic and neutral nanoparticles [208]. Extensive interactions of positively charged nanoparticles with anionic mucin proteoglycans was believed to be responsible for their entrapment in mucus. Although surface charge is not the only determinant of the behavior of nanocarriers, one can expect that nanocarriers with close to neutral surface charge could have optimal *in vitro* and *in vivo* performance.

Studies have shown that charge dependent *in vitro* and *in vivo* behavior of nanocarriers can be easily modulated by decorating surface of the nanocarriers with moieties (such as PEG) that can provide steric barrier. It has been observed that surface charge of negatively charged nanocarriers (surface charge: −40 mV) can be brought close to neutral by use of PEGylation [212,213,223]. PEGylation can also reduce cytotoxicity and/or clearance of positively charged nanocarriers [212,213,223,255]. In fact, recently, positively charged dendritic micelles with a very high PEG density showed minimal interaction with the cells (indicative of minimal or no cytotoxicity) [256]. Surface PEGylation of nanocarriers has been widely used for reducing their opsonization and MPS uptake, which leads to longer *in vivo* circulation of nanocarriers. Interestingly, PEGylation of nanocarriers has also shown to improve their mucosal transport. Hanes et al. have shown that decorating surface of anionic polymeric nanocarriers with PEG can significantly improve their transport through cervicovaginal mucus [222, 257]. Saltzman et al. have shown that surface PEGylated PLGA nanoparticles have longer retention time in vaginal cavity than non-PEGylated nanocarriers [94]. It should be noted that PEG chain length (or molecular weight of PEG used for PEGylation) and density of PEG chains on nanocarriers are also very important factors governing *in vitro* and *in vivo* behavior of nanocarriers. Typically, very low (≤1000 Da) or very high (>20,000 Da) molecular weight PEGs have not been found to be suitable for increasing *in vivo* circulation and/or transport through cervicovaginal mucus [258–260]. Polymeric nanoparticles coated with vitamin E TPGS (PEG chain length 1000 Da) get trapped in cervicovaginal mucus whereas increasing chain length of PEG in vitamin E TPGS to 5000 Da significantly increased mucosal transport of nanocarriers [260]. In another study, the authors compared mucosal transport of nanoparticles coated with PEG-2000 and PEG-10000. It was observed that nanocarriers coated with PEG-2000 had significantly higher mucosal transport as compared to that of PEG-10000 coated nanocarriers [257]. It should be noted that PEG chain length required for optimal *in vitro* and *in vivo* behavior would also depend upon material properties, surface charge and type of nano-architecture.

7. Importance of route of administration

The route of administration can have an immense impact on the disposition of the therapeutic agent and/or nanocarriers. Additionally, the route of administration can have considerable effect on the extent of therapeutic efficacy, immunological effects and/or duration of action depending upon the anatomical site and its physiology [214]. It is important to understand various barriers and challenges associated with various route of administration while selecting appropriate route for drug delivery. In order to achieve prophylaxis against infectious diseases including HIV, drugs or vaccines have been administered through various routes such as intramuscular, intravenous, peritoneal, dermal, subcutaneous, nasal, oral, vaginal and rectal [261–263]. Table 3 discusses various advantages and disadvantages associated with the different routes

Table 3
Brief overview of advantages and disadvantages associated with various routes of administration.

Route of administration	Advantages	Disadvantages
Oral	• Easy, economical and convenient • Non-invasive • No need of special training • Can be used to deliver aqueous or non-aqueous vehicles	• Therapeutic agents are exposed to harsh gastrointestinal environment • Therapeutic agents undergo first-pass metabolism • Possibility of slow and erratic absorption • Requires higher dose for efficacy
Intravenous	• Instantaneous absorption • Requires lower dose for efficacy • Precise dose delivery • Ease of dose titration • Can avoid first-pass effect	• Invasive nature • Needs special training • Not suitable for sustained drug delivery • Only aqueous solutions can be delivered
Intramuscular	• Can be used to deliver aqueous or non-aqueous vehicles • Useful to achieve sustained drug delivery • Needs less skill than intravenous administration	• Invasive and painful • Needs special training • Variable absorption • Possibility of drug precipitation at the site of injection
Subcutaneous	• Can be used to deliver aqueous or non-aqueous vehicles or suspensions • Useful to achieve sustained drug delivery • Minimal skill required • Avoidance of first-pass effect	• Variable absorption from various subcutaneous sites • Limitations on injection volume • Painful • Potential of tissue damage
Nasal	• Non-invasive nature • No special training needed • Rapid systemic absorption • Possibility of brain targeting • Avoidance of first-pass effect	• Limited volume can be administered • Limitations on use of pharmaceutical excipients
Intradermal	• Useful to achieve sustained drug delivery • Can avoid first-pass effect • Targeting of Langerhans cells • May results in sustained drug delivery	• Invasive • Limited volume can be administered • Special training required
Vaginal	• Useful for achieving local drug concentration • May be used for systemic delivery • No special training required	• Drugs exposed to acidic environment • Limitation of volume • Limitations on use of pharmaceutical excipients
Rectal	• Useful for achieving local drug concentration • May avoid first-pass effect • Useful in pediatric or geriatric patients	• Limitation of volume • Limitations on use of pharmaceutical excipients • Variable absorption • Low patient acceptability

of administration. It is evident that each route of administration has pros and cons. The choice of route of administration depends on many factors such as type of infectious disease, properties of therapeutic agent, type of prophylaxis (systemic or local), concentration required for prophylaxis and type of immune responses (systemic and/or mucosal). There are relatively few studies that compare the effect of various routes of administration on the extent of prophylaxis and until today, there is no study that compares all the routes of administration at once. The majority of these studies have focused on studying the effect of route of administration on the extent of systemic and/or mucosal immune responses for various vaccines. Based on these studies, it is still difficult to predict most appropriate vaccination strategy among local vaccination (vaginal or rectal), systemic (intramuscular) and distant mucosal vaccination (nasal) to obtain desired level of protection against HIV infection. Conventionally, intramuscular (i.m.) route is widely used for delivery of vaccines. However, for an infection like HIV or HSV-2 which requires mucosal immunization (or Th1 response), i.m. route may not always be desirable. Jazayeri et al. compared immune responses to DNA vaccine encoding HSV-gD2 (glycoprotein D of Herpes Simplex Virus-2) after i.m. and s.c. immunization [264]. Interestingly, the type and extent of immune response was found to be dependent on route of administration. It was observed that s.c. administration of vaccine elicited significantly higher IL-2 production and cytotoxic T-lymphocytes (Th1 response) as compared to i.m. injection. The i.m. route showed significantly higher HSV-2 neutralizing antibodies (Th2 response) as compared to s.c. administration [264]. In an another study, immune responses to PLGA nanoparticles containing plasmid DNA encoding HIV gp160 were compared after oral and i.m. administration. Interestingly, oral route yielded systemic as well as mucosal immune responses whereas i.m. route yielded only systemic immune responses [265]. These observations indicate that i.m. route may not always be desirable for mucosal immunization. Additionally, an interesting study evaluated the impact of type of nanocarrier and route of administration on the immune responses [266]. Cationic liposomes and trimethyl chitosan nanoparticles (TMC-NPs) containing ovalbumin with or without adjuvant were administered via i.m., s.c. and intradermal (i.d.) routes and immune responses were monitored. Liposomes and TMC-NPs containing ovalbumin (OVA) with or without adjuvant elicited similar OVA-IgG1 titers (Th2 response) irrespective of route of administration. However, OVA-IgG2a and IFN-γ titers (Th1 response) were found to be dependent upon route of administration, type of nanocarrier and/or presence of adjuvant [266]. IFN-γ titers were highest for TMC-NPs containing OVA with or without adjuvant administered by the s.c. route and lowest for the i.m. route whereas no such differences were observed in case of liposomes. OVA-IgG2a titers were highest for cationic liposomes containing OVA after administration by the i.d. route and lowest for the s.c. route. However, no such differences were found in case of TMC-NPs [266]. Recently, a small scale human clinical trial evaluated difference in immune responses after delivery of DNA vaccine and recombinant replication-defective adenovirus type 5 (rAd5) vaccine boost by i.m., s.c. and i.d. routes [267]. The trial could not identify best route of administration for vaccines. Thus, choice of i.m., i.d. and s.c. as route of administration for mucosal immunization may have to be evaluated on a case-by-case basis.

Sexual transmission is the most common cause for HIV infection and vaccination strategies that can induce significant mucosal immunization in vagina and/or rectum are desirable. There have been

few studies to identify best route of administration for generating immunization in vaginal and/or rectal mucosa. Arias et al. evaluated immune responses in the vagina after nasal, vaginal and rectal administration of carnauba wax nanoparticles containing HIV gp140 antigen [180]. Interestingly, intranasal administration of nanoparticles elicited potent systemic immune response and mucosal immune response in the vagina whereas vaginal and rectal administration showed very weak immune response [175]. It is well known that vaginal and rectal mucosa can offer a barrier for effective delivery of antigens. Furthermore, vaginal acidic pH can also have a negative influence on antigen stability [175,268]. In addition, vaginal and rectal mucosal tissues have dearth of follicle-associated epithelium (FAE). On the contrary, intranasal administration enables delivery of antigen to nasopharynx associated lymphoid tissue (NALT) containing large population of microfold cells (M cells) and FAE bearing high densities of immunologically active cells [175,268]. Thus, intranasal delivery can offer greater antigen delivery and presentation to immune cells as compared to vaginal and rectal administration, which may be reflected in the immune responses. Pattani et al. developed microneedles containing HIV gp140 antigen and primed animals with microneedles by the i.d. route [268]. Afterwards, animals were boosted with antigen by intranasal (i.n.), intra-vaginal and s.c. route and systemic and vaginal immunization were evaluated. Once again, the nasal route was found to be superior as compared to the other routes of administration [268]. Buffa et al. have noted similar observations for delivery of HIV gp140 [269]. Thus, nasal administration could be beneficial for acquiring highest level of immunization in vaginal mucosa but further evidence with different antigens and different nanocarriers would be required to validate these observations.

In the case of prophylaxis using antiretroviral drugs, oral, subcutaneous and vaginal routes have mainly been explored. Oral route is the most convenient and preferred route of administration. However, oral route also presents maximum barriers and challenges for drug delivery. In order to show an optimal effect, therapeutic agent has to survive in the harsh acidic environment of stomach and various digestive enzymes and be absorbed at an optimal level. Thus, often-high doses of therapeutic agents have to be administered to achieve effective systemic as well as mucosal concentrations. Currently, a once a day oral administration of Truvuda® (tablet containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxilfumarate) has been approved for pre-exposure prophylaxis [4]. It is well known that NRTIs such as emtricitabine and tenofovir can have dose related side effects such as mitochondrial toxicity. As sexual contact is major mode of transmission of HIV infection, it is advisable to develop strategies that can offer effective concentrations of antiretroviral drugs in vaginal and/or rectal tissues. Albeit orally delivered antiretroviral drugs would be compartmentalized into vaginal and rectal tissues but the dose required to achieve effective concentration would be much higher as compared to local and/or systemic delivery. In view of this, researchers compared vaginal and rectal delivery of tenofovir with oral administration of tenofovir [4,270]. Vaginal and rectal delivery yielded dramatically higher mucosal concentrations of tenofovir diphosphate (active metabolite of tenofovir) as compared to oral administration of tenofovir. Nutall et al. compared pharmacokinetics of the 1% tenofovir gel in macaques after intravaginal and intrarectal administration. Interestingly, tenofovir concentrations (in vaginal fluid) achieved after intravaginal administration was considerably lower than concentrations (in rectal fluid) obtained after intrarectal administration [271]. These studies clearly indicated importance of route of administration in the prophylaxis using antiretroviral drugs. Furthermore, s.c. route has also been compared to oral route. Garcia-Lerma et al. compared once weekly s.c. administration of tenofovir and emtricitabine with daily oral dosing of tenofovir and emtricitabine in macaques [272]. Interestingly, once weekly s.c. administration of antiretroviral drugs yielded similar degree of protection from HIV infection as compared to daily oral dosing of antiretroviral drugs. Thus, simple change in route of administration can have dramatic impact on the HIV prophylaxis. It is important to note that s.c. route is invasive but requires minimal training whereas oral route is very convenient for patients. A recent multinational study compared attitudes and acceptance of patients towards oral and parenteral pre-exposure prophylaxis [273]. Interestingly, the survey indicated willingness to experience inconvenience and expense associated with parenteral delivery indicating potential for development of long-lasting parenteral formulations of antiretroviral drugs for HIV prophylaxis. Finally, it should be kept in mind that there are several other factors in addition to route of administration that would govern direction for an optimal HIV prophylaxis.

8. Future perspectives and conclusion

HIV/AIDS is responsible for death of more than 25 million individuals worldwide since its discovery and every year more than 2 million individuals acquire HIV infection. As a result, prevention of HIV infection has become a global priority. Until now, development of conventional prophylactic modalities like HIV vaccines has not been successful due to intricate structural features of HIV. Hence, antiretroviral agents and nucleic acid-based therapeutics are being actively explored as prophylactic modalities for HIV. Recent clinical trials have shown that oral or local (vaginal) administration of antiretroviral agents like tenofovir offers moderate prevention from HIV infection. Extreme hydrophilicity or hydrophobicity, poor permeability, poor chemical or enzymatic stability are some of the major reasons for moderate success of antiretroviral agents as prophylactic modalities. Nanotechnology has demonstrated a potential to bring change in this scenario. Over the years, various nano-architectures have been developed and evaluated for augmenting prophylactic activity of HIV vaccines, antiretroviral agents and nucleic acid therapeutics. These nano-architectures have shown ability to improve solubility, permeability, stability and pharmacokinetics (systemic or local) of the prophylactic modalities. The ability of certain nano-architectures such as polymeric nanoparticles, lipid nanoparticles and nanofibers to offer sustained release of prophylactic modality is of great benefit to reduce number of dosing and to improve patient adherence. Smartly assembled nano-architectures such as nanofibers and dendrimeric-based hydrogels could be useful for sustained and local delivery of hydrophilic antiretroviral modalities like tenofovir and emtricitabine. It is also possible to develop nano-architectures with inherent ability to inhibit HIV by intelligent manipulation of composition and surface characteristics. Combination of antiretroviral agents has demonstrated a great success in improving management of HIV infections. Recently, evaluation of vaginal rings containing combination of antiretroviral agents (acting on different stages of HIV replication) has been initiated. In view of this, it would be interesting to develop nano-architectures containing a combination of antiretroviral drugs or prophylactic modalities (antiretroviral agent + siRNA). The performance of nano-architectures is also dependent on the route of administration selected for prophylaxis.

At the moment, only one nanotechnology based product (VivaGel®, a Carbopol based gel containing dendrimers with ability to inhibit HIV-1 entry) is being evaluated in Phase I clinical trials. Although nanotechnology has a great potential in HIV prophylaxis, several aspects need to be considered and optimized for successful translation of nanotechnology from lab to clinical settings. Various aspects such as biocompatibility, safety and cost of materials, regulatory status of the materials ease of scale-up and large-scale-

manufacture, reproducibility of *in vitro* and *in vivo* effects of large-scale batches are very important for successful translation of nanotechnology from lab to clinical settings. It is also important that the developed nanotechnology should offer significant cost to benefit ratio in order to gain wide acceptability. This is very important as the majority of HIV affected individuals are from economically poor and underdeveloped countries. A recent Phase I trial has shown that VivaGel® could be effective in preventing HIV infection at least 3 h before coitus. However, it is expected that nanotechnology based prophylactic modalities should offer coitus-independent and long-term (more than 1 day) prophylaxis in order to justify the added cost associated with use of nanotechnology. In view of this, it would be interesting to know the fate of VivaGel®, the first nano-microbicide.

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Review

Nanotech-derived topical microbicides for HIV prevention: The road to clinical development

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ABSTRACT

More than three decades since its discovery, HIV infection remains one of the most aggressive epidemics worldwide, with more than 35 million people infected. In sub-Saharan Africa, heterosexual transmissions represent nearly 80% of new infections, with 50% of these occurring in women. In an effort to stop the dramatic spread of the HIV epidemic, new preventive treatments, such as microbicides, have been developed. Nanotechnology has revolutionized this field by designing and engineering novel highly effective nano-sized materials as microbicide candidates. This review illustrates the most recent advances in nanotech-derived HIV prevention strategies, as well as the main steps required to translate promising *in vitro* results into clinical trials.

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Contents

1. HIV prevention strategies: an urgent necessity . . . 34
2. History of the microbicide pipeline. . . . 35
3. Nanotechnology: a new field in the design of microbicides . . . 37
4. Moving towards pre-clinical development of nanotech-derived microbicides: the main stages . . . 38
   4.1. Identification of novel candidates: *in vitro* screening . . . 40
      4.1.1. Characteristics of ideal nanotech-derived microbicide candidates . . . 40
      4.1.2. Antiviral assays . . . 40
      4.1.3. Safety and biocompatibility of nanoparticles . . . 41
      4.1.4. HIV-transmission inhibition assays . . . 42

Abbreviations: AIDS, acquired immunodeficiency syndrome; ARV, antiretroviral; ASPIRE, a study to prevent infection with a ring for extended use; BLT, bone marrow liver thymic mice; CAP, cellulose acetate phthalate; CAPRISA, centre for the AIDS programme of research in South Africa; CBA, carbohydrate-binding agents; CCR5, C–C chemokine receptor type 5; CD34, cluster of differentiation 34 marker; CD4, cluster of differentiation 4 marker; CXCR4, C-X-C chemokine receptor type 4; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; EC50, half maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IL, interleukin; IPM, international partnership for microbicides; LDH, lactate dehydrogenase; LFA, leukocyte function-associated antigen; MIP, macrophage inflammatory protein; MTN, microbicide trials network; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); N-9, nonoxynol-9; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PAMAM, poly(amido amine); PBMC, peripheral blood mononuclear cell; PEG, poly(ethylene glycol); PI, protease inhibitor; PLGA, poly-lactic-co-glycolic acid; PrEP, pre-exposure prophylaxis; RANTES, regulated on activation, normal T cell expressed and secreted protein, also known as CCL5 (Chemokine (C–C motif) ligand 5); SHIV, simian–human immunodeficiency virus; STD, sexually transmitted disease; TC50, toxic concentration 50; TEER, trans epithelial electric resistance; TFV, tenofovir; TNF, tumor necrosis factor; UNAIDS, joint United Nations programme on HIV and AIDS; VOICE, vaginal and oral interventions to control the epidemic; XTT, (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide); 7AAD, 7-aminoactinomycin D.

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4.1.5. Investigation of nanoparticle inhibitory mechanisms . . . 42
4.1.6. Combination assays . . . 42
4.1.7. Broad spectrum activity of topical microbicides . . . 43
4.2. *Ex vivo* and *in vivo* evaluation: a *proof-of-concept* of compound efficacy prior to human clinical trials . . . 43
5. Future perspectives, obstacles and conclusions . . . 44
Acknowledgments . . . 44
References . . . 44

1. HIV prevention strategies: an urgent necessity

As announced in the 2013 UNAIDS report, approximately 35.3 million people are living with HIV worldwide (UNAIDS, 2013). Although during the last few years international and national health programs have achieved a progressive reduction in new HIV infections, 2.5 million new infections and 1.7 million AIDS-related deaths still occurred worldwide. HIV heterosexual transmission is responsible for approximately 80% of these new infections and is one of the most difficult routes of transmission to control. During transmission, HIV-1 must enter or cross the stratified epithelium of the vagina or the columnar epithelium of the uterine cervix. It is unknown whether infectious virions penetrate mucosal tissues far enough to reach epithelial or subepithelial target cells, which cells are initially infected in addition to CD4+ T cells and to what extent trans-presentation by non-infected dendritic cells contributes to HIV-1 dissemination (Fackler et al., 2014).

Women constitute over 50% of the HIV infected population, although in some countries, such as South Africa, this percentage rises to approximately 60% (Carter et al., 2013). The problem is even more alarming in sub-Saharan Africa, where more than six out of every ten infected individuals is female, and young women aged 15–24 years are twice as likely to be infected with HIV compared to men of the same age. Furthermore, HIV/AIDS has become the main cause of death among women aged 15–44 years. In many cases, men and women do not share the responsibility of reducing the risk of HIV transmission because women are excluded from sex-related decision-making, do not receive in-depth sex education or have unequal access to preventive methods. That translates into 3000 new infections in women every day.

For over 20 years, the scientific community has been working hard to develop new preventive strategies to decrease HIV infection rates, especially in women. To date, despite a great deal of effort, there are no effective vaccines against HIV infection (Munier et al., 2011). Thus, it is urgent and imperative to develop prevention systems capable of stopping the spread of HIV in the most disadvantaged and vulnerable populations, especially during sexual intercourse (Anton, 2012; Stephenson, 2011). It is known that behavioral and structural interventions, such as the use of condoms or circumcision, are effective methods in the prevention of HIV sexual transmission (Padian et al., 2008; Quinn, 2007). Other pre-exposure prophylaxis (PrEP) HIV-preventive strategies, such as the oral administration of antiretroviral (ARV) drugs or topical microbicides, also seem to be feasible and effective approaches against the spread of HIV (Abdool Karim and Baxter, 2014; Balzarini and Van Damme, 2005; Buckheit et al., 2010; Granich et al., 2010).

Microbicides are compounds that when applied topically to the vagina and/or rectum can reduce the risk of HIV infection. Microbicides are advantageous over other prevention methods, such as condoms, because they are easier to use and provide users, especially women, with the ability to decide on their utilization. Microbicides are extremely advantageous to highly vulnerable groups, such as sex workers or women who suffer from sexual aggression. Due to their great potential, much effort in the past and present has been put into developing vaginal or rectal microbicides, including gels, rings and other formulations, capable of reducing the risk of HIV sexual transmission (Tables 1–3).

Table 1
Ongoing and planned Phase III and Phase II/IIb clinical trials of microbicide candidates. The only candidate to show efficacy to date is the antiretroviral drug tenofovir formulated in 1% gel form. One trial has shown benefit; another has not. Confirmatory research is ongoing in different trials. A new formulation of tenofovir gel is also being evaluated for rectal used, as a possible HIV prevention tool for anal sex. Other leading candidate in large efficacy trials is dapivirine ring. Currently, there are two ongoing efficacy trials of the dapivirine ring recruiting women from communities across several countries in East and Southern Africa. TDF – Tenofovir disoproxil fumarate; TFV – Tenofovir; FTC – emtricitabine; MSM – men who have sex with men. (Adapted from http://www.avac.org/).

Trial name	Phase	Start date	Locations	Population	Candidate(s)	Status/expected completion
Phase III (safety and effectiveness)						
FACTS 001	Phase III	October 2011	South Africa	2900 women	1% TFV gel	Ongoing/December 2014
IPM 027 (The Ring Study)	Phase III	April 2012	Rwanda, South Africa, Malawi	1650 women	4-week vaginal dapivirine ring	Ongoing/August 2015
MTN 020 (ASPIRE)	Phase III	July 2012	Malawi, South Africa, Uganda, Zimbabwe	3476 women	4-week vaginal dapivirine ring	Ongoing/December 2014
CAPRISA 008	Phase III, II	October 2012	South Africa	700 women	1% TFV gel	Ongoing/February 2015
Phase II, IIb (safety, adherence, acceptability, feasibility, effectiveness)						
MTN 017	Phase II	September 2013	Peru, South Africa, Thailand, United States, Puerto Rico	186 transgender, MSM	Daily oral TDF/FTC, Reformulated rectal 1% TFV gel	Ongoing/June 2016
MTN 019	Phase II	July 2011		384 women	1% TFV gel	Ongoing/August, 2015
MTN 023/IPM 030	Phase II	March 2014	United States	HIV-negative adolescent females	Dapivirine vaginal ring	Ongoing
MTN 024/IPM 031	Phase II	December 2013	United States	96 postmenopausal women	Dapivirine vaginal ring	Ongoing/May 2014
FACTS 002	Phase II	To be determined	South Africa	60 young women (16–17 years)	Vaginal TFV 1% gel	Ongoing

Table 2
Current Phase I microbicide clinical trials. The majority of microbicide candidates currently in clinical trials are formulated with ARV drugs and most of them have been gels. However, there are several other candidates in early phases of development. In addition, it may be useful to develop non-ARV based candidates and candidates delivered in other ways (e.g., a film or fast dissolving tablets).

Trial name	Phase	Start date	Locations	Population	Candidate(s)	Status/expected completion
Phase I (safety, adherence, acceptability, feasibility)						
CONRAD A10-114	Phase I	March 2012	United States, Dominican Republic	72 women, HIV negative	1% TFV gel, DMPA	Ongoing/January 2014
CONRAD 122	Phase I	To be determined	United States	40 men and women	SILCS diaphragm and 1% TFV gel	Protocol in development
CONRAD 118	Phase I	December 2013	United States	54 women, HIV negative	1% TFV gel, Contraceptive Vaginal Ring, Metronidazole, Terconazole	Ongoing/November 2014
CONRAD 117	Phase I	February 2013	United States	48 women, HIV negative	TFV/FTC and TFV only fast dissolve vaginal tablets	Ongoing/January 2014
CONRAD 114	Phase I	March 2012	Dominican Republic, United States	72 women	1% TFV gel, Depo-provera, Oral contraceptive	Ongoing
Project Gel	Phase I	March 2014	United States, Puerto Rico	240 MSM	1% TFV rectal gel once daily for 7 days and placebo rectal gel prior to rectal intercourse	Ongoing/March 2014
NNRTI Microbicide Gel	Phase I	March 2014	United States	30 women, HIV negative	PC-1005 gel, HEC gel	Ongoing/March 2015
MTN-014	Phase I	April 2014	United States	28 women	1% tenofovir reduced-glycerin gel	Ongoing
IPM-034	Phase I	November 2013	Belgium	40 women	Dapivirine Ring	Ongoing/May 2014
Population Council #558	Phase I	March 2014	United States	women	PC-1005 gel	Ongoing/March 2015
MTN-011	Phase I	November 2012	United States	80 women, men, HIV negative	1% TFV gel	Ongoing/April 2015
CONRAD 123	Phase I	2014		women	TFV gel/SILCS Diaphragm	Protocol in development
CONRAD 128	Phase I	Q4 2013		90 women	TFV and TFV/LNG vaginal ring	Pending

Table 3
Open label and other clinical trials of microbicide candidates. Various other clinical studies and sub-studies are currently ongoing to better understand, for instance, the adherence and use of the study products or the sexual behavior of users during their participation in the trials.

Trial name	Phase	Start date	Locations	Population	Candidate(s)	Status/ expected completion
Open label						
MTN 025	Open label/phase IIIb ASPIRE follow on		Malawi, South Africa, Uganda, Zimbabwe		Vaginal dapivirine ring	Planned
MTN 016 (EMBRANCE)	Open label/pregnancy exposure registry	October 2009	South Africa, Uganda, United States, Zimbabwe	800 women, men, HIV negative	N/A	Ongoing/ May 2013
IPM-33	Open label	August 2013	United States	160 women/men	No ring, condom only/ placebo vaginal ring + female condom used	Ongoing
IPM-29	Open label	February 2013	United States	140 women/men	Silicone elastomer vaginal ring	Ongoing/ October 2013
Other						
Effects of Tenofovir on CAPRISA samples	Pre-clinical	Q4 2013	Eastern Virginia Medical School/To be determined	To be determined	TFV	Planned 2014
MTN 003C	MTN 003 (VOICE) community sub-study	July 2010	South Africa	275 Participants (includes women in VOICE, their male partners, CAB members and community stakeholders)	N/A	Primary analysis completed
MTN 003B	MTN 003 (VOICE) bone mineral density sub-study	November 2009	Uganda, Zimbabwe	518 women	Oral TDF; oral TDF/FTC	Primary analysis completed
MTN 015	Seroconverter protocol	August 2008	Malawi, South Africa, Uganda, Zambia, Zimbabwe	500 HIV-positive women	N/A	Ongoing/ March 2014

2. History of the microbicide pipeline

Many agents with antiviral activity have been evaluated as microbicidal candidates. The first tested microbicides were surfactants, which are compounds that act as detergents and are able to destroy the integrity of lipid membranes. The most well known is the non-specific microbicide nonoxynol-9 (N-9) (Roddy et al., 1998; Van Damme et al., 2002). This compound was able to destroy the virus by damaging the viral envelope (Bourinbaiar and Fruhstorfer, 1996). However, clinical trials showed that N-9

increased the risk of HIV infection (Roddy et al., 1998). Another surfactant, C31G (SAVVY), which was shown to be safe *in vitro* and exhibited broad-spectrum activity against bacteria and herpes simplex virus-2 (HSV-2), was analyzed against HIV in three independent clinical trials in Ghana and Nigeria (Ballagh et al., 2002; Bax et al., 2002; Feldblum et al., 2008; Krebs et al., 1999; Mauck et al., 2004; Thompson et al., 1996). These trials were suspended because the rates of infection were higher in the surfactant-treated group compared to the placebo group. Currently, the surfactant candidate sodium lauryl sulfate (Invisible Condom; Université Laval, Quebec, Canada) is being investigated (Bestman-Smith et al., 2001; Mbopi-Keou et al., 2010; Piret et al., 2002). It works as an 'invisible condom' that could cover the vaginal wall as a liquid at room temperature and subsequently transform into a gel at body temperature, inhibiting the transmission of HIV-1 and other sexual transmitted diseases (STD). The results from a randomized, double blind, placebo-controlled Phase II study in Cameroonian women revealed that sodium lauryl sulfate was well tolerated and acceptable (Mbopi-Keou et al., 2010). Further phases of clinical development are planned (Gupta, 2013).

Together with surfactants, many polyanionic compounds have been analyzed as microbicides (Baranova et al., 2011; Pirrone et al., 2011). The anti-HIV activity of these compounds is associated with the establishment of electrostatic interactions between the viral envelope proteins and the anionic functional groups of the compounds. This interaction prevents the binding of the virus to the target cell, blocking viral entry. Three of these polyanionic compounds, Carrageenan (Carraguard), cellulose sulfate and PRO2000, were effective *in vitro* and in preclinical trials, although none showed any efficacy in clinical trials in humans (Gibson and Arts, 2012; Huskens et al., 2009; Keller et al., 2010; Pirrone et al., 2011; Reina et al., 2010). Surprisingly, in some cases, such as with cellulose sulfate, higher rates of HIV infection were found in the treated group compared to the placebo group (Tao et al., 2008). Several rationales were proposed to explain the failure of these polyanionic compounds, including their lower inhibitory effects in comparison to other antivirals, poor absorption by the mucosa, reduced efficacy in the presence of seminal plasma, or the induction of local inflammation and changes in vaginal microflora, all of which could facilitate HIV-1 infection (Pirrone et al., 2011).

Currently, the leading candidates in the microbicide pipeline are ARV (Chirenje et al., 2010; Granich et al., 2010; Klasse et al., 2008; Omar and Bergeron, 2011; Shattock and Rosenberg, 2012). These compounds exhibit potent and specific anti-HIV activity and are safe, as demonstrated by their continuous use in highly active antiretroviral therapy (HAART). Currently, more than thirty ARV targeting different steps of the viral cycle have been approved by the U.S. Food and Drug Administration (FDA) for use in HAART (Fig. 1). HIV inhibitors, including entry inhibitors, nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI, respectively) or integrase and protease inhibitors have been used as potential microbicide candidates. The only candidate to show high efficacy to date is the NRTI Tenofovir (TFV) (Abdool Karim et al., 2010; Gengiah et al., 2012; Mertenskoetter and Kaptur, 2011). TFV formulated as a 1% gel was tested in the CAPRISA 004 trial in 889 South African women, resulting in a 39% overall reduction in the women's risk of contracting HIV infection via vaginal sex (Network, 2010). However, other clinical trials failed, such as VOICE, a five-armed proof-of-concept clinical trial that enrolled more than 5000 women in South Africa, Uganda and Zimbabwe (Friend and Kiser, 2013). The VOICE study was prematurely stopped in 2011 due to lack of efficacy. It concluded that none of the interventions tested (daily oral TFV, daily oral TFV/emtricitabine or daily TFV gel) reduced the risk of HIV transmission. Some reasons have been provided to explain the apparent lack of efficacy, particularly the low adherence. Most participants did not use the tested interventions daily as recommended. Thus, confirmatory research is ongoing, and trials with other formulations of TFV are planned (Network, 2013a). For instance, CAPRISA 008 will assess the feasibility and effectiveness of providing the TFV gel in a clinic setting. A new reduced glycerin formulation of the TFV gel is being evaluated for rectal use in the MTN 017 trial as an HIV prevention system for rectal transmission (Network, 2013b). This is particularly relevant to homosexual communities and other men who have sex with men, especially when the risk associated with HIV transmission through unprotected receptive anal intercourse is 1.7% per act (versus 0.08% in case of unprotected vaginal intercourse). This is due to anatomical differences between vaginal and rectal tissues. The rectal epithelium is formed by a single layer of cells as opposed to the multilayer squamous epithelium found in the vagina and ectocervix. In addition, the gastrointestinal tract is populated with a large number of HIV-1 infectable cell subsets (van Marle et al., 2007). All these factors make the rectal route a more susceptible route for HIV infection. Several rectal microbicide clinical trials testing others antiretrovirals such as UC781 have been conducted, as deeply reviewed by Rohan et al. (2013).

Another candidate in large-scale efficacy trials is the NNRTI dapivirine. It was formulated as a vaginal ring, and currently there are two ongoing phase III clinical trials (ASPIRE and IPM 027) in Africa (Microbicides, 2012; Network, 2012). Another phase I clinical trial, MTN 013/IPM 026, has studied the effectiveness of the combination of dapivirine and maraviroc as a microbicidal vaginal ring (Fetherston et al., 2012). Results were presented this year at the 21st Conference on Retroviruses and Opportunistic Infections (CROI). MTN-013/IPM 026 found that the ring was safe in women. In addition, dapivirine was able to block HIV infection, though levels of maraviroc were not sufficient to have a similar effect (Chen et al., 2014, (CROI)).

Because CCR5-tropic viral strains are dominant during HIV-1 sexual transmission, entry inhibitors, such as CCR5 antagonists, have also been evaluated as microbicide candidates (Maeda et al., 2012). CCR5 antagonist CMPD167, a cyclopentane-based compound used as a vaginal gel that has provided protection from vaginal SHIV challenge in macaques, and maraviroc, a small molecule that binds to the CCR5 co-receptor and blocks HIV-1 entry into cells, are being tested in humans. A proof-of-concept study in macaques showed that maraviroc could play a role in protecting women from HIV-1 infection if developed as a vaginal microbicide (Veazey et al., 2010). A major challenge in considering the use of CCR5 inhibitors as topical microbicides is their inability to block the entry of X4-tropic viruses. Although infection via the CXCR4 co-receptor is less important in sexual transmission, X4-tropic viruses are present in vaginal fluids and semen during acute HIV infection (Grivel et al., 2011). Thus, the development of broad spectrum topical microbicides capable of preventing HIV transmission regardless of viral tropism is crucial.

There are several other novel ARV-based and non-ARV-based candidates in the early phases of preclinical development. For instance, the cyclic antimicrobial peptide Retrocyclin RC-101 (Gupta et al., 2013), the NRTI saquinavir (Stefanidou et al., 2012) and rilpivirine (De Clercq, 2012), the fusion inhibitor cyanovirin (Tsai et al., 2003) and zinc-derived compounds (Kenney et al., 2011) are also under development. Interestingly, griffithsin, a carbohydrate-binding agent (CBA) extracted from red algae, is expected to enter in a phase I safety and acceptability trial in the next year (MTN-038) (Emau et al., 2007; Hillier, 2013). A summary of the clinical trials of microbicide candidates is shown in Tables 1–3. The outcomes of these trials currently underway will significantly impact on the development and search of new candidates. However, there are some limitations that could explain the modest success of the use of antiretroviral drugs in preventive approaches

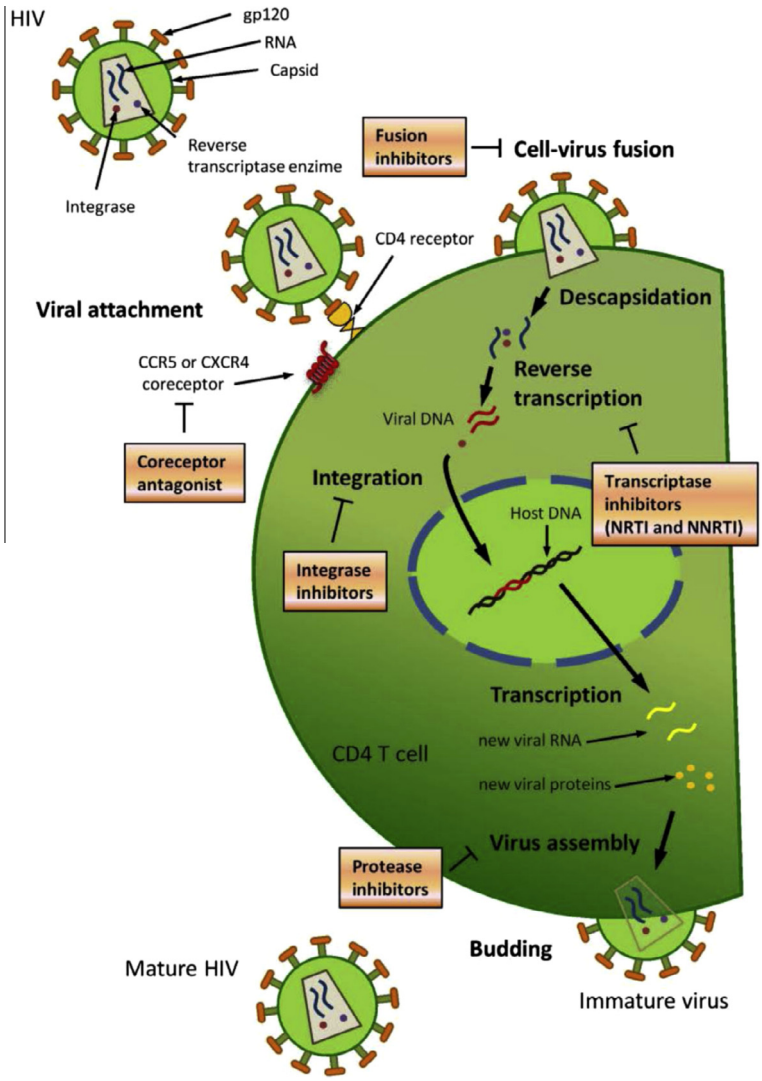


Fig. 1. Therapeutic targets of the HIV life cycle. Viral entry requires the binding of gp120 to the cellular receptor CD4 and a co-receptor (CCR5 or CXCR4). Co-receptor binding causes changes in gp120 and the gp41 transmembrane peptide, leading to membrane fusion. CCR5 inhibitors, such as maraviroc (Celsentri®), prevent viral binding to this co-receptor. Fusion inhibitors as Enfuvirtide (Fuzeon®) bind to gp41 and prevent the conformational changes required for viral cell fusion. In the cytoplasmic compartment, the internalized viral core and viral enzyme reverse transcriptase copies the viral RNA into a DNA strand which is transported into the nucleus. Analogue reverse transcriptase inhibitors and non-nucleoside inhibitors (NRTI and NNRTI), such as tenofovir (Viread®), abacavir (Ziagen®) and efavirenz (Sustiva®), inhibit this process. In the nucleus, the viral integrase incorporates the viral DNA into the cellular genome, a step blocked by integrase inhibitors, such as raltegravir (Isentress®). The integrated proviral DNA is transcribed to generate complete viral RNA sequences, and multiple mRNAs are translated into proteins using cellular machinery. The viral RNA and viral proteins are transported to the cell membrane, where they are assembled with cellular factors within the viral particle. Budding of viral particles occurs simultaneously with the cleavage of precursor Gag and Gag-Pol by the viral protease, resulting in a mature virion. Protease inhibitors, such as atazanavir (Reyataz®) and darunavir (Prezista®), block the budding step and prevent the formation of mature virions.

against HIV, such as their extreme hydrophilic and hydrophobic nature or their low enzymatic and chemical stability.

3. Nanotechnology: a new field in the design of microbicides

In recent years, nanotechnology has emerged as a new research area capable of providing solutions to the limitations found in the development of preventive strategies against HIV. This field is focused on designing, engineering and fabricating new materials at the nanosize level. According to the National Nanotechnology Initiative, nanotechnology involves study of materials/architectures of size 1–100 nm in at least one dimension. Nevertheless, materials with size up to several hundred nanometers are also included under nanotechnology. These materials can be classified into several groups, such as liposomes, polymers, dendrimers, nanocrystals, nanofibers and metal nanoparticles, according to the material used in their assembly (Table 4). In biomedical applications, nanotechnology has enabled the development of novel nanodrugs and compounds with useful pharmacological advantages in the therapy and diagnosis of cancer and infectious diseases, including HIV (Kim et al., 2010a; Kim and Read, 2010; Siccardi et al., 2013).

Various nanosystems have been used in different strategies for the treatment and prevention of HIV-1 infection (Date and Destache, 2013; Mahajan et al., 2012; Mallipeddi and Rohan, 2010; Mamo et al., 2010). Some nanoplatforms, due to their nanometric size, are versatile drug delivery systems because of their capacity to overcome corporal barriers and deliver drugs to specific cells or intracellular compartments. Different methods of cargo delivery include encapsulation in the particle core or absorption onto their functionalized surface, thereby improving the pharmacological properties of several ARV. For instance, nanosuspensions of rilpivirine stabilized by polyethylene–polypropylene glycol and

PEGylated tocopheryl succinate ester resulted in extended sustained release of the compound in animal models, suggesting that nanoscale drug delivery may potentially reduce dosing frequency and improve adherence (Baert et al., 2009).

Nanoplatforms can also be used to control the release of nucleic acids in gene therapy against HIV, or as new antiviral agents due to their intrinsic antiviral properties (Amiji et al., 2006; Briz et al., 2012; Cordoba et al., 2013b,c; Chonco et al., 2012; das Neves et al., 2010; de Las Cuevas et al., 2012; Farokhzad, 2008; Jimenez et al., 2010; Mallipeddi and Rohan, 2010; Perise-Barrios et al., 2014; Sanchez-Nieves et al., 2014; Weber et al., 2008, 2012; Meng et al., 2011; Steinbach et al., 2012). These nano-architectures have also been used in different approaches to design novel topical microbicides (Date and Destache, 2013; Ensign et al., 2014). Polymeric biodegradable nanoparticles, liposomes, nanofibers and inorganic nanoplatforms are being explored as platforms for vaginal drug delivery.

TFV and tenofovir disoproxil fumarate nanoparticles composed of a blend of poly-lactic-co-glycolic acid (PLGA) and pH-responsive polymethylmethacrylate (Eudragit) polymer, dapivirine-loaded poly(epsilon-caprolactone) nanoparticles, raltegravir-efavirenz-loaded PLGA nanoparticles or PLGA nanoparticles with encapsulated PSC-RANTES (an antagonist of the CCR5 co-receptor) have already been tested in animal models against HIV (Belletti et al., 2012; Chaowanachan et al., 2013; das Neves et al., 2012a, 2013; Ham et al., 2009; Zhang et al., 2011; Ensign et al., 2014). PSC-RANTES encapsulation within PLGA nanoparticles provided sustained release and increased uptake into *ex vivo* cervical tissue compared to soluble PSC-RANTES. Other major study has shown that PSC-RANTES is effective and provides potent protection against vaginal challenge in rhesus macaques, indicating its potential as microbicide candidate (Lederman et al., 2004).

Liposomes composed of phospholipid bilayer structures were investigated as antiviral agents due to discovery that certain lipid compositions can bind to HIV particles and modulate their infectivity. Moreover, lipid-based nanoparticles have been designed for vaginal delivery of drugs or small interfering RNA to silence HIV gene expression and prevent the establishment of primary infection (Malavia et al., 2011; Wang et al., 2012).

The ability of nanofibers, which are spaghetti-like compounds made from materials, such as polysaccharides, peptides or polymers with diameters ranging from 1 to 1,000 nm, to deliver different microbicides for HIV prevention has been explored. Maraviroc-loaded nanofibers, nanofibers made of cellulose acetate phthalate (CAP) that acts as an HIV entry inhibitor and CAP fibers loaded with TFV have shown great potential in inhibiting HIV infection (Gunn and Zhang, 2010; Huang et al., 2012).

Furthermore, the antiviral properties of inorganic metals, such as silver, gold and zinc, have been exploited in the search for novel topical microbicides. Polyvinyl pyrrolidone-coated silver nanoparticles inhibited viral fusion and demonstrated antiviral activity against various HIV strains, including drug-resistant strains (Lara et al., 2010a,b). Gold nanoparticles contain free thiol groups on their periphery that permit their easy functionalization with various biomolecules. Multivalent oligomannosides-coated gold nanoparticles and anionic sulfate-functionalized gold nanosystems have demonstrated inhibitory activity against HIV *in vitro* (Di Gianvincenzo et al., 2010; Vijayakumar and Ganesan, 2012).

Dendrimers are the most advanced group of nanotech-derived microbicides (Lazniewska et al., 2012; Parboosing et al., 2012; Svenson and Tomalia, 2005). Dendrimers are a class of hyper-branched polymeric nano-structures that are much smaller than traditional nanoparticles, with narrow polydispersity and precise numbers of terminal groups. Dendrimer structure and composition, including the number of branching units and the composition and number of surface functional groups, can be engineered to obtain compounds with the desired physicochemical and biological properties. The peripheral functional groups can be designed to directly interact with target cells or pathogens, conjugate drugs, or complex with other biomolecules, such as peptides or siRNA.

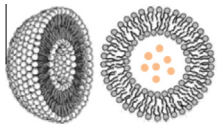
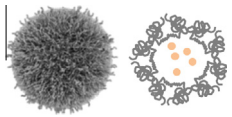
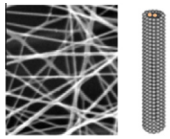
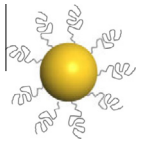
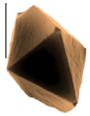
One dendrimer based-compound, VivaGel, is the only nanotech-derived microbicide candidate that has reached human clinical trials (Bernstein et al., 2003; Gong et al., 2005; Jiang et al., 2005; Telwatte et al., 2011; Tyssen et al., 2010). The active ingredient in VivaGel is the SPL7013 dendrimer, which is a polylysine anionic dendrimeric agent. It has demonstrated great potency against both HIV-1 and HSV-2 (Price et al., 2011; O'Loughlin et al., 2010), although the phase I clinical trial evaluating its antiviral activity and microbiota tolerance revealed evidence of mild irritation after repeated vaginal use (McGowan et al., 2011). VivaGel is not currently being tested in clinical trials as a topical microbicide against HIV; nevertheless, it is in phase II trials as a treatment for bacterial vaginosis. Other dendrimeric compounds are in development for use as vaginal microbicides (Date and Destache, 2013; Navath et al., 2011). Dendrimers containing a carbosilane core and different anionic ended functional groups have been studied as anti-HIV compounds (Chonco et al., 2012; Galan et al., 2014; Garcia-Gallego et al., 2012; Rasines et al., 2012; Vacas Cordoba et al., 2013). These dendrimers have shown high biosafety in human epithelial cell lines derived from the female genital tract and in primary human blood cells (PBMC), blocked the entry of both X4- and R5-tropic HIV-1 isolates into epithelial cells while protecting the epithelial monolayer from cell disruption, reduced HIV-1 infection of PBMC and were safe in mouse and rabbit models, with no promotion of irritation or vaginal lesions (Chonco et al., 2012; Vacas Cordoba et al., 2013). Anionic dendrimers comprising benzhydryl amide cores and lysine branches and functionalized with naphthalene disulfonic acid or 3,5-disulfobenzoic acid groups have demonstrated activity against HIV-1 and HSV-2 infection, preventing viral entry and HIV envelope-mediated cell-to-cell fusion (Telwatte et al., 2011; Tyssen et al., 2010). Polycationic "viologen" based dendrimers have also shown anti-HIV activity by interaction with the CXCR4 co-receptor (Asaftei et al., 2012). Other dendrimers, such as four generation-PAMAM branched-naphthalene disulfonic surface groups ending with SPL2923 or polysulfonated dendrimer BRI2923, have shown inhibitory effects against HIV-1 not only at the entry step but also at later stages of the viral replicative cycle (Witvrouw et al., 2000). Multivalent dendrimeric compounds coated with carbohydrates expressed on immune cells inhibited HIV-1 infection by targeting viral entry, whereas multivalent-glycodendrimeric compounds prevented HIV transmission via DC-SIGN on dendritic cells (Garcia-Vallejo et al., 2013; Rosa Borges et al., 2010).

Although nanoparticles are under development as useful and powerful tools in the field of antiviral microbicides, they display a series of limitations that should be addressed because they can affect the transfer of these platforms to clinical trials. These limitations include toxicity, the appearance of non-desirable biological interactions, bioaccumulation, the enzymatic degradation of these nanosystems, their penetrance and absorption features into different tissues and their high manufacturing cost; this last limitation is especially a problem for scale-up production (Mamo et al., 2010).

4. Moving towards pre-clinical development of nanotech-derived microbicides: the main stages

Rigorous pre-clinical evaluation of vaginal or rectal candidate microbicides is essential for the selection of the best compounds for continued development. This involves a series of basic steps designed to evaluate and guarantee safety and efficacy (Buckheit

Table 4
Important features and schematic structure of nanosystems used in the development of nano-based HIV topical microbicides. Different nano-architectures (liposomes, dendrimers, polymeric nanoparticles, nanocrystals, nanofibers and inorganic nanoparticles) are being explored as novel microbicide candidates. The most advanced in the microbicide pipeline are liposomes, polymers and dendrimers. Liposomes are spherical vesicles that comprise one or more lipid bilayer structures enclosing an aqueous core. They are high-potency carriers that protect encapsulated drugs from degradation. Polymeric nanoparticles are basically solid polymers with drugs embedded in the polymer matrix or with an inner space loaded with the drug of interest. Dendrimers are highly branched polymers with a controlled three-dimensional structure around a central core that can be easily functionalized. (Adapted from Date and Destache (2013), *Biomaterials*).

Nanocompound	Architecture	Features	Candidates explored *in vivo*
Liposomes		• Biocompatible, completely biodegradable and non-immunogenic • Encapsulation of hydrophobic, amphipathic and hydrophilic drugs • Ability to protect encapsulated drugs from the external environment and to act as sustained release depots • Available technologies for large-scale production	• Anti CCR5 siRNA-loaded liposome (Kim et al., 2010a) • Cardiolipin liposome (Malavia et al., 2011)
Dendrimers		• Controlled synthesis and narrow polydispersity • Different molecules/drugs could be conjugated to a single dendrimer • Due to their hyperbranched architecture, they can interact in a multivalent way • They can be tailored by manipulating their structure or composition and number of surface functional groups to obtain compounds with desired properties	• Vivagel (SPL7013 dendrimer) (O'Loughlin et al., 2010) • Sulfonated carbosilane dendrimer 2G-S16 (Chonco et al., 2012) • Tenofovir-emtricitabine-loaded thiopyridine terminated G4-PAMAM dendrimer (Navath et al., 2011)
Polymeric nanoparticles		• There are a wide range of biodegradable and non-biodegradable materials for desired application • Sustained release of encapsulated agents • Improved pharmacokinetic, solubility and stability of encapsulated agents • They can be used in targeted-delivery by tailoring their surface	• PSC-RANTES loaded PLGA-nanoparticles (Ham et al., 2009) • siRNA-loaded PLGA nanoparticles (Steinbach et al., 2012) • Tenofovir-loaded pH-sensitive PLGA nanoparticles (Zhang et al., 2011) • Tenofovir-loaded chitosan nanoparticles (Meng et al., 2011) • Tenofovir loaded hybrid chitosan/PLGA nanoparticles (Belletti et al., 2012) • Dapivirine-loaded polycaprolactone nanoparticles (das Neves et al., 2012a,b)
Nanofibers		• A wide variety of materials can be assembled into nanofibers • Large surface area and many active surface sites • Delivery and sustained drug release • Easy production process: electrospinning	• Tenofovir-loaded cellulose acetate phthalate nanofibers (Huang et al., 2012)
Inorganic nanoparticles		• High surface-to-volume ratios • Manipulable optical and surface characteristics • Multiple ligands/drugs could be conjugated to surface using appropriate chemistry • Applications in electrooptic devices, biomedical imaging, diagnostic or therapeutic field	• Silver nanoparticles coated condoms (Mohammed Fayaz et al., 2012)
Nanocrystal		• Drug molecules are micronized in nano-scale crystals using top-down/bottom-up techniques • Nanocrystals are composed of 100% drug; there is no carrier material • Increase of dissolution velocity by surface area enlargement • Increase in saturation solubility and bioavailability • There are feasible technologies for large-scale production	

and Buckheit, 2012). There are different parameters that need to be studied in several *in vitro* and *in vivo* models (Fig. 2).

4.1. Identification of novel candidates: in vitro screening

4.1.1. Characteristics of ideal nanotech-derived microbicide candidates

The discovery and development of new nanocompounds as microbicides with antiviral properties is difficult because these agents need to exhibit a series of desirable properties. They must be non-toxic, safe for prolonged use and highly effective against HIV-1 and other STDs (Fig. 3). They must also be easy to use, odorless and colorless, inexpensive and easy to manufacture and scale-up. Moreover, they must minimally damage the mucosa, be safe to the microflora found at the target mucosal tissue and not interfere with the efficacy and safety of other therapeutic agents, such as contraceptives. Because microbicides should diminish viral transmission both vaginally and rectally, different formulations need to be designed to prevent HIV transmission by these routes (Morrow and Hendrix, 2010; Turpin, 2011). The microbicide's effectiveness should not be influenced by factors, such as seminal plasma or pH changes, that occur in the vagina during sexual intercourse. Thus, rational design of these nanoplatforms could be crucial for optimal performance in the vaginal and rectal environments (Fahmy et al., 2008). Properties such as mucus-penetration ability and sustained drug release capacity are important for improving vaginal drug delivery in HIV prophylactic approaches (Ensign et al., 2014). For instance, PEG surface density has been shown to be important for efficient mucus penetration (Cu and Saltzman, 2009; Tang et al., 2009). Recent progress in the design of biodegradable compounds, together with the regulation of nanosystem design and preparation, makes it possible to precisely manipulate the nanoparticle's architecture, molecular weight and chemical composition, resulting in predictable tuning of their biocompatibility as well as their delivery.

4.1.2. Antiviral assays

Initial screening assays provide assessments of the compound's efficacy (EC_{50}: 50% reduction in HIV production from treated and untreated cells) and allow the identification of inhibitory compounds with direct antiviral activity or with the capacity to inhibit a specific step of the HIV life cycle. Human epithelial cell lines from appropriate target tissues, such as the vaginal mucosa-derived VK2/E6E7, uterine mucosa-derived HEC-1A or colorectal epithelium Caco-2 cell lines are commonly used in the study of transepithelial infection process (Gali et al., 2010a,b). Although infection of epithelial cells has been previously described, it is most probably not productive (Dezzutti et al., 2001). True early target cells of sexually acquired HIV include intra-epithelial cells such as Langerhans cells, subepithelial macrophages or CD4+ T lymphocytes. These PBMCs subets and the CCR5-expressing TZM-bl reporter cell line

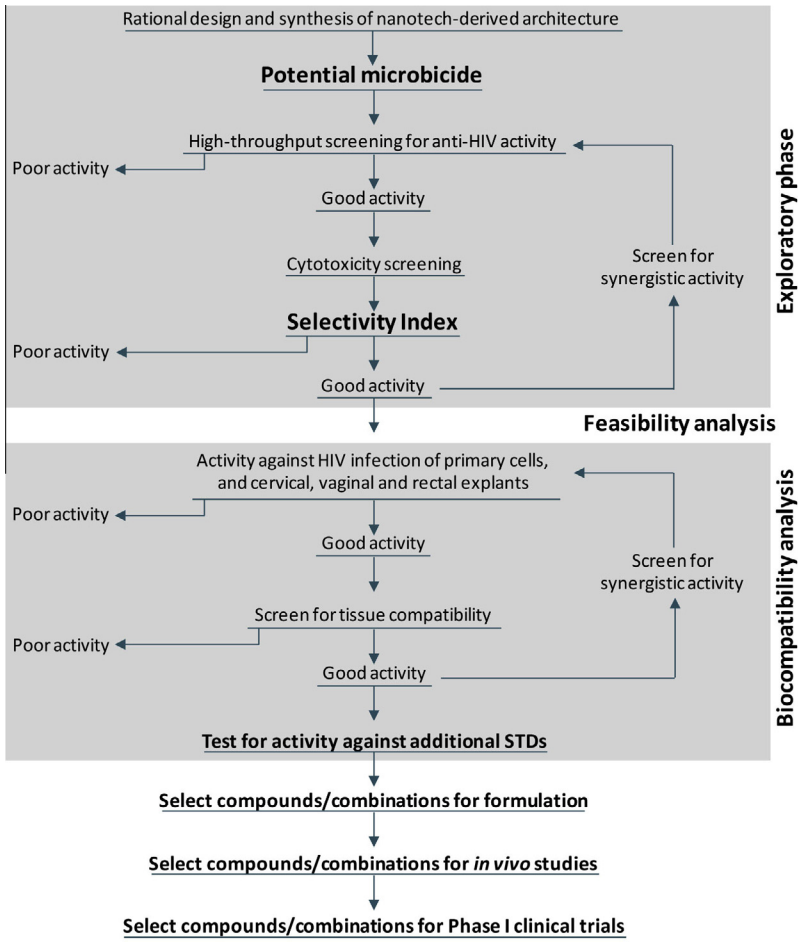


Fig. 2. Algorithm for selection and evaluation of HIV microbicide candidates. It comprises the main steps for a rationale preclinical development of novel nano-based anti-HIV agents, including the evaluation of compound efficacy and toxicity in relevant cell-based systems, the range and mechanism of action of candidate compounds, the interactions of the compound in combination with other approved antiretrovirals, *in vitro* and *in vivo* pharmacokinetic, pharmacodynamics and safety evaluations and efficacy studies in sensitive models such as non-human primate or humanized mice. Adapted from Science Working Group of the Microbicide Initiative, *The Science of Microbicides: Accelerating Development*, 2002.

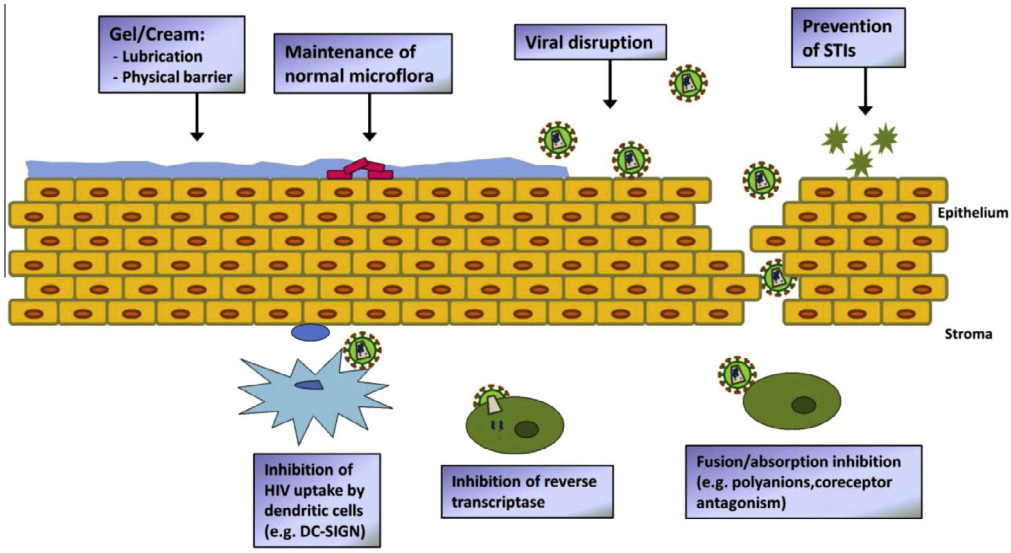


Fig. 3. Potential activity of topical microbicides in vaginal epithelium. Microbicides should have not only anti-HIV activity, but they must be effective against other STDs that can increase the risk of HIV transmission by increasing the risk of epithelial inflammation and ulceration. Polyanionic compounds can disrupt the viral envelop or block cell-virus fusion. An acidic pH is necessary for natural vaginal protection against pathogens. Therefore it is important that microbicides do not alter the normal vaginal microflora. When the virus crosses the epithelial barrier it is taken up dendritic cells prior to infection of T cells and the recruitment of additional susceptible cells (adapted from Shattock et al., *Nat Rev Microbiol,* 2003).

are the best choice in the primary evaluation of microbicide candidates.

Because R5-tropic viral isolates are mainly involved in HIV sexual transmission, the evaluation of the antiviral activity of nanoparticles against these viral strains is relevant because the majority of sexually-transmitted infections are via the CCR5 co-receptor. CD4+ T cells can be found along the lamina propia in the human vagina (endo- and ectocervix), often under the basal membrane (Edwards and Morris, 1985). Most are memory T cells that have increased CCR5 expression compared to T cells circulating in the peripheral blood (Hladik and McElrath, 2008; Hladik et al., 2007; Zhang et al., 1998). Nevertheless, it is important to remember that CXCR4-tropic viral strains are also present in vaginal fluids and semen. Therefore, to assure broad spectrum and potent antiviral activity, microbicide candidates should be tested against CCR5-, CXCR4- and dual-tropic viral strains. The most common R5-tropic laboratory strains are HIV-$1_{JR\text{-}CSF}$, HIV-1_{BaL} and HIV-$1_{NL(AD8)}$ (B subtype) and the most common dual-tropic strain is HIV-1_{CBL23}. All of these strains infect human primary cells and cell lines productively. However, to evaluate the efficacy of nanoparticles, testing their antiviral activity against different subtypes of primary clinically isolated viral strains is also necessary. Owing to the difficultly involved in using primary viral strains, working with established laboratory viral strains is an easier and quicker first step of *in vitro* primary screening of novel candidates.

4.1.3. Safety and biocompatibility of nanoparticles

Once the inhibitory potency of new compounds is established, it is important to evaluate their safety and 50% toxic concentration (TC_{50}) in different primary cells and cell lines. Different commercial assays are available for assessing nanoplatform toxicity in cells (Duncan and Izzo, 2005), the most common being the MTT assay. Other closely related colorimetric assays, including XTT and MTS, are also used. Studying cell membrane integrity is another common method to measure cell viability and cytotoxic effects. Vital dyes are frequently employed, such as trypan blue, 7-aminoactinomycin D (7-AAD) or propidium iodide. Alternatively, enzymatic assays can be used to monitor the passage of molecules though the membrane, such as the LDH assay.

Nanoparticle cytotoxicity depends on different features, such as nanoparticle size, surface charge and structure hydrophobicity/hydrophilicity as reviewed by Date and Destache (2013). For instance, in cationic nano-architectures, the chemical structure of used cationic lipids can dramatically impact their efficacy, cellular uptake and toxicity. Nanosystems composed of cationic lipids with single alkyl chain were more toxic and incapable of transport through cervicovaginal mucus in comparison to nano-architectures composed of cationic lipids with two alkyl chains (das Neves et al., 2012b).

Molecular weight is another important property that governs performance and toxicity of nano-architectures. In case of polyethylenimines (PEI), polymer molecular weight has an enormous influence on their transfection capability, antiviral activity and cytotoxicity. Size dependent toxicity has been also observed for silver nanoparticles. 15 nm sized-silver nanoparticles were significantly more toxic to macrophages than 30–55 nm silver nanoparticles (Carlson et al., 2008). On the contrary, PLGA nanoparticles did not show size dependent toxicity in macrophages, showing that, other factors, such as material type, are also crucial in size dependent responses (Xiong et al., 2013).

Surface characteristics such as surface charge, hydrophilicity or surface functional groups have significant impact on nanoparticles behavior. Several studies have shown that positively charged nanocarriers have higher cytotoxicity as compared to anionic and neutral nanocarriers. Oral administration of positively charged dendrimers to mice was found to cause adverse effects such as hemobilia and splenomegaly, whereas negatively charged dendrimers did not show adverse effects and were well tolerated even at 10-fold higher dose (Thiagarajan et al., 2013).

The decoration of nanosystem surfaces with biocompatible groups and moieties, such as sugars or as PEG, could also improve nanocompound biocompatibility. Therefore, a thorough understanding of the relationships between the physicochemical properties and the behavior of nanomaterials in biological systems is highly necessary for designing safe and efficacious nano-based microbicides. Novel techniques such as quantitative structure–activity relationship (QSAR) methods can help to establish such relationships (Burello and Worth, 2011).

Another important point to consider when developing a vaginal microbicide is that the compound should not alter the normal vaginal flora or have spermicidal activity. Normal vaginal flora is composed of different *Lactobacillus* species, mainly *Lactobacillus crispatus* and *Lactobacillus iners* but also *Lactobacillus jensenii* and *Lactobacillus* , as well as other minority populations such as *Atopobium vaginae, Megasphaera* or *Leptotrichia* species (Martin, 2012). Finally, it is important that the compounds remain stable and maintain their antiviral activity in an acidic medium, such as that present in the human female vagina (pH $\approx$ 4–5.8). Factors, such as seminal plasma or pH changes, that occur in the vagina during the sexual act can diminish the effectiveness of microbicides. An acidic pH is necessary for natural protection of the vagina against pathogens. Therefore, it is important to test the effectiveness of compounds in an acidic medium and in the presence of seminal plasma to probe their stability, safety and efficacy.

4.1.4. HIV-transmission inhibition assays

Sexual transmission is one of the main routes of HIV transmission, mediated by exposure to infected cells or seminal fluid secretions in the mucosa. Currently, whether primary HIV infection is due to transmission of cell-free virus or cell-associated viruses is not well understood. The vaginal or rectal epitheliums are the first sites of deposition for cell-free or cell-associated viruses contained in the semen during sexual intercourse. The use of transwell permeable supports has been proposed as a model of these physiological conditions that can be used to study the potential of microbicide candidates to block the transmission of cell free or cell-associated HIV particles through the genital epithelium (Chonco et al., 2012; Gali et al., 2010a). This dual-chamber system consists of a tight epithelial cell layer or explants from vaginal, uterine cervical, endometrial or rectal mucosa grown on a transwell insert, and a basal chamber with PBMC. This model can be used to monitor whether treatment of the epithelial cells with the microbicide candidate prevents transmission of cell-free or cell-associated virus through the epithelial cell monolayer, simulating natural viral transmission in the vaginal/rectal epithelium. The infection of indicator cells, such as PBMC, in the lower chamber of the transwell is used to evaluate HIV transepithelial transmission. Moreover, transepithelial electrical resistance (TEER, Ωxcm^2) across the monolayer of epithelial cells can be monitored using a voltmeter and an electrode to investigate whether alterations of tight junction dynamics in the cell cultures are induced by treatment with the compounds. Several studies have shown the importance of using human vaginal or rectal explants to test the antiviral characteristics of the compounds in a physiologically relevant manner (Gali et al., 2010b; McElrath et al., 2010; Rohan et al., 2010). Interestingly, some nanoparticles have been already tested using these models. Polyanionic carbosilane dendrimers G3-S16 and G2-NF16 were able to prevent the transmission of cell-associated R5-HIV particles through transepithelial monolayers in transwell assays and they could block HIV internalization inside epithelial cells showing ability to protect the integrity of the monolayers (Vacas Cordoba et al., 2013).

4.1.5. Investigation of nanoparticle inhibitory mechanisms

Following determination of the potency and biosafety of microbicide candidates, the next relevant step is to investigate the mechanisms by which these compounds inhibit infection. Ideal agents are those that block HIV-1 infection at early stages of the HIV-1 life cycle prior to viral integration, primarily by impeding HIV attachment, entry, fusion or reverse transcription. To date, the majority of nanotech-based microbicides act as HIV entry inhibitors. Due to their mechanism, they prevent the integration of the viral genome into the DNA of target cells, avoiding the primary infection and the establishment of a viral latent reservoir. Nevertheless, other nanocompounds with a late mode of action such as protease inhibitors (PI) could be also very efficacious blocking HIV sexual infection. PI are successfully used in HAART and present some advantages for their development as microbicide candidates. They exhibit a low adsorption in epithelium that may be advantageous in ensuring high local drug concentrations with minimal systemic exposure. Moreover, PI are the agents with the highest genetic barrier to resistance among ARV and due to their delayed antiviral activity, inhibiting maturation but not viral integration, an increased window of protection could be offered by these microbicide candidates (Herrera and Shattock, 2012), even exhibiting efficacy after post-coital application. Molecular modeling is an easy and potent tool to evaluate the targets on which the compounds exert their effects (Xue et al., 2014). For instance, molecular modeling was useful to establish the interaction between carbosilane dendrimer 2G-S16 and gp120 and CD4 proteins (Chonco et al., 2012). Atomistic molecular dynamics simulations of complexes 2G-S16/CD4 and 2G-S16/gp120 showed that dendrimer bound to cationic areas in those proteins such as gp120-V3 loop region, ultimately blocking gp120–CD4 interaction and thus the attachment step. Furthermore, molecular modeling can also be used to tailor and improve candidates in the search for more effective compounds. Other mechanistic studies, including cell-based and biochemical and enzymatic assays, can define the mode of action of the tested nanocompound. These include time-of-addition assays, in which a variety of antiretrovirals targeting different stages of the viral life cycle are compared to the activity of the tested compound at different time points, or gp120 capture ELISAs, which study the inhibitory activity of compounds on gp120–CD4 binding (Lara et al., 2010a).

4.1.6. Combination assays

The combination of at least two families of ARV is more effective than the use of monotherapy for HIV treatment. Therefore, the combination of different compounds should be taken into consideration when designing new microbicide strategies (Anton, 2012; Balzarini and Schols, 2012). However, combination therapy has various significant disadvantages such as multiple drug–drug interactions, the potential development of multidrug resistances or the additive toxicity from the combination therapy leading to poor adherence. Nanotechnology can help to overcome these limitations. Nanomaterials have emerged as promising tools to increase the delivery of antiretroviral drugs or improve their pharmacokinetic and pharmacodynamic properties, certainly generating positive interactions. Polyanionic carbosilane dendrimers combined with different ARV, such as maraviroc or TFV, show synergistic activity against several laboratory and primary HIV isolates (Cordoba et al., 2013a; Sepulveda-Crespo et al., 2014; Vacas-Cordoba et al., 2014). Efavirenz and saquinavir were loaded into PLGA nanoparticles in another combinatorial microbicide candidate study. The drug released from the nanoparticles was effective at inhibiting R5-tropic HIV in the human TZM-bl cell line (Chaowanachan et al., 2013). These combinatorial strategies have the potential to block HIV-1 infection at different stages of the HIV-1 life cycle, and may explain the positive interaction outcomes that are commonly observed. Moreover, targeting multiple stages within the HIV-1 replication cycle may decrease the risk of transmission of resistant viral isolates as well as reduce the development of resistance. Decreasing the amounts of antiviral compounds used *in vivo* might result in the reduction of adverse effects, limiting local toxicity and inflammation caused by topical application of the microbicide before sexual intercourse. Several software packages are available to analyze and statistically evaluate the outcome of combination assays (Buckheit and Lunsford, 2009; Chou and Talalay, 1984).

4.1.7. Broad spectrum activity of topical microbicides

A desirable consideration in the development of new microbicides is not only effectiveness against diverse primary isolates, including clinical, drug resistant and multidrug resistant HIV viral strains, but also against other sexually transmitted pathogens, such as herpes simplex virus, hepatitis viruses, human papillomavirus and causative agents of bacterial and fungal vaginosis including *Candida albicans*, *Neisseria gonorrhoeae* or *Gardnerella vaginalis*. Therefore, it is crucial to evaluate the range of action of topical microbicide agents.

It is especially important to develop effective microbicides against herpes simplex type 2 (HSV-2), the virus that causes genital herpes. HSV-2 infection is the most prevalent STD worldwide, with more than 500 million people infected and approximately 20 million new infections occurring each year. Moreover, genital herpes is an important co-factor for HIV-1 transmission; individuals infected with HSV-2 have a threefold higher risk of acquiring HIV-1 infection (Thurman and Doncel, 2012). Several mechanisms may account for this HSV-2-mediated facilitation of HIV-1 infection. HSV-2 infection causes ulceration of the genital mucosa, increasing the risk of HIV-1 acquisition. Furthermore, latent HSV-2 infection increases the levels of CD4 and CCR5 expression on lymphocytes and macrophages and the levels of DC-SIGN on dendritic cells in the genital mucosa, increasing the probability of HIV infection (Rebbapragada et al., 2007; Sartori et al., 2011).

As opposed to HIV, which primarily infects cells of the immune system, HSV-2 infects epithelial cells, neurons and other cell types. Therefore, distinct attachment and entry pathways are involved in HSV-2 entry into target cells. This attachment includes interactions between several glycoproteins in the viral envelope (gB, gD, gH, and gL) and a variety of cell surface molecules, including heparan sulfate moieties (Spear and Longnecker, 2003). Compounds capable of inhibiting this initial association and binding, as well as subsequent membrane fusion, could be potential microbicide candidates.

The prediction algorithm and follow-up steps used to evaluate the antiviral activity of nanostructures against HSV-2 are the same as those used for HIV infection. However, several distinct *in vitro* and *in vivo* models have been successfully developed to evaluate inhibitory potency, such as the use of epithelial Vero cells for infection or plaque reduction assays for viral titration (Blaho et al., 2005). Some nanotech-derived architectures, such as membrane interacting peptide-functionalized dendrimers or zinc acetate gel, have been tested as potential anti-HSV-2 microbicides (Kenney et al., 2013; Luganini et al., 2011).

Several nanoparticles have shown a broad spectrum of activity. For instance, phosphatidylcholine liposomes containing the microbicide octylglycerol were effective *in vitro* against HSV-1, HSV-2 and HIV-1 (Wang et al., 2012). SPL7013 dendrimer-based VivaGel demonstrated antiviral activity against HIV and HSV in animal models and antibacterial activity preventing bacterial vaginosis (Price et al., 2011), and silver nanoparticle-coated condoms were also able to inhibit HIV and HSV infection and demonstrated antibacterial and antifungal activities against several microorganism such as *Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Klebsiella pneumoniae, Candida tropicalis, Candida krusei, Candida glabrata,* and *Candida albicans* (Mohammed Fayaz et al., 2012).

4.2. Ex vivo and in vivo evaluation: a proof-of-concept of compound efficacy prior to human clinical trials

Because HIV replication is restricted to humans, and the level of biological complexity of vaginal and rectal transmission of HIV cannot be mimicked in cell or tissue culture models, the research community has been searching for alternative *in vivo* study strategies for a long time. Sensitive animal models, that express the specific receptor and coreceptors that HIV-1 uses for attachment and entry in target cells (CD4, CCR5, or CXCR4), such as non-human primates and humanized mice (Brehm et al., 2010; Denton and Garcia, 2011; Veazey et al., 2012) play an important role in preclinical testing of microbicides, specially on safety, pharmacokinetic and efficacy testing. However, these animal models are not yet validated for US FDA (U.S. Food and Drug Administration) for predicting efficacy in humans (Administration, 2012). To date, the *in vivo* test based on the rabbit remains the only model recommended by the US FDA for the safety evaluation of vaginal products.

The most widely used non-human primates models are macaques, including the rhesus (*Macaca mulatta*), pigtail (*Macaca nemestrina*), and cynomolgus (*Macaca fascicularis*) sub-species. These species have anatomical, immunological, and reproductive physiological properties broadly comparable to those of humans and they can be efficiently infected by different HIV strains.

In case of rodents, their cells are non-permissive for the replication of HIV-1. Although rats, mice and rabbits, are certainly useful for preclinical safety studies of microbicide candidates, their reproductive cycles and tissues are quite different from those of humans or primates. Nevertheless, several humanized mouse models have been engineered recently to study HIV infection *in vivo:* (i) mice with single or multiple human transgenes; (ii) immunodeficient mice transplanted with human cells or tissues (healthy or diseased); (iii) immunodeficient mice transplanted with human CD34+ cells; or (iv) a combination of these three types. Currently, the most common model is the recently developed NOD/SCIDγc−/−, Rag2−/−γc−/− and blood, liver and thymus human (h-BLT) mouse model, which is reconstituted with human CD34 cells (NSG/BLT). In this model, immunodeficient mice are transplanted with human bone marrow (containing T cell progenitors), liver (for fetal immune development), and thymus (for T cell maturation) cells. Following engraftment, the mice are allowed to recover for 12 weeks prior to assessment for engraftment efficiency. These mice can completely reconstitute and self-renew all major human hematopoietic lineages (T and B cells, monocyte/macrophage, dendritic and NK cells), essentially reproducing a functional human immune system that responds to X4- and R5-tropic HIV-1 infection. The female reproductive tract of humanized BLT mice is reconstituted with functional human immune cells, rendering them susceptible to vaginal HIV-1 infection and allowing for testing of microbicide candidates.

Recently, several laboratories have used this mouse model to evaluate the effectiveness of novel HIV microbicide candidates (Chateau et al., 2013; Denton et al., 2008, 2010, 2011; Di Fabio et al., 2003; Neff et al., 2011; Veselinovic et al., 2012; Wheeler et al., 2011). To test the ability of microbicides to prevent HIV infection, these studies focused on the evaluation of the presence of HIV particles in treated mice through different molecular techniques. Viral infection and viral loads are commonly assessed by Q-RT-PCR and western blot and flow cytometry is used to measure the levels of CD4+ T cells in the peripheral blood of monitored mice.

Although humanized mice are certainly useful, they present some obstacles, like the high cost and complexity of the surgical techniques required to generate them. Non-human primate models, due to their similarities to humans, remain the best option for safety and efficacy testing of new microbicide candidates.

These *in vivo* models, together with *ex vivo* explant cultures, are also highly useful in evaluating the biosafety profiles of new nano-based microbicides in the final steps of their preclinical development, prior to toxicological analysis in humans. To study the impact of the compounds in the mucosal tissues, compounds must be tested vaginally and/or rectally in order to evaluate potential toxic effects though direct observation of lesions in the vaginal or

rectal area and the quantification of pro-inflammatory cytokines. Histopathological analysis of vaginal tissues can also be performed using semi-quantitative clinical scores to determine a vaginal irritation index (Eckstein et al., 1969). Epithelial lesions are evaluated based on the existence of hyperplasia or hyperkeratosis and ulceration, and/or inflammatory infiltrate in the epithelium. The inflammation process is characterized by an increased infiltration of lymphocytes compared to healthy mucosal tissues. Therefore, analysis of the lymphocyte score could help determine microbicide efficacy. Pro-inflammatory chemokines and cytokines plays an important role in HIV infection, owing to the recruitment and activation of CD4+ T cells to the mucosa (Fichorova et al., 2004). As mentioned previously, the concentration of inflammatory chemokines and cytokines in culture supernatants, serum samples or vaginal secretions is important in assessing the biosafety of microbicides. Furthermore, immunohistochemical studies on vaginal explants could be performed to study the levels of several biomarkers of cervicovaginal inflammation, such as IL-1, IL-6, TNFα, IL-8, macrophage inflammatory protein (MIP) or RANTES.

Some nanoplatforms have been tested as topical microbicides in animal models. RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevented HIV infection in BLT mice (Kim et al., 2010b). SPL7013 gel was protective against vaginal challenge with X4- and R5-tropic simian humanized immunodeficiency virus (SHIV) in a macaque model (Jiang et al., 2005). Moreover, activity against HSV-2 infection was demonstrated in mice and guinea pigs (Bernstein et al., 2003).

5. Future perspectives, obstacles and conclusions

Nanomedicine has provided new tools and rationales in the development and search for new and more powerful PrEP strategies, particularly in the design and engineering of more potent topical microbicides. The rational design and manipulation of diverse nanosystems, including dendrimers, liposomes and polymeric nanoparticles, has provided new microbicide candidates against HIV and other STDs with improved solubility, stability and pharmacokinetic and adherence properties. Furthermore, the combination of these nanoplatforms with other inhibitory drugs targeting different stages in the virus life cycle is essential for the development of more powerful and effective microbicides and for the improvement of existing ones. Since one of the most important problems in microbicide development is the lack of adherence in patients, a critical point to consider is the optimization of the nano-based microbicide formulation (Wong et al., 2014). Topical gel, intravaginal rings, films, tablets, ovules and other novel formulations such as female condoms coated with these nanostructures could be used. In each case, the formulation should be designed to maximize the activity, pharmacokinetics, safety, acceptability and adherence to the regimen of the different candidates. However, other limitations such as the adherence measurement or the comprehension of microbicide instructions for use in participating patients should be also improved to overcome adherence problems as reviewed in depth by Woodsong et al. (2013). In addition, novel routes of administration must be explored in order to discover novel nanotech-based prophylactic strategies against HIV.

Although there are benefits and advantages of using nanotechnology in the development of novel microbicides to stop the spread of HIV worldwide, there are features and challenges that must be overcome in the future for successful translation of nano-based microbicides from *in vitro* and *in vivo* assays to clinical settings. These include the biocompatibility, safety, stability and cost of nanocompounds, as well as their scale-up for large-scale production. More efficient and ecologically sustainable production methods or searching for active natural nanostructures like vegetable polymers, that can be large-scale produced in biofactories, could be essential points to reduce the production cost of nano-based microbicides. Plant systems offer unique advantages in comparison with conventional techniques (bacteria or yeast) or chemical synthesis such as lower production costs or an easy scale-up and purification process. These economic aspects are especially important because the main target of these products are the most vulnerable populations that live mainly in poor and developing countries, particularly in Africa.

Because the majority of microbicides have failed in the last phases of human clinical trials, it is critical to begin the evaluation of topical microbicide candidates in conditions as similar as possible to physiological conditions that occur during sexual intercourse at the moment of HIV transmission. Pivotal points are the study of the impact of microbicides on vaginal and rectal fluid, on mucosal immune-cross-talk and the consequences of physical trauma during sex on the use of the microbicides.

Currently, several nanosystems are under investigation as potential microbicides, with several on the threshold of clinical trials in humans. The encouraging results obtained in the CAPRISA 004 clinical trial and with VivaGel are proof-of-concepts that topical microbicides, and specifically nanotechnology-derived systems, can be effective approaches in the context of blocking HIV-1 sexual transmission. These results support further clinical trials and *in vivo* research studies to develop a powerful strategy in the fight against the spread of the HIV/AIDS epidemic worldwide.

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Novel Approaches to Measuring Adherence

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BRIEF REPORT

Strong Correlation Between Concentrations of Tenofovir (TFV) Emtricitabine (FTC) in Hair and TFV Diphosphate and FTC Triphosphate in Dried Blood Spots in the iPrEx Open Label Extension: Implications for Pre-exposure Prophylaxis Adherence Monitoring

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Self-reported adherence to pre-exposure prophylaxis (PrEP) has limitations, raising interest in pharmacologic monitoring. Drug concentrations in hair and dried blood spots (DBS) are used to assess long-term-exposure; hair shipment/storage occurs at room temperature. The iPrEx Open Label Extension collected DBS routinely, with opt-in hair collection; concentrations were measured with liquid chromatography/tandem mass spectrometry. In 806 hair-DBS pairs, tenofovir (TFV) hair levels and TFV diphosphate (DP) in DBS were strongly correlated (Spearman coefficient $r = 0.734$; $P < .001$), as were hair TFV/DBS emtricitabine (FTC) triphosphate (TP) ($r = 0.781$; $P < .001$); hair FTC/DBS TFV-DP ($r = 0.74$; $P < .001$); hair FTC/DBS FTC-TP ($r = 0.587$; $P < .001$). Drug detectability was generally concordant by matrix. Hair TFV/FTC concentrations correlate strongly with DBS levels, which are predictive of PrEP outcomes.

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The utility of pre-exposure prophylaxis (PrEP) for human immunodeficiency virus (HIV) prevention has been demonstrated in multiple trials. The efficacy of PrEP is highly dependent on adherence to the tenofovir disoproxil fumarate (TDF)/emtricitabine (FTC)–based regimen. However, adherence assessed via self-report or pill counts inconsistently predicts prevention outcomes [1, 2]. Pharmacologic exposure measures, wherein drug levels are monitored in a biomatrix such as plasma, peripheral blood mononuclear cells (PBMCs), or dried blood spots (DBS), are of significant interest to the field [3]. Indeed, substantial discordance between self-reported measures of adherence and drug detection in PrEP trials, such as the global iPrEx study [4], FEM-PrEP [1], Partners PrEP [5, 6], and the Vaginal and Oral Interventions to Control the Epidemic (VOICE) trial [2], have highlighted the critical need to incorporate objective pharmacologic exposure measures into PrEP demonstration projects and real-world settings.

Plasma or PBMC concentrations of tenofovir (TFV)/FTC or their phosphorylated forms have been most frequently incorporated into PrEP trials as pharmacologic measures [1, 2, 7]. Measurements of plasma antiretroviral concentrations represent more recent dosing (eg, 1–14 preceding days, depending on assay sensitivity) [6] and may be susceptible to "white-coat effects," wherein adherence improves transiently before visits [8]. Exposure measures for drugs with longer half-lives, such as TFV and its phosphorylated forms, would ideally provide information over prolonged periods. For example, TFV diphosphate (DP) levels in PBMCs [4, 9] relay information on exposure over longer periods (7–14 days), although processing, isolating and counting PBMCs are costly and technically challenging. DBS—made from whole blood—are easier to collect and process than PBMCs [10], and drug levels in DBS (which consist mainly of red blood cells), provide longer-term exposure information (half-life 17 days) [10]; DBS assays, however, require standardization against hematocrit abnormalities for interpretation. Importantly, TFV or TFV-DP concentrations in all 3 matrices (plasma, PBMCs, and DBS) have shown strong relationships with the protective efficacy of PrEP in either global iPrEx [4, 9] or the Open Label Extension follow-up study (iPrEx OLE) [11].

Given the need for low-cost, feasible, longer-term measurements of exposure for PrEP, analyzing concentrations of

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TFV/FTC in small hair samples has been of emerging interest. Hair collection is noninvasive, nonbiohazardous, and does not require phlebotomy or cold storage, and hair levels reflect uptake from the systemic circulation over weeks to months. Hair concentrations of TFV are strongly and linearly related to dose [12], and hair levels of both TFV and FTC correlate with plasma and PBMC concentrations [3]. Hair and DBS collection both provide feasibility advantages in resource-limited settings, and concentrations in these 2 matrices represent longer-term exposure, although the correlation between hair and DBS measures is unknown. We report for the first time the correlation between drug levels and concordance of drug detection in hair and DBS in a large cohort receiving PrEP.

METHODS

Participants and Sample Collection

The iPrEx Open Label Extension (OLE) enrolled 1603 HIV-negative men who have sex with men and transgender women previously enrolled in 3 PrEP trials [11], of whom 1225 initiated PrEP. Visits were performed at baseline and at weeks 4, 8, 12, 24, 36, 48, 60, and 72. Whole blood was routinely collected for DBS preparation, as described elsewhere [10], at 4 and 8 weeks after PrEP initiation and then every 12 weeks. Hair samples were collected from consenting participants who opted in for this substudy every 12 weeks after PrEP initiation by cutting approximately 100 strands of hair as close as possible to the scalp in the occipital region and labeling the distal end. As described elsewhere [3], each hair sample is stored and shipped in aluminum foil at room temperature. The study protocol was approved by Institutional Review Boards of all participating sites, and all participants provided written informed consent in their preferred language.

Laboratory Methods

The DBS from all relevant visits were stored at −20°C within 24 hours of collection and shipped on dry ice to our University of Colorado laboratory. At the time of analyses, a 3-mm DBS punch sample was extracted and analyzed for TFV-DP and FTC-triphosphate (TP) concentrations by validated liquid chromatography/tandem mass spectrometry (LC/MS-MS) methods, as described elsewhere [10]. The dynamic range of the assay was 2.5–2000 fmol per sample for TFV-DP and 0.1–200 pmol per sample for FTC-TP.

After storage and shipment at ambient temperature to our University of California, San Francisco laboratory, the proximal 1.5 cm of each hair samples (representing approximately 6 weeks of exposure) was cut finely with scissors and 5 mg was processed and analyzed using LC/MS-MS as described elsewhere [3, 12]. These assays have been validated from 0.002 to 0.400 ng/mg hair for TFV and from 0.02 to 4 ng/mg for FTC [12]. Of note, our assays for measuring TFV and FTC in hair samples have been peer reviewed and approved by the Division of AIDS Clinical-Pharmacology and Quality-Assurance (CPQA) program.

Statistical Analysis

Spearman correlation coefficients and scatterplots were examined to assess relationships between TFV and FTC levels in hair and TFV-DP and FTC-TP concentrations in red blood cells measured via DBS. The concordance of drug detection for each combination was also determined for paired samples.

RESULTS

Hair Collection in iPrEx OLE

Concentration results from 806 paired hair-DBS samples from 217 participants were available for comparison. Among 1603 HIV-negative individuals enrolled in iPrEx OLE, DBS were analyzed among all seroconverters and a random sample of seronegative participants, as described elsewhere [11]. All available hair and DBS samples from seroconverters and the random sample of non-seroconverters were used in the present analysis. Although 75% of participants in the overall study agreed to hair sampling, not enough hair samples were collected from the seroconversion visit in the 28 participants who acquired HIV during PrEP to perform a meaningful analysis of the protective effect of hair concentrations on HIV acquisition (12 declined or didn't have enough hair, 6 had hair collected but only at HIV RNA–negative time points; only 10 had hair collected at HIV RNA–positive time points).

Correlation Between DBS and Hair Drug Concentrations

Scatterplots of the correlation between hair TFV or FTC levels and DBS TFV-DP or FTC-TP levels in the 806 paired samples are depicted in Figure 1. TFV concentrations in hair and TFV-DP levels in DBS were strongly correlated ($r = 0.734$; $P < .001$), as were hair TFV levels and DBS FTC-TP concentrations ($r = 0.781$; $P < .001$). FTC concentrations in hair were more strongly correlated with DBS TFV-DP levels ($r = 0.742$; $P < .001$) than with DBS FTC-TP levels ($r = 0.587$; $P < .001$).

Levels of TFV/ FTC Hair Concentrations Estimated to Confer Protective Efficacy

In iPrEx OLE [11], the protective levels of TFV-DP in DBS were quantified as 700 fmol per punch sample. From the correlation analysis, these DBS levels correspond to a median hair TFV concentration of 0.023 ng/mg (95% confidence interval [CI], .019–.025) and a hair FTC concentration of 0.37 ng/mg (95% CI, 28–.58).

Concordance of Detection of Hair and DBS Drug Levels

A comparison of detectability of the relevant antiretroviral or metabolite in the 2 matrices is shown in Table 1. Concentrations of drugs were mostly concordant in terms of detectability, with

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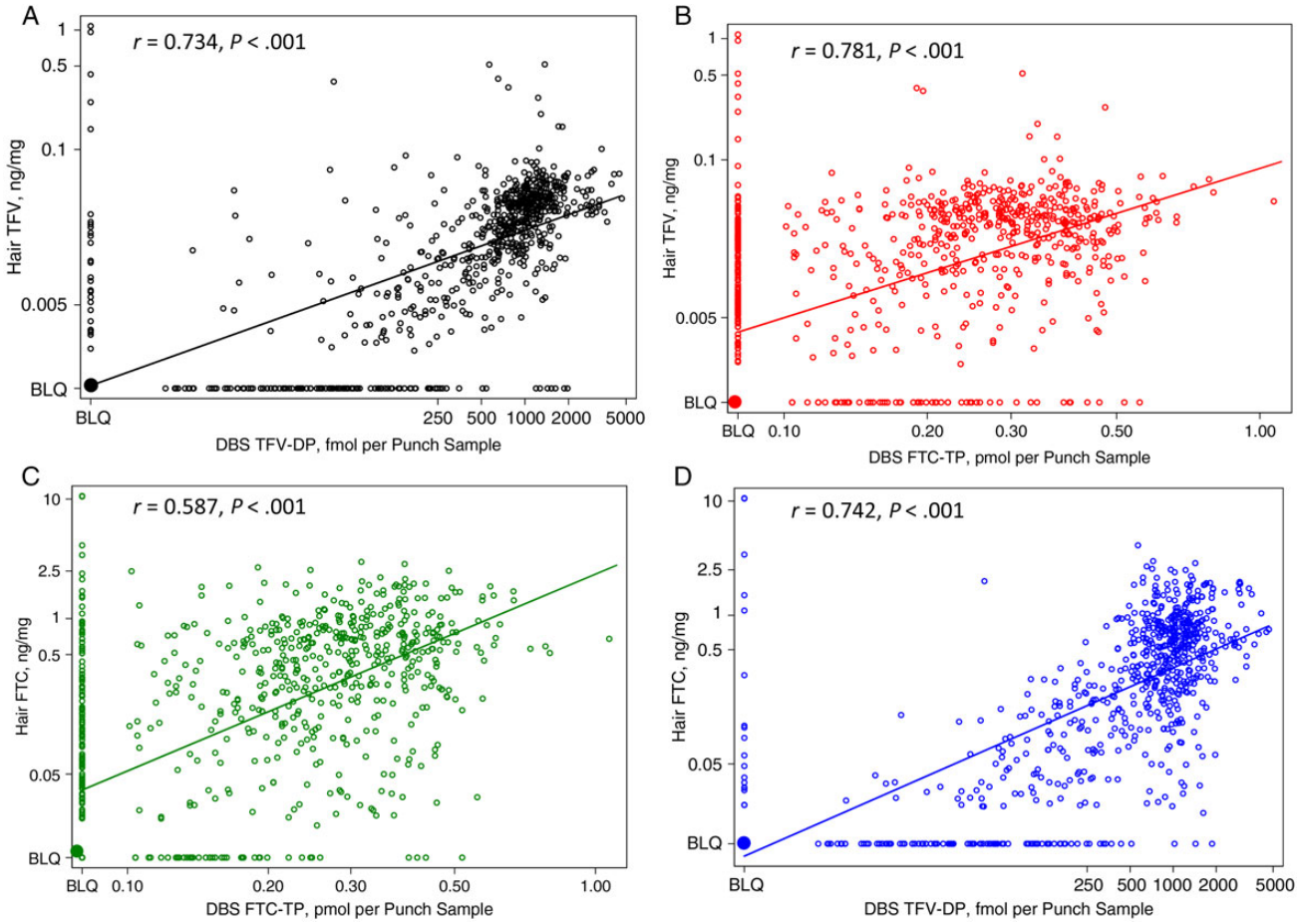

Figure 1. Scatterplots showing correlations between hair tenofovir (TFV) and dried blood spot (DBS) TFV-diphosphate (DP) levels (*A*); hair TFV and DBS emtricitabine (FTC)-triphosphate (TP) levels (*B*); hair FTC and DBS FTC-TP levels (*C*); and hair FTC and DBS TFV-DP levels (*D*). Abbreviation: BLQ, below limit of quantification.

concordance of 82% for hair TFV and DBS TFV-DP levels, 90% for hair TFV and DBS FTC-TP levels, 81% for hair FTC and DBS FTC-TP levels, and 85% for hair FTC and DBS TFV-DP levels.

DISCUSSION

Given that pharmacologic measures of drug exposure have been crucial for trial interpretation in several PrEP studies [1, 2, 4], the incorporation of objective markers of drug adherence/exposure into PrEP settings is of growing interest. Easily stored samples that do not require a cold chain, such as hair, could be useful for drug monitoring in resource-constrained settings. Our study showed strong and significant correlations between concentrations of PrEP drugs in hair and DBS, as well as high levels of concordance (>80% for all combinations) for drug detectability in the 2 matrices. The current study is the first to analyze correlations between antiretroviral concentrations in hair and DBS.

Table 1. Concordance of Drug Detection of PrEP Drugs and Their Metabolites in Hair and DBS (806 Paired Samples)

Drug Level	Detectable in Hair and DBS, %	Detectable in Hair but Not DBS, %	Detectable in DBS but Not Hair, %	Undetectable in Hair or DBS, %
TFV in hair, TFV-DP in DBS	72	3	15	10
TFV in hair, FTC-TP in DBS	71	5	5	19
FTC in hair, FTC-TP in DBS	62	14	5	19
FTC in hair, TFV-DP in DBS	74	2	13	11

Abbreviations: DBS, dried blood spots; DP, diphosphate; FTC, emtricitabine; PrEP, pre-exposure prophylaxis; TFV, tenofovir; TP, triphosphate.

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Although plasma measures of TFV and/or FTC have been most commonly analyzed in PrEP trials [1, 2, 4–7], drug concentrations in plasma represent recent drug ingestion (eg, yes/no for taking drug or not). However, TFV and its moieties exhibit long half-lives in various matrices, enabling assessment of longer-term measures of cumulative drug exposure, such as those in DBS [10] and hair, that may be more relevant for PrEP monitoring. PBMC measurements of TFV-DP/ FTC-TP have proved useful [9], but PBMCs are generally difficult to collect, process, and transport. DBS are easy to prepare from whole-blood samples [10], although they must be shipped and stored at cold temperatures to allow measurement. Hair samples are easy to collect in real-world settings, and samples are stored and shipped at room temperature under nonbiohazardous conditions. However, it should be noted that current assays to quantify drug levels for PrEP, including those in both DBS and hair, use LC/MS-MS, which requires expensive equipment. Widespread applicability of biologic adherence measures in the context of PrEP rollout will rely on the availability of lower-cost methods to analyze drug levels, currently in development for hair assays [13].

Although whole blood was routinely collected at all time points in iPrEx OLE, hair collection was performed only on an opt-in basis; sampling required participants to sign an extra consent form and was only performed quarterly. The lack of hair sampling at relevant visits for participants who seroconverted during PrEP impeded our ability to directly analyze this measure in relationship to HIV seroconversion. However, from the correlation data [11], we can estimate that hair TFV concentrations of 0.023 ng/mg (95% CI, .019–.025 ng/mg) and hair FTC concentrations of 0.37 ng/mg (95% CI, .28–.58 ng/mg) are likely to confer protective benefit in the context of PrEP. The TFV hair concentration estimated to confer protection in this study is similar to that achieved by volunteers taking 4 doses of TFV per week in a separate study (median value, 0.023 ng/mg) [12].

Hair collection has been shown to be feasible and acceptable (>95%) among HIV-infected or at-risk individuals in Africa [3, 14]. A recent study from rural Kenya showed that community information campaigns and field staff training early in protocol development was likely to enhance hair collection rates [14]. A qualitative study from South Africa on the acceptability of hair collection underscored this point [15]. Of note, TFV concentrations in pubic hair samples were not reliably associated with dose in another study [12], which corroborates other findings that pubic hair, with its erratic growth rates and urine contamination, is not an informative biomatrix.

Two phase II PrEP studies in Africa collected multiple biologic samples for exposure monitoring (plasma, PBMCs, and hair) and correlated drug levels in hair with drug or metabolite concentrations in plasma and PBMCs [3]. These studies did not collect DBS. In these phase II PrEP studies, the correlation between TFV levels in hair and plasma after 8 weeks was moderate ($r = 0.41$), as was the correlation between FTC levels in hair and plasma ($r = 0.51$). The coefficients for correlation between TFV or FTC levels in hair and their relevant phosphorylated concentrations in PBMCs at 8 weeks were similar ($r = 0.43$ and $r = 0.50$, respectively). In the current study, the correlation between TFV concentrations in hair and TFV-DP levels in DBS (both longer-term measures of exposure) was high ($r = 0.734$), and DBS measures of TFV-DP in the overall cohort were strongly associated with protective efficacy [11], implying that TFV hair levels may also have predicted efficacy.

The more moderate correlation observed between hair FTC hair and DBS FTC-TP levels may reflect the relatively short half-life of FTC-TP in DBS (approximately 30 hours). The discordance between drug detection in hair and DBS in 19% of pairs could be secondary to differential half-lives of TDF/FTC-based PrEP in different matrices; another study will examine the elimination kinetics of TFV in hair and DBS in the context of directly observed therapy (ClinicalTrials.gov, NCT02022657). Undetectable drug concentrations in hair (25% of samples for TFV) suggest no appreciable dosing over a significant duration before a visit.

The current study adds to the growing literature around the utility of hair monitoring in TDF/FTC-based PrEP. A large ongoing PrEP demonstration trial in 3 US cities has incorporated multiple pharmacologic measures of drug exposure, including hair assays, and will provide further data on the utility of hair measures in monitoring adherence. Future studies of pharmacologic monitoring in PrEP should probably examine the predictive utility of multiple measures of exposure simultaneously, because each measure reflects a different duration of exposure. Given the well-acknowledged limitations of self-report in monitoring adherence to PrEP, novel biologic measures of assessing exposure, including hair assays, should be further examined in real-world settings.

Notes

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Potential conflicts of interest. Gilead Sciences donated emtricitabine/tenofovir disoproxil fumarate for participants in the study but provided no other financial support and did not contribute to data interpretation or manuscript development. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Strong Relationship between Oral Dose and Tenofovir Hair Levels in a Randomized Trial: Hair as a Potential Adherence Measure for Pre-Exposure Prophylaxis (PrEP)

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Abstract

Background: Pre-exposure prophylaxis (PrEP) trials using tenofovir-based regimens have demonstrated that high levels of adherence are required to evaluate efficacy; the incorporation of objective biomarkers of adherence in trial design has been essential to interpretation, given the inaccuracy of self-report. Antiretroviral measurements in scalp hair have been useful as a marker of long-term exposure in the HIV treatment setting, and hair samples are relatively easy and inexpensive to collect, transport, and store for analysis. To evaluate the relationship between dose and tenofovir concentrations in hair, we examined the dose proportionality of tenofovir in hair in healthy, HIV-uninfected adults.

Methods: A phase I, crossover pharmacokinetic study was performed in 24 HIV-negative adults receiving directly-observed oral tenofovir tablets administered 2, 4, and 7 doses/week for 6 weeks, with a ≥3-week break between periods. Small samples of hair were collected after each six-week period and analyzed for tenofovir concentrations. Geometric-mean-ratios compared levels between each pair of dosing conditions. Intensive plasma pharmacokinetic studies were performed during the daily-dosing period to calculate areas-under-the-time-concentration curves (AUCs).

Results: Over 90% of doses were observed per protocol. Median tenofovir concentrations in hair increased monotonically with dose. A log-linear relationship was seen between dose and hair levels, with an estimated 76% (95% CI 60–93%) increase in hair level per 2-fold dose increase. Tenofovir plasma AUCs modestly predicted drug concentrations in hair.

Conclusions: This study found a strong linear relationship between frequency of dosing and tenofovir levels in scalp hair. The analysis of quantitative drug levels in hair has the potential to improve adherence measurement in the PrEP field and may be helpful in determining exposure thresholds for protection and explaining failures in PrEP trials. Hair measures for adherence monitoring may also facilitate adherence measurement in real-world settings and merit further investigation in upcoming PrEP implementation studies and programs.

Trial Registration: ClinicalTrials.gov NCT00903084.

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Introduction

Recent studies have provided new hope for effective HIV biomedical prevention strategies [1–4]. The efficacy of pre-exposure prophylaxis (PrEP), where at-risk HIV uninfected individuals take antiretroviral medications daily to prevent infection, has been demonstrated in several recent trials. The iPrEx study demonstrated that daily administration of emtricita-

bine/tenofovir disoproxil fumarate (FTC/TDF) PrEP decreased HIV-1 acquisition in men who have sex with men [2]. The Partners PrEP trial and Botswana TDF2 study subsequently verified and extended these findings in serodiscordant couples and seronegative heterosexual adults respectively [3,4]. Most recently, a trial in HIV-uninfected injection drug users (IVDU) in Thailand showed a reduction in HIV incidence with the use of daily tenofovir [5]. Cumulative data from these trials led to the approval of FTC/TDF as PrEP for uninfected individuals at risk of HIV infection by the Food and Drug Administration [6] and/or the development of interim guidance for clinicians prescribing PrEP in different populations [7–9].

Importantly, several trials have highlighted the critical relationship between adherence and PrEP efficacy. For example, the efficacy of FTC/TDF in iPrEx rose from 44% overall to an estimated 92% among those with detectable blood drug levels [2]. Furthermore, two large PrEP trials in sexually active African women [10,11] were unable to demonstrate significant efficacy of daily FTC/TDF in reducing HIV acquisition, likely due in large part to low adherence to study drug.

Incorporating measures of drug exposure as biomarkers of PrEP adherence has been critical to interpreting PrEP trials. In iPrEx, although mean adherence by self-report was 95%, drug was detected in only 8% of seroconverters compared with 54% of matched active-arm controls who remained uninfected [2]. Self-reported adherence was similarly high in both FEM-PrEP [10] and VOICE [11], but random plasma tenofovir (TFV) levels among women on active drug revealed target (≥10 nanograms per milliliter) or detectable TFV concentrations in only 26% and 29% of women, respectively. The limitations of self-report and other commonly used adherence measures in HIV treatment monitoring are well-described [12], and self-reported adherence may be particularly inaccurate in clinical trials [13]. Biomarkers of drug exposure can serve as surrogates of adherence because non-adherence is the most frequent cause of low drug levels. Pharmacologic parameters may be especially critical to monitor in HIV-negative individuals on antiretroviral prophylaxis since HIV viral loads cannot serve as indicators of adherence in noninfected persons.

Due to TFV's extended intracellular half-life (~150 hours) [14], an ideal adherence biomarker would reflect average drug exposure over several weeks to months. Single plasma concentrations, which represent only a small window of exposure (dosing in the last few days) [15–17], are subject to significant day-to-day variation [15] and "white-coat" effects [18,19] and are imperfect indicators of exposure. As the concentration of drugs in hair reflects uptake from the systemic circulation over an extended time window (weeks to months) [20,21], hair analysis provides an advantage over plasma monitoring in assessing average drug exposure over a longer period of time. We have demonstrated that hair concentrations of antiretrovirals (ARVs) are the strongest independent predictor of virologic success in large prospective cohorts of HIV-infected patients [22–25]. Analyzing antiretroviral levels in hair may be a promising approach to objectively quantify adherence to PrEP regimens as well. Although the measurement of intracellular tenofovir diphosphate (TFV-DP) concentrations in peripheral blood mononuclear cells (PBMCs) has been incorporated into previous PrEP trials as a long-term measure of exposure, hair levels may provide feasibility advantages in the field since methods to process, isolate and count PBMCs are costly, technically challenging, and difficult to institute widely. Unlike phlebotomy, hair collection is noninvasive and does not require specific skills, refrigeration, or sterile equipment. Hair can be stored at room temperature prior to analysis and shipped without biohazard precautions.

To widely employ hair as a biological marker of drug exposure in HIV prevention settings, the relationship between different dosing strategies and drug concentrations in this matrix must be established. Moreover, individual variation in pharmacokinetic (PK) parameters (e.g. plasma oral clearance measured as areas-under-the-concentration-time-curve or AUCs) can contribute to variability of antiretroviral drug levels in various compartments [26], so the effect of these parameters on hair drug concentrations must be determined. We performed a phase I randomized crossover study with the objective of examining the impact of varying dosing patterns of oral TDF (2, 4 and 7 doses per week using directly observed dosing) and individual plasma AUCs on TFV concentrations in hair in healthy, HIV-negative adults. These dosing frequencies were chosen to represent low, moderate, and high adherence to PrEP. Furthermore, modeling studies suggest TFV-DP levels achieved with these dosing patterns were associated with increasing levels of PrEP efficacy [27]. In this study, we found a strong relationship between dose and hair levels of TFV, paving the way for further study of hair measures as an adherence monitoring tool in PrEP trials and implementation projects.

Methods

Study Design and Participant Selection

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

STRAND was an open-label, randomized, three-period PK study conducted between October 2009 and March 2011 at the University of California San Francisco (UCSF) Clinical Research Center and the San Francisco Department of Public Health (SFDPH) Bridge HIV research clinic. To facilitate directly observed dosing, participants were recruited from UCSF (students, staff, and faculty) and from neighborhoods adjoining SFDPH. Tablets of TDF (300 milligrams, mg) were provided by Gilead Sciences (Foster City, CA). As several PrEP trials were evaluating TDF alone as a PrEP agent [3,5,28] and no significant pharmacokinetic interactions have been observed between tenofovir and emtricitabine [29], TDF tablets alone were chosen for evaluation to minimize potential toxicity to healthy subjects in this study. All subjects provided written informed consent prior to study procedures and the study was approved by the UCSF Committee on Human Research.

Eligible subjects were healthy male or female volunteers age 18 or older without serious or active medical conditions. Participants were all HIV-negative based on HIV rapid antibody testing and were hepatitis B surface antigen negative at enrollment. Enrolled participants could not be on nephrotoxic agents, had to have an estimated creatinine clearance ≥60 mL/min, serum creatinine levels below the upper limit of normal, urine dipstick tests that were negative or trace positive for glucose and protein, adequate hepatic and hematologic function, and negative urine pregnancy tests (for women). Female volunteers were required to use effective contraception during the study.

All participants had to be willing to undergo directly observed dosing (DOT), provide personal cell phone numbers for unobserved modified DOT (mDOT) visits, have dark (brown or black) hair (to minimize interindividual variability in this proof-of-concept study), a minimum occipital scalp hair length of 3 centimeters (cm), and not have used hair dyes or hair permanent products in the past 3 months. Participants at high risk of HIV

infection, including those reporting injection drug use (IDU); vaginal or anal intercourse with an HIV-positive partner, IDU partner, or with more than 4 partners of the opposite sex; and/or transactional sex in the prior 6 months were excluded.

Study Visit and Dosing Procedures

Subjects were randomly assigned to one of 6 dosing sequences and completed three 6-week dosing periods of 2, 4 and 7 doses per week, with a break of at least 3 weeks between periods (**Figures 1** and **2**). Based on an average hair growth rate of 1 centimeter per month [30], a minimum of 3-weeks was chosen for the washout period. Each sequence arm included both sexes equally for a total of 12 male and 12 female participants. The 2 doses/week were taken on Tuesday and Wednesday, and the 4 doses/week on Monday, Tuesday, Thursday, and Friday. All scheduled doses on Monday to Friday were directly observed by study staff, and weekend doses were confirmed by text messaging or phone using previously-described mDOT procedures [31,32]. A one-week supply of back-up medication was provided for participants unable to complete a directly-observed dose; these doses were confirmed via phone or text message at dosing. Dosing visits occurred at UCSF, SFDPH, or another location per participant convenience. Participants returned to the clinic for follow-up at a start, mid-point (after 3 weeks of dosing), and end-point (at the end of each 6 week dosing period) visit for safety and adverse event monitoring, symptom-directed physical exams, and rapid HIV testing.

At enrollment and end-point visits, approximately 150–200 strands of scalp hair were collected; additionally, a similar number pubic hair strands were collected on an opt-in basis. After 4 weeks of dosing during the 7 doses/week period (at steady state for plasma TFV), participants were admitted to the UCSF Clinical Research Center for intensive 24-hour plasma PK evaluations to calculate individual PK parameters (plasma AUC for oral clearance and C_{max}). The participant's usual diet was ascertained prior to the PK visit, and simulation of the usual diet was undertaken during PK sampling. An initial blood level was drawn (called the "0" timepoint) prior to an observed dose of TFV, followed by blood collection at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, and 24 hours post-dose for measurement of TFV plasma concentration. Concentrations could not be measured for one participant at the 0.5 hour time and another participant at the 1.5 hour time; these were interpolated by fitting quadratic functions to the log-concentrations using the two previous and two subsequent times.

Sample Collection and Processing

Several small thatches of hair (~150–200 strands) were cut as close as possible to the scalp in the occipital region and/or pubic region, and the distal portion labeled [22]. The proximal section of the scalp or pubic hair sample (about 1.5 cm) was cut down and chopped to 1–2 mm length segments with scissors and 5 mg were weighed, processed and analyzed using liquid chromatography/tandem mass spectrometry (LC/MS-MS). The tenofovir in the cut hair sample was extracted with 50% methanol/water containing 1% trifluroacetic acid, 0.5% hydrazine dihydrochloride, and internal standard in a 37°C shaking water bath overnight (>12 hours) and then analyzed by a modified LC-MS/MS method [33]. The relative error (%) and precision (coefficients of variation (CV)) for spiked quality control hair samples at low, medium and high concentrations were all <15% (CV of 10.5%, 8.4%, and 6.0%, respectively). Each sample was analyzed once due to the limited number of clinical hair samples. The method to analyze TFV in hair was validated from 0.002 nanograms (ng)/mg to 0.400 ng/mg hair, with a lower limit of quantitation at 0.002 ng/mg [34,35].

Plasma was collected in EDTA tubes for drug concentration measurement for the intensive pharmacokinetic studies. Plasma was analyzed for TFV via standard techniques of LC/MS/MS [36]. The linearity of the concentration curves is in the range of 10 to 1000 ng/milliliter (mL), and the lower limit of quantification is 10 ng/mL.

Statistical Analyses

All analyses were conducted using SAS (Version 9.2, SAS Institute, Cary, NC) and Stata (Version 12.1, College Station, TX). The primary outcome variable was concentration of TFV in hair at each end-point visit. Median drug levels with 95% confidence intervals (CIs) were calculated, and geometric mean ratios with 95% CIs were used to compare levels between each pair of dosing conditions. We used geometric means to avoid overweighting large ratios and to match the logarithmic transformation used for the multivariate modeling. Spaghetti plots were used to depict drug levels for each participant, with each line representing an individual. The chosen sample size (n = 24) was not based on formal power considerations, but was feasible and expected to provide useful new information about typical relationships between TDF dosing and drug concentrations, as well as a preliminary assessment of how well hair levels could distinguish between daily versus intermittent dosing.

Linear mixed models with random intercepts were used for multivariable analyses of predictors of logarithmically transformed hair levels. Plasma AUC_{0-24}, a measure of the subject's oral clearance (dose*F/CL), was calculated using the linear-log trapezoidal method, and C_{max} was defined as the largest observed TFV concentration in plasma over the 24 hours. The primary predictor variables were dosing condition (2, 4, and 7 dose/week), log-transformed plasma AUC, and log-transformed C_{max}. We examined linearity assumptions for continuous predictors by adding quadratic terms to the models.

Results

Sociodemographics, Safety, and Dosing:

Twenty-four participants met eligibility criteria and were enrolled (**Figure 1**). Demographics and baseline data for the 23 participants who provided one or more hair samples are shown in **Table 1**.

There were 167 adverse events (AEs) reported during the study, with each participant reporting at least 1 AE. Most AEs were either mild, grade 1 (128 events, 76%) or moderate, grade 2 (37 events, 22%). The most commonly reported AEs were headache (54%), upper respiratory infection (38%), nausea (29%), abdominal gas (17%), soft/loose stools (17%), and fatigue (17%). Only one participant experienced a grade 3 AE (traumatic jaw fracture) and one experienced a grade 4 AE (metastatic breast cancer), both adjudicated as unrelated to study drug. Four participants withdrew before completing all study procedures: one after the first TDF dose due to a grade 2 hypersensitivity reaction (urticarial rash with tongue tingling) occurring prior to PK data collection, one before completing the 4 and 7 doses/week periods due to headaches, one before completing the 2 doses/week period due to use of post-exposure prophylaxis and elevated pancreatic enzymes, and one before completing the 7 doses/week period due to the new cancer diagnosis described above.

Over 90% of doses were observed according to protocol: 249/267 (93%) doses in the 2 doses/week arm, 473/517 (91%) doses in the 4 doses/week arm, and 773/856 (90%) in the 7 doses/week

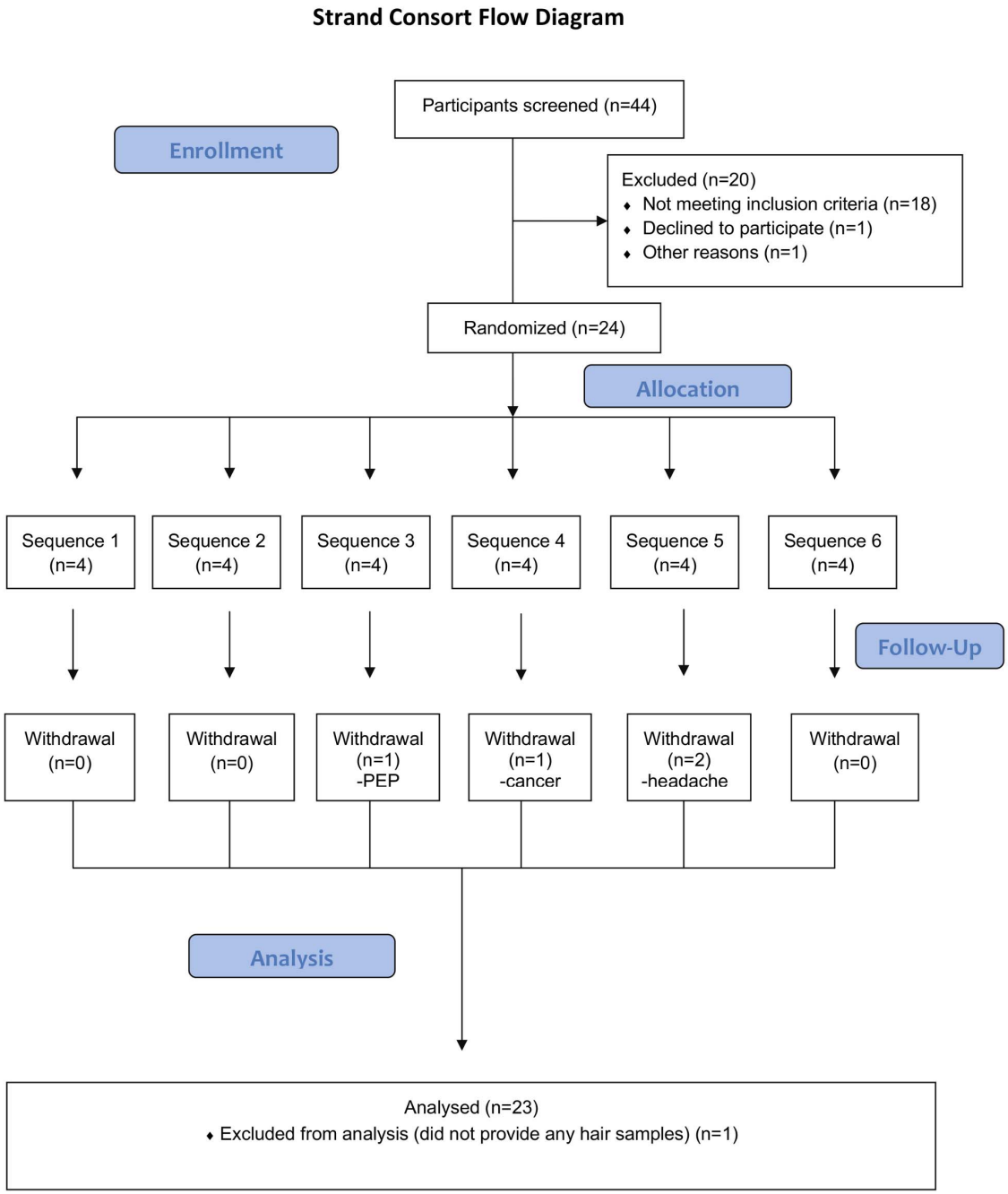

Figure 1. CONSORT flowchart for STRAND study.
doi:10.1371/journal.pone.0083736.g001

arm. Of these 1495 doses observed on protocol, 82% were DOT and 18% were mDOT visits. An additional 130 (7.9%) doses were taken from participants' back-up medication supplies (confirmed via phone/text message). Two participants were unable to attend several consecutive DOT visits during their 7 doses/week period due to travel; all 31 of these doses were confirmed on time via phone or text. Combining per protocol doses and confirmed doses from back-up supplies, 1624/1640 (99%) of expected doses were taken according to prescribed schedules Overall, 55/67 (82%) of dosing periods had 100% on-time completion rates, and only 2 periods had <90% completion: one participant missed 3/12 (25%) doses during the 2 doses/week period, and another participant missed 4/36 (11%) doses in the 4 doses/week period.

TFV concentrations in hair

Median TFV levels in scalp hair increased monotonically from 2 to 4 to 7 doses/week (**Table 2**). There was minimal overlap in TFV hair concentrations between 2 versus 7 doses/week, with some overlap between the 2 versus 4 and 4 versus 7 doses/week dosing periods. The geometric mean ratios were close to the ratios of the doses: 4 versus 2 doses/week averaged 1.76 (versus 2.0 ratio of doses); 7 versus 4 doses/week averaged 1.60 (versus 1.75 ratio of

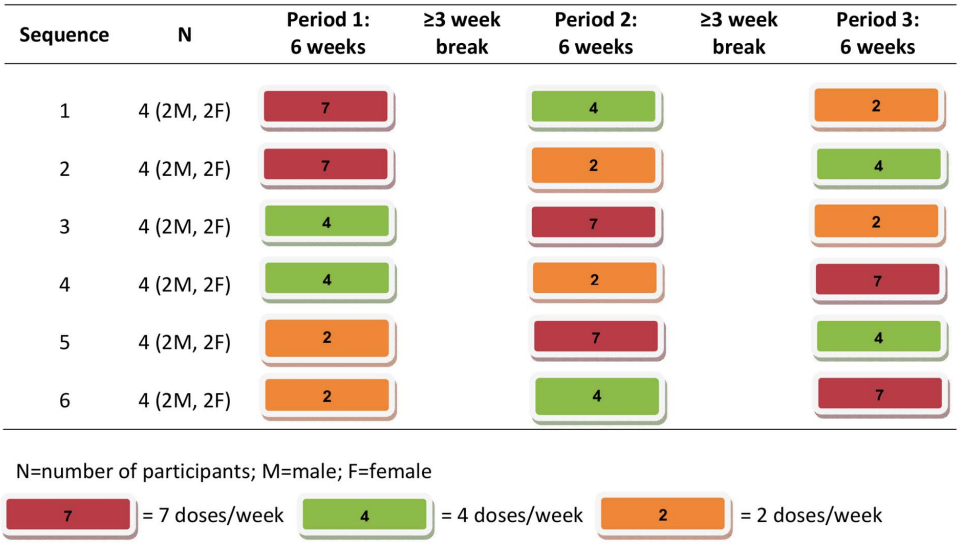

Figure 2. Dosing scheme for the STRAND Study.
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doses); 7 versus 2 doses/week averaged 2.85 (versus 3.5 ratio of doses).

Figure 3 depicts the scalp hair TFV concentrations for the 2, 4, and 7 doses/week periods for each individual. A concentration of TFV in hair of 0.021 ng/mg reliably distinguished between 2 versus 7 doses/week (before rounding, all levels for 2 doses/week were <0.021 ng/mg, and all levels for 7 doses/week were >0.021 ng/mg). Tenofovir hair concentrations were higher for 7 versus 4 doses/week and 4 versus 2 doses/week in all but 3 participants (**Table 3**). These 3 participants did not have fewer observed doses or other signs of altered adherence. Coefficients of variation were 0.27 for hair at 2 doses/week, 0.34 at 4 doses/week, and 0.25 at 7 doses/week.

In a model with log(doses per week) and log(AUC) as predictors, a log-linear relationship was seen between doses per week and TFV scalp hair level, with an estimated 76% (95% CI 60–93%, $p<0.0001$) increase in hair level per 2-fold dose increase. Adding a random dose effect to this model estimated minimal inter-individual variability in dose effect (estimated random effect variance of zero), suggesting very similar dose effects across most subjects. TFV plasma AUCs, ranging from 1210 to 5610 ng×h/mL, were a moderate predictor of TFV hair level, with a 23% increase in hair level per 2-fold increase in AUC ($p = 0.035$).

Table 1. Demographics of participants who provided hair samples (n = 23).

Variable	N	%
Sex		
Female	12	52%
Male	11	48%
Race/Ethnicity		
Non-Hispanic White	12	52%
Asian/Pacific Islander	3	13%
Latino/Hispanic	7	30%
Other	1	4%
Education		
Some college	9	39%
Bachelors	9	39%
Graduate	5	22%

Variable	N	Mean	SD	IQR
Age (in years)	23	34.2	9.0	26.6–41.1
Creatinine Clearance*	23	129.4	31.1	107.2–154.6
Weight (in kg)	23	73.9	13.2	64.1–86.3

*Estimated by the Cockcroft-Gault equation [57].
N = number of participants; SD = standard deviation; kg = kilograms. IQR = interquartile range.
doi:10.1371/journal.pone.0083736.t001

Table 2. Median drug levels and geometric mean ratios in hair after 6 weeks of use, by dosing period.

	Hair (scalp)	
Dosing period	**N**	**Median (range) TFV levels (ng/mg)**
2 doses/week	22	0.012 (0.008 to 0.021)
4 doses/week	22	0.023 (0.011 to 0.042)
7 doses/week	21	0.038 (0.021 to 0.053)
Period comparison		**Geometric mean ratios (95% CI)**
4 vs. 2 doses/week	21	1.76 (1.45 to 2.13)
7 vs. 4 doses/week	21	1.60 (1.34 to 1.90)
7 vs. 2 doses/week	20	2.85 (2.46 to 3.32)

N = number; TFV = tenofovir; ng/mg = nanogram/milligram; CI = confidence interval.
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Allowing for different residual variance at different dose levels (heteroscedasticity) resulted in similar dose and AUC estimates. Also, similar results were observed when restricting analyses to the 7 doses/week period where intensive PK parameters were measured (27% increase in hair level per 2-fold increase in AUC, $p = 0.042$). C_{max} appeared to have little positive effect on hair level when controlled for dose and AUC (−20% per 2-fold increase, 95% CI −43% to +10%, $p = 0.16$). There was no statistically significant effect of total body weight ($p = 0.35$), estimated creatinine clearance ($p = 0.52$), or sex ($p = 0.89$) on TFV hair concentrations.

Pubic hair TFV levels varied erratically and did not monotonically increase in 5/11 subjects who had at least two on-drug pubic hair samples. In addition, levels were exceedingly high in 6 samples (~30–300 times higher than TFV levels in scalp hair).

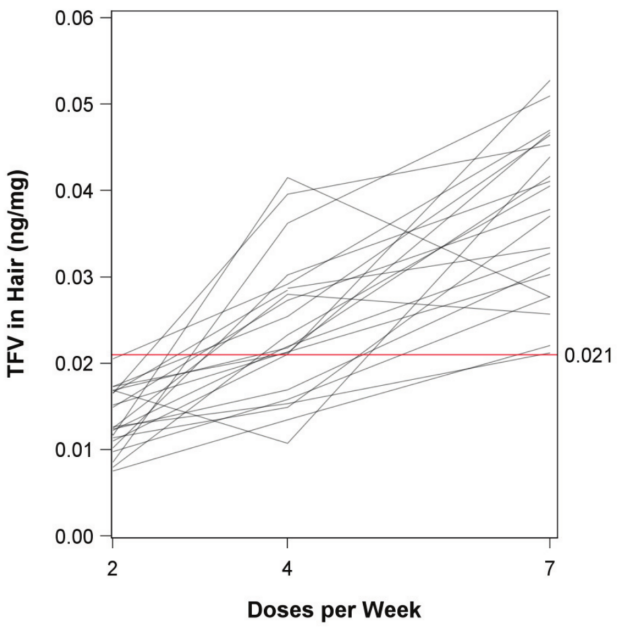

Figure 3. Spaghetti plot of tenofovir (TFV) concentrations. Hair TFV concentrations for each subject are shown for each dosing period. Each line represents drug concentration data from one participant (at 2, 4, and 7 doses/week). The red line distinguishes between hair scalp concentrations for 2 vs. 7 doses/week. ng/mg = nanogram/milligram.
doi:10.1371/journal.pone.0083736.g003

Discussion

Accurate adherence measures are critical to the interpretation of PrEP clinical trials and may play an important role in monitoring PrEP use in clinical practice. An effective pharmacologic measure of PrEP adherence in a particular matrix must demonstrate a clear relationship between dose and drug levels. Our analysis provides evidence of a proportional relationship between dose and hair levels of TFV in small samples of scalp hair from healthy, HIV-uninfected individuals. Specifically, there was an estimated 76% increase in TFV hair level with doubling of dose, with similar dose effects across subjects. Hair concentrations exhibited linearity with dose in the range of 2 to 7 doses per week, with minimal inter-individual variation in dose effect. A hair concentration of 0.021 ng/mg distinguished between 2 versus 7 doses/week dosing. This discrimination ability may have useful clinical applications in monitoring PrEP use, as a previous PK modeling study demonstrated that estimated protective efficacy for taking FTC/TDF 2 doses/week was 76% (95% CI 56 to 96%), compared with a projected 99% (95% CI 96 to >99%) for 7 doses/week [27]. There were 3/23 participants in which TFV dose was not monotonically increasing. In all 3 cases, these discrepancies were between 2 vs. 4 doses/week or 4 vs. 7 doses/week.

Pubic hair samples were not useful in measuring TFV concentrations and varied erratically in this study, with several high outlier values. Similar results have been reported for pubic hair levels of opiates [37], amphetamines [38], benzodiazepines [39], and rodenticidal toxins [40], and may reflect urinary contamination of pubic hair or the slower growth rate of pubic hair. Furthermore, individual PK parameters, including oral clearance as measured by plasma AUC, and C_{max}, had modest predictive value on drug levels in hair. The limited predictive value of PK parameters in this study may have been due to within-person variation over time in PK parameters (such that a single PK measure may not completely represent systemic drug exposure over the entire study period), or other factors that may influence hair levels, such as differences in hair growth rate and drug in sweat that came in contact with sampled hair [41].

Antiretroviral drug levels in hair have been shown to be highly correlated with treatment outcomes in HIV-infected patients, demonstrating stronger predictive value than self-reported adherence [22,24] or single plasma ARV concentrations [23,24]. Similarly, objective biological markers of PrEP adherence have been used to predict protective efficacy in PrEP trials [2–4]. While qualitative drug detection overall has demonstrated utility in

Table 3. Three participants where hair levels did not demonstrate monotonic increases.

Patient ID	Hair TFV (ng/mg) at 2 doses/week	Hair TFV (ng/mg) at 4 doses/week	Hair TFV (ng/mg)at 7 doses/week
039	0.017	0.011	0.044
051	0.013	0.028	0.026
063	0.012	0.042	0.028

ID = identification number; TFV = tenofovir; ng/mg = nanogram per milligram.
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predicting PrEP efficacy across a number of PrEP studies [42], quantitative drug levels may provide further value in predicting protective effects and could also be used in establishing potential threshold drug levels for protection. For example, a regression analysis in iPrEx estimated that a tenofovir-diphosphate (TFV-DP) concentration of 16 femtomole (fmol)/10^6 PBMCs was associated with a 90% reduction in HIV acquisition [27]. Similarly, women in the CAPRISA study of 1% vaginal tenofovir gel who had a tenofovir concentration of >1000 ng/mL in cervicovaginal fluid samples had a significant reduction in HIV acquisition compared with those on placebo, in contrast to women with tenofovir concentrations of ≤1000 mg/mL, who did not [43]. Quantitative drug levels can also be used to understand PrEP failures. In the randomized phase of the iPrEx study, TFV-DP concentrations in peripheral blood mononuclear cells was detected in only 3/42 HIV seroconverters at the visit with first evidence of HIV infection, and quantitative TFV-DP levels were low (and below the range expected for daily dosing) in these individuals at this time point [27]. Finally, quantitative drug levels could be tested in real-time or near-time and be used to identify individuals who may need additional adherence support while taking PrEP in upcoming PrEP studies and demonstration projects. For example, having an undetectable or low TFV hair drug level after PrEP initiation could trigger further evaluation of patterns of adherence and/or potential individual PK parameters affecting TFV clearance. Patients with evidence of low adherence could receive a more intensive adherence intervention and more frequent drug monitoring in PrEP implementation programs. Important next steps for the use of hair as a biological marker of PrEP exposure will be to establish the acceptability of hair collection in real-world PrEP delivery settings, determine the relationship between hair and PrEP efficacy in upcoming PrEP studies, assess the impact of different dosing patterns (e.g. occasional missed doses vs. longer drug holidays) on hair TFV levels, and evaluate the use of hair levels in interpreting PrEP breakthrough infections. These data will help determine whether analysis of hair drug levels could be used as a primary monitoring tool of adherence in upcoming PrEP studies and programs.

As tenofovir's long intracellular half life could afford TDF-based PrEP regimens some PK forgiveness [44], the protective effect of PrEP is more likely to be affected by longer treatment interruptions rather than occasional missed doses, a scenario similar to missed doses to non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimens in HIV-infected individuals with low to moderate adherence [45]. Strategies to improve the utility of hair analyses to detect drug interruptions, such as segmental hair analysis (analysis of short, contiguous segments of hair from the scalp end) [46], warrant further investigation. Given the consistent growth rate of hair in the occipital region of the scalp at approximately 1 centimeter/month, analysis of shorter segments of hair (e.g. proximal 0.5 cm of hair growth) may be useful in monitoring brief periods of PrEP use (e.g. preceding 2 weeks of PrEP use).

Our study had several strengths, including use of a randomized cross-over study design, high adherence to mDOT procedures ensuring dosing fidelity, and intensive PK sampling to evaluate key PK parameters that could influence drug concentrations in various compartments. This study also had several limitations. First, participation was limited to individuals with dark hair, as we aimed to reduce inter-individual variability and maximize the ability of hair to discriminate between different adherence patterns in this proof-of-concept study. While this may limit the generalizability of our findings, many PrEP studies are conducted in international settings where most individuals have dark hair. Second, oral clearance at steady state was only measured once during the 7 doses/week period to reduce participant burden, which did not allow for assessment of intra-subject variability at the other dosing regimens. However, the predictive value of plasma AUC on tenofovir hair levels did not substantially improve in analyses predicting hair levels only after the 7 doses/week period. Third, weekend or holiday doses were not directly observed due to feasibility issues; however, these doses were confirmed by text message or phone. This mDOT approach has been demonstrated to be effective in prior studies [31,47]. Fourth, likely due to our limited recruitment venues to facilitate DOT dosing, we did not enroll African-American participants into this proof-of-concept study. As African-Americans are disproportionately impacted by HIV [48] and are likely be a prioritized group for PrEP, future studies evaluating the PK of TFV and FTC in hair should include this important population. Another limitation is that hair samples were only analyzed once, due to the limited quantity of hair collected from subjects. Finally, this study measured drug levels after 6 weeks of dosing, which may not represent "steady state" concentrations in the proximal 1.5 cm of hair due to growth rate variability. Additional studies are needed to evaluate whether TFV levels in proximal hair samples continue to increase with longer dosing periods (e.g. 3–6 months).

In summary, this study reports a consistent relationship between tenofovir dose and hair in HIV-negative men and women. These measures have already demonstrated utility for other ARVs in the HIV treatment setting and the finding of a strong dose relationship for tenofovir in hair paves the way for evaluation of its use in monitoring adherence in tenofovir-based PrEP demonstration projects and real-world PrEP settings. Unlike phlebotomy, hair collection is inexpensive, noninvasive and does not require specific skills, sterile equipment, or specialized storage conditions. While this hair PK study collected 150–200 strands of hair, ~100 strands are typically needed for TFV level analysis. Hair can be stored for prolonged durations prior to analysis at room temperature and shipped without biohazardous precautions or cold chain requirements. These features may make this monitoring tool particularly promising in resource-poor settings, where high acceptability of hair collection has been demonstrated [49–51]. While the current

equipment used for the LC-MS/MS method of hair analysis is expensive, methods to analyze hair using thin layer chromatography (TLC), a simple and inexpensive analytical tool that has been used to detect ARVs in human plasma, saliva, and umbilical cord blood [52–54] are currently being developed or have been developed [55] and may be performed by local laboratories in resource-poor settings. A low-cost, point-of-care hair assay [55] could also facilitate real or near-time testing of hair samples, and results could be used to inform PrEP adherence counseling.

Quantitative, time-integrated measures of long-term drug exposure in hair may provide more salient adherence information than qualitative (e.g. detectable/undetectable) results or drug levels in single plasma samples. Furthermore, our results may be useful in interpreting data from intermittent PrEP trials. Such dose reduction strategies are expected to lower costs and toxicity, but would be difficult to evaluate without validated biomarkers of adherence. Given that adherence is the ''Achilles' heel'' of PrEP [56], this novel biomarker examining drug levels in hair has the potential to improve adherence measurement in PrEP trials, implementation studies, PrEP adherence promotion strategies, and real-world use.

Supporting Information

Checklist S1 CONSORT Checklist.
(DOCX)

Protocol S1 Trial Protocol.
(DOCX)

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Author Contributions

Conceived and designed the experiments: AL, YH, PB, PLA, KG, RG, SB, RG, and MG contributed to the design, analysis, and interpretation of the data, drafting and/or revising of the article. Performed the experiments: QY and YH conducted the hair assays for this paper. Analyzed the data: AL, CJ, PB and MG were primarily responsible for analyzing the data. Contributed reagents/materials/analysis tools: KS helped provide data relevant to this manuscript. Wrote the paper: All authors (AL, QY, YH, CJ, PB, KS, PLA, KG, RG, SB, RG, and MG) contributed to the drafting or revising of the article and approved the final version of the manuscript. Contributions as above.

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Emtricitabine-tenofovir exposure and pre-exposure prophylaxis efficacy in men who have sex with men

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Abstract

Drug concentrations associated with protection from HIV-1 acquisition have not been determined. This study evaluated drug concentrations among men who have sex with men in a substudy of the iPrEx trial,(1) a randomized placebo controlled trial of daily oral emtricitabine/tenofovir disoproxil fumarate pre-exposure prophylaxis (PrEP). Any detectable drug in blood plasma and viably cryopreserved peripheral blood mononuclear cells (vPBMCs) was less frequent in HIV-infected cases at the visit when HIV was first discovered compared with controls at the matched time point of the study (8% vs 44%, P<0.001) and in the 90 days prior to that visit (11% vs 51%, P<0.001). An intracellular tenofovir-diphosphate (TFV-DP) concentration of 16 fmol per million vPBMCs was associated with a 90% reduction in HIV acquisition relative to the placebo arm. Directly observed dosing in a separate study, STRAND, yielded TFV-DP concentrations that, when analyzed with this iPrEx model, corresponded with HIV-1 risk reduction of 76% for 2 doses per week, 96% for 4 doses per week, 99% for 7 doses per week. Prophylactic benefits were

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Author contributions: PL Anderson helped design the pharmacology studies, oversee the analytical pharmacology work, and analyze the data. DV Glidden helped design the analyses and perform the statistical modeling. A Liu designed and conducted STRAND and assisted with the design and conduct of iPrEx. S. Buchbinder, J Lama, JV Guanira, O Montoya-Herrera, VG Veloso, S Chariyalertsak, K Mayer, LG Bekker, EG Kallás assisted with the design and conduct of iPrEx. LR Bushman oversaw the analytical pharmacology work. V McMahan coordinated the iPrEx study. RM Grant designed and conducted iPrEx, helped design the pharmacology studies, and analyze the data. All authors assisted with writing the manuscript.

Competing interests: Study drugs were donated by Gilead Sciences.

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observed over a range of doses and drug concentrations, suggesting ways to optimize PrEP regimens for this population.

INTRODUCTION

UNAIDS estimates there are 2.6 million new HIV-1 infections per year, despite widespread awareness of the modes of transmission and the protective benefits of condom use.(2) Men who have sex with men carry a disproportionate burden of infection on all continents.(3) The iPrEx trial demonstrated that randomization to daily oral emtricitabine/tenofovir (TFV) disoproxil fumarate (FTC/TDF) PrEP decreased HIV-1 acquisition by 44% compared with placebo among such men, with greater reductions in HIV-1 risk associated with higher reported adherence and detectable drug in the blood. (1)

In a predefined pharmacology substudy of iPrEx, FTC, TFV in plasma and/or FTC-triphosphate (FTC-TP), TFV-DP in vPBMC was detected in 22 of 43 of seronegative subjects (51%) versus 3 of 34 HIV-infected subjects from the active arm (9%) ($P<0.001$). (1) Predicted efficacy in iPrEx increased from 44% to over 90% when detectable drug was accounted for. Thus, adherence to daily FTC-TDF was critical for PrEP efficacy in iPrEx, and the same has been found in other PrEP studies.(4–7) However, the quantitative relationship between drug concentration and/or level of adherence with PrEP efficacy has not been determined. The present study expands on the predefined pharmacology substudy in iPrEx and quantifies the concentrations of drugs associated with protection from HIV-1 acquisition, as well as the frequency of PrEP use required to achieve those concentrations.

RESULTS

Overview

Intracellular tenofovir-diphosphate, the active form of tenofovir, was analyzed in vPBMCs arising from two studies, STRAND and iPrEx. TFV-DP in vPBMC from the STRAND study were used to establish expected concentrations from 2, 4, or 7 doses/week of directly observed TDF therapy, and TFV-DP in vPBMC from iPrEx was used to estimate concentrations associated with decreased HIV acquisition. PrEP efficacy was then quantified for 2, 4, and 7 doses/week by analyzing TFV-DP concentrations from STRAND with the iPrEx HIV risk reduction model. The vPBMC collection, processing and storage for iPrEx and STRAND used identical laboratory protocols, and the drug concentration testing was performed in the same laboratory using identical laboratory methods.

Expected TFV-DP from STRAND

The STRAND study administered oral TDF to 24 HIV-negative adults (FTC was not included), each of whom received 6 weeks of 2, 4, or 7 doses per week. Dosing was directly observed Monday through Friday, including all the 2 and 4 doses per week, and participants provided confirmation of the date and time of doses taken on Saturday and Sunday by text message or telephone contact on the day of use. TFV-DP in vPBMC was measured at the end of the 6 weeks of each dosing regimen. The median (IQR) values were 11 fmol/M (6 to 13) fmol/M for 2 doses per week, 32 fmol/M (25 to 39) for 4 doses per week, and 42 fmol/M (31 to 47) (Figure 1). The median (IQR) times from the last dose to vPBMC collection were 24 hours (20 to 141), 25 hours (22 to 62), and 24 hours (21 to 25) hours, respectively. TFV-DP was quantifiable in all STRAND participants at all visits.

iPrEx analyses

The iPrEx study provided an opportunity to identify vPBMC concentrations associated with different levels of protection from HIV-1 acquisition. Plasma and/or vPBMC were tested for

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FTC-TDF in all 48 seroconverters at the visit when HIV-1 infection was first detected, either by plasma viral RNA, by serum antibodies, or both. The median time between the last HIV-negative test and the first evidence of HIV was 33 days (interquartile range of 28 to 48 days). Each HIV infected case was matched to 3 seronegative controls in the FTC-TDF arm by study site, and duration on study. One of the 3 controls was selected having reported high-risk sexual practices, to assure comparable HIV-1 exposure with cases, and the other two were selected randomly. The cases and controls were comparable with respect to HIV risk factors, level of schooling, and alcohol use (Table 1). Both plasma and PBMC were tested in 42/48 (88%) of cases at the visit when HIV-1 infection was first detected and 144/144 (100%) of controls at the matched time point on study. Plasma and/or PBMC were also tested from other longitudinal study visits in the cases and controls. Drug detection was defined as any quantifiable moiety among plasma TFV, FTC, and intracellular TFV-DP and FTC-TP.

Drug detection in plasma and vPBMC in iPrEx was concordant in >95% of all pairwise comparisons, indicating that plasma and vPBMC analysis yielded similar estimates of the proportion of groups having detectable drug.

Drug detection at the visit with first evidence of HIV-infection

Any drug moiety was detected 5.5-fold less frequently (8% vs 44%; P<0.001) in HIV-positive cases at the visit when HIV infection was first detected compared with the matched time point in HIV-negative controls (Fig. 2). Lower drug detection among cases vs controls (11% vs. 51%; P<0.001) was also observed within the 90 days before the HIV infection visit or matched time point in controls. At time points more than 90 days before HIV was first detected, the proportion of cases and controls with any detectable drug was comparable (36% vs 41%; P=0.77), indicating that HIV infection occurred during periods of low drug exposure in the active arm of iPrEx. These drug detection rates suggest that a substantial fraction of iPrEx participants were dosing with fewer than 2 doses per week, given the 100% TFV-DP detection rate for 2, 4, and 7 doses per week in STRAND (fig 1).

TFV-DP concentrations and risk of HIV-acquisition

Of the iPrEx seronegative controls having quantifiable TFV-DP (36% of the total), the median concentration was 16 fmol/M (IQR 9 to 27) which was between the median concentrations observed for 2 and 4 doses per week in STRAND (Fig. 1). Only 18% of seronegative controls had TFV-DP concentrations in the range associated with daily dosing in STRAND. This suggests that TFV-DP concentrations below those associated with daily dosing were exerting antiviral effects to give the 44% overall efficacy in the group randomized to FTC/TDF versus placebo. Only 3 HIV infected cases had detectable TFV-DP at the time HIV was first detected (the concentrations at the time of infection are not known). These concentrations were in the range of those associated with 2 doses per week in STRAND and none were in the range of daily dosing.

The TFV-DP concentration in vPBMC associated with HIV-1 acquisition was estimated using a Cox proportional hazards model that assessed the risk of HIV infection in the iPrEx trial as a function of TFV-DP (or FTC-TP) concentrations in active arm participants. Exponential regression was also used in a second analysis. Multiple imputation(8, 9) was used to construct a dataset with drug concentrations at each visit when an HIV-1 test was performed. If a drug concentration was not available, imputation was employed using participant and visit level data (age, site, time on study, numbers of partners, creatinine clearance, alcohol use, secondary education, HSV, sexually transmitted disease, pharmacy drug dispensation records and unprotected receptive anal intercourse at baseline and followup) to estimate the missing concentration. This approach allowed HIV-1 risk to be

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estimated for the entire cohort, to estimate how drug exposure as a time dependent variable associated with HIV risk compared with the placebo arm. Drug concentrations below the limit of quantitation (BLQ) were set to 0 fmol/M.

The relationship between drug concentration and HIV infection risk was significant for both TFV-DP (p=0.016) and FTC-TP (p=0.004) among those assigned to receive oral FTC/TDF. Compared with the placebo HIV incidence, the TFV-DP concentrations associated with 50%, 90% and 99% reduced HIV-1 acquisition were 3 (95% CI: <1 to 7), 16 (95% CI: 3 to 28), and 33 (6 to 60) fmol/M vPBMC, respectively. For FTC-TP, the corresponding values were 0.82 (0.1 to 1.6), 3.7 (1.2 to 6.1), and 7.7 (2.6 to 12.9) pmol/M viable cells, respectively. Median (IQR) FTC-TP in cases at the visit when HIV was first detected and at the matching visit in controls were 1.8 (0.9 to 5.1) and 2.9 (1.6 to 5.2) pmol/M, respectively. However, neither TFV-DP nor FTC-TP was independent of the other in a model that included both, therefore further analyses focused upon TFV-DP so that TFV-DP concentrations from STRAND could be utilized.

Dosing and risk of HIV-acquisition

The exponential regression shown in Figure 3 demonstrated that the risk of HIV infection was comparable among participants in the active arm with undetectable TFV-DP compared to the placebo arm (Relative hazard = 0.78; 95% CI: 0.49 to 1.06; p=0.19). Similar to the Cox analysis above, a TFV-DP concentration of 16 fmol/M vPMBCs was associated with 90% HIV risk reduction (EC90) relative to placebo. For perspective, the concentrations from the STRAND study are with colored panels in Figure 3; the EC_{90} (16 fmol/M) was 38% of the median TFV-DP concentration observed in those taking 7 doses per week (42 fmol/M).

Sensitivity analyses were conducted to evaluate the robustness of the estimated vPBMC EC_{90} TFV-DP. An analysis that adjusted for unprotected receptive anal intercourse (the main risk factor for HIV acquisition in this population), and other factors also used for multiple imputation, yielded an estimate of 15 (95% CI: 3 to 27) fmol/M. Allowing drug concentrations below the limit of quantitation to vary uniformly between 0 and 5 yielded an estimate of 20 (95% CI: 7 to 33) fmol/M. Using the averaged drug concentrations from the visit closest to HIV infection with values in the previous 90 days yielded an estimate of 23 (5 to 41) fmol/M. An analysis that brought drug detection at subsequent seroconversion time points to the time of the first detection of HIV-1 RNA yields an estimate of 19 (95% CI: 4 to 33). Adjustment of TFV-DP (from 4.19 to 13.4 fmol/M) in one person whose blood specimen was available 7 days after stopping oral FTC/TDF at seroconversion yielded an estimate of 20 (95% CI: 4 to 36). Estimating a TFV-DP concentration associated with a 90% HIV risk reduction in cases versus controls was also possible using conditional logistic regression without multiple imputation. This analysis yielded a similar estimate for the vPBMC EC_{90} of 16 fmol/M vPBMC (95% CI: 8 to 44). All estimates were comparable with the initial EC_{90} estimate of 16 fmol/M and well within the range of concentrations achieved with 4 to 7 tablets per week in STRAND.

The fraction of case and control time points that exceeded the EC_{90} over time is shown in Figure 4. At the visit when HIV was first detected, no cases (0/42) had TFV-DP ≥ 16 fmol/M compared with 18% (26/144) of seronegative controls at the matched time point (P<0.001; Fig. 4). The proportion of iPrEx participant time points with TFV-DP at or above the EC_{90} decreased among cases (P=0.02) and controls (P<0.001) over time. Reported risk behavior associated with exposure to HIV-1 also decreased overtime in iPrEx.(1)

TFV-DP concentrations arising from 2, 4, and 7 directly observed doses per week in STRAND were analyzed with the exponential regression model from iPrEx described above. The estimated PrEP efficacy was 76% (95% CI: 56 to 96%) for 2 doses per week,

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96% (95% CI: 90 to >99%) for 4 doses per week, 99% (95% CI: 96 to >99%) for 7 doses per week. The proportions of people who attained the TFV-DP EC_{90} were 14% for 2 tablets per week, 90% for 4 tablets per week, and 100% for 7 tablets per week.

DISCUSSION

Drug exposure in HIV-infected cases in iPrEx was critically low at the time of first laboratory evidence of HIV infection, providing a likely explanation for HIV acquisition in these participants. Other evidence indicated negligible drug exposure near the time of HIV infection in cases: plasma HIV-1 RNA levels were comparable in the placebo and active arms, and there was no evidence of TDF or FTC resistance among emergent infections in the active arm.(1) Drug detection in controls was higher than in cases, but was not commensurate with daily dosing in the majority. Only 44% of controls had any detectable drug moiety at the matching time point of the case. Only 18% of seronegative controls had TFV-DP concentrations above 16 fmol/M, a level achieved by 90% of STRAND participants taking 4 or more doses per week, and a level associated with a 90% HIV infection risk reduction.

The minimum protective drug concentrations in the blood, and the required numbers of tablets per week required to achieve those concentrations, may differ depending on the route and frequency of exposure to HIV.(10) For men who have sex with men, the tissue of greatest relevance to the acquisition of HIV-1 infection is the rectal mucosa. Oral dosing has been shown to deliver 20 to 100-fold higher TFV-DP in rectal compared with blood or vaginal/cervical cells/tissue.(11, 12) TFV-DP delivery to penile tissue, relevant for male insertive exposures, has not been determined to our knowledge. The uniquely high delivery of TFV-DP to rectal mucosa suggests that pharmacology findings relevant for MSM, such as in this study, may not directly extrapolate to parenteral, vaginal or penile exposures.

This study demonstrated that the EC_{90} in vPBMC (16 fmol/M) was 38% the median from daily dosing in STRAND (42 fmol/M). With this information, a rectal mononuclear cell EC_{90} can be estimated. The rectal mononuclear cell concentration observed with daily oral dosing was reported to be 1846 fmol/M (95% CI: 931 to 3659).(11) Assuming that the kinetics of TFV-DP in rectal mononuclear cells are similar to PBMC, the EC_{90} in rectal mononuclear cells would be 38% of this value, or approximately 700 fmol/M rectal cells (95% CI: 350 to 1400). This estimate makes several assumptions that require further validation, but nevertheless provides a starting point for a target cell concentration in tissue to translate into animal or ex vivo systems for validation.(13, 14) The threshold identified here is analogous to the TFV concentration threshold of 1000 ng/mL in vaginal fluid identified as protective in the CAPRISA 004 study and in ex vivo assays.(5, 14)

Drug concentrations were measured as close to the HIV infection as possible (at the time HIV infection was first discovered and within 90 days of this time point). Through use of multiple imputation, drug concentrations were assigned to all active arm participants at the time of HIV infection in placebo cases. However, the model could not account for variations in dosing patterns and relationship to timing of HIV exposure and transmission risk.

Confounding is also possible in this analysis, as there may be factors that link higher adherence with lower exposure to HIV. The finding that HIV acquisition among active arm participants with undetectable drug concentrations was not higher than the placebo rate argues against confounding (Fig. 3). In addition, the statistical analysis adjusted for several markers of HIV incidence, including numbers of sexual partners, unprotected anal intercourse, sexually transmitted diseases, age, level of schooling, and substance use.

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vPBMC were available for drug analysis, whereas freshly processed and lysed PBMC are traditionally used for cell pharmacology studies.(15) Measurements of TFV-DP in vPBMC from STRAND were a median of 48% (IQR: 38% to 67%) that of freshly lysed PBMC also collected in that study. Processing vPBMC also introduces additional variability in TFV-DP/FTC-TP measures. Despite this added variation, levels of drug concentrations in blood were found to be strongly associated with reduced HIV risk in the active arm compared with placebo. This finding suggests that drug concentration monitoring could inform HIV acquisition risk in persons taking FTC/TDF for PrEP. Other specimens, such as hair, or dried blood spots, may afford more convenient long term measures of drug exposure which will be particularly useful if they can be correlated with protective drug concentrations in vPBMC.(16, 17)

This study identified a relationship between systemic drug exposure and reduction of HIV acquisition risk in one important population. Protective TFV-DP concentrations were readily achieved with 4 or more doses per week. This study focused on TFV-DP because independent relationships for FTC-TP could not be identified in iPrEx and STRAND did not include FTC-TP so expected concentrations for non-daily dosing are not known. FTC co-administration is not expected to affect intracellular concentrations of TFV-DP in PBMC.(18) Importantly the TFV-DP EC_{90} identified in iPrEx was in the presence of FTC-TP, indicating that reaching these protective TFV-DP concentrations with FTC-TDF dosing is relevant for PrEP efficacy in MSM. Nevertheless, future studies should aspire to evaluate the contribution of FTC-TP to PrEP efficacy.

Alternative dosing regimens, such as pre- and post-intercourse dosing, warrant controlled clinical trials to evaluate the acceptability, behavioral feasibility, and pharmacokinetics of non-daily regimens. The 95% confidence interval for the estimate of the TFV-DP EC_{90} was 3 to 28 fmol/M vPBMC. The lower bound (3 fmol/M vPBMC) would be achievable after a single dose,(19) and 28 fmol/M was just below the median achieved with 4 doses per week. While animal studies indicate that both pre- and post-exposure dosing are important,(20) more information is needed to define the timing and duration of drug exposure that is required to prevent infection. Dose optimization in the absence of a surrogate marker of protection would require prohibitively large trials, so better definition of pharmacological parameters associated with differing dosing strategies (eg: dose and dosing interval) and protection will be essential to move the PrEP field forward. Ultimately, recommendations for daily PrEP use may be more robust, as daily regimens encourage routinization, and afford drug concentrations that are expected to persist in the protective range even if some doses are missed. The low fraction of iPrEx participants with TFV-DP in the range of daily dosing suggests that demonstration projects should optimize ways to promote daily dosing.

In conclusion, a target TFV-DP concentration for MSM was identified. This threshold enables further studies in MSM that evaluate new ways of promoting the consistent use of PrEP, the key determinant of efficacy.

MATERIALS AND METHODS

iPrEx Trial

The design, conduct, and outcomes of the iPrEx trial have been published previously.(1) Briefly, the iPrEx initiative was a randomized, double-blinded, controlled trial of daily FTC-TDF versus placebo in HIV-negative men and transgender women 18 years or older who have sex with men, meeting behavioral criteria that put them at risk for sexual acquisition of HIV. Two thousand four hundred ninety nine participants were randomized and followed monthly through a median of 87 weeks of therapy (IQR 61 to 125). Eighty-three infections

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were observed in the placebo arm versus 48 infections in the FTC-TDF arm (efficacy, 42% (95% CI 18% to 60%).(21)

Plasma specimens were stored every 12 weeks and viably cryopreserved PBMC specimens were stored every 24 weeks and at the time of study discontinuation or seroconversion. Each participant from the active arm who contracted HIV during the study was included in this pharmacology substudy (cases). For each visit week when HIV was detected in cases, samples were selected from 3 HIV negative controls from the active arm at the same site; 2 randomly, and 1 from among those reporting unprotected receptive anal intercourse (URAI) in a recall period prior to the specimen collection. The latter control was chosen to enrich the control sample for people exposed to HIV, to better match the HIV infected cases. Specimens were tested from the time of first evidence of HIV infection in the cases, and the nearest visit week in controls, as well as longitudinally at other available time points during the treatment period.

STRAND Study

The STRAND study was an open label, randomized, 6 sequence, 3-period, single-site, cross-over trial in 24 HIV-negative adults (12 men, 12 women) that tested the effects of three different oral TDF dosing regimens on TFV concentrations in hair, plasma, and PBMC. The 6 sequences were different orders of the 3 dosing strategies: 7 doses/week, 4 doses/week, and 2 doses/week. The 2 doses/week were dosed on Tuesday and Wednesday and 4 doses/week on Monday, Tuesday, Thursday, and Friday. Each dosing period lasted 6 weeks. All doses scheduled Monday through Friday were directly observed by study staff and the doses on the weekend were confirmed by telephone or text message. Stored viably cryopreserved PBMC (using the same method as in iPrEx) were collected at the end of each 6 week dosing period. Freshly processed/lysed PBMC were available from a subset of participants. The study was funded by the US National Institutes of Health and was approved by the UCSF Committee on Human Research.

Analytical pharmacology

An LC/MS/MS assay was validated for the determination of TFV and FTC in human plasma. (22) The method includes protein precipitation and stable isotopic internal standards. The linearity of the concentration curves were in the range 10 ng/mL to 1500 ng/mL for both analytes (250 μL plasma extracted). The lower limit of quantification was 10 ng/mL for both analytes.

The viable PBMC processing procedure involved a quick thaw in a 37°C water bath and mixing by inversion (1 minute). Cells were immediately transferred to a 15 ml centrifuge tube that already contained 10 ml of pre-warmed (37°C) PBS followed by gentle mixing by inversion. Cells were then pelleted and the supernatant discarded. If RBC contamination was visible, the cells were treated with a RBC lysing buffer. Cells were then counted with an automated hemocytometer. Viability and total cell count were recorded; cells were kept on crushed ice through the process. Cells were washed and lysed. Median (IQR) viability at the time HIV was discovered was 66% (56% to 76%) overall, 71% (56% to 79%) in cases and 64% (56% to 73%) in controls.

A validated liquid chromatography tandem mass spectrometry (LC–MS/MS) assay was used for the determination of TFV-DP and FTC-TP from lysed intracellular matrix.(15) The method utilized a strong anion exchange isolation of mono-(MP), di-(DP), and tri-phosphates (TP) from intracellular matrix. The TP fraction was then dephosphorylated to the parent moiety yielding a molar equivalent to the original nucleotide analog intracellular concentration. The analytical portion included desalting/concentration by solid phase

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extraction and detection by LC–MS/MS. The quantifiable linear range for TFV-DP was 2.5–2000 fmol/sample, and that for FTC was 0.1–200 pmol/sample. Stable labeled isotopic internal standards facilitated accuracy and precision in various cell matrices. (15) Two million total cells were typically extracted, constituting the "sample", and results were corrected for cell viability and normalized to fmol or pmol per million (M) viable cells.

Stored viable PBMC samples such as in this study have been used for the measurement of intracellular TFV-DP in previous studies (see appendix in reference 1).(1) These studies demonstrated that values in viably cryopreserved PBMC (median approximately 40 fmol/M viable cells) were lower than those measured in pharmacology studies among HIV-infected subjects where PBMC samples were processed and lysed immediately (median approximately 90 fmol/M cells).(23, 24)

To evaluate viable cells in more detail, an internal study called iOptimum enrolled 10 HIV-infected participants for a single blood draw to assess the TFV-DP and FTC-TP in viable cryopreserved cells. Self-reported adherence in the prior 30 days was 63 to 100% and viral loads were largely suppressed (range, <40 to 240 cp/mL), suggesting good adherence to treatment. Aliquots of viable cells were stored in liquid nitrogen for 4, 12 and 24 weeks. Freshly lysed cells were also collected. Both TFV-DP and FTC-TP were quantifiable in all viable PBMC samples, and above the lower limit of quantitation by several fold. The median (IQR) ratio of viable PBMC/PBMC at weeks 4 and 12 in liquid nitrogen storage were 0.3 (0.22 to 0.38). The median (IQR) value for 24 weeks in liquid nitrogen was 0.56 (0.38 to 0.83), which was similar to STRAND 0.48 (0.38 to 0.67). Median (IQR) time in liquid nitrogen for iPrEx and STRAND samples were 70 (46 to 108) weeks and 57 (52 to 65) weeks, respectively. We have observed no loss in viable cell concentrations for storage times in liquid nitrogen through 119 weeks (2.3 years).(1)

Statistical Methods

All analyses were performed using Stata 12.1.(25)

Model for the Effect of Intracellular Drug Concentration

This analysis fits a (stratified by site) Cox proportional hazards model to the entire cohort with a model

$$h_k(t;Z(t),\mathrm{rx})=h_{ok}(t)\exp(\beta_1\mathrm{rx}+\beta_2 Z(t)) \quad (1)$$

$h_k(t)$ is the hazard of HIV infection at the kth site and rx = 1 if the participant is assigned to FTC/TDF and 0 if the participant is assigned to placebo. The variable Z(t) is a time-dependent covariate for a quantitative drug concentration (e.g., TFV-DP) where Z(t) is set to be a drug concentration of 0 if the participant is on the placebo group. We examined quadratic and logarithmic transformations of the concentration Z(t) but neither improved the fit.

The primary analyses have used models stratified by clinic site to avoid confounding the association between drug concentrations and HIV acquisition. Stratification was incorporated into the design of the nested case control study by matching controls by study site. Stratification can be highly efficient relative to an unstratified model even if confounding by site is weak.(26)

The model (1) uses the placebo group as the baseline hazard function permitting comparison the hazard of HIV acquisition at a given drug concentration to the placebo group. For

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instance, the concentration Z(t) which is associated with a d% reduction is risk relative to placebo is

$$\{\log(1-d/100)-\beta_1\}/\beta_2.$$

The concentration associated with a d% reduction in risk relative to a 0/BLQ concentration on the FTC/TDF arm is

$$\log(1-d/100)/\beta_2$$

Here, β_1, is the log hazard ratio of a 0/BLQ level on the FTC/TDF arm (relative to placebo), β_2 quantifies the change in risk with the concentrations of drug and $h_{0k}(t)$ is the baseline hazard of HIV seroconversion on the placebo arm.

We also fit

$$\alpha(t;Z,\mathrm{rx})=\alpha\exp\{\alpha_1\mathrm{rx}+\alpha_2 Z(t)\} \quad (2)$$

where this exponential model is parameterized to yield the annualized incidence of HIV. The fit of this model to the data yielded similar results to the fit of model (1) and was used as the basis of the annualized HIV incidence graphed in Figure 3.

Note that the models (1) and (2) require a complete set of drug concentrations for participants in the active arm. We must confront two type of missing data. First, only a randomly selected subset of time points had drug concentrations tested. Second, intracellular drug (TFV-DP, FTC-TP) was only tested every 6 months and at the time of HIV seroconversion. Hence, even if a control has drug concentration tested, the information is sparse and may not correspond to an ideal time match to their HIV case.

We used two strategies for fitting model (1) with missing data in the drug concentrations: multiple imputation and conditional logistic regression. Conditional logistic regression functions only as a sensitivity analysis since it is unable to use the placebo group as a reference and does not handle the uncertainty in drug concentrations in the controls due to the long periods of time between measurement of drug concentrations.

Multiple Imputation

The concentration of drug for the HIV-infected cases at the time of HIV infection was taken as the intracellular concentration observed at the first laboratory evidence of HIV infection. Six participants lacked an intracellular specimen at the first laboratory evidence of HIV infection -- all had a plasma specimen at the first evidence of infection and all were BLQ (below the limit of quantitation) for TFV and FTC. In the case control study of 355 plasma specimens which are BLQ for TFV and FTC, 96% were BLQ for TFV-DP and 95% were BLQ for FTC-TP. Hence, the TFV-DP and FTC-TP for these 6 seroconverters were set as BLQ.

For controls, we attempted to infer the drug concentration at the identical day of follow up as seroconverters from the same site. We used the closest intracellular drug concentration within 45 days as the drug concentration if one was available and considered that as a measured drug concentration. If there was no tested drug concentration within 45 days, then

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drug concentrations were imputed. Varying the 45 day window had little effect on the results.

If the visit had plasma but not intracellular concentrations (vPBMC), we used whether the plasma had detectable or not in our imputation model. We performed multiple imputations (9) of TFV-DP, FTC-TP and any detection of drug in plasma or PBMC using medication possession ratio (defined as the number of tablets dispensed at the prior visit divided by days since the last visit at which medication was dispensed), study week, unprotected receptive anal intercourse (URAI) at baseline, URAI at follow-up (most recent report on or after the time point), baseline herpes simplex virus (HSV) status, number of male sexual partners at screening, participant report of a sexual transmitted infection in the six months prior to enrollment, secondary education, age at enrollment (in years) and baseline (estimated) creatinine clearance (by Cockcroft-Gault equation). Plasma and PBMC concentrations have monotone missingness hence chained imputation was used to create joint imputations. The imputation model for detection of drug in plasma was based on logistic regression and imputations for TFV-DP and FTC-TP were based on predictive means matching.(8) Predictive means matching used a regression model to identify observed values in the data which form the most plausible value for the imputations, yielding non-parametric imputations that must follow the observed distribution of the drug concentrations. This method seamlessly imputed concentrations of TFV-DP and FTC-TP that were below the limit of quantitation. This permitted us to form imputations in a first stage of analysis and to vary the strategy for quantifying BLQ values in a second stage. We performed 20 imputations per observation.

Conditional Logistic Regression

Analyses using conditional logistic regression mimics a (stratified) Cox proportional hazards model among participants on the active arm of the form

$$h_k(t;Z)=h_{0k}(t)\exp\beta_3 Z(t) \quad (3)$$

where k is the clinic site and Z(t) is a time-dependent covariate for drug concentration. Prentice and Breslow demonstrated that by measuring covariates in cases and a sample of (time matched) controls, it was possible to fit (3) using a conditional logistic regression model. (27) Hence, the coefficient in the conditional logistic regression is a valid estimator of the log hazard ratio β_3 which is the risk reduction compared to a drug concentration of 0 on the active arm. As a sensitivity analysis, we compared the results of our estimated protective concentrations by calculating the intracellular drug concentrations associated with a d% reduction in risk relative to a 0/BLQ concentration on the FTC/TDF arm as

$$\log(1-d/100)/\beta_3$$

Inferred Protection

Based on estimates for model 1 fit using multiple imputation, we estimated the protective effect of dosing regimens used in STRAND. For this, we combined the parameter estimates from model (1) with the observed TFV-DP from the 2, 4, and 7 tablets per week regimens in STRAND. The estimated relative risk reduction was approximated by

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$$100\left(1-n_D^{-1}\sum_{i=1}^{n_D}\exp\{\widehat{\beta}_1+\widehat{\beta}_2 Z_{iD}\}\right)$$

where (Z_{1D},,....., Z_{nDD}) were the n_D observed TFV-DP concentrations for D tablet per week regimen and the parameter estimates were taken from fitting model 1 using multiple imputation with associated 0.95 level Wald-based confidence intervals.

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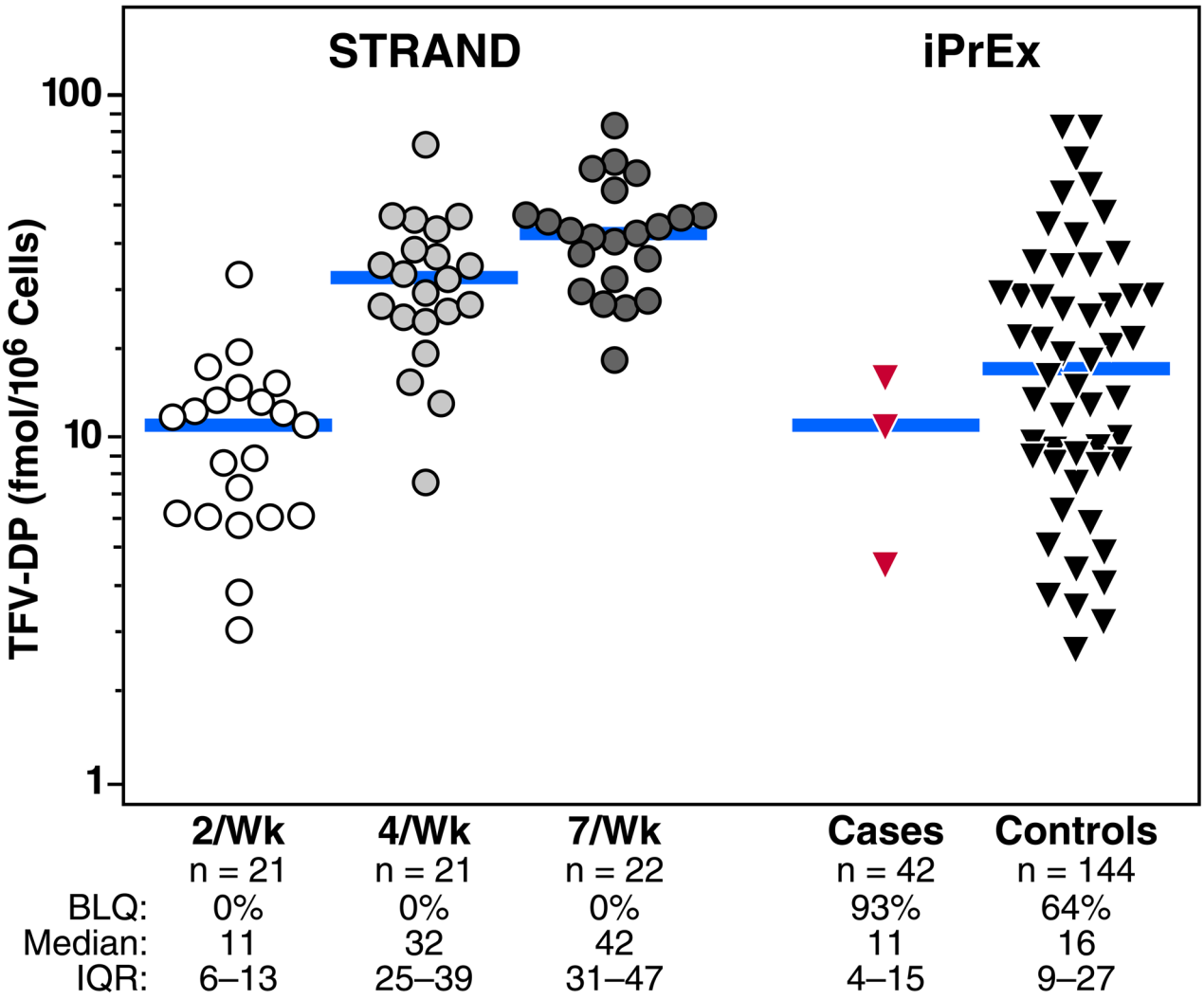

Fig 1.

Observed TFV-DP concentrations from STRAND and iPrEx. The values observed in the STRAND study are shown on the left for 2 doses/week (open circles), 4 doses/week (light-toned circles), and 7 doses/week (dark-toned circles). iPrEx values on the right included those from the visit with first evidence of HIV infection in cases (red triangles) and the matched study visit in HIV-negative controls (dark-toned triangles). The bars represent the medians. The numbers of participants tested, the proportion of vPBMC tested with detectable TFV-DP; the median concentrations among values in the detectable range; and the interquartile ranges are listed below the x-axis.

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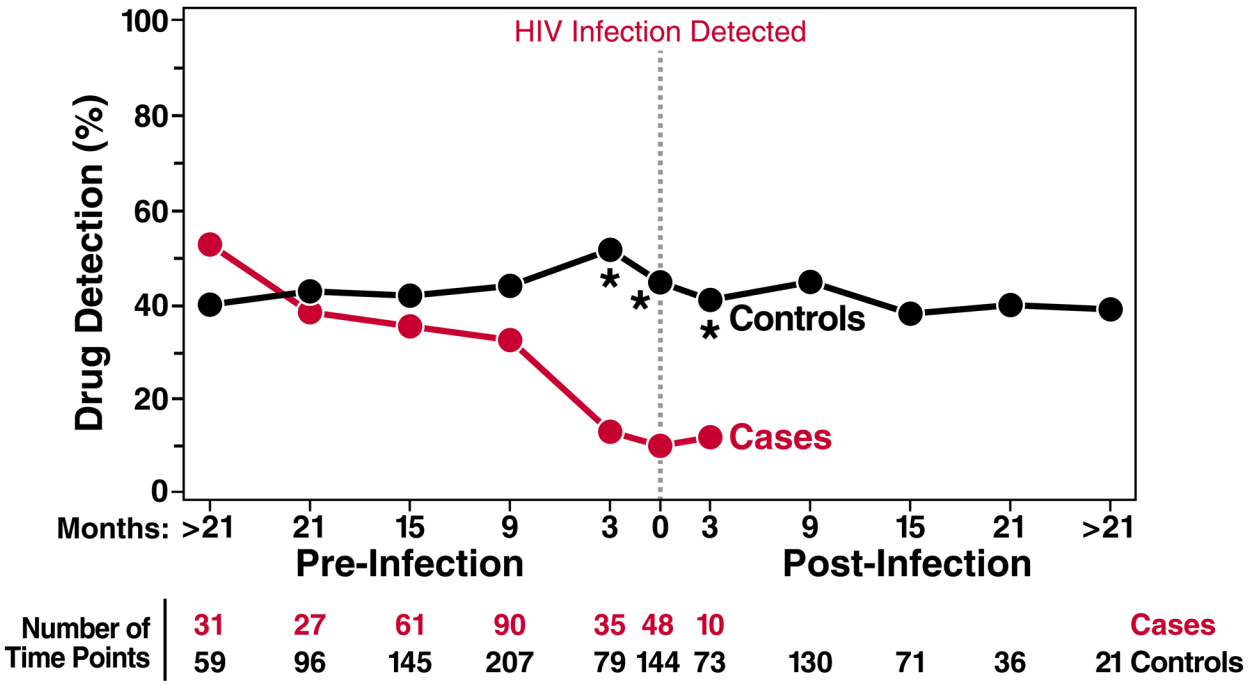

Fig 2.

Longitudinal drug detection relative to the time of first HIV-1 detection. The % of case and control time points with any drug detection in plasma or PBMC including TFV, FTC, TFV-DP, or FTC-TP, at the time of first laboratory evidence of HIV in cases or the matched time point in controls (dashed vertical line), and at pre- and post-HIV infection time points. The pre- and post-HIV infection time points were divided into those within 3 months (90 days) before or after HIV infection, and those within 6 month intervals distal to these time points. The number of time points with either plasma or vPBMC available for testing is listed for each time period.

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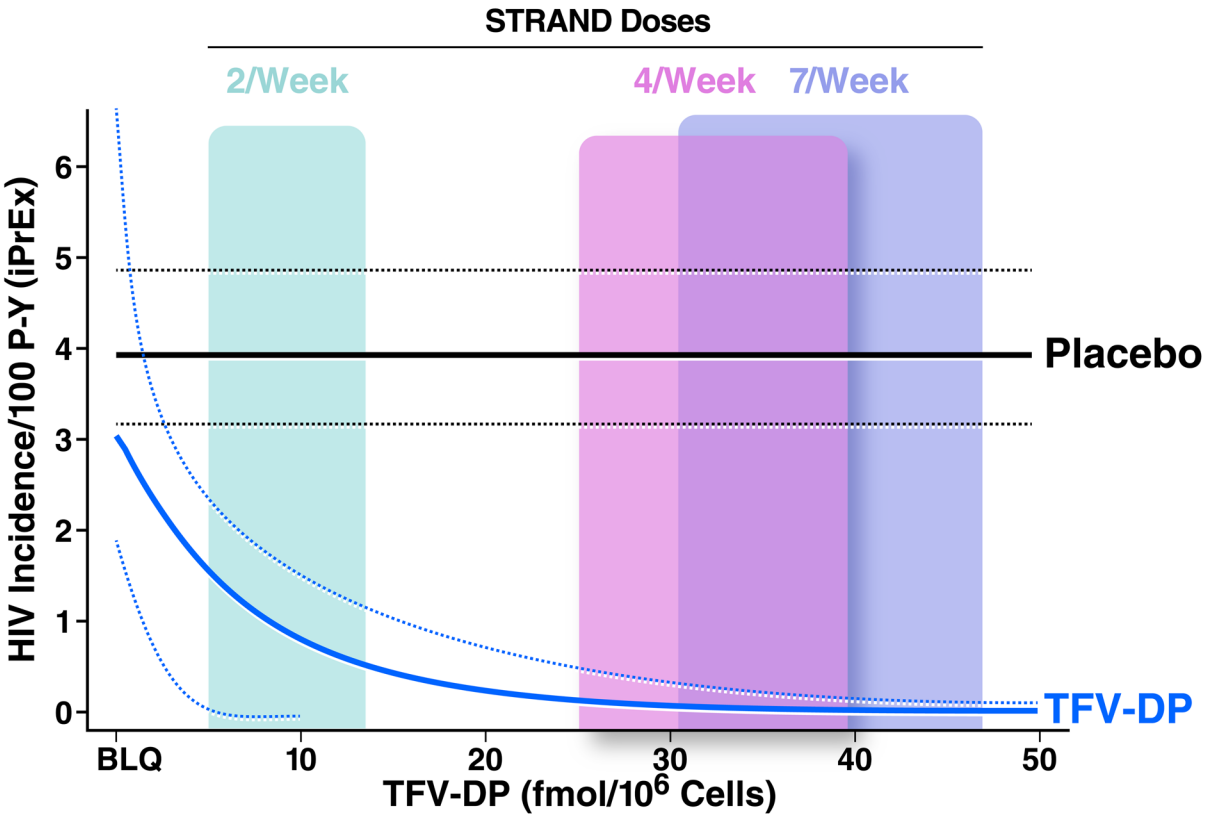

Fig. 3.

Estimated HIV incidence from exponential regression model of TFV-DP concentrations (fmol/M vPBMC) in iPrEx. The placebo HIV infection rate is shown as a horizontal line at 3.9 infections per 100 person years, with the relative rate in the active arm according to TFV-DP concentrations (x-axis). Dashed lines represent the 95% confidence intervals. The IQR of TFV-DP concentrations associated with directly observed dosing in STRAND are provided as colored panels overlying the curves.

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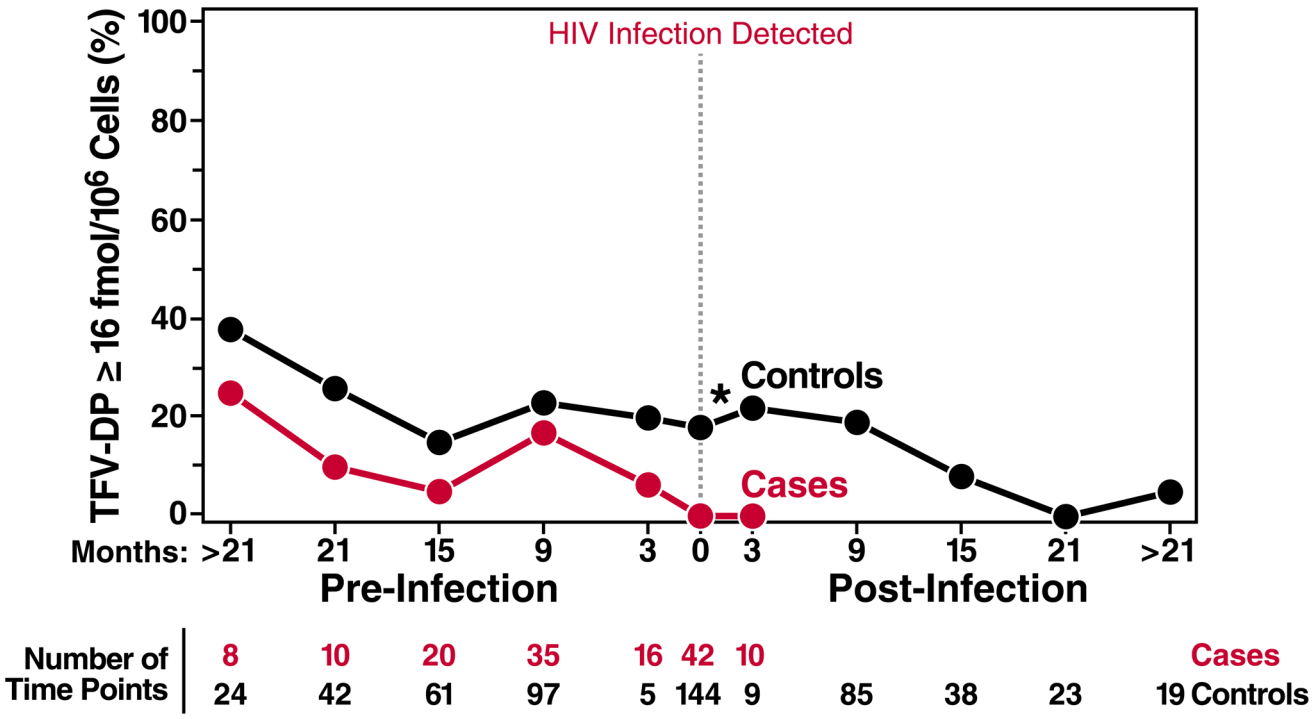

Fig 4.

The % of case and control time points with TFV-DP ≥ 16 fmol/M viable PBMC (estimated EC90). Dark-toned circles represent HIV-negative controls, and red circles represent HIV infected cases. The x-axis represents time relative to the visit with first evidence of HIV infection in the case and the matching time point in controls. Pre- and post-HIV infection time points were divided into those within 3 months (90 days) before or after HIV infection, and those within 6 month intervals distal to these time points. The number of time points with vPBMC tested for TFV-DP is listed for each time period. *indicates significant differences between cases and controls (P<0.05).

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Table 1

Demographic and HIV risk characteristics at baseline among analysis groups in the iPrEx study

	Cases N=48	Controls N=144	p-value
Baseline	*mean (sd)*		
Age in years	24 (6)	27 (8)	0.01
Calculated CrCl ml/min	125 (23)	121 (22)	0.30
Male Partners In past 6 months	12 (32)	13 (46)	0.78
	n (%)		
Secondary School	31 (66%)	109 (76%)	0.19
Alcohol Use (≥5 drinks per day on days when drinking)	24 (52%)	74 (54%)	0.87
Unprotected Receptive Anal Intercourse	34 (71%)	109 (76%)	0.57
History of sexually transmitted infection in past 6 months	15 (31%)	52 (36%)	0.60
Herpes seropositive	24 (50%)	69 (48%)	0.87
At Time of HIV Detection in Cases	*mean (sd)*		
Weeks on Study	63 (35)	65 (36)	0.73
	n (%)		
Unprotected Receptive Anal Intercourse	19 (40%)	74 (51%)	0.18

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Pre-exposure Prophylaxis (PrEP) for HIV Infection: How Antiretroviral Pharmacology helps to Monitor and Improve Adherence

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Abstract

Pre-exposure prophylaxis (PrEP) with antiretroviral drugs is a novel biomedical intervention that can prevent HIV transmission among high-risk populations. As findings from multiple PrEP studies have suggested that adherence is vital to achieve the full prevention benefits of PrEP, it is important to understand the clinical pharmacology and pharmacokinetic (PK) properties of PrEP antiretrovirals, the association of PK and PrEP efficacy and the potential for drug concentration measurement to be used as a tool to monitor PrEP adherence. In this review, we examine studies related to PrEP adherence with attention to the clinical pharmacology and pharmacokinetics of current and novel PrEP agents. Studies of animal models, pharmacokinetics and clinical trials related to PrEP and adherence were reviewed. In summary, when combined as part of a comprehensive prevention strategy that includes use of condoms and risk reduction counseling, PrEP has tremendous promise as a adjunctive biomedical HIV prevention intervention, providing that adherence is maintained.

Keywords

Pre-exposure prophylaxis; adherence; pharmacology; pharmacokinetics

I. Introduction

Given the failure of the most promising HIV vaccine candidates, most experts agree that combination prevention strategies will be needed to control the HIV epidemic. In addition to encouraging early and effective therapy for those already diagnosed with HIV infection--to interrupt transmission to others as well as for the benefit of reducing HIV disease progression--newer strategies have focused on the use of antiretroviral agents in high-risk, HIV-uninfected populations. Because of the improved tolerability, ease of administration and efficacy of newer agents, several recent clinical trials have evaluated single and dual antiretroviral agents for pre-exposure prophylaxis (PrEP). These studies confirmed the findings in animal models that PrEP, when used correctly, can prevent HIV-infection in high-risk populations.

On July 16, 2012, the Food and Drug Administration (FDA) approved a once daily fixed dose combination of emtricitabine/tenofovir disoproxil fumarate (FTC/TDF) to reduce the risk of HIV infection in uninfected individuals who are at high risk of HIV infection and

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who may engage in sexual activity with HIV-infected partners.[1] Several randomized controlled trials (RCTs) of FTC/TDF for PrEP have shown a statistically significant reduction in HIV acquisition among at-risk individuals.[2–5] However, not all studies support the use of PrEP; in two studies, there was no reduction in the risk of HIV transmission in high-risk heterosexual women.[6,7] In both of these studies, inadequate adherence to PrEP medication reduced potential benefit of PrEP and undermined the trials' ability to determine the efficacy. In fact, data from all the PrEP studies suggest that maintaining adherence to the drug is critical to realize the full prevention benefits of PrEP. In this paper, we will review studies related to PrEP adherence with a focus on defining how the clinical pharmacology of oral PrEP can be used to understand efficacy and as a potential adherence measurement.

II. Pharmacology of TDF and FTC

Both TDF and FTC are nucleos(t)ide analogue reverse transcriptase inhibitors (NRTIs) which must be taken up into cells and sequentially phosphorylated in the cell to the pharmacologically active diphosphate (DP) and triphosphate (TP) anabolites. Thus, the clinical pharmacology of TDF and FTC is dependent on their intacellular half-life.[8,9] The longer intracellular NRTI anabolite half-life enables less frequent dosing; the strategy of NRTI dosing based on the intracellular half-life has been confirmed by many clinical studies.[10–12] The half-lives of intracellular tenofovir-DP (~150h) and emtricitabine-TP (~39h) are the longest for the NRTI class, a potentially favorable pharmacological characteristic for PrEP from an adherence perspective.[9,13] However, these long half-lives could be harmful in terms of clinical toxicities and drug and food interactions.

Animal models of simian immunodeficiency (SIV), HIV or SHIV (SIV/HIV chimera) transmission have shown valuable proof-of-concept information regarding the prophylactic efficacy of oral and topical PrEP with ARV drugs through varying routes of mucosal exposure. In one study, a model of rectal infection to assess the efficacy of TDF, FTC or FTC/TDF at equivalent human exposure found that FTC/TDF was more protective compared to either TDF or FTC alone.[14] Other animal models have been used to explore the efficacy of intermittent oral dosing with TDF or FTC/TDF. Studies in macaques showed protection from oral or rectal SIV/SHIV exposures by a two-dose subcutaneous regimen of TFV or TFV/FTC.[14,15]

Understanding the distribution and persistence of virus, introduced during an HIV exposure, can help to ensure that ARV agents used for PrEP are available at the site of infection for adequate time to prevent infection. Two studies used simulations of rectal and vaginal sex acts and a virus surrogate to model the distribution of cell-free and cell-associated HIV that would be present in the colon and vagina following sex. The investigators found that rectally dosed HIV surrogates concentrated in the recto-sigmoid colon and vaginally dosed HIV surrogates concentrated in the peri-cervical area, and both were usually cleared within 24 hours.[16,17] These studies suggest that optimal PrEP medications deliver HIV-inhibiting ARV concentrations to the peri-cervical region or into the recto-sigmoid prior to and for at least 24 hours after sex.

Mucosal tissue distribution after PrEP delivery has been examined to determine drug concentrations in relevant anatomic compartments. In Microbicide Trials Network (MTN)-001, a randomized cross-over trial in which African and US high-risk women received daily oral TDF, TFV vaginal gel, or both; several important findings emerged. There was 60-fold greater serum TFV concentrations after oral dosing compared with vaginal dosing and greater than 130-fold higher vaginal tissue TFV-DP (the intracellular active form of the drug) concentrations with vaginal compared to oral dosing.[18]

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In the CAPRISA 004 trial, which tested tenofovir gel in heterosexual women, the HIV incidence rate in women with tenofovir cervicovaginal concentrations greater than 1000 ng/mL was significantly lower than the HIV incidence in the placebo group.[2] Active treated subjects with cervicovaginal concentrations less than 1000 ng/mL had HIV incidence rates close to that in the placebo group. These tissue (i.e., cervicovaginal) tenofovir concentrations may reflect drug exposure at the actual time of HIV exposure; however, other non-measured confounders between these three groups of women might have accounted for some of the variations in HIV risk. Nevertheless, these data suggest that cervicovaginal fluid concentrations of tenofovir greater than 1000 ng/mL were required to prevent HIV infection. The cervicovaginal concentration of 1000 ng/mL is more than ten times the concentration seen with oral tenofovir disoproxil fumarate and emtricitabine.[19]

Similar to vaginal dosing, in the Rectal Microbicide Program-02/MTN-006 which compared a single oral TDF dose to a rectal TFV gel dose, plasma TFV concentrations were 23-fold greater after oral compared to rectal dosing, and tissue TFV-DP concentrations were 10-fold greater after rectal compared to oral dosing.[20] In a single dose oral TDF-FTC study of 6 men and 6 women with 2 weeks of post-dose sampling, rectal biopsy homogenates in men showed TFV-DP concentrations more than 100 times greater than TFV-DP in vaginal homogenates in women twenty-four hours after dosing. Interestingly, FTC-TP was 10-fold greater in vaginal tissue compared with rectal tissue but was not detectable in either after 2 days. Although the significance of this difference is unknown, these findings suggest that different PrEP agents may be more effective for different HIV routes of exposure. [21] Taken together, these studies highlight the differences in plasma, lumen and tissue concentrations of antiretroviral agents and that these concentrations are dependent upon patient sex, drug used and method of delivery. All of these factors need to be considered when selecting PrEP agents for clinical use and assessed when interpreting the results of randomized clinical trials. However, these studies should be interpreted cautiously as definitive association of tissue concentrations with efficacy of PrEP has not been demonstrated.

III. Pharmacokinetic Considerations

In addition to the value of drug concentrations and pharmacokinetics (PK) to the selection of ARV for PrEP and in the design of PrEP efficacy studies, drug concentration measures and PK can be useful for interpreting the results of studies and as surrogate measures of patient adherence to therapy. Characterization of the PK of FTC, TDF and their triphosphate anabolites in plasma, red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs) is essential to developing informative pharmacologic adherence metrics. In general, long half-life drugs are viewed favorably as they may offer “pharmacokinetic forgiveness” during times of suboptimal adherence and may be less impacted by drug-drug or drug-food interactions. This idea of “pharmacokinetic forgiveness” is most important once concentrations are at steady state, which is reached in approximately 4 half-lives of continuous dosing.[22]

The key element in being able to accept a pharmacologic metric as a surrogate for patient adherence to medication is to establish a relationship between measured drug concentration and response to therapy. In the case of PrEP studies, a concentration-prevention association must be established. PK data from several oral PrEP RCTs offer evidence of concentration-response. In the Pre-exposure Prophylaxis Initiative (iPrEx) study, the detection of any of TVF and FTC in blood plasma and PBMCs was associated with 92% relative risk reduction in HIV transmission compared to 44% reduction in transmission for all participants randomized to the drug.[3] Thus, in the sub-group of subjects with presumed better PrEP adherence based on detection of drug in their specimens, the benefit of PrEP was increased from 44% to 92%. Relatively sparse PK sampling showed clear demonstration of

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concentration-response in the Partners PrEP trial with a small increase in relative risk reduction in both TDF and TDF/FTC arms from 67 to 86% and 75 to 90%, respectively, in the overall cohort compared to subjects who had detectable TFV levels.[4] In the CDC TDF2 study, TVF and FTC were more commonly detected in nonseroconverters (~80%) compared with seroconverters (50%) for both drugs.[5]

From these oral PrEP RCTs, the TFV plasma concentrations were different not only among subjects but also among studies. TFV concentration data contain significant heterogeneity due in part to variability between individuals which is further increased by variation in the timing of the previous dose of medication relative to the time of concentration measurement. In addition, the confidence intervals of relative risk reduction are also quite large in some studies due to few seroconversions or small sample size. However, the variation seen in concentrations among RTCs goes beyond the variability that would be expected if due soley to either inter-individual variability or prescribed daily dosing time in the day prior to blood testing.

Not all studies that use oral TDF or TDF/FTC have demonstrated protection against HIV compared to placebo controls. While reduction in HIV transmission was seen in the iPrEX, Partners' PrEP and TDF2 trials, no reduction in transmission was found in FEM-PrEP and Vaginal and Oral Interventions to Control the Epidemic (VOICE) studies. Similarly, the use of tenofovir gel for topical protection against HIV transmission was effective in Centre for AIDS Programme of Research in South Africa (CAPRISA) 004 when used peri-coitally but not in VOICE study when it was used daily.[19] A potential unifying mechanism underlying the lack of efficacy in the studies which failed to show benefit is poor adherence to administered treatment. Further, it is the objective measures, i.e., measured drug concentrations, and not self-reported adherence measures that highlighted the lack of benefit. For example, in each treatment arm in VOICE, participants reported ~90% medication adherence, with product-return data suggesting similarly high levels of adherence. Plasma TFV levels, however, were detectable in 30% or fewer women in all three arms that were given active drug.[7] Together, these findings not only underscore the importance of adherence to achieve adequate drug levels but the difficulty with which it is to accurately measure adherence, an integral monitoring strategy for effective PrEP implementation.

IV. Selection of Antiretroviral (ARV) Agents for PrEP

FTC and TDF quickly emerged as viable and practical PrEP agents based upon safety and tolerability from studies in HIV-infected subjects, potential cost-effectiveness, penetration into target tissues and expected HIV resistance profile if HIV infection were to occur during PrEP use. In addition, these agents have favorable pharmacological characteristics that allow for once daily and potentially less frequent dosing, few drug interactions and theoretical effectiveness during periods of missed doses.[22] The combination of FTC/TDF was the first oral agent to demonstrate clinical efficacy in reducing HIV transmission in randomized, controlled trials. A major drawback of the use of TDF and FTC is their central role in current treatment strategies for HIV-infected patients; if HIV infection were to occur during PrEP exposure with these agents, treatment efficacy of TDF/FTC containing regimens might be reduced due to the development of HIV drug resistance at the time of infection. Thus, antiretroviral drugs that are less commonly used for HIV treatment would have an advantage for use in PrEP.

Additional drugs under consideration for use as PrEP include maraviroc, long-acting rilpivirine and the investigational drugs S/GSK1265744, ibalizumab and dapivirine. Maraviroc, a CCR5 antagonist, has a low rate of adverse events, remains active for several

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days and concentrates in vaginal secretions[23] and rectal tissue[24]. Mutations associated with drug resistance are infrequent during treatment of HIV-infected patients--the main mechanism of viral escape has been emergence of low levels of non-CCR5 containing virus among pre-existing quasispecies within treated patients. As most transmitted virus is CCR5 tropic, this mechanism of resistance is posited to be less problematic for prevention strategies.[25] As there is limited clinical data in HIV-uninfected persons, a phase 2 study of maraviroc is currently underway to assess safety and tolerability.

A parenteral, long-acting form of rilpivirine (RPV-LA), a nonnucleoside reverse transcriptase inhibitor (NNRTI), was developed in an effort to improve treatment adherence for HIV-infected patients and for potential use as a PrEP agent. The greatest advantage to this drug for PrEP use is its very long half-life. Preclinical studies in rats and dogs demonstrated effective concentrations of the drug being measured for more than 3 weeks and 3 months, respectively.[26] A long half-life and concentration of rilpivirine in tissues was seen in men and women in a phase Ia study of a single 600 mg intramuscular dose of long-acting RPV nanosuspension. RPV persisted for more than 80 days and had a terminal half-life in women of 33 days and 35 days in men. Vaginal fluid and rectal tissue concentrations were higher than matched plasma concentrations in women and men, respectively.[27]

An early investigational HIV integrase inhibitor, S/GSK1265744, appears to be a promising agent for PrEP. Although safety and efficacy data in HIV-uninfected individuals is currently being analyzed[28], S/GSK1265744 has a 30-hour half-life[29] and has been formulated as a long-acting parenteral form. This nanosuspension formulation of S/GSK1265744 has a half-life of 21 to 50 days after a single injection in HIV-negative human subjects, suggesting that once monthly or even less frequent dosing may be possible.[30] The long acting preparation of GSK1265744, when given to rhesus macaques exposed to Simian-human Immunodeficiency Virus (SHIV), was found to completely prevent infection in test animals from repeated rectal SHIV challenge.[31]

Finally, ibalizumab is an investigational CD4 attachment inhibitor that can be given as weekly or biweekly injections.[32] Its novel mechanism of action, initial favorable safety profile and pharmacokinetics favoring infrequent dosing make it a promising PrEP agent. However, lack of data on tissue distribution and the observation of drug resistance when used as monotherapy in HIV-infected individuals warrant further exploration.

Vaginal rings containing ART are a new mode of drug delivery for HIV prevention. Phase III trials are underway of a vaginal ring which slowly releases dapivirine, a potent NNRTI. This ring, which is used monthly, has the potential to give women a convenient, discreet, long-acting method of self-protection against HIV.[33] In a monkey study, an intravaginal ring impregnated with tenofovir (TDF) replaced every 4 weeks completely protected female monkeys from SHIV.[34] This study showed for the first time that a ring can deliver enough of the widely used TDF to offer complete protection for monkeys against SHIV.

V. Non-pharmacological Measurements of Adherence

Adherence to prescribed treatment can be difficult to assess. Monitoring adherence to ART continues to be challenging as there is no gold standard and most measures rely on patient self-report. The most objective and accurate measure of adherence is directly observed therapy (DOT). Directly administered antiretroviral therapy (DAART) is a strategy that has been used to improve adherence among HIV-infected individuals; DAART has been shown to be effective in drug users, in methadone clinic attendees and in subject that are incarcerated.[35–37] However, directly observed therapy is invasive, expensive and time-consuming and may be difficult to implement in a real world setting.

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Various self-report adherence questionnaires have been used to assess recent ART adherence including the AIDS Clinical Trials Group four-day recall Instrument[38], the Visual Analog Scale (VAS)[39], the Likert scale[40,41] and the Morisky Self-reported Medication Taking Scale (MSMTS).[42] However, self-reported adherence questionnaires are subjective tools that assume rather than prove the patient's actual medication intake. In addition, self-reported medication adherence is frequently over-reported, and lack of standardization limits the ability to interpret findings across studies.[43]

Electronic drug monitoring (EDM) devices, which record each time a pill bottle has been opened, were initially praised for their potential to provide a less biased estimate than self-report. However, EDM may underestimate adherence because they can be inconvenient and patients may remove more than one dose at a time, i.e., when a patient uses a multiple day pill container. Other disadvantages that reduce the success and accuracy of EDM include cost, loss of the devise and malfunction.[44]

A new adherence approach developed by Proteus Digital Health, Inc. consists of an ingestible indicator (which can be attached to a medication or used in an over-encapsulation) and an on-body wearable sensor which, together, electronically confirm unique medication ingestions and record the date/time of the ingestion. The recorded ingestion events can be synced from the monitor worn by the patient with a paired cell phone and uploaded to a patient management web site. The adherence results can be shared with family and care providers. Results from a feasibility study using an early prototype in active TB patients suggested that the system is able to correctly identify ingestible indicators with high accuracy, poses a low risk to users and may have high patience acceptability.[45] Some cited concerns about this device include cost and patient privacy.

VI. Pharmacological Measurements of Adherence

One objective way to assess adherence is to measure plasma drug concentrations. Both FTC and TDF concentrations in plasma remain detectable throughout the dosing interval. While many factors contribute to variability in plasma concentrations, completely undetectable plasma concentrations of FTC suggest non-adherence, if the subject is correctly taking a once daily TDF/FTC medication regimen. The reported half-lives for FTC-TP (39 hours)[46] and TFV-DP (150 hours)[47] suggest that both should be detectable for 7 to 14 days if dosing is consistent. Thus, undetectable concentrations can be assumed to represent missed doses rather than pharmacokinetic variability.[48]

However, there are several limitations to using blood plasma concentrations as adherence metrics. Most importantly, plasma concentrations only reflect adherence to the most recently ingested doses. If a patient knows they will have a clinic visit the next day, they may 'remember' to take a dose of medication the day prior to the visit and render the concentration detectable during the clinic visit (i.e., the "white coat phenomena"). Hence, plasma sampling represents short-term adherence and can be influenced by patient knowledge of concentration monitoring. Despite this limitation, in clinical trials, multiple random samples of plasma drug concentrations, drawn throughout the study follow-up, have been associated with both self-reported adherence and with study outcomes of HIV RNA suppression in HIV-infected subjects[49] and transmission in HIV-uninfected subjects[2–5].

Venous sampling itself has many drawbacks including the need for trained personnel and specialized equipment for sample processing. In addition, measuring trough levels is often recommended for drug monitoring which can be difficult with a once daily dosing regimen (e.g., when the subject normally takes medication at night, drawing blood prior to the next dose is logistically challenging). If a qualitative measurement is taken to obviate the need for trough level monitoring, the information that can be obtained is the presence of drug or not,

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which is less useful for adherence monitoring than a quantitative measure; however, if undetectable levels are documented, then lapses in adherence can be objectively inferred.

Novel measures are being developed to monitor antiretroviral adherence in both HIV-infected and uninfected populations. The use of dried blood spots (DBS) is an easy way to obtain stable, transportable specimens for drug analysis for the purpose of therapeutic drug monitoring or assessment of medication adherence in a routine clinical setting. DBS can be collected by patients with a finger stick after which blood is collected on filter paper cards.[50] DBS contain tens of millions of RBC and thus could offer an easy and efficient means to measure TFV, FTC and/or TFV-DP for adherence monitoring. [51–53] TFV-DP has a long half-life (17 days) in RBC; concentrations remain quantifiable in RBC for at least 30 days after drug discontinuation. Thus, the RBC concentration of TFV-DP is unlikely to be undetectable from a single missed dose and may be a good objective marker of longer term adherence to TDF.

As discussed above, poor adherence to PrEP medications may be largely responsible for decrements in effectiveness of TDF-based PrEP regimens in randomized studies. A sub-study from the iPrEx trial examined plasma and intracellular (cryopreserved peripheral blood mononuclear cells (PBMC)) drug concentrations for association with reduction in HIV transmission. Among subjects in the active TDF-containing PrEP arms, the presence of TFV in plasma samples was lower in cases who acquired HIV than in controls who were exposed but did not acquire HIV, suggesting that infected subjects were less adherent to TDF regimens than those who were not infected. Additionally, a statistical model was developed to define the risk of HIV transmission based on the intracellular PBMC concentration of TFV-DP. From this model, the risk of contracting HIV was reduced by ≥90%, relative to placebo, among subjects taking TDF that had a TFV-DP concentration ≥15.6 fmol/million PBMC.[54] Although PBMC TFV-DF concentrations were good markers of adherence and were highly associated with HIV acquisition, the sample collection and preparation for viable PBMC precludes their routine use in clinical or expanded research studies. Studies are currently underway to examine intracellular RBC levels in HIV uninfected individuals taking PrEP as a possible measurement of adherence monitoring.[55] Various combinations of pharmacologic metrics of adherence (e.g., detectable FTC plasma and quantitative RBC TVF-DF concentrations) will be explored in order to best characterize TDF/FTC adherence behavior and determine the most useful in therapeutic drug monitoring as an adherence measure.

Another recent study, which characterized the pharmacokinetics of TFV-DP and FTC-TP in RBCs and PBMCs, used complex PK-PD modeling to predict theoretical reduction in HIV transmission from 3 dosing strategies. TFV-DP levels were measured in subjects assigned to 2, 4 or 7 TDF/FTC doses per week. The model predicted that 4 or more doses of TDF/FTC per week would lower the risk of HIV acquisition by more than 95%. Additional analyses from the models were used to define DBS RBC concentration thresholds that might be used as surrogates for adherence in future studies.[56]

Other pharmacologic approaches to adherence monitoring have been explored. TFV levels in scalp hair have been evaluated and showed a clear and consistent correlation with dose. Using hair samples to monitor PrEP medication levels is a promising marker of TFV dosing and exposure with feasibility advantages over other expensive and labor intensive methods.[57] Concentrations of TVF-DP and FTC-TP measured in the upper layer packed cells (ULPC) from whole blood centrifugation to isolated PBMCs had been used as a possible alternative marker of adherence; ULPC based concentrations were found to correlate significantly with PBMC concentrations and may offer a simply-collected, surrogate measure of ARV adherence.[58] Finally a breath test for adherence to antiretroviral

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vaginal gel was created by adding ester taggants to vaginal gels that would generate exhaled alcohol and ketone metabolites. A recent study found this breath test to be both physiologically and scientifically feasible.[59]

VII. Conclusions

Clinical pharmacology studies help inform our understanding of concentration-response in PrEP RCTs and suggest that differences in anatomic site of HIV risk and adherence are important variables that will impact PrEP as an HIV prevention strategy. Maintaining high levels of adherence to ART, among those who are HIV-infected, has consistently demonstrated a strong relation to better treatment outcomes and survival. In the burgeoning arena of PrEP, adherence is again emerging as a critical component to the efficacy and application of PrEP. As the prophylactic benefits of PrEP are substantially strengthened by adherence, effective PrEP implementation programs will need to focus on this behavioral variable. In order to directly address adherence on both a per-patient and global scale, we will not only need to find the most appropriate agent for PrEP but we will also need an objective, standardized measure of adherence that will allow for monitoring of PrEP adherence in order to assure the safety and effectiveness during implementation of PrEP.

IIIX. Expert Opinion

In the past few years, we have made great strides toward adding PrEP as an option into a multimodal biomedical HIV prevention strategy. We have identified several potential PrEP agents, many of which have been or are currently being studied in clinical trials. We have a strong understanding of the clinical pharmacology which guides our interpretations of concentration-response in PrEP RCTs. The approval of FTC/TDF by the FDA has made PrEP more readily available outside of the clinical research arena. In communities at high risk for HIV acquisition, PrEP awareness and interest are increasing, although post-approval data is lacking.

Several studies support the use of PrEP (iPrEx[3], Partners PrEP[4], CAPRISA-004[2], TDF2[5]) by demonstrating significant reductions in the relative risk of HIV infection in active versus control arms overall. This benefit of lowering the risk of HIV infection is augmented when sub-groups of subjects with detectable plasma drug concentrations are compared to control. However, more sobering news came with the results of FEM-PrEP[60] and VOICE[7], in which no reduction in the risk of HIV transmission was seen. Poor adherence appeared to explain the lack of benefit seen in these studies. Furthermore, even though women in VOICE self-reported high rates of medication adherence, less than one third of subjects had detectable plasma drug levels, suggesting not only medical noncompliance but also imperfection in adherence monitoring that relied on self-report.

Given the mixed results from the landmark PrEP studies, the implications of these findings should be used to design and interpret future studies. New animal models of PrEP need to be explored that allow for a greater understanding of tissue-specific concentrations and the relationship between tissue concentration and efficacy. In particular, additional animal models are needed that assess vaginal challenge after oral dosing. In women, where the concentration of tenofovir in vaginal tissue and fluid is lower than plasma concentrations after oral dosing, future tenofovir clinical trials should attempt to maintain high drug concentrations in the vagina. Combinations of oral and topical ARVs and other innovative formulations should be investigated. Studies of new PrEP formulations, such as use of long acting parenteral preparations and intravaginal rings, will need to carefully examine the association between the drug concentrations achieved at the site of viral exposure and the efficacy of preventing HIV infection.

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As RCTs have shown the potential efficacy of PrEP, we must press on to perform demonstration projects to understand real-world implementation issues, help develop best practices and define effectiveness of PrEP strategies in the context of the combination HIV prevention agenda. These projects are focused on populations most vulnerable to HIV (gay and bisexual men, transgender women, high-risk heterosexual women and men including those in serodiscordant partnerships and sex workers) and thus who could benefit most from HIV prevention efforts. PrEP, as a biomedical prevention intervention, has great potential as long as medications are well tolerated and easy to adhere to and are accompanied by medical and behavioral interventions to ensure adherence, minimize risk compensation and monitor for adverse events.

Although currently only TDF + FTC is approved for use as PrEP, additional agents are under investigation that are safe, well tolerated, potentially have a high barrier to resistance and penetrate into target tissues. Long-acting injectable antiretroviral formulations, such as RPV-LA and S/GSK1265744, and intravaginal rings containing dapivirine or TDF show promise to mitigate the issues of poor adherence. However, until such agents are available, more objective adherence measurement tool are needed that do not rely on patient self-report. Measurement and monitoring using intracellular TFV-DP in dried RBC may provide such a measure. As RBC concentrations reflect days to weeks of TDF exposure, they may be a more accurate and sensitive measure of longer term adherence than self-report or random plasma TFV or FTC plasma concentrations, which only reflect medication adherence over the past 2–4 days. Further, the sample is easy to obtain, does not require complex processing at the local laboratory and is stable for shipping to central labs. However, measurement of RBC intracellular TFV-DP concentrations are currently specialized, expensive, are not commercially available and few labs are capable of performing the assay. Thus, current use of PrEP will have to rely on traditional, imperfect adherence assessment while the more objective pharmacologic measures will be reserved for research and demonstration projects.

Clearly, successful implementation of PrEP at the population level will need to go beyond simply providing medications. A potential future step will be the integration of PrEP into a broader comprehensive care program as part of both a preventative health and treatment as prevention strategy. Ideally, a PrEP intervention strategy will follow general guiding principals that will also integrate into a local context and suit the unique needs and experiences of vulnerable populations.

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Key points

- Antiretroviral agents are now being used prophylactically with the goal to decrease the risk of HIV infection in uninfected individuals who are at high risk of acquiring HIV.
- Various antiretroviral medications have been selected as putative PrEP agents based on their pharmacodynamic properties.
- Oral agents already FDA approved for treatment including maraviroc and raltegravir or new and promising long-acting investigation agents including long-acting rilpivirine, S/GSK1265744, ibalizumab and dapivirine may serve as alternate PrEP agents.
- Findings in several randomized controlled trials have emphasized the importance of medication adherence for efficacy.
- Adherence is difficult to measure but newer approaches, including dried blood spots, have been developed that are more objective and may better approximate actual medication use.

Trial Design in the Era of PrEP and TasP

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Study Design Considerations for Evaluating Efficacy of Systemic Preexposure Prophylaxis Interventions

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Background: The development of interventions for systemic preexposure prophylaxis (PrEP) faces several significant challenges following the US Food and Drug Administration's approval of emtricitabine/tenofovir (FTC/TDF) for HIV prevention. This development is particularly complex because of inconsistency of efficacy results of FTC/TDF PrEP trials for HIV prevention.

Methods: Possible designs for a PrEP phase 3 efficacy trial are obtained by considering scenarios for potential experimental PrEP and control regimens, including consideration of placebo and active controls, longer acting PrEP and alternate dosing schedules.

Results: Noninferiority (NI) trials with hazard ratio NI margins ranging from 1.10 to 1.25 can be justified in the contexts of the 3 PrEP trials demonstrating efficacy of FTC/TDF. However, these HIV endpoint trials may require extremely large number of participants, particularly in settings where FTC/TDF has been shown to reduce the risk of HIV acquisition. NI trials also are often difficult to interpret because they depend on previous placebo-controlled efficacy results. Superiority trials for PrEP are plausible in settings where FTC/TDF efficacy is not yet established, possibly due to low adherence (ie, women at risk as in FemPrEP and VOICE): a new product with potential for higher adherence and potency would be a promising candidate in this setting.

Conclusions: Following Food and Drug Administration's approval of FTC/TDF for PrEP, trials to establish efficacy of new PrEP regimens require stringent design standards, together with rigorous debate about adherence within study populations and many important ethical issues.

Key Words: preexposure prophylaxis, noninferiority, efficacy, phase 3, clinical trials, adherence

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INTRODUCTION

The US Food and Drug Administration approved daily dosing of emtricidabine/tenofovir (FTC/TDF) for prevention of HIV acquisition in July 2012. Although this provides an important advance in HIV prevention, there is need for more than 1 option for preexposure prophylaxis (PrEP). In settings where daily FTC/TDF results have been disappointing, there is particular interest in new products that could improve prevention efficacy through improved adherence or potency. Furthermore, FTC/TDF is currently a first-line choice for treatment of HIV infection, raising concerns about potential for resistance and competing resource demands for a drug used for both treatment and prevention.

The development of new interventions for PrEP faces several significant challenges. A study evaluating the effectiveness of a PrEP intervention requires HIV seroincidence endpoints because we do not have evidence to justify a biomarker as a replacement endpoint. To use a biomarker endpoint, we would need to establish that the effect of PrEP on the biomarker reliably predicts whether PrEP has a clinically meaningful effect on the risk of HIV acquisition. Trials with HIV endpoints may be large. This is particularly likely in settings where FTC/TDF has been shown to reduce the risk of HIV acquisition and then would be used as the standard of care (SOC) in the trial of the new intervention because the overall seroincidence rates in the trial could be below 2 per 100 person-years. Even larger sample sizes may be required if we conduct noninferiority (NI) trials, that is, trials designed to determine whether we can rule out that use of a new intervention rather than FTC/TDF would cause an unacceptable increase in the risk of HIV acquisition. The inconsistency of efficacy results for FTC/TDF, across recent PrEP trials in Table 1,[1–5] makes NI trials with FTC/TDF as the active control regimen problematic because an NI trial is appropriate only when the effect of the active control in the NI trial setting is (1) large and (2) precisely estimated from previous randomized trials conducted in similar settings. In settings where FTC/TDF has been shown to provide little, if any, reduction in the risk of HIV acquisition, superiority designs could be justified for drugs that could plausibly achieve higher adherence or potency.

In this manuscript, we will explore potential study designs for randomized efficacy trials evaluating experimental PrEP regimens for prevention of HIV acquisition.

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TABLE 1. Settings, Population and Principal Results From Clinical Trials Evaluating the Daily Oral TDF/FTC Regimen for PrEP for Risk of HIV Acquisition

Study and Setting (% Total Population)	Risk/Gender	Adherence, %	No. Events	Efficacy (95% CI), %
Partners PrEP; Kenya, Uganda (100)	Discordant heterosexual couples	~80	13 vs. 52	75 (55 to 87)
TDF2; Botswana (100)	Heterosexual men and women	~80	9 vs. 24	63 (22 to 83)
iPrEx; Peru, Brazil, Ecuador (82)	Men who have sex with men	~50	48 vs. 83	42 (18 to 60)
FemPrEP; South Africa, Kenya (98)	Heterosexual women	~35	33 vs. 35	6 (−69 to 41)
VOICE; South Africa (81)	Heterosexual women	~29	61 vs. 60	−4 (−50 to 30)

A FRAMEWORK FOR CONSIDERING PREP PHASE 3 EFFICACY TRIALS

Designs for PrEP phase 3 efficacy trials are obtained by considering potential experimental and control PrEP regimens.

1. Potential experimental PrEP regimens:
   a. Oral daily drug(s) other than FTC/TDF.
   b. A long-acting formulation (oral or injectable) of an experimental agent(s).
   c. Oral FTC/TDF with a different dosing strategy (eg, event-based dosing; event-based dosing + weekly dosing).
2. Potential control regimens:
   a. Active control: Daily FTC/TDF is provided as the control; daily FTC/TDF is assumed to be a SOC regimen.
   b. Add-on placebo control: Placebo is the control; participants in both arms of the trial have access to background use of daily FTC/TDF with level of use matched to the clinical setting of interest.
   c. Placebo control: Placebo is the control, where there is justification that background management does not include use of daily FTC/TDF.

The potential scenarios for a PrEP phase 3 efficacy trial are obtained by combining the choice of experimental and control PrEP regimens (Table 2).

POTENTIAL SCENARIOS FOR A PREP PHASE 3 TRIAL

We discuss the 8 scenarios in Table 2, considering strengths and weaknesses of superiority and NI designs, as well as anticipated level of interest or priority.

Scenario A: Experimental Oral Drug Versus Daily FTC/TDF

In this scenario, daily FTC/TDF is assumed to be SOC for PrEP, most justified where adherence rates and levels of efficacy for that regimen are high, as in the Partners PrEP trial setting of discordant heterosexual couples in committed relationships.[1] If an experimental oral drug is of interest, even though its efficacy might be only similar to or modestly better than that of FTC/TDF, it could be evaluated in a FTC/TDF-controlled NI trial. By design, the intention of such an NI trial is to determine whether we can rule out that the efficacy of the experimental oral drug is "unacceptably worse" than daily FTC/TDF in preventing HIV acquisition. The NI trial requires specification of a threshold, called the "NI margin," for what constitutes unacceptable loss of efficacy. NI is established if the estimated relative effect of the experimental drug to FTC/TDF is sufficiently favorable and precise that the confidence interval for that relative efficacy does not include the NI margin. High adherence to a daily FTC/TDF active control regimen is necessary in an NI trial because justification for the NI margin includes an assumption that the historical randomized trials establishing efficacy of FTC/TDF (where adherence was necessarily high enough to prevent a substantial proportion of infections) provide an unbiased estimate of FTC/TDF's effect (relative to a hypothetical placebo) in the NI trial.[6–9]

In NI trials in Scenario A, participants randomized to the experimental arm receive the new oral agent instead of the FTC/TDF control, even though there is substantive evidence establishing FTC/TDF meaningfully reduces the risk of HIV acquisition. For such a trial to be ethical, there needs to be strong previous proof-of-concept for efficacy of the new oral agent.[9]

The sample size of an NI trial can be very large (Table 3). Suppose we decide (1) it is unacceptable for the relative rate of HIV acquisition on the new oral agent to be 1.25-fold higher than on FTC/TDF [ie, the NI margin is selected to be a hazard ratio (HR) of 1.25] and (2) the probability of a false-positive conclusion should be only 2.5% when the HR truly is 1.25. In this setting, if we want to rule out the prespecified NI margin with 90% probability when the experimental drug and FTC/TDF truly have the same effect on HIV acquisition, the sample size and duration of follow-up in the NI trial need to be sufficiently large that 844 trial participants have HIV acquisition events (Table 3). This would be reduced to 211 HIV acquisition events if we believe that the new oral agent is somewhat better than daily FTC/TDF and require 90%

TABLE 2. Schema for Potential Experimental and Control Arms in PrEP Trials

Control/Experimental Regimen	2a: New Daily Oral Drug(s)	2b: Long-Acting Drug (Oral or Injectable)	2c: Different Oral FTC/TDF Dosing Strategy
1a: FTC/TDF daily as active control	Scenario A	Scenario B	Scenario C
1b: Placebo add-on to FTC/TDF daily	Scenario D	Scenario E	Not applicable
1c: Placebo add-on to "Standard-of-care"	Scenario F	Scenario G	Scenario H

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TABLE 3. Sample Sizes and Critical Observed Effects for NI Trials of PrEP

HR to be Ruled out to Establish NI, for a Trial With 90% Power Under True HR	Total No. HIV Events	Maximum Estimated HR to Conclude NI	Sample Size, When 2.25 Events per 100 Person-years and 2 Year Follow-up
Rule out 1.10 against true 1.00	4628	1.0384	104,015
Rule out 1.10 against true 0.90	1044	0.9743	23,654
Rule out 1.10 against true 0.80	415	0.9075	9403
Rule out 1.10 against true 0.67	171	0.8151	3800
Rule out 1.20 against true 1.00	1265	1.0748	28,430
Rule out 1.20 against true 0.90	508	1.0084	11,510
Rule out 1.20 against true 0.80	256	0.9392	5800
Rule out 1.20 against true 0.67	124	0.8439	2756
Rule out 1.25 against true 1.00	844	1.0922	18,969
Rule out 1.25 against true 0.90	390	1.0250	8766
Rule out 1.25 against true 0.80	211	0.9543	4742
Rule out 1.25 against true 0.67	108	0.8572	2400

NI margins ranging from 1.10 (justified by iPrEX) to 1.25 (justified by Partners PrEP). The HR compares an experimental regimen to active control for HIV acquisition. Calculations are conducted assuming the analysis have a 2.5% probability of a (one sided) false-positive error rate.

probability to rule out the NI margin when the experimental drug to daily FTC/TDF HR is 0.80 (Table 3).

Scenario A is of interest when adherence rates and levels of efficacy of FTC/TDF are high and an experimental oral drug has strongly favorable evidence from proof-of-concept trials. There is interest in this setting in part because a new oral agent could preserve daily FTC/TDF for first-line HIV treatment and reduce the risk of emerging resistance. However, given the above considerations of ethics and feasibility, Scenario A may be a low priority unless there is favorable evidence for the new oral agent from, for example, a previously completed superiority trial (eg, Scenario D or F.)

Scenario B: Long-Acting Formulation (Injectable or Oral) of Experimental Agent(s) Versus Daily FTC/TDF

In Scenario B, as in Scenario A, daily oral FTC/TDF is assumed as an effective SOC regimen for PrEP. Because participants receiving the experimental long-acting agent are not receiving daily FTC/TDF in a randomized head-to-head comparison trial, the ethics and level of priority of this scenario again is dependent on the strength of evidence from proof-of-concept trials of the long-acting experimental agent.

Even where the daily FTC/TDF control regimen has been proven effective, it might be possible to conduct a superiority trial for a new PrEP drug because exploratory analyses in the Partners PrEP and iPrEx trials suggested that 85% to 90% efficacy might be achieved with higher adherence. To illustrate sample size requirements, a trial with 88 patients having HIV acquisition events would be required to rule out equal effectiveness of experimental and FTC/TDF regimens with 90% "true-positive" probability, when the experimental regimen truly reduces relative risk of HIV infection by 50% (assuming a 2.5% "false-positive" probability if regimens are truly equally effective). If this trial was conducted in settings where HIV acquisition would be approximately 2% per year, then approximately 2250 participants would need to be enrolled, followed up for an average of 2 years.

Alternatively, an NI trial design might be preferred. Suppose we derive the NI margin using the Partners PrEP or the Botswana TDF2 trial to estimate the effect of FTC/TDF (vs. hypothetical placebo) in the NI trial, which assumes adherence levels in the NI trial will be as high as was seen in those trials. Using the "95–95" approach for derivation of the NI margin[7,8] and the Partners PrEP result to estimate that FTC/TDF provides a 75% (95% CI: 55 to 87) reduction in the risk of HIV acquisition, and requiring the experimental regimen to preserve at least 75% of FTC/TDF's effect, the NI margin is an experimental to FTC/TDF HR of 1.25. In such a setting, a trial with 108 patients having HIV acquisition events will have 90% power to rule out that the experimental regimen to daily FTC/TDF HR is 1.25 when the experimental regimen truly provides a 33% reduction in the risk of HIV acquisition relative to FTC/TDF [requiring only 2.5% probability of a false-positive conclusion when the HR truly is 1.25 (Table 3)]. Applying the same "95–95" approach in the men who have sex with men setting and using the iPrEX trial to estimate FTC/TDF provides a 42% (18%–60%) reduction in the risk of HIV acquisition; even if the margin ensures preservation of only 50% of FTC/TDF's effect, only a modest 1.10 NI margin for experimental to daily FTC/TDF HR would be justified. In this setting, approximately 171 HIV acquisition events would be required (Table 3).

Suppose, however, that adherence to daily FTC/TDF is not expected to be high. If FTC/TDF still is used as an active control, the experimental regimen needs to be established to be superior to conclude it is effective. This is plausible if the experimental regimen is a long-acting drug. If an experimental regimen is shown to be superior to daily FTC/TDF active control when adherence to FTC/TDF is not high, trial results may not generalize to settings with high adherence to FTC/TDF.

Scenario C: Alternative Dosing of Oral FTC/TDF Versus Daily FTC/TDF

A trial to assess potential superiority of an alternate dosing strategy for FTC/TDF, compared with daily, could be justified if it were thought that the new dosing might substantially reduce the risk of HIV acquisition, perhaps by achieving substantially higher adherence. However, if alternate dosing for FTC/TDF became an established standard regimen in real world settings (eg, it became common practice to use

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FTC/TDF shortly before and after risky sex) or if alternate dosing would be expected to reduce the risk of serious toxicities, conducting a trial to verify that the alternate dosing was not inferior to the daily regimen could be justified. However, since Scenario C does not achieve replacement of FTC/TDF for PrEP, it likely would not be a priority to conduct a large-scale clinical trial simply to establish that an alternate dosing strategy would be noninferior to daily dosing, keeping in mind that establishing NI does not mean the alternative dosing strategy is "at least as good as" daily FTC/TDF but rather that it is "not unacceptably worse than" daily FTC/TDF.[7,8]

Scenarios D and E: New Oral Drug, or Long-Acting Experimental Drug (Injectable or Oral) Versus Placebo, Where Trial Participants Have Access to Background Use of Daily FTC/TDF

Daily FTC/TDF is available for use in the clinical setting addressed by these scenarios. However, we want to improve the level of protection against HIV acquisition by developing a long-acting drug or a new drug with mechanisms of action complementary to FTC/TDF. This approach is particularly appealing in settings where adherence to FTC/TDF was not high, such as in iPrEx, and yet relatively high levels of adherence to the new intervention are expected. In this setting, superiority trials would be conducted, with careful assessment of drug–drug interactions. Such trials could be of moderate to high priority, especially Scenario E, because a drug with a long-acting formulation should have a favorable adherence profile.

An important decision is whether daily oral FTC/TDF should be supplied by the trial in Scenarios D and E. The following considerations should guide this decision. First, to enhance the clinical relevance and interpretability of results, the use of FTC/TDF as a background intervention should match that in the clinical setting of interest. Second, by the principle of "distributive justice,"[9] the trial should provide results that are relevant and informative for the population in which the trial is conducted. By not supplying daily FTC/TDF to trial participants, efficacy and safety results from the trial become directly relevant to the context of FTC/TDF as it is currently delivered. However, it is appropriate to supply daily FTC/TDF to all trial participants if it is expected that the use of FTC/TDF in clinical practice will evolve so that the level of use in the trial matches what will become standard use of FTC/TDF after the trial is completed.

Scenarios F and G: New Oral Drug, or Long-Acting Experimental Drug (Injectable or Oral) Versus Placebo, Where Background Management Properly Does Not Include Daily FTC/TDF

In settings where patients are not willing or able to adhere to daily FTC/TDF, or where communities are not willing or able to provide or support daily FTC/TDF for prevention, finding safe and effective PrEP regimens that are affordable and feasible is a moderate to high priority. Lack of community willingness to support daily FTC/TDF for PrEP could reflect a desire to reserve this regimen for first-line HIV treatment.

To satisfy the unmet needs in these settings, a new drug has to overcome the barriers encountered by daily FTC/TDF. Hence, drugs with long-acting formulations are of greater interest (Scenario G) than alternative daily oral products (Scenario F). Either scenario requires a superiority trial, with a potential need to establish super-superiority by statistically ruling out modest levels of efficacy, such as a 30% reduction in the risk of HIV acquisition.

Of note, it is ethical to conduct placebo-controlled trials of new PrEP agents only in populations where it is anticipated that the new agent, if effective, would become available for use. In particular, it is not acceptable to evaluate a new agent in a population if the reasons that preclude availability of FTC/TDF would also apply to the new agent.

Scenario H: Alternative Dosing Strategy for FTC/TDF Versus Placebo, Where Background Management Properly Does Not Include Daily FTC/TDF

For this scenario to arise, an alternative dosing strategy for FTC/TDF would be viewed as promising, even though daily FTC/TDF regimen is judged to be unaffordable, impractical, or not effective. For illustration, from a closely related field: clinical trials are ongoing to evaluate effectiveness of coital use of a tenofovir gel microbicide in women,[10] even though daily use of the same product was demonstrated to be ineffective.[5,11,12]

To justify a trial of alternative dosing strategies for FTC/TDF, we would need data establishing proof-of-concept that alternate dosing addresses the deficiencies of daily FTC/TDF. The level of priority of this superiority trial would depend on the strength of those previous data.

DISCUSSION

An important goal in HIV prevention research is the identification of safe and effective PrEP regimens that are affordable and feasible and do not interfere with the effects of drugs integral to HIV treatment. With initial progress that has resulted in the availability of FTC/TDF for PrEP, it is likely that subsequent trials will require larger sample sizes and longer duration, whether we pursue settings where FTC/TDF has been shown to reduce the risk of HIV acquisition to relatively low levels or settings where FTC/TDF has failed to provide clinically meaningful protection.

NI trials provide a potentially appealing study design in settings where FTC/TDF has been established to be effective, yet there is still interest in alternative PrEP interventions that are better tolerated, more convenient, less costly, or not involving drugs commonly used for HIV treatment, even if these alternative interventions would have efficacy that is only similar to or slightly better than that of FTC/TDF. Unfortunately, NI trials often require large sample sizes and often yield results that are difficult to interpret due to uncertainty about the validity of their strong underlying assumptions. One key assumption is that historical trials can

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provide an unbiased estimate of the active control's effect for the NI trial. When experimental and FTC/TDF regimens demonstrate similar efficacy in an NI trial, if we cannot rely on the validity of that key assumption, how can we distinguish whether the regimens are similarly effective or similarly ineffective? For illustration, suppose the FemPrEP trial conducted in heterosexual women in South Africa and Kenya[4] had been a comparison between an experimental PrEP drug that truly provided no benefit, versus FTC/TDF as the active control. Since daily FTC/TDF provided essentially no benefit in that setting (Table 1), the regimens would have had similar rates of HIV acquisition. If the placebo-controlled evaluation of FTC/TDF provided by the Centers for Disease Control and Prevention's TDF2 PrEP trial conducted in heterosexual men and women in Botswana[3] had been used as the historical trial providing an estimate of the effect of FTC/TDF in the setting of this hypothetical FemPrEP trial (Table 1), we would have concluded that this experimental drug was very beneficial even though it was, in reality, ineffective.

The influence of level of adherence will be an important consideration in the design and interpretation of NI trials of PrEP regimens. A requirement of NI trials is that the active comparator regimen has strong efficacy that is precisely estimated by historical trials conducted in settings similar to the setting of the NI trial. As indicated by the evidence from Table 1, the level of adherence has strong influence on efficacy for PrEP regimens, such as FTC/TDF. Hence, it is likely that NI trials of PrEP regimens will be appropriate only when (1) the active comparator (ie, FTC/TDF) is a potent agent, (2) the active comparator's efficacy was established in a setting of high adherence, and (3) there is reliable evidence that the level of adherence to the active comparator will be equally high in the setting of the NI trial. It is problematic that it is unknown why the participants in Partners PrEP and TDF2 were highly adherent to FTC/TDF while the women enrolled in FemPrEP and VOICE were not. The reasons for these differences must be better understood to be able to justify the NI trial assumption that an unbiased estimate of efficacy of the active comparator in the NI trial can be obtained from data in the historical trials. Without that justification, NI trials will not be interpretable.

Superiority trials provide a clearer path to obtaining reliable evidence of clinically meaningful advances. There are important populations at demonstrated high risk of HIV acquisition where daily FTC/TDF has been found to be inadequately effective. A new product with high potential to achieve protection, either because adherence requirements were less onerous or the drug was more potent, may be the highest priority for moving into efficacy evaluation. Conducting such a superiority trial against the evolving background of PrEP SOC would seem to be addressing a particularly relevant public health question.

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Statistical issues in trials of preexposure prophylaxis

David T. Dunn[a] *and David V. Glidden*[b]

Purpose of review
We discuss selected statistical issues in the design and analysis of preexposure prophylaxis (PrEP) trials. The general principles may inform thinking for other interventions in HIV prevention.

Recent findings
To date, four different designs have been used to determine the effectiveness of PrEP: randomized, double-blind, placebo-controlled; randomized, open-label, immediate or delayed access; nonrandomized comparison of HIV incidence according to the level of drug detected; comparison of the observed HIV incidence to the expected rate using historical control data. Open-label trials of PrEP, which assess public health effectiveness, complement the placebo-controlled trials which established the biological efficacy of TDF/ FTC. Future trials of PrEP will be highly challenging to design since a no PrEP group is difficult to justify and the natural control regimen, TDF/FTC, is highly efficacious.

Summary
Standard statistical paradigms for noninferiority trials should be reconsidered for evaluating alternative PrEP regimens.

Keywords
intention-to-treat, noninferiority, open-label, placebo-controlled, risk compensation

INTRODUCTION

Randomized controlled trials of preexposure prophylaxis (PrEP) have given rise to specific statistical challenges both in design and analysis. In this article we focus in depth on three issues: assessing the influence of risk compensation, dealing with patients with acute HIV infection at study enrolment, and the design of future studies in the context of a highly efficacious preexisting regimen.

Risk compensation and the limitation of placebo-controlled trials

'Risk compensation' is the adjustment of behaviour in response to a perceived reduction in risk, a critical issue in the public health implementation of PrEP because of the potential for increased risky sexual behaviour which could counteract biological efficacy [1]. Placebo-controlled randomized trials are regarded as the gold standard for establishing the biological efficacy of an experimental drug. A key rationale for using placebo in trials of PrEP agents has been to avoid bias because of differential exposure to HIV caused by different sexual behaviour in the randomized groups; this contrasts with the real-life situation, where individuals know if they are taking an active drug. A frequently unappreciated point is that risk compensation cannot be assessed by standard within or between group comparisons in a placebo-controlled trial [2]. The European Medicines Agency stated that 'The behavioural impact of PrEP on risk compensation and condom replacement cannot be assessed in prelicensure placebo-controlled trials' and that 'it is mandatory that the marketing authorisation application contains a risk management plan that adequately covers the public health impact of the PrEP intervention' [3].

In an imaginative analysis to gain insights into risk compensation in the Preexposure Prophylaxis Initiative (iPrEx) trial, Marcus *et al.* [4] compared patients who believed they were taking active drug

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KEY POINTS

- Open-label randomized trials are the only design that can reliably assess risk compensation.
- The proportion of prevalent acute infections can provide insights into underlying HIV incidence in the trial population.
- New paradigms are required for noninferiority trials of experimental PrEP regimens.

($n = 553$) with patients who believed they were taking placebo ($n = 223$). Patients who believed they were receiving active drug had higher number of receptive partners at baseline, but the difference between the two groups did not increase during follow-up after study drug was initiated. There was also no difference at any time point in the percentage of receptive anal intercourse partners using condoms. These results were interpreted as no evidence of risk compensation. However, this study has several limitations: confidence intervals were relatively wide (the analysis excludes 1429 patients who did not predict their treatment assignment); the accuracy of self-reported data on sexual practices; and the fact that groups were based on perceived assignment rather than certain knowledge as pertains in real-life. A further limitation is that risk compensation is a function of how effective an individual considers the intervention to be, and the very high biological efficacy of tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) was not known at the time the study was conducted.

Grant *et al.* [5▪] assessed and presented a detailed analysis of a cohort study of MSM enrolled from three previous randomized controlled trials of PrEP (including iPrEx) that were offered open-label PrEP. The authors assessed risk compensation by looking at longitudinal changes in behaviour, comparing patterns among men who accepted the offer of PrEP and those who declined it. Self-reported total number of sexual partners, noncondom receptive/insertive anal intercourse decreased during follow-up in both groups and to a similar extent. Syphilis incidence was also similar in the two groups. However, the fact that the control group was not randomized limits the interpretability of these data.

The most robust data on risk compensation to date were obtained in PROUD, a pragmatic, open-label trial which attempted to mimic how PrEP would be administered in routine clinical practice [6▪▪]. Eligible patients were randomized to receive daily TDF/FTC either immediately ($n = 275$) or after a deferred period of 1 year ($n = 269$). Data from the first year of follow-up allowed direct assessment of risk compensation. Patients were asked to complete monthly questionnaires and daily diaries about sexual behaviour but the completion rates of these were low, particularly in the deferred group. Accordingly the investigators reported cross-sectional analyses of sexual behaviour based on baseline and 1 year questionnaires only. No differences were found in terms of the total number of different anal sex partners but there was marginal evidence of a larger proportion of PrEP recipients at 1 year who reported receptive anal sex with 10 or more partners without a condom. An indirect, but more objective measure of risky sexual behaviour, is the diagnosis of other sexually transmitted infections (STIs) [7]. PROUD reported a slightly higher rate of diagnosis with any bacterial STI in the immediate PrEP group (57%) than in the deferred group (50%). However, after adjustment for the number of screens, there was no evidence of a difference between the groups in the frequency of bacterial STIs, either individually or overall.

A potentially important effect which could impact negatively on the cost–effectiveness of PrEP is that some men who have been using condoms consistently may stop doing so because they are able to access PrEP. Such men have not been eligible for PrEP trials to date and are unlikely to be formally eligible in PrEP implementation programmes. However, setting rigid criteria for PrEP access is not realistic, and if this phenomenon is real it will be difficult to detect it.

Acute HIV infections at study enrolment in the analysis

A clinical challenge with PrEP is the window period between exposure to HIV and the (assay-dependent) detection of infection, meaning that PrEP is inevitably initiated in some individuals who are already infected. The procedure used in most trials has been to perform a point-of-care serological test for HIV on the day of enrolment and to store an additional plasma sample that is retrospectively tested for HIV RNA, the earliest marker for HIV infection, if the patient had a reactive HIV antibody test at their first (or early) follow-up visit [8–12]. In real-life clinical practice, procedures are usually less stringent than in trials. United States guidelines recommend 'At a minimum, clinicians should document a negative antibody test result within the week before initiating (or reinitiating) PrEP medications' [13]. Also, samples may not be routinely stored, precluding the possibility of retrospective testing.

The primary efficacy analyses of trials have generally excluded patients with detectable HIV

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Table 1. Impact on effect measures of including or excluding patients with acute HIV infection at enrolment in the PROUD trial

	ITT (all patients)		Modified ITT (excluding acute cases)	
	Immediate	Deferred	Immediate	Deferred
No. of infections	5	21	3	20
Follow-up (person-years)	243.5	222.1	243.5	222.1
Incidence rate*	2.1	9.5	1.2	9.0
Rate difference*	7.4		7.8	
Number needed to treat	13.5		12.9	
Efficacy (%)	78		86	

Three patients (two Immediate, one Deferred) tested nonreactive by a third-generation rapid test on the day of enrolment but reactive with a joint antigen/antibody assay. ITT, intention to treat.

RNA at enrolment [modified intention-to-treat, (mITT)] on the grounds that PrEP cannot possibly avert infection in these individuals. (PrEP may have a postexposure prophylaxis effect but only if initiated within 48–72 h of exposure.) From an effectiveness rather than an efficacy perspective a full ITT analysis including all patients is arguably the more relevant [14]. In particular, analyses of safety outcomes should be intention-to-treat (ITT), particularly those relating to drug resistance, as viral mutations are particularly likely to emerge during acute infection under selective drug pressure.

In practice, ITT and mITT analyses in most studies produce very similar results as the number of prevalent acute infections is generally much smaller than the number of incident infections. However, it can make a material difference in studies where adherence to PrEP is high. For example, of the five infections in the immediate PrEP arm in PROUD, two occurred at enrolment. Here, the estimated efficacy is 78% under an ITT analysis compared with 86% under an mITT analysis (Table 1). Note that there is little effect on the rate difference (or number-needed-to-treat), the most relevant measure for public health.

Finally, in the following section we raise the possibility of using the number of prevalent acute infections (antibody negative/HIV RNA-positive result on enrolment sample) to measure the underlying 'force of infection' in the trial population. The method is described in the footnote to Fig. 1, which shows the inferred baseline incidence plotted against the observed incidence of infection among patients who were allocated to placebo in trials that tested enrolment samples for HIV RNA. With one exception, it over-estimates incidence is overestimated by a factor of between 2 and 3. There are two main possible explanations for this. First, the calculation is highly sensitive to the assumption about the mean interval between detectable circulating viral RNA and detectable circulating antibody, which may be incorrect. Second, patients may have been motivated to join the trial because they had recently been at especially high risk of exposure to HIV. Nonetheless, in large trials this approach can provide a rough estimate of the underlying rate of infection.

Future studies and the challenge of a highly-efficacious control regimen

Although TDF/FTC is the only drug currently approved by Food and Drug Administration for prevention, there is a pipeline of other agents,

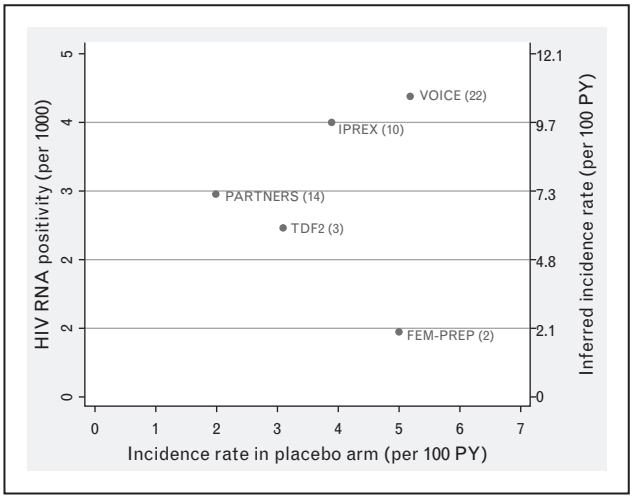

FIGURE 1. Proportion of patients with acute infection at enrolment plotted against incidence of infection observed in placebo recipients. Limited to studies that tested enrolment plasma samples retrospectively for HIV RNA. Value in brackets is number of acute infections observed in each study. The inferred incidence of infection at enrolment (plotted on right-hand vertical axis) was estimated by dividing the proportion of patients who were HIV RNA-positive/antibody negative by the mean interval between the detection of HIV RNA and antibody [20]. We assumed a value of 15.1 days using data from Eller *et al.* [21].

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Table 2. Outcomes in hypothetical study – low-incidence population

Group	Total follow-up	HIV infections	Rate (per 1000 PY)	Effectiveness compared to N (%) (95% CI)
Control (C)	5000	8	1.6	60 (5, 85)
Experimental (E)	5000	15	3.0	25 (−54, 64)
No treatment (N)	5000	20	4.0	–

CI, confidence interval.

particularly long-acting agents [15]. Given the proven biological efficacy of TDF/FTC, there are ethical barriers to conducting future clinical trials that include a no PrEP comparison group. Possible exceptions to this are populations where PrEP is not policy or where adherence to daily TDF/FTC is uncertain.

Donnell *et al.* comprehensively reviewed study designs for PrEP interventions, assuming daily TDF/FTC to be the control regimen [16▪▪]. They considered three different experimental regimens: a new daily drug, a long-acting drug, and a different TDF/FTC dosing strategy. For the first of these scenarios, a noninferiority design would be the natural choice. The study explored noninferiority margins of 1.10, 1.20, and 1.25 on a hazard ratio scale. For the highest noninferiority margin of 1.25, and assuming the experimental intervention to be equally effective to TDF/FTC, the authors show that a trial would have to accumulate a total of 844 HIV events to be sufficiently powered; this translates to a sample size of approximately 19 000 subjects for HIV incidence of 2.25/100 person-years and 2 years follow-up on average – an infeasible undertaking.

Further calculations were made under the assumption that the experimental agent is more effective than TDF/FTC, to enable smaller, more realistic sample sizes. However, in the face of strong evidence that TDF/FTC confers very high protection if adequate drug concentrations are achieved [17], this assumption is plausible only in comparisons with long-acting drugs in a population likely to experience barriers to adherence to a daily oral medication.

The large number of required events for noninferiority studies is driven mainly by the use of the hazard ratio (which is based on the multiplicative scale) for assessing noninferiority. From a public health perspective, the rate difference is the more important metric as it translates directly to the number needed to treat [18], and this concept can be utilized in the comparison of drugs as well as to a comparison of drug versus no treatment.

Suppose we did a clinical trial to compare an experimental preventive intervention (E) to daily TDF/FTC (control, C) in a group of 5000 volunteers. The trial randomizes 2500/arm and follows them for a total of 2 years, yielding the results in Table 2. The HIV rate ratio (relative to C) is 1.88 [95% confidence interval (CI) 0.74, 5.1]. Thus, the rate of HIV could be as much as five times higher for E and would clearly exceed any noninferiority margin. The rate difference is much narrower: 1.4 (95% CI −0.4 to 3.3)/1000 person-years. For every thousand people getting E rather than C for 1 year, the best estimate is that there would be 1.4 more infections (or 3.3 at most).

We now argue that information on the number of infections under the condition of no-treatment (N) is essential context, noting this group is not actually observed. Suppose, HIV incidence under N is 4.0/1000 person-years. The effectiveness of E compared to N is 25% (95% CI −54% to 64%) and the effectiveness of C compared to N is 60% (5–85%). It is helpful to compare the effectiveness estimate for E and C on the additive scale: 60 − 25% = 35% (95% CI −14 to 84%), which represents that proportional increase in the number of infections using E rather than C relative to the number of infections in the absence of PrEP. Thus given 5000 person-years follow-up we would expect 20 infections with no PrEP and 7 (15–8) more infections with the use of E rather than C (7/20 = 35%); this would seem to represent an appreciable loss of efficacy.

Consider an alternative scenario where the trial population is at 10 times higher risk of HIV and is highly adherent to both E and C (Table 3). Under this scenario, the effectiveness of E compared to N is 93% (95% CI 87–96%) and the effectiveness of C compared to N is 96% (92–98%). The HIV rate ratio is unchanged (1.88 = (1–93%)/(1–96%)), but the difference in effectiveness on the additive scale is much smaller: 96 − 93% = 3% (95% CI −1 to +8%). Given 5000 person-years follow-up, we still expect seven more infections with the use of E rather than C but this time against a background of 200 infections in the absence of PrEP. In this scenario, E would seem to be an acceptable alternative to C.

The fact that underlying HIV incidence as well as adherence to PrEP can vary greatly between populations implies the need to anchor any comparison to the number of HIV infection we would have

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Table 3. Outcomes in hypothetical study – high-incidence population

Group	Total follow-up	HIV infections	Rate (per 1000 PY)	Effectiveness compared to N (%) (95% CI)
Control (C)	5000	8	1.6	96 (92, 98)
Experimental (E)	5000	15	3.0	93 (87, 96)
No treatment (N)	5000	200	40.0	–

PY, person-years.

observed in the absence of PrEP. We propose, for wider discussion, the use of a two-part noninferiority definition:

$$\text{(i) } \lambda_E - \lambda_C < \Delta \text{ and (ii) } (\lambda_E - \lambda_C)/\lambda_N < \rho,$$

where λ_E, λ_C, and λ_N are estimates of HIV incidence in the E, C, and N groups respectively and the noninferiority margins (Δ, ρ) are appropriately chosen. (To simplify exposition, we have avoided attaching probabilistic statements to the lower confidence limits.)

For instance, in the low-incidence scenario the upper CI for $\lambda_E - \lambda_C$ is 3.3/1000 and the upper bound on $(\lambda_E - \lambda_C)/\lambda_N$ is 0.84 (or 84% more of total infections). In the high-incidence scenario, the upper CI for $\lambda_E - \lambda_C$ remains 3.3/1000 whereas the upper bound on $(\lambda_E - \lambda_C)/\lambda_N$ is now 0.08 (or 8% more of total infections). The first part of the definition is fully rigorous is the sense that it is intention-to-treat and does not rely on an external estimate of λ_N, but this is required for the second part of the definition. The Partners Demonstration project estimated this based on the placebo rate of HIV in the cohort prior to the treatment period [19]. An alternative approach could be to use the proportion of patients with HIV RNA detected in their enrolment sample, as described earlier. A final possibility is to use external data in the population from which the study patients are recruited, although this can be misleading. The PROUD study observed an HIV incidence of 9.0/100 person-years in the deferred group, which was approximately seven-fold higher than a national estimate of 1.34/100 person-years for MSM attending sexual health clinics [6▪▪]; this underscores that it may be difficult to assemble control groups that accurately reflect the HIV risk of individuals who seek participation in a trial.

CONCLUSION

Placebo-controlled and open-label trials of PrEP have addressed fundamentally different questions. The former evaluates the biological efficacy of the PrEP agent studied; the latter attempts to evaluate real-life effectiveness, reflecting the impact of risk compensation and actual adherence. Future trials of PrEP are highly challenging to design since daily TDF/FTC, the natural control regimen, is highly efficacious. New statistical paradigms for noninferiority trials are required, with statisticians and expert clinicians working closely together to develop these.

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Conflicts of interest

There are no conflicts of interest.

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