Antiviral Research 88S (2010) S10–S18

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Preclinical evaluation of anti-HIV microbicide products: New models and biomarkers

Gustavo F. Doncel*, Meredith R. Clark

CONRAD, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, VA, 23507, United States

ARTICLE INFO

Article history:
Received 18 September 2010
Accepted 28 September 2010

Keywords:
Microbicide
Antiviral
Drug development
Non-clinical studies
HIV prevention
Pre-exposure prophylaxis

ABSTRACT

A safe and effective microbicide product designed to prevent sexual transmission of HIV-1 rests on a solid foundation provided by the proper selection and preclinical characterization of both its active pharmaceutical ingredient (API) and formulation. The evaluation of API and formulation physicochemical properties, drug release, specific antiviral activity, cell and tissue toxicity, organ toxicity, pharmacokinetics, and pharmacodynamics and efficacy provides information to understand the product, make go/no go decisions in the critical path of product development and complete a regulatory dossier to file an investigational new drug (IND) with the US Food and Drug Administration. Incorporation of new models, assays and biomarkers has expanded our ability to understand the mechanisms of action underlying microbicide toxicity and efficacy, enabling a more rational selection of drug and formulation candidates. This review presents an overview of the models and endpoints used to comprehensively evaluate an anti-HIV microbicide in preclinical development. This article forms part of a special supplement on presentations covering HIV transmission and microbicides, based on the symposium "Trends in Microbicide Formulations", held on 25 and 26 January 2010, Arlington, VA.

Contents

1. Introduction

According to UNAIDS (UNAIDS, 2009) around 33 million people are living with HIV. More than half are women and 2.1 million are children under 15 years of age. In 2008, there were 2.7 million new infections and 2.0 million people died of AIDS. During the same year, 4.0 million infected people in low- and middle-income countries were put on treatment. In spite of the success of this intervention, it is clear that treatment alone is insufficient to stem the tide of the epidemic. To be successful, it is now widely recognized that HIV-1 prevention programs must incorporate biomedical, behavioral, and structural strategies (Horton and Das, 2008; Stover et al., 2002).

Microbicides or topical pre-exposure prophylaxis (PrEP) are a promising biomedical prevention strategy that has recently shown

* Corresponding author. Tel.: +1 757 446 5026; fax: +1 757 446 8998.
E-mail addresses: DoncelGF@evms.edu (G.F. Doncel), mclark@conrad.org (M.R. Clark).

0166-3542/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2010.09.018

proof-of-concept (Karim et al., 2010). Women who were randomized to use 1% tenofovir gel had 39–50% lower chances of getting infected when compared to those randomized to placebo gel. Adherence to the protocol, application of one dose of gel before and after sex, may have been an important factor in the seemingly lower effectiveness of the gel at 30 months of use when compared to 12 months of use.

Several microbicide candidates have been previously tested with no positive results (Feldblum et al., 2008; Halpern et al., 2008; Karim, 2010; Peterson et al., 2007; Van Damme et al., 2008; Van Damme et al., 2002). Surfactants, polyanions and acid buffers were among those which failed to protect women against HIV-1 infection in clinical trials. Lessons learned from the development and testing of these candidates have led to more stringent criteria to qualify candidates and a reduction of the development pipeline. Antiretroviral compounds are now at the forefront of microbicide/PrEP development (McGowan, 2010; Nuttall et al., 2010). Although they are very potent and specific, some having demonstrated successful viral load reduction in the clinic, they face concerns about genesis of resistance, low bioavailability when applied topically to the genital or rectal mucosa, and possible collision with their use in therapeutics (Mellors, 2010).

There is a clear need to expand the existing pipeline of microbicide candidates with more potent and specific compounds, which display new mechanisms of action and present a high genetic barrier to resistance. Preferably these compounds will not be the same as those in use as therapeutics. Equally important is to develop improved formulations for coitally associated and coitally independent modes of administration. Formulation characteristics will impact a range of microbicide properties from biological activity to acceptability and use. The qualities of an active pharmaceutical ingredient (API) and its formulation will determine the safety, efficacy and acceptability of a microbicide product, and, in conjunction with cultural and behavioral factors, will define the ultimate effectiveness and health impact of this biomedical HIV prevention approach.

Critical to the identification of a successful microbicide product is a rational selection of candidates to be tested in clinical trials through a comprehensive preclinical evaluation algorithm. This algorithm includes assays to characterize physico-chemical (P/C) properties of APIs and formulations, release rates, specific antiviral activity, toxicity, pharmacokinetics, pharmacodynamics and efficacy. Based on an enhanced understanding of mucosal HIV transmission and the challenges faced by microbicides (Hladik and Doncel, 2010), new models, assay endpoints and biomarkers have recently been reported, which provide new insights into the mechanisms underlying microbicide-tissue interaction, safety and efficacy (Cummins and Doncel, 2009; Denton et al., 2008; Lackman-Smith et al., 2008; Mesquita et al., 2009; Parikh et al., 2009; Rohan et al., 2010; Van Herrewege et al., 2006; Watson et al., 2008). Many of these novel studies are not an essential part of the investigational new drug (IND)-enabling pathway. However, they expand our understanding of the mode of action of microbicides, paving the way for improving the next generation of compounds. In this manuscript, we review the preclinical assessment of microbicide candidates, putting the emphasis on new models and biomarkers.

This paper forms part of a group of seven reviews covering presentations from the Trends in Microbicide Formulations Workshop that was held on 25–26 January, 2010 in Arlington, Virginia, USA. The other articles discuss HIV transmission (Hladik and Doncel, 2010), gel, film, and tablet formulations (Garg et al., 2010), intravaginal rings (Malcolm et al., 2010), clinical evaluation of microbicides (Morrow and Hendrix, 2010), dual protection (Friend and Doncel, 2010) and novel approaches to microbicide delivery and safety assessment (Whaley et al., 2010).

2. Preclinical evaluation of microbicide candidates

The physico-chemical and biological properties of active pharmaceutical ingredients (APIs) and their formulations are the foundation of safe, efficacious and acceptable microbicides. Hence, the initial selection of the API and its primary formulation is crucial. Certain undesirable properties of an API can be compensated or masked by an appropriate formulation. However, there is a risk in proceeding with inadequate APIs or formulations, as they may fail later in development, costing more time and money. It is important to have a set of criteria that inform Go/No Go decisions prior to entering in clinical trials. This set of criteria is based on API and formulation parameters derived from a comprehensive evaluation of their *in vitro* P/C properties, drug release rates, specific activity, and cell and tissue toxicity, which in turn represent the base for animal studies focused on organ toxicity/safety, pharmacokinetics (PK), and pharmacodynamics (PD) and efficacy. A solid preclinical foundation will help navigate clinical testing successfully, ultimately leading to a safe and effective microbicide (Fig. 1).

In addition to the more classical IND-enabling studies, new models, assays and biomarkers have been developed and adapted to the evaluation of genital and rectal microbicides. Fig. 2 shows examples of these studies in three critical areas of preclinical evaluation, cervico-vaginal (CV) safety, efficacy and PK/PD. Experience with previous microbicide candidates has led to the inclusion of assays evaluating the impact of genital environmental factors such as low pH, seminal plasma, CV secretions and microflora on microbicide safety and efficacy. Microbicide-induced inflammatory mediators and alteration of innate immunity have also been recently incorporated to the standard testing. Given the need of antiretroviral

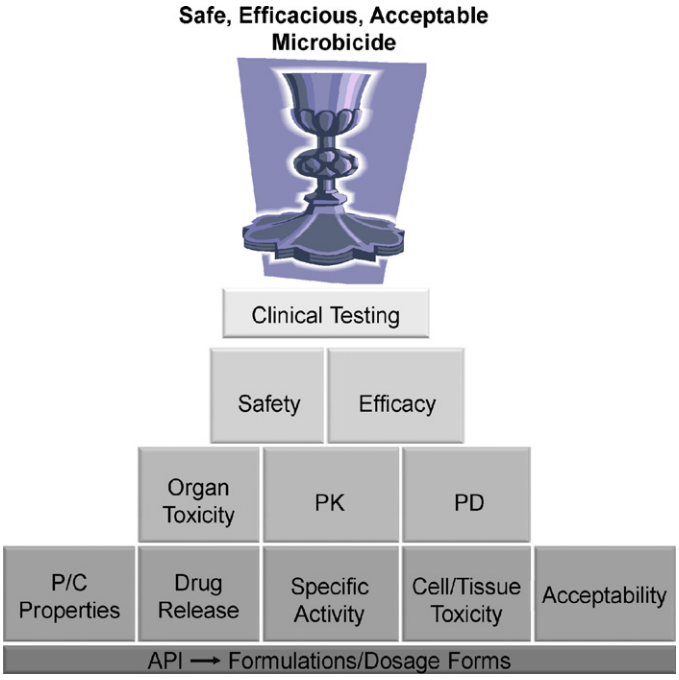

Fig. 1. Building blocks of microbicide preclinical evaluation. Starting with appropriate selection of noncytotoxic, specific, potent and stable active pharmaceutical ingredients and formulating them according to the intended delivery mode, a successful path to the "Holy Grail" of the microbicides field, i.e., a safe, efficacious and acceptable product, goes through the *in vitro* assessment of physico-chemical (P/C) properties and their correlation with acceptability, drug release, specific antiviral activity, and cell and tissue toxicity. It then continues with animal toxicity, pharmacokinetics (PK) and pharmacodynamics (PD), and finishes with cervico-vaginal safety and efficacy leading to an early phase clinical study. This sequential and integrated series of studies provides information for a better understanding of the microbicide as well as its preclinical regulatory package.

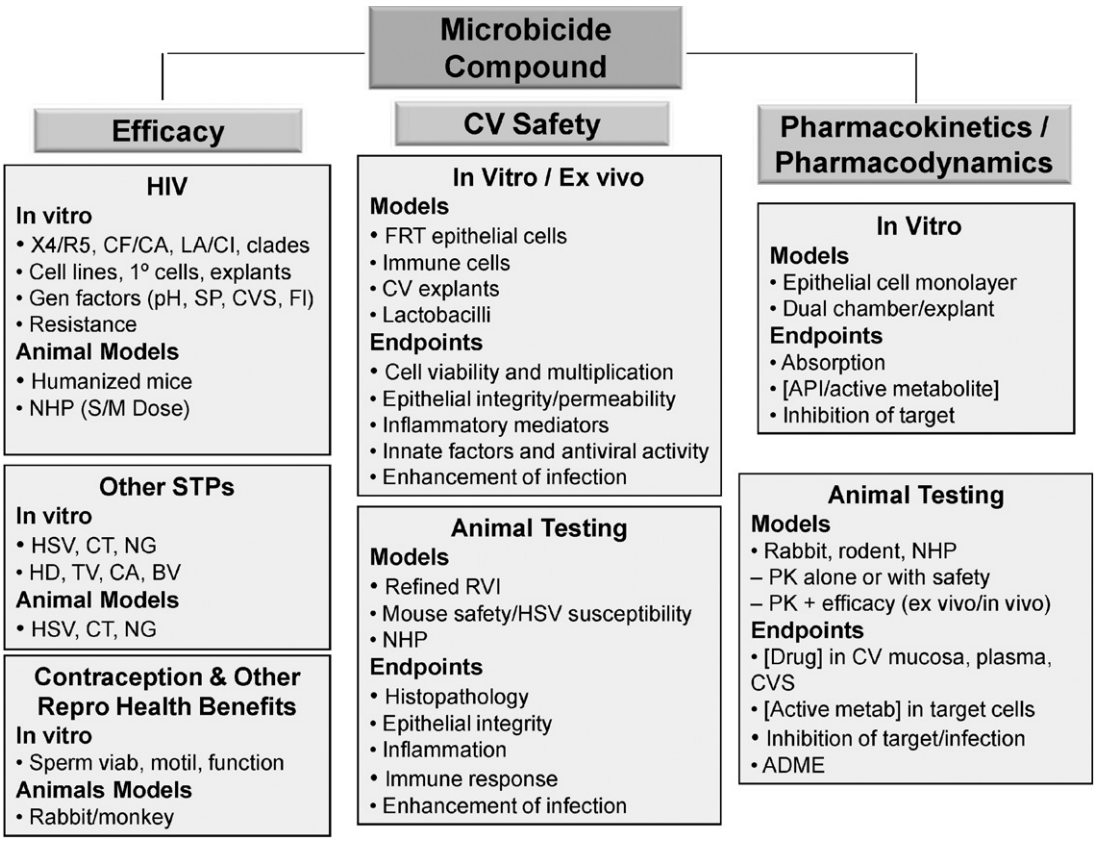

Fig. 2. Expanded preclinical testing of microbicide candidates for efficacy, cervico-vaginal safety and pharmacokinetics and pharmacodynamics. Cervico-vaginal (CV) safety, efficacy and pharmacokinetics (PK) and pharmacodynamics (PD) are three essential components of microbicide preclinical evaluation. Studies typically progress from cell-based to explant-based to animal-based testing models. New endpoints, models and biomarkers have recently been incorporated, and although many of them are not required to file an investigational new drug (IND), they certainly provide information that enables a better understanding of the properties of the microbicide in development. Abbreviations: X4 (CXCR4), R5 (CCR5), CF (cell-free HIV), CA (cell-associated HIV), LA (lab-adapted), CI (clinical isolates), 1° cells (primary immune cells, e.g., peripheral blood mononuclear cells, macrophages), Gen (genital), SP (seminal plasma/semen), CVS (CV secretions), FI (vaginal microflora), NHP (nonhuman primates), S/M (single and multiple compound and virus applications), STPs (sexually transmitted pathogens), HSV (herpes simplex virus), CT (*Chlamydia trachomatis*), NG (*Neisseria gonorrhoeae*), HD (*Hemophilus ducreyii*), TV (*Trichomonas vaginalis*), CA (*Candida albicans*), BV (bacterial vaginosis), viab (viability), motil (motility), FRT (female reproductive tract), RVI (rabbit vaginal irritation model), API (active pharmaceutical ingredient), ADME (absorption, distribution, metabolism, excretion).

compounds, in particular reverse transcriptase inhibitors, to enter target tissues and cells and, in some cases be activated inside them, PK/PD evaluations are now essential components of preclinical and early clinical microbicide evaluation.

3. Physico-chemical (P/C) properties

Early and extensive evaluation of a microbicide candidate's P/C properties is critical for the efficient development of a new microbicide product. P/C property analysis can be broken down in a systematic manner, beginning with preformulation testing of the drug substance and leading to formulation development studies, including drug product characterization and long term stability testing (Table 1). Briefly, preformulation testing entails several types of studies, including solubility testing, stability testing of the drug substance in various drug states (e.g., dry, dispersed, and dissolved) and across a range of pHs, forced degradation studies under an assortment of extreme conditions (e.g., light, heat, acid/base, and oxidative stress), and compatibility studies with dosage form-inspired excipients and/or in combination with other drugs. Equally comprehensive, formulation studies include characterization of both the drug in the dosage form (e.g., drug state, drug content/uniformity, post-processing stability, and determination of drug-related impurities) and the dosage form itself (e.g., pH, mechanical/rheological properties, and appearance).

Formulation scientists are faced with many questions at the onset of a microbicide development program. For instance, what is the ideal dosage form for a new drug candidate? Should the drug be formulated in the dissolved or dispersed state? Will the drug stay in that drug state over the intended shelf-life? Will any P/C properties negatively impact the acceptability of the product? Through the systematic characterization of these P/C properties above, several product development risks can be assessed at the earliest possible stage to better enable a more successful and accelerated microbicide development path (Garg et al., 2003a,b). Subsequently, this early P/C property assessment strategy has become increasingly standard procedure for formulation development groups in the microbicides field and became the focus of several presentations at the Microbicides 2010 Conference (Damian et al., 2010; Fetherston et al., 2010; McBride et al., 2010; Wang et al., 2010a,b).

Not to be excluded from this discussion is the increasing use of new models that utilize P/C properties as inputs to predict the performance of a microbicide product. For example, rheological parameters may be used to not only predict *in vivo* spreading, coating and retention of a vaginal gel (Katz et al., 2010; Kieweg and Katz, 2007; Szeri et al., 2008) but to rationally design new gel formulations still early in formulation development (Mahalingam et al., 2010). Moreover, ongoing work by Dr. Morrow and colleagues is identifying specific links between acceptability and P/C properties of a microbicide product (Morrow et al., 2010). As these links are established for different dosage forms, acceptability-optimizing

Table 1
Standard tests for evaluating the physico-chemical properties of a microbicide candidate.

Preformulation studies (drug substance)	Formulation studies (drug product)	Stability of drug product
Solubility	pH	Chemical stability
Stability	Osmolality	Physical stability
Chemical	Mechanical properties	Drug state
Physical	Viscosity (gels)	Dosage form
Forced degradation	Stiffness (films, IVRs)	Long-term storage conditions:
Compatibility	Hardness/friability (tablets)	25 °C/60% RH or 30 °C/65% RH
Excipients	Appearance	Accelerated studies
Aqueous, as function of:	Odor	40 °C/75% RH
pH	Drug content/uniformity	>40 °C (IVRs)
Oxidative stress	Drug related impurities	Other conditions:
Drug state (dissolved/dispersed)	Particle size distribution	Light
Drug combinations	Unit dose/volume	Aqueous (rings)
	Product dimensions	5 °C (shipping deviations)
	Water content (films, tablets)	
	Others? (dosage form dependent)	

formulations can be designed through target P/C property specifications.

4. Drug release and permeability

With next generation microbicide products focusing heavily on the development of potent antiretrovirals formulated in various dosage forms, evaluating the ability of that formulation to deliver the drug when and where it is needed is an essential building block to the microbicide product development pathway. Typically, *in vitro* dissolution studies are performed early, with the intent to compare candidate formulations and theoretically begin to predict *in vivo* release profiles.

Dissolution studies may be performed using one or more experimental setups, selection of which is generally dictated by the type of dosage form being tested. For example, testing of semisolid gels typically use two-compartment Franz cell dissolution chambers, while testing of intravaginal rings (IVRs) more commonly employ orbital shaker/incubator systems which are more suitable to longer duration studies (Gupta et al., 2008a; Johnson et al., 2010; Malcolm et al., 2005). Depending on the P/C properties of a drug, the dissolution media is often modified with organic solvents or solubilizers to maintain sink conditions. However, the selection of dissolution media – and the interpretation of their results – should be made with great caution, particularly if based on historical *in vitro–in vivo* release correlation (IVIVRC) data for a previously tested drug. The relationship of P/C properties to drug release under sink conditions *in vitro* is not fully understood and has yet to provide universally predictive release profiles for the diversity of microbicide drug candidates, particularly for testing of intravaginal rings (Lowry et al., 2010; Malcolm et al., 2010). Moreover, large compositional changes to a formulation (e.g., silicone versus polyurethane IVRs) can also have a large impact on the ability to compare drug release profiles due to potential interactions with the release media and/or setup. Some researchers are therefore exploring the development of new *in vitro* drug release models that better mimic the *in vivo* drug release environment. One such example is a two compartment model developed specifically for silicone intravaginal rings in which a low-volume donor fluid, semi-permeable membrane and receptor fluid sink intend to represent vaginal secretions, vaginal tissue and systemic circulation, respectively (McConville et al., 2010a).

Even more mimetic yet still testable at these early product development stages are the use of tissue permeability studies. These studies provide information regarding the ability of a formulation, administered either neat or diluted with cervico-vaginal fluid simulants, to deliver the API to its target site. In the case of many antiretrovirals, this means into target cells within the genital tissues. Moreover, the rate of flux of a drug across the tissue may provide early knowledge of the expected level of systemic exposure *in vivo*. Several *in vitro* and *ex vivo* models have served this purpose, most notably human ectocervical and colorectal explants (Rohan et al., 2010). More recently, the EpiVaginal tissue (MatTek Corp., Ashland, MA), a three-dimensional vaginal-ectocervical tissue culture model originally developed for early product safety evaluations (Ayehunie et al., 2006; Trifonova et al., 2006) may also be used. The EpiVaginal model maintains good tissue viability and integrity for several days, making it an attractive option for testing sustained dosage forms such as the IVR (Ho et al., 2010a,b; McConville et al., 2010b). These studies are increasingly being used to screen new formulations, and parallel safety and efficacy testing in these same models provide unusually early IND-enhancing data that further accelerate the microbicide product development timeline to the clinic.

5. Activity against HIV-1 and other sexually transmitted pathogens

Microbicide specific anti-HIV-1 activity translates into efficacy and it should be assessed very early in the development of a compound. Both API and formulations should be evaluated sequentially in cell-based, explant-based and animal-based models. Primary testing includes API activity against HIV-1 X4 and R5, cell-free and cell-associated, lab-adapted and clinical isolate viruses in a single-round infection assay format using CD4/CCR5/CXCR4 expressing cell lines and a short compound-cell-virus incubation (1–2 h) (Doncel and Mauck, 2004; Lackman-Smith et al., 2008). A low 50% inhibitory concentration (IC_{50}), preferably in the nano- to sub-nanomolar range, and a high selectivity index (relating cytotoxicity and antiviral activity), preferably higher than 10^4, are recommendable. Wild-type HIV-1 from different clades, especially those dominant in high HIV prevalence areas, and resistant mutants are evaluated next using peripheral blood mononuclear cells (PBMCs). The impact of genital factors such as low pH, seminal plasma, CV secretions and microflora follows using single-round infection assays (Lackman-Smith et al., 2008). Cervical and colorectal explants are especially suited for evaluating full-strength, undiluted gel formulations, a step prior to animal testing (Abner et al., 2005; Cummins et al., 2007; Fletcher et al., 2005; Greenhead et al., 2000). The study of induction of resistant virus by the candidate compound involves the use of escalating suboptimal doses of drug and the genotypic characterization of the escape mutants. The ability of such compound to "sterilize" an HIV infected culture, preventing the escape of resistant virus for at least 30 days constitutes the basis of a more stringent determination of a microbicide IC_{50} (Watson et al., 2008). In an attempt to more closely

mimic *in vivo* sexual transmission conditions, another stringent way to assess a microbicide candidate is to apply compound and then cell-associated virus over a cervical epithelial cell monolayer growing in the upper compartment of a dual—chamber system, evaluating the microbicide's HIV inhibitory activity on dendritic cell/T cell complexes in the bottom chamber (Van Herrewege et al., 2006). Combination studies determine the existence of synergism, additivism or antagonism between pairs of selected compounds, and provide the basis for combination microbicide products (Buckheit et al., 2010; Chou and Talalay, 1984; Liu et al., 2005).

Antiviral data from the *in vitro* assessment of a successful microbicide candidate should be confirmed in at least one animal model of genital/rectal mucosal HIV infection. The two primary models to evaluate microbicide efficacy in animals use non-human primates and humanized mice (see below).

Although this review focuses on the preclinical assessment of specific anti-HIV microbicide candidates, it is important to have in mind that there is ongoing R&D on dual-protection technologies (Friend and Doncel, 2010) including products that, in addition to anti-HIV activity, display activity against other sexually transmitted pathogens (STPs, e.g., HSV-2) or sperm (i.e., they are contraceptives). Due to the intertwined nature of STP transmission and its contextual similarities with mammalian fertilization (Doncel, 2006), activity against sperm and main STPs should also be evaluated during preclinical development (Doncel and Mauck, 2004; Lard-Whiteford et al., 2004).

6. Cell and tissue toxicity—microbicide safety *in vitro*

First and foremost microbicide APIs should not be significantly cytotoxic. Given the expected chronic exposure of the CV mucosa to the compound, microbicide candidates should not display tissue toxicity at concentrations at which they are intended to be used in humans, even after repeated applications. Clinical experience with cytotoxic compounds such as nonoxynol-9 has proved their deleterious mucosal effects and confirmed the suspicion that they can enhance rather than reduce HIV transmission (Doncel et al., 2004; Fichorova et al., 2001; Roddy et al., 1993; Van Damme et al., 2002). Cytotoxicity involves alteration of cell viability, multiplication and/or function. It is typically evaluated as a companion to antiviral activity assays to rule out artifactual effects, but it should also be evaluated using cervical, vaginal and rectal cells and tissue explants. As indicated before, explants and organotypic cervical and vaginal tissue constructs (e.g., EpiVaginal) are ideal to evaluate formulations (Ayehunie et al., 2006; Cummins et al., 2007; Hjelm et al., 2010). Given that immune cells are an essential component in mucosal transmission of HIV, the impact of microbicides on these cells, directly or indirectly through their actions on epithelial cells, should also be determined (Milligan et al., 2004; Milligan et al., 2005). Finally, lactobacilli are essential in maintaining a healthy vaginal microflora. Analysis of the effects of compounds on lactobacillus growth is an integral part of the preclinical assessment of microbicides (Moncla et al., 2010).

In addition to the standard endpoints of cell and tissue toxicity, the peculiarities of HIV mucosal infection (Hladik and Doncel, 2010) have highlighted the importance of evaluating the induction of inflammatory mediators such as cytokines and chemokines (Doncel et al., 2004; Fichorova, 2004; Li et al., 2009). Recently, differential gene expression analysis of vaginal epithelial cells has revealed about 80 genes significantly upregulated in response to pro-inflammatory compounds (Zalenskaya et al., 2010). A dozen or so have been confirmed by mRNA and protein expression and constitute new biomarkers of genital epithelial alteration. Inflammatory and innate immune mediators are part of the natural cervico-vaginal defensive mechanisms triggered by microbial activation of Toll-like receptors (TLRs) (Nasu and Narahara, 2010). Interference with TLR mediated responses have been demonstrated for certain polyanionic microbicide candidates (Trifonova et al., 2009). Mucosal alteration not only leads to secretion of pro-inflammatory mediators that in turn recruit HIV target cells to the mucosal portals of entry, but may also be manifested by enhanced permeability to pathogens, including HIV, and decreased innate antimicrobial activity. Epithelial tight junctions regulate the permeability of the epithelium and can be compromised by the action of microbicides (Gali et al., 2010; Mesquita et al., 2009). Innate factors such as SLPI and human beta defensins are involved in the mucosal defense against pathogen invasion (Cole, 2006). The integrity of defensive mechanisms such as epithelial junctions, innate factors and lactobacilli needs to be evaluated preclinically with both APIs and formulations. Enhancement of HIV infection should be avoided and may be evaluated using *in vitro* assays and animal models.

7. Organ toxicity—microbicide safety *in vivo*

The nonclinical toxicology testing plan required for the development of a topical microbicide entails numerous IND-enabling and enhancing studies conducted at each step of the clinical development pathway (i.e., pre-phase I to pre-NDA). If the microbicide candidate is a new chemical entity not previously tested in humans, then evaluation begins with general toxicology studies of the drug substance as well as the drug product (Lard-Whiteford et al., 2004; Lard et al., 1994). Oral and/or intravenous toxicity studies are commonly performed to maximize systemic exposure, however vaginal or rectal dosing (i.e., route of intended clinical administration) is required. These studies are typically performed in two animal models (rodent and non-rodent) and evaluate a number of general animal health/behavior parameters as well as blood and urine chemistry analysis, and tissues and organs macroscopically and microscopically. Multiple dose levels are typically evaluated with the objective of identifying a maximum tolerated (or maximum feasible) dose level, helping to select the dose level of the formulated product. Single (acute) and multiple, repeated (subchronic) dosing regimens are standard. Safety pharmacology studies are also required if systemic absorption is determined. These studies specifically assess drug effects on vital organs and systems (e.g., central nervous system, respiratory system, cardiovascular system).

Other toxicology tests required early in the development of a new microbicide include genotoxicity and hypersensitivity studies. Genotoxicity tests intend to evaluate a drug's mutagenic potential and must be performed on both bacteria (e.g., AMES test) and mammalian cells (e.g., mouse lymphoma assay, micronucleus test). Hypersensitivity studies, such as the guinea pig maximization test, evaluate the immunogenic response following repeated cutaneous exposures. Guidances for these IND-enabling studies are available from the International Conference on Harmonisation (ICH, 2009). In the case of intravaginal rings, similar toxicology studies are required following medical device guidances issued by the International Organization of for Standardization (ISO, 2009).

Given the intended use in young, child-bearing aged women, reproductive toxicology is essential for the nonclinical development of vaginal microbicides. Both segment I (reproductive toxicity in rodents) and segment II (teratology in rodents and non-rodents) are recommended to be performed early, preferably before human exposure, while segment III (peri-/post-natal toxicity) may be performed in parallel with Phase III clinical trials (Lard-Whiteford et al., 2004). Also conducted at these late clinical stages are chronic toxicology and carcinogenicity studies in rodent and/or non-rodent species.

In addition to standard toxicology, vaginal products are evaluated for their direct impact on cervico-vaginal tissues. Typically the rabbit vaginal irritation (RVI) model is used for this purpose (Eckstein et al., 1969). USFDA recognizes the rabbit as the non-rodent species of choice for vaginal irritation studies. This model, however, fails to provide adequate characterization of the inflammatory response elicited by certain compounds. We have improved the RVI model by including additional endpoints such as phenotype and activation status of infiltrating leukocytes, mucosal expression of lymphocyte homing receptors, tight junctions and biomarkers of inflammation (Doncel et al., 2004; Fichorova, 2004; Trifonova et al., 2007). The model has also incorporated a cervico-vaginal lavage that provides materials for the analysis of pro- and anti-inflammatory cytokines and innate factors at multiple time points (Aranha et al., 2008; Fichorova, 2004; Gupta et al., 2008b). Although not a requirement for an FDA IND, the refined RVI model provides important information about microbicide-induced mucosal inflammatory reactions. Similar endpoints may be evaluated and measured in a mouse model (Wilson et al., 2009). The amount of compound to be administered vaginally, however, is very small (~5 μL) and the epithelium of the vagina is stratified and keratinized, making it more resistant to the effect of vaginal formulations. On the positive side, this epithelium may be thinned out by the use of a progestin (e.g., depo medroxyprogesterone acetate), rendering the animals susceptible to genital infection by HSV-2. This model has been used to determine potential enhancement of HSV infection by candidate microbicides (Cone et al., 2006; Segarra et al., 2010; Wilson et al., 2009). Repeated application of surfactants like nonoxynol-9 increases the susceptibility of the animal to be infected by HSV-2 vaginally.

Assessment of vaginal and rectal safety of a microbicide may also be performed in non-human primates (NHP). In addition to colposcopic evaluation of the cervical and vaginal mucosa, CVLs and tissue biopsies provide materials to evaluate cellular and soluble markers of inflammation and tissue alteration (Patton et al., 2004; Patton et al., 2007). The NHP model can also be used to measure enhancement of infection after repeated applications of the compound (Doncel and Miller, manuscript in preparation).

Sheep are increasingly being used for the evaluation of local toxicity and toxicology of microbicide products, particularly for fixed-size solid dosage forms such as IVRs (Nuttall et al., 2010). Methods of evaluation in sheep typically include colposcopy, histology of post-treatment biopsies and toxicology through plasma sampling. New and cutting edge to the *in vivo* preclinical assessment of vaginal and rectal microbicides is the use of non-invasive imaging techniques for evaluating local toxicity *in situ* and with increased sensitivity when compared to colposcopy. Optical coherence tomography (OCT) has recently been demonstrated to detect treatment-induced changes in vaginal and rectal epithelial thickness similar to histological findings (Vincent et al., 2010; Vincent et al., 2009).

8. Pharmacokinetics of microbicides in animal models

Nonclinical pharmacokinetic studies intend to characterize the absorption, distribution, metabolism and excretion (ADME) profile of a drug. Ideally, these studies should be performed on the formulated drug product using the intended route of administration. Often however, the extent of systemic exposure of a microbicide following vaginal or rectal routes of administration is low (Nel et al., 2010; Schwartz et al., 2008). Consequently, the design of many microbicide-driven PK studies focuses more on the absorption and local distribution of drug, with bioanalysis of collected genital tissues and vaginal fluids (e.g., cervico-vaginal lavage, swab, sponge), in addition to plasma samples. Depending on tissue availability, isolated $CD4^+$ cells and/or iliac lymph nodes may also be analyzed for drug concentrations.

There are a handful of animal models typically used for PK testing of topical microbicides. The rabbit model is a common first study, given their low cost, high availability, and dual use in the RVI model. Although typically used to evaluate gels, rabbits are also suitable for testing other dosage forms such as small tablets, suppositories and IVR segments. For testing of the latter, surgically implanted IVR segments may be recovered at the end of the study and residual drug content may be extracted for determination of *in vivo* drug release (Clark et al., 2010; Moss et al., 2010). It should be noted, however, that there are several anatomical and physiological differences between the rabbit and human vagina (Barberini et al., 1991, 1992; Rodriguez-Antolin et al., 2009); therefore extrapolation of rabbit vaginal PK data to human vaginal levels should be interpreted with caution.

Non-human primates (NHPs) are often considered the penultimate animal PK model, with several species to choose from, including cynomologus, pig-tailed and rhesus macaques. There are pros and cons to each of these NHP models, including animal availability, size appropriateness for testing different dosage forms, the impact and/or need for Depo Provera® treatment (Malcolm et al., 2010), and whether parallel animal challenge models may be available. Moreover, each of these models requires cross-validation of bioanalytical methods, therefore careful and early planning of these studies is required.

Clearly, there is a large and relatively underexplored testing space between rabbits and NHPs for PK evaluation of new microbicide candidates. Certainly as new animal safety and efficacy models are developed, their dual use for PK testing is likely. Sheep are one such example, particularly for the testing of full-sized IVRs and other alternative dosage forms. Due to their size, tissue biopsies can be collected from multiple sites and at multiple time points from a given animal. Swabs of vaginal secretions may also be collected readily.

9. Efficacy and pharmacodynamic evaluation of microbicides

Non-human primate models for vaginal infection of HIV are the best established models to evaluate microbicide efficacy. Using simian immunodeficiency virus (SIV), a chimera between SIV and the envelope of HIV (SHIV) and a further modification of this virus with an HIV reverse transcriptase (to make the virus susceptible to nonnucleoside reverse transcriptase inhibitors), the model can evaluate most of the microbicide candidates in development. The viral inoculum and challenging schedule vary, and there is no agreement as to which variant predicts human efficacy more accurately. The spectrum goes from low viral titer and multiple applications to a single dose of highly concentrated virus, with and without an injection of Depo Provera® to thin out the vaginal epithelium and sensitize the model (Boadi et al., 2005; Miller et al., 1992; Subbarao et al., 2007; Veazey et al., 2005). Although there is no validated cut-off, it is recommendable to get at least statistically significant 90% protection from infection in this model.

Humanized mice, especially those obtained by the xenografting of human $CD34^+$ stem cells and pieces of fetal thymus and liver (BLT mice), represent an alternative to the use of NHP for microbicide efficacy evaluation (Denton and Garcia, 2009). The generation of animals is very laborious and technically demanding, and the population of human immune cells in the vagina (targets for HIV infection) is not yet quantitatively robust. The model, however, does allow for vaginal and rectal HIV infection when high-titer virus is used. Furthermore, it has been validated by demonstrating the

Table 2
List and timing of investigational new drug (IND)- and new drug application (NDA)-enabling studies.

Pre-phase I	Pre-phase II/III	Pre-NDA
Chemistry, manufacturing, controls (CMC)	Antiviral activity	Reproductive toxicology
Antiviral activity	*Ex vivo*	Segment III
In vitro	*In vivo*	Carcinogenicity
Cytotoxicity	Chronic toxicology	General toxicity for impurity
General toxicology	Hypersensitivity	
Acute		
Subchronic		
Genotoxicity		
Safety pharmacology		
Rabbit vaginal irritation (RVI)		
Reproductive toxicology		
Segments I–II		

Phase I and II/III refer to different stages of clinical testing.

efficacy of pre-exposure prophylaxis with tenofovir/emtricitabine in preventing vaginal and rectal infection by HIV-1 (Denton et al., 2008).

Very recently, we have reported the use of an *ex vivo* technique in women as a surrogate marker of microbicide pharmacodynamic activity and efficacy (Schwartz et al., 2010). A similar technique had been previously employed in men for testing a rectal microbicide (Anton, 2010). The technique consists of administering the microbicide vaginally, taking a biopsy of the tissue 4–8 h later (depending on PK), and infecting the explants *in vitro* with a low dose of HIV-1_{BaL}, a lab-adapted R5 virus. Following the production curve of p24 throughout multiple time points and analyzing tissue integrated HIV, one can compare the values pre- and post-treatment, expecting a significantly reduced infection in the post-treatment biopsies.

We are currently refining this technique and incorporating it into the NHP model. Another measure of PD activity is the evaluation of antiviral activity of CV fluid at multiple time points after a single-dose application of the microbicide. The *ex vivo* evaluation of residual anti-HIV activity, typically using a single-round infection assay (e.g., TZM-bl cells), may provide information about the distribution of the compound and its activity in the presence of CV secretions, semen (if intercourse has taken place), and microflora (Keller et al., 2010; Keller et al., 2006; Mauck et al., 2010). Unlike tenofovir, a microbicide that proved to be efficacious in humans, PRO2000 suffered a significant loss in activity after intercourse with ejaculation.

10. IND-enabling studies

On the path to a new drug application (NDA) with the USFDA, the first step is to assemble a regulatory preclinical dossier to support an investigational new drug (IND) application. Typically the dossier for a vaginal microbicide should contain information about chemistry, manufacturing and controls (CMC), microbiology (mostly antiviral activity), and the initial part of pharmacology and toxicology. Not all the information in these categories is required for the first filing of the IND. The requirements increase as the compound progresses through clinical stages from phase I to phase III trials (Table 2). For early phase I clinical trials designed to provide information on drug product feasibility following limited exposure (e.g., single or repeat dose PK and PD, biodistribution using imaging techniques and safety for seven days or less), then filing an exploratory IND is an option that generally has reduced and more flexible nonclinical testing requirements than traditional INDs (FDA, 2006). Additional information on IND and NDA requirements may be found at the FDA website (www.fda.gov) and in Lard-Whiteford et al. review (2004).

11. Summary and conclusions

A comprehensive preclinical evaluation of a microbicide candidate is essential to the selection of a successful product. Although drugs can and certainly do fail during clinical stages of development, many of these failures are due to incomplete or inadequate preclinical characterization. The building blocks of a safe and efficacious microbicide start with the selection of a stable, specific, potent and non-cytotoxic API and follow with the development of an appropriate formulation and delivery system that provide good PK and PD characteristics. Evaluation of these parameters through a sequential, integrated algorithm including *in vitro* (cells), *ex vivo* (explants) and *in vivo* (animal models) studies provides the necessary information to understand the mode of action of a microbicide candidate and file a preclinical dossier with the regulatory authorities. New models, assay endpoints and biomarkers are being developed and incorporated into this algorithm. Examples of them are evaluation of drug release and tissue permeability using explants and reconstructed tissue, antiviral activity in the presence of genital environmental factors such as semen, induction of cervico-vaginal mucosal inflammatory responses, interference with mucosal innate antimicrobial activity, enhancement of HIV infection, tissue and HIV target cell concentrations of active drug, pharmacodynamic surrogates, and animal model efficacy. As more microbicide candidates are tested in human clinical trials, validation of these new tools will allow us to simplify the preclinical testing algorithm and streamline the critical path to product development.

Acknowledgements

This work was supported by CONRAD intramural funds (GD, MC) from the US Agency for International Development (grant GPO-8-00-08-00005-00) and the Bill and Melinda Gates Foundation (grant 41266). The views of the authors do not necessarily represent those of their funding agencies. The authors are also grateful to Nancy Gonyea for her assistance in the preparation of this with manuscript.

References

Abner, S.R., Guenthner, P.C., Guarner, J., Hancock, K.A., Cummins Jr., J.E., Fink, A., Gilmore, G.T., Staley, C., Ward, A., Ali, O., Binderow, S., Cohen, S., Grohskopf, L.A., Paxton, L., Hart, C.E., Dezzutti, C.S., 2005. A human colorectal explant culture to evaluate topical microbicides for the prevention of HIV infection. J. Infect. Dis. 192, 1545–1556.

Anton, P., 2010. Bridging preclinical observations to phase I clinical trials. In: Microbicides 2010, Pittsburgh, PA.

Aranha, C.C., Gupta, S.M., Reddy, K.V., 2008. Assessment of cervicovaginal cytokine levels following exposure to microbicide Nisin gel in rabbits. Cytokine 43, 63–70.

Ayehunie, S., Cannon, C., Lamore, S., Kubilus, J., Anderson, D.J., Pudney, J., Klausner, M., 2006. Organotypic human vaginal-ectocervical tissue model for irritation

studies of spermicides, microbicides, and feminine-care products. Toxicol. In Vitro 20, 689–698.
Barberini, F., Correr, S., De Santis, F., Motta, P.M., 1991. The epithelium of the rabbit vagina: a microtopographical study by light, transmission and scanning electron microscopy. Arch. Histol. Cytol. 54, 365–378.
Barberini, F., De Santis, F., Correr, S., Motta, P.M., 1992. The mucosa of the rabbit vagina: a proposed experimental model for correlated morphofunctional studies in humans. Eur. J. Obstet. Gynecol. Reprod. Biol. 44, 221–227.
Boadi, T., Schneider, E., Chung, S., Tsai, L., Gettie, A., Ratterree, M., Blanchard, J., Neurath, A.R., Cheng-Mayer, C., 2005. Cellulose acetate 1,2-benzenedicarboxylate protects against challenge with pathogenic X4 and R5 simian/human immunodeficiency virus 2. AIDS 19, 1587–1594.
Buckheit Jr., R.W., Watson, K.M., Morrow, K.M., Ham, A.S., 2010. Development of topical microbicides to prevent the sexual transmission of HIV. Antiviral Res. 85, 142–158.
Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.
Clark, M., Loxley, A., Ho, E., Tuitupou, A., McConnell, J.R., Okoh, O., Goldman, L., Morgan, C., Mitchnick, M., Kiser, P., Friend, D., 2010. In vitro and in vivo release of antiretrovial UC781 from polyurethane and ethylene vinlyacetate copolymer intravaginal ring segments. In: Annual Meeting and Exposition of the Controlled Release Society, Portland, OR.
Cole, A.M., 2006. Innate host defense of human vaginal and cervical mucosae. Curr. Top. Microbiol. Immunol. 306, 199–230.
Cone, R.A., Hoen, T., Wong, X., Abusuwwa, R., Anderson, D.J., Moench, T.R., 2006. Vaginal microbicides: detecting toxicities in vivo that paradoxically increase pathogen transmission. BMC Infect. Dis. 6, 90.
Cummins Jr., J.E., Doncel, G.F., 2009. Biomarkers of cervicovaginal inflammation for the assessment of microbicide safety. Sex Transm. Dis. 36, S84–91.
Cummins Jr., J.E., Guarner, J., Flowers, L., Guenthner, P.C., Bartlett, J., Morken, T., Grohskopf, L.A., Paxton, L., Dezzutti, C.S., 2007. Preclinical testing of candidate topical microbicides for anti-human immunodeficiency virus type 1 activity and tissue toxicity in a human cervical explant culture. Antimicrob. Agents Chemother. 51, 1770–1779.
Damian, F., Fabian, J., Clark, M.R., Elgendy, H., Doncel, G.F., Friend, D., McCormick, T., Kiser, P., 2010. Preclinical stability and excipient compatibility leading to the development of combination gel formulations of UC781 and tenofovir. In: Microbicides 2010, Pittsburgh, PA.
Denton, P.W., Estes, J.D., Sun, Z., Othieno, F.A., Wei, B.L., Wege, A.K., Powell, D.A., Payne, D., Haase, A.T., Garcia, J.V., 2008. Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT Mice. PLoS Med. 5, 16.
Denton, P.W., Garcia, J.V., 2009. Novel humanized murine models for HIV research. Curr. HIV/AIDS Rep. 6, 13–19.
Doncel, G., Mauck, C., 2004. Vaginal microbicides: a novel approach to preventing sexual transmission of HIV. Curr. HIV/AIDS Rep. 1, 25–32.
Doncel, G.F., 2006. Exploiting common targets in human fertilization and HIV infection: development of novel contraceptive microbicides. Hum. Reprod. Update 12, 103–117.
Doncel, G.F., Chandra, N., Fichorova, R.N., 2004. Preclinical assessment of the proinflammatory potential of microbicide candidates. J. Acquir. Immune Defic. Syndr. 37 (Suppl. 3), S174–S180.
Eckstein, P., Jackson, M.C., Millman, N., Sobrero, A.J., 1969. Comparison of vaginal tolerance tests of spermicidal preparations in rabbits and monkeys. J. Reprod. Fertil. 20, 85–93.
FDA, 2006. Guidance for Industry, Investigators and Reviewers: Exploratory IND Studies http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078933.pdf.
Feldblum, P.J., Adeiga, A., Bakare, R., Wevill, S., Lendvay, A., Obadaki, F., Olayemi, M.O., Wang, L., Nanda, K., Rountree, W., 2008. SAVVY vaginal gel (C31G) for prevention of HIV infection: a randomized controlled trial in Nigeria. PLoS One 3, 1474.
Fetherston, S., Malcolm, K., Woolfson, D., Lacey, C., Shattock, R., 2010. Formulation development of matrix-type silicone elastomer vaginal rings containing protease inhibitors. In: Microbicides 2010, Pittsburgh, PA.
Fichorova, R.N., 2004. Guiding the vaginal microbicide trials with biomarkers of inflammation. J. Acquir. Immune Defic. Syndr. 37 (Suppl. 3), S184–S193.
Fichorova, R.N., Tucker, L.D., Anderson, D.J., 2001. The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. J. Infect. Dis. 184, 418–428.
Fletcher, P., Kiselyeva, Y., Wallace, G., Romano, J., Griffin, G., Margolis, L., Shattock, R., 2005. The nonnucleoside reverse transcriptase inhibitor UC-781 inhibits human immunodeficiency virus type 1 infection of human cervical tissue and dissemination by migratory cells. J. Virol. 79, 11179–11186.
Friend, D.R., Doncel, G.F., 2010. Combining prevention of HIV-1, other sexually transmitted infections and unintended pregnancies: development of dual-protection technologies. Antiviral Res. 88, S47–S54.
Gali, Y., Arien, K.K., Praet, M., Van den Bergh, R., Temmerman, M., Delezay, O., Vanham, G., 2010. Development of an in vitro dual-chamber model of the female genital tract as a screening tool for epithelial toxicity. J. Virol. Methods 165, 186–197.
Garg, S., Goldman, D., Krumme, M., Rohan, L.C., Smoot, S., Friend, D., 2010. Advances in development, scale-up and manufacturing of microbicide gels, films, and tablets. Antiviral Res. 88, S19–S29.
Garg, S., Kandarapu, R., Vermani, K., Tambwekar, K.R., Garg, A., Waller, D.P., Zaneveld, L.J., 2003a. Development pharmaceutics of microbicide formulations Part I: preformulation considerations and challenges. AIDS Patient Care STDS 17, 17–32.
Garg, S., Tambwekar, K.R., Vermani, K., Kandarapu, R., Garg, A., Waller, D.P., Zaneveld, L.J., 2003b. Development pharmaceutics of microbicide formulations Part II: formulation, evaluation, and challenges. AIDS Patient Care STDS 17, 377–399.
Greenhead, P., Hayes, P., Watts, P.S., Laing, K.G., Griffin, G.E., Shattock, R.J., 2000. Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides. J. Virol. 74, 5577–5586.
Gupta, K.M., Pearce, S.M., Poursaid, A.E., Aliyar, H.A., Tresco, P.A., Mitchnik, M.A., Kiser, P.F., 2008a. Polyurethane intravaginal ring for controlled delivery of dapivirine, a nonnucleoside reverse transcriptase inhibitor of HIV-1. J. Pharm. Sci. 97, 4228–4239.
Gupta, S.M., Aranha, C.C., Mohanty, M.C., Reddy, K.V., 2008b. Toll-like receptors and cytokines as surrogate biomarkers for evaluating vaginal immune response following microbicide administration. Mediators Inflamm., 2008 (epub).
Halpern, V., Ogunsola, F., Obunge, O., Wang, C.H., Onyejepu, N., Oduyebo, O., Taylor, D., McNeil, L., Mehta, N., Umo-Otong, J., Otusanya, S., Crucitti, T., Abdellati, S., 2008. Effectiveness of cellulose sulfate vaginal gel for the prevention of HIV infection: results of a Phase III trial in Nigeria. PLoS One 3, 3784.
Hjelm, B.E., Berta, A.N., Nickerson, C.A., Arntzen, C.J., Herbst-Kralovetz, M.M., 2010. Development and characterization of a three-dimensional organotypic human vaginal epithelial cell model. Biol. Reprod. 82, 617–627.
Hladik, F., Doncel, G.F., 2010. Preventing mucosal HIV transmission with topical microbicides—challenges and opportunities. Antiviral Res. 88, S3–S9.
Ho, E.A., Clark, J.T., Damian, F., Tuitupou, A.L., Clark, M.R., Friend, D., Kiser, P., 2010a. The development of a combination tenofovir and UC781 intravaginal ring. In: Trends in Microbicide Formulations Symposium, Arlington, VA.
Ho, E.A., Tuitupou, A.L., Elgendy, H., Clark, M.R., Friend, D., Kiser, P., 2010b. A polyurethane intravaginal ring for the delivery of UC781. In: Microbicides 2010, Pittsburgh, PA.
Horton, R., Das, P., 2008. Putting prevention at the forefront of HIV/AIDS. Lancet 372, 421–422.
ICH, 2009. International Conference on Harmonization M3 Guideline: guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3 (R2).
ISO, 2009. ISO 10993-1:2009. Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process.
Johnson, T.J., Gupta, K.M., Fabian, J., Albright, T.H., Kiser, P.F., 2010. Segmented polyurethane intravaginal rings for the sustained combined delivery of antiretroviral agents dapivirine and tenofovir. Eur. J. Pharm. Sci. 39, 203–212.
Karim, A.S.S., 2010. Results of effectiveness trials of PRO 2000 gel: lessons for future microbicide trials. Future Microbiol. 5, 527–529.
Karim, Q., Karim, S.S., Frohlich, J.A., Grobler, A.C., Baxter, C., Mansoor, L.E., Kharsany, A.B., Sibeko, S., Mlisana, K.P., Omar, Z., Gengiah, T.N., Maarschalk, S., Arulappan, N., Mlotshwa, M., Morris, L., Taylor, D., 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science 329, 1168–1174.
Katz, D., Peters, J.J., Clark, M.L., Friend, D., Kiser, P., 2010. Multivariate computational model to help rate microbicide gel performance: combining vaginal distribution, drug delivery and acceptability. In: Microbicides 2010, Pittsburgh, PA.
Keller, M.J., Mesquita, P.M., Torres, N.M., Cho, S., Shust, G., Madan, R.P., Cohen, H.W., Petrie, J., Ford, T., Soto-Torres, L., Profy, A.T., Herold, B.C., 2010. Postcoital bioavailability and antiviral activity of 0.5% PRO 2000 gel: implications for future microbicide clinical trials. PLoS One 5, 8781.
Keller, M.J., Zerhouni-Layachi, B., Cheshenko, N., John, M., Hogarty, K., Kasowitz, A., Goldberg, C.L., Wallenstein, S., Profy, A.T., Klotman, M.E., Herold, B.C., 2006. PRO 2000 gel inhibits HIV and herpes simplex virus infection following vaginal application: a double-blind placebo-controlled trial. J. Infect. Dis. 193, 27–35.
Kieweg, S.L., Katz, D.F., 2007. Interpreting properties of microbicide drug delivery gels: analyzing deployment kinetics due to squeezing. J. Pharm. Sci. 96, 835–850.
Lackman-Smith, C., Osterling, C., Luckenbaugh, K., Mankowski, M., Snyder, B., Lewis, G., Paull, J., Profy, A., Ptak, R.G., Buckheit Jr., R.W., Watson, K.M., Cummins Jr., J.E., Sanders-Beer, B.E., 2008. Development of a comprehensive human immunodeficiency virus type 1 screening algorithm for discovery and preclinical testing of topical microbicides. Antimicrob. Agents Chemother. 52, 1768–1781.
Lard-Whiteford, S.L., Matecka, D., O'Rear, J.J., Yuen, I.S., Litterst, C., Reichelderfer, P., 2004. Recommendations for the nonclinical development of topical microbicides for prevention of HIV transmission: an update. J. Acquir. Immune Defic. Syndr. 36, 541–552.
Lard, S.L., Hastins, K.L., Feigal, D., 1994. Considerations in the development of vaginal products intended to prevent the sexual transmission of HIV http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/InvestigationalNewDrugINDApplication/Overview/ucm077750.htm.
Li, Q., Estes, J.D., Schlievert, P.M., Duan, L., Brosnahan, A.J., Southern, P.J., Reilly, C.S., Peterson, M.L., Schultz-Darken, N., Brunner, K.G., Nephew, K.R., Pambuccian, S., Lifson, J.D., Carlis, J.V., Haase, A.T., 2009. Glycerol monolaurate prevents mucosal SIV transmission. Nature 458, 1034–1038.
Liu, S., Lu, H., Neurath, A.R., Jiang, S., 2005. Combination of candidate microbicides cellulose acetate 1,2-benzenedicarboxylate and UC781 has synergistic and complementary effects against human immunodeficiency virus type 1 infection. Antimicrob. Agents Chemother. 49, 1830–1836.
Lowry, D., Fetherston, S., McConville, C., Woolfson, D., Malcolm, K., 2010. Silicone elastomer rings for sustained delivery of HIV microbicides: correlating historical in vitro and in vivo release data. In: Microbicides 2010, Pittsburgh, PA.

Mahalingam, A., Smith, E.K., Fabian, J., Damian, F., Peters, J.J., Clark, M.R., Friend, D., Katz, D., Kiser, P., 2010. Design of a semisolid vaginal microbicide gel by relating composition to properties and performance. Pharm Res (epub).

Malcolm, K., Lowry, D., Green, L., Shattock, R., Mitchnik, M., Geer, L., Klasse, P.J., Moore, J., Veazey, R., 2010a. Pre-treatment with Depo-Provera modifies the pharmacokinetics of CMPD167 in rhesus macaques following vaginal ring administration. In: Trends in Microbicide Formulations Symposium, Arlington, VA.

Malcolm, R.K., Edwards, K.L., Kiser, P., Romano, J., Smith, T., 2010b. Advances in microbicide vaginal rings. Antiviral Res. 88, S30–S39.

Malcolm, R.K., Woolfson, A.D., Toner, C.F., Morrow, R.J., McCullagh, S.D., 2005. Long-term, controlled release of the HIV microbicide TMC120 from silicone elastomer vaginal rings. J. Antimicrob. Chemother. 56, 954–956.

Mauck, C., Weiner, D.H., Lai, J., 2010. Colposcopy: still useful in microbicide safety trials? In: Microbicides 2010, Pittsburgh, PA.

McBride, M., Boyd, P., Fetherston, S., McCoy, C., Malcolm, K., Woolfson, D., Edwards, K.L., Ampofo, S., 2010. Preclinical stability evaluation of vaginal rings containing dapivirine and maraviroc. In: Microbicides 2010, Pittsburgh, PA.

McConville, C., Friend, D., Woolfson, D., Malcolm, K., 2010a. A two compartment in vitro release model for the testing of HIV microbicide formulations. In: Microbicides 2010, Pittsburgh, PA.

McConville, C., Woolfson, D., Elgendy, H., Friend, D., Malcolm, K., 2010b. Safety testing of silicone elastomer matrix vaginal rings containing UC781 and Tween 80 using Epivaginal (VEC-606) tissues. In: Microbicides 2010, Pittsburgh, PA.

McGowan, I., 2010. Microbicides for HIV prevention: reality or hope. Curr. Opin. Infect. Dis. 23, 26–31.

Mellors, J., 2010. Antiretrovirals for treatment and prevention—two trains on a collision course? In: M2010, Pittsburgh, PA.

Mesquita, P.M., Cheshenko, N., Wilson, S.S., Mhatre, M., Guzman, E., Fakioglu, E., Keller, M.J., Herold, B.C., 2009. Disruption of tight junctions by cellulose sulfate facilitates HIV infection: model of microbicide safety. J. Infect. Dis. 200, 599–608.

Miller, C.J., Alexander, N.J., Gettie, A., Hendrickx, A.G., Marx, P.A., 1992. The effect of contraceptives containing nonoxynol-9 on the genital transmission of simian immunodeficiency virus in rhesus macaques. Fertil. Steril. 57, 1126–1128.

Milligan, G.N., Chu, C.F., Young, C.G., Stanberry, L.R., 2004. Effect of candidate vaginally-applied microbicide compounds on recognition of antigen by $CD4^+$ and $CD8^+$ T lymphocytes. Biol. Reprod. 71, 1638–1645.

Milligan, G.N., Young, C.G., Meador, M.G., Chu, C.F., Stanberry, L.R., 2005. Effects of candidate vaginally-applied microbicide compounds on innate immune cells. J. Reprod. Immunol. 66, 103–116.

Moncla, B.J., Pryke, K., Rohan, L.C., 2010. Microbicide testing for effects on lactobacillus species: appropriate methodology. In: Microbicides 2010, Pittsburgh, PA.

Morrow, K., Hendrix, C., 2010. Clinical evaluation of microbicide formulations. Antiviral Res. 88, S40–S46.

Morrow, K., Kiser, P., Rosen, R.C., Katz, D., 2010. The LINK between gel properties and user preceptions: implications for rational design of microbicides. In: Microbicides 2010, Pittsburgh, PA.

Moss, J.A., Baum, M.M., Malone, A.M., Friend, D., Smith, J., Kopin, E., Kennedy, S., Nguyen, C.T., Cortez, C., Sinha, P.K., Smith, T.J., 2010. Vaginal ring delivery system for multiple microbicides. In: Trends in Microbicide Formulations Symposium, Arlington, VA.

Nasu, K., Narahara, H., 2010. Pattern recognition via the toll-like receptor system in the human female genital tract. Mediators Inflamm., 2010 (epub).

Nel, A.M., Smythe, S.C., Habibi, S., Kaptur, P.E., Romano, J.W., 2010. Pharmacokinetics of 2 dapivirine vaginal microbicide gels and their safety vs. hydroxyethyl cellulose-based universal placebo gel. J. Acquir. Immune Defic. Syndr., 2010 (epub).

Nuttall, J., Kashuba, A., Wang, R., 2010. The pharmacokinetics of tenofovir following intravaginal and intrarectal administration of tenofovir gel to rhesus macaques. In: Microbicides 2010, Pittsburgh, PA.

Parikh, U.M., Dobard, C., Sharma, S., Cong, M.E., Jia, H., Martin, A., Pau, C.P., Hanson, D.L., Guenthner, P., Smith, J., Kersh, E., Garcia-Lerma, J.G., Novembre, F.J., Otten, R., Folks, T., Heneine, W., 2009. Complete protection from repeated vaginal simian-human immunodeficiency virus exposures in macaques by a topical gel containing tenofovir alone or with emtricitabine. J. Virol. 83, 10358–10365.

Patton, D.L., Sweeney, Y.C., Tsai, C.C., Hillier, S.L., 2004. *Macaca fascicularis* vs. *Macaca nemestrina* as a model for topical microbicide safety studies. J. Med. Primatol. 33, 105–108.

Patton, D.L., Sweeney, Y.T., Balkus, J.E., Rohan, L.C., Moncla, B.J., Parniak, M.A., Hillier, S.L., 2007. Preclinical safety assessments of UC781 anti-human immunodeficiency virus topical microbicide formulations. Antimicrob. Agents Chemother. 51, 1608–1615.

Peterson, L., Nanda, K., Opoku, B.K., Ampofo, W.K., Owusu-Amoako, M., Boakye, A.Y., Rountree, W., Troxler, A., Dominik, R., Roddy, R., Dorflinger, L., 2007. SAVVY (C31G) gel for prevention of HIV infection in women: a Phase 3, double-blind, randomized, placebo-controlled trial in Ghana. PLoS One 2, 1312.

Roddy, R.E., Cordero, M., Cordero, C., Fortney, J.A., 1993. A dosing study of nonoxynol-9 and genital irritation. Int. J. STD AIDS 4, 165–170.

Rodriguez-Antolin, J., Xelhuantzi, N., Garcia-Lorenzana, M., Cuevas, E., Hudson, R., Martinez-Gomez, M., 2009. General tissue characteristics of the lower urethral and vaginal walls in the domestic rabbit. Int. Urogynecol. J. Pelvic Floor Dysfunct. 20, 53–60.

Rohan, L.C., Moncla, B.J., Kunjara Na Ayudhya, R.P., Cost, M., Huang, Y., Gai, F., Billitto, N., Lynam, J.D., Pryke, K., Graebing, P., Hopkins, N., Rooney, J.F., Friend, D., Dezzutti, C.S., 2010. In vitro and ex vivo testing of tenofovir shows it is effective as an HIV-1 microbicide. PLoS One 5, e9310.

Schwartz, J., Doncel, G., Asin, S.N., Thurman, A.R., Kimble, T., Chandra, N., Rollenhagen, C., Cooper, J., Ju, S., Archer, D., 2010. Pilot pharmacokinetic and pharmacodynamic study of UC781 vaginal gel. In: Microbicides 2010, Pittsburgh, PA.

Schwartz, J.L., Kovalevsky, G., Lai, J.J., Ballagh, S.A., McCormick, T., Douville, K., Mauck, C.K., Callahan, M.M., 2008. A Randomized six-day safety study of an antiretroviral microbicide candidate UC781, a non-nucleoside reverse transcriptase inhibitor. Sex Transm. Dis. 35, 414–419.

Segarra, T.J., Fakioglu, E., Wilson, S., Boukari, H., Doncel, G.F., Herold, B., 2010. Bridging the gap between preclinical studies and the performance of microbicides in large scale clinical trials: a reanalysis of cellulose sulfate. In: Microbicides 2010, Pittsburgh, PA.

Stover, J., Walker, N., Garnett, G.P., Salomon, J.A., Stanecki, K.A., Ghys, P.D., Grassly, N.C., Anderson, R.M., Schwartlander, B., 2002. Can we reverse the HIV/AIDS pandemic with an expanded response? Lancet 360, 73–77.

Subbarao, S., Ramos, A., Kim, C., Adams, D., Monsour, M., Butera, S., Folks, T., Otten, R.A., 2007. Direct stringency comparison of two macaque models (single-high vs. repeat-low) for mucosal HIV transmission using an identical anti-HIV chemoprophylaxis intervention. J. Med. Primatol. 36, 238–243.

Szeri, A.J., Park, S.C., Verguet, S., Weiss, A., Katz, D.F., 2008. A model of transluminal flow of an anti-HIV microbicide vehicle: combined elastic squeezing and gravitational sliding. Phys. Fluids 20, 83101.

Trifonova, R.T., Bajpai, M., Pasicznyk, J.M., Chandra, N., Doncel, G.F., Fichorova, R.N., 2007. Biomarkers of leukocyte traffic and activation in the vaginal mucosa. Biomarkers 12, 608–622.

Trifonova, R.T., Doncel, G.F., Fichorova, R.N., 2009. Polyanionic microbicides modify Toll-like receptor-mediated cervicovaginal immune responses. Antimicrob. Agents Chemother. 53, 1490–1500.

Trifonova, R.T., Pasicznyk, J.M., Fichorova, R.N., 2006. Biocompatibility of solid-dosage forms of anti-human immunodeficiency virus type 1 microbicides with the human cervicovaginal mucosa modeled ex vivo. Antimicrob. Agents Chemother. 50, 4005–4010.

UNAIDS, 2009. 09 AIDS epidemic update http://data.unaids.org/pub/Report/2009/JC1700_Epi_Update_2009_en.pdf.

Van Damme, L., Govinden, R., Mirembe, F.M., Guedou, F., Solomon, S., Becker, M.L., Pradeep, B.S., Krishnan, A.K., Alary, M., Pande, B., Ramjee, G., Deese, J., Crucitti, T., Taylor, D., 2008. Lack of effectiveness of cellulose sulfate gel for the prevention of vaginal HIV transmission. N. Engl. J. Med. 359, 463–472.

Van Damme, L., Ramjee, G., Alary, M., Vuylsteke, B., Chandeying, V., Rees, H., Sirivongrangson, P., Mukenge-Tshibaka, L., Ettiegne-Traore, V., Uaheowitchai, C., Karim, S.S., Masse, B., Perriens, J., Laga, M., 2002. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet 360, 971–977.

Van Herrewege, Y., Michiels, J., Waeytens, A., De Boeck, G., Salden, E., Heyndrickx, L., van den, M.G., de Bethune, M.P., Andries, K., Lewi, P., Praet, M., Vanham, G., 2006. A dual chamber model of female cervical mucosa for the study of HIV transmission and for the evaluation of candidate HIV microbicides. Antiviral Res. 74, 111–124.

Veazey, R.S., Klasse, P.J., Schader, S.M., Hu, Q., Ketas, T.J., Lu, M., Marx, P.A., Dufour, J., Colonno, R.J., Shattock, R.J., Springer, M.S., Moore, J.P., 2005. Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion. Nature 438, 99–102.

Vincent, K.L., Bell, B.A., Vargas, G., Johnston, K., Bourne, N., Motamedi, M., 2010. Image-based noninvasive evaluation of sheep vaginal and rectal tissue response following topical applications of microbicides. In: Microbicides 2010, Pittsburgh, PA.

Vincent, K.L., Bourne, N., Bell, B.A., Vargas, G., Tan, A., Cowan, D., Stanberry, L.R., Rosenthal, S.L., Motamedi, M., 2009. High resolution imaging of epithelial injury in the sheep cervicovaginal tract: a promising model for testing safety of candidate microbicides. Sex Transm. Dis. 36, 312–318.

Wang, L., Dezzutti, C.S., Moncla, B.J., Parniak, M.A., Mallipeddi, R., Friend, D., Rohan, L.C., 2010a. Formulation of tenofovir containing a quick dissolve film product. In: Microbicides 2010, Pittsburgh, PA.

Wang, L., Schnaare, R.L., Anton, P., Rohan, L.C., 2010b. Preformulations studies for a UC781 rectal microbicide product. In: Microbicides 2010, Pittsburgh, PA.

Watson, K.M., Buckheit, C.E., Buckheit Jr., R.W., 2008. Comparative evaluation of virus transmission inhibition by dual-acting pyrimidinedione microbicides using the microbicide transmission and sterilization assay. Antimicrob. Agents Chemother. 52, 2787–2796.

Whaley, K.J., Hanes, J., Shattock, R., Friend, D.R., 2010. Novel approaches to vaginal delivery and safety of microbicides: biopharmaceuticals, nanoparticles and vaccines. Antiviral Res. 88, S55–S66.

Wilson, S.S., Cheshenko, N., Fakioglu, E., Mesquita, P.M., Keller, M.J., Herold, B.C., 2009. Susceptibility to genital herpes as a biomarker predictive of increased HIV risk: expansion of a murine model of microbicide safety. Antiviral Ther. 14, 1113–1124.

Zalenskaya, I.A., Yousefieh, N., Joseph, T., Schriver, S.D., Cote, L., Doncel, G., 2010. Gene expression profile of vaginal epithelial cells exposed to pro-immunoinflammatory agents—identification of novel biomarkers of mucosal safety. In: Microbicides 2010, Pittsburgh, PA.