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Preclinical assessments of vaginal microbicide candidate safety and efficacy☆



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ABSTRACT

Sexually transmitted infections like HIV, HPV, and HSV-2, as well as unplanned pregnancy, take a huge toll on women worldwide. Woman-initiated multipurpose prevention technologies that contain antiviral/antibacterial drugs (microbicides) and a contraceptive to simultaneously target sexually transmitted infections and unplanned pregnancy are being developed to reduce these burdens. This review will consider products that are applied topically to the vagina. Rectally administered topical microbicides in development for receptive anal intercourse are outside the scope of this review. Microbicide and microbicide/contraceptive candidates must be rigorously evaluated in preclinical models of safety and efficacy to ensure that only candidates with favorable risk benefit ratios are advanced into human clinical trials. This review describes the comprehensive set of *in vitro*, *ex vivo*, and *in vivo* models used to evaluate the preclinical safety and antiviral efficacy of microbicide and microbicide/contraceptive candidates.

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1. Introduction

The origins of sexually transmitted infections (STIs), also known as venereal infections, may date back as far as the very beginning of civilization. The etymology of the name venereal is related to the goddess Venus, synonymous of love and fertility, perhaps to describe the need for

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intimate sexual contact to spread the infectious agents although the consequences of acquiring such infections are far from pleasant [1]. The etiological agents of STIs are bacteria, protozoans, viruses and chlamydias among which are: *Neisseria gonorrhoeae*, *Treponema pallidum*, *Trichomonas vaginalis*, *Chlamydia trachomatis*, human immunodeficiency virus (HIV), herpes simplex virus 2 (HSV-2), human papillomavirus (HPV) and hepatitis B virus (HBV).

According to the World Health Organization (WHO) estimates, 499 million curable STIs occur each year in the world [2]. This number excludes pathogens like HIV, HSV-2 and HPV, the three sexually transmitted viral infections with the greatest impact on human health. All three viruses cause chronic or latent infections, which cannot be eliminated through any antiviral treatment, resulting in high morbidity or mortality. Additionally, infections by HSV-2 or HPV can increase the risk of HIV infection [3,4] making novel strategies to prevent their transmission a priority area of research to improve the welfare of millions of human beings, most of them living in third world countries.

Vaccines are available for the prevention of HPV 6, 11, 16 and 18 types [5]. However, there are some limitations that may require additional prevention tools for HPV infections. These limitations include the lack of protection against other HPV types associated with anogenital infections (36 types), the need of cold chain distribution and storage and low worldwide vaccine coverage, in part due to a very high cost [6]. Changes in sexual behavior through counseling, availability of condoms for men or women, the use of antiretroviral (ARV) therapy in HIV infected persons in serodiscordant couples, male circumcision and treatment of STIs can reduce the risk of acquiring HIV or other STIs. But despite having all these tools, infections by sexually transmitted pathogens have stabilized or increased in various parts of the world and new strategies are needed to fight STIs. A more recent idea proposes the use of microbicides, which are novel topical products containing active pharmaceutical ingredients (APIs) to block the infection by these pathogens. These APIs may be delivered intravaginally or intrarectally, using different delivery systems including gels, creams, films, suppositories, probiotics, nanofibers or intravaginal rings (IVRs). The vaginal formulations may also include contraceptives, opening the field of multipurpose prevention technologies (MPTs), to not only prevent HIV and/or other STIs but also unintended pregnancy [7].

The novelty of this approach imposes a very cautious and rigorous preclinical evaluation of safety and efficacy in order to move forward the most promising microbicides and microbicide/contraceptives into clinical trials. These studies, mostly guided by prerequisites suggested or imposed by regulatory agencies that approve studies in humans, help to follow a rational and ethical approach that will finally allow the start of clinical trials. This review will focus on the preclinical assessment of safety and efficacy of vaginal microbicide and microbicide/contraceptive candidates with particular emphasis on preclinical models to evaluate efficacy against HIV, HSV-2 and HPV, as well as safety. It is important to emphasize the equal importance of development of rectal microbicides to prevent these STIs also transmitted through rectal intercourse. However, this review will focus on vaginal models as part of this special issue devoted to vaginal drug delivery.

2. The female reproductive tract as a media for establishing HIV, HSV-2 and HPV infections

The design of an effective vaginal microbicide requires understanding the steps that the viruses follow to establish infection in the female genital tract (Fig. 1). Female genital mucosa consists of stratified squamous epithelial tissue in the vagina and ectocervix, while the endocervix is composed of columnar epithelium. The vaginal mucus and intact vaginal epithelial tissues provide the first barriers that HIV must overcome before it can infect the host cells, the CD4 + T cells [8]. However, the loss of tissue integrity as a result of ulcerative genital infections (as caused by HSV-2, for example) or abrasions that occur

during intercourse or possibly transcytosis [9], can allow HIV to enter epithelial tissues and establish productive infection in target cells. The main cellular targets are CD4 + T lymphocytes, CD4 + cells of the macrophage lineage and dendritic cells (DCs). DCs efficiently capture HIV and transmit captured or newly produced virus to T cells [10], where the DC–T cell communication drives robust virus replication [11]. Interestingly, HSV-2 may modulate its microenvironment after infecting DCs to drive HIV infection in the DC–T cell mixtures [12] (Goode et al., in preparation). HIV starts its replication cycle by interacting with receptors (CD4, $\alpha 4\beta 7$) [13,14] and coreceptors (CCR5, CXCR4) [15] on the surface of target cells. This process of adsorption and subsequent entry into the cells, followed by reverse transcription and integration, are all targets being exploited to develop potential microbicides that may block infection [16–38].

Epithelial cells are also the first type of cells that HSV-2 and HPV encounter, although unlike with HIV, epithelial cells constitute the primary target and site of replication of these viral infections. HSV-2 has several viral glycoproteins in the virion surface that play an important role in adsorption and entry to epithelial cells, which makes them attractive targets to develop microbicidal compounds that could block these steps [39]. The adsorption and entry are a complex process that involves interaction with heparan sulfate and other receptors like herpesvirus entry mediator (HVEM) and nectin to then induce conformational changes that result in fusion with the cell membrane [39]. The next steps are translocation of the nucleocapsid to the nucleus, viral gene expression and genome replication. Inhibition of viral enzymes that participate in these steps, like the viral DNA polymerase, could help prevent HSV-2 infection [39]. After replicating initially in epithelial cells, HSV-2 is transported retrogradely along the axon of sensory neurons to establish latency in the sensory ganglion. The latency is kept under surveillance of the immune system that also controls the virus present in the mucosa [39]. Under certain conditions, including stress, immunodeficiency or immunosuppression, the virus is reactivated and taken through anterograde transport back to the genital mucosa where once again HSV-2 replicates [39]. During this replication, HSV-2 may or may not cause lesions in epithelial tissues and be transmitted to another susceptible host [39]. HPV limits its tropism to epithelial cells and requires damaged epithelia, where the basement membrane is exposed, to start the infection. The virus first attaches to heparan sulfate on the basement membrane and then undergoes a conformational change followed by cleavage (perform by furin and/or PC5/6) of the structural protein L2. The cleavage results in a modified virion that can now attach to a secondary receptor in basal keratinocytes and enter these cells [40]. Although heparan sulfate has been suggested as the universal receptor for attachment to the basement membrane, other studies have shown that infection of human keratinocytes with tissue-derived HPV 31 does not require heparan sulfate [41]. After entering basal keratinocytes the virus continues through a complex cycle of replication that requires cellular factors that are present at different stages of the epithelium differentiation. During this process viral enzymes like DNA polymerase may constitute interesting targets to prevent infection, but compounds like cidofovir, known to inhibit this particular enzyme [42], may not have a favorable enough toxicity profile to warrant its use as a potential microbicide [43].

3. Selecting a microbicide candidate

The first step in the development of microbicides is to identify APIs that may block STIs. Candidates must have a good therapeutic index (TI), inhibit virus replication at low, non-toxic concentrations *in vitro*, have a good resistance profile, be stable, and have the potential for reasonable pricing. Fig. 2 shows a go/no-go chart that combines the preclinical assessment of safety, efficacy and quality of microbicide candidates, highlighting in green boxes the steps that will be discussed in the next sections.

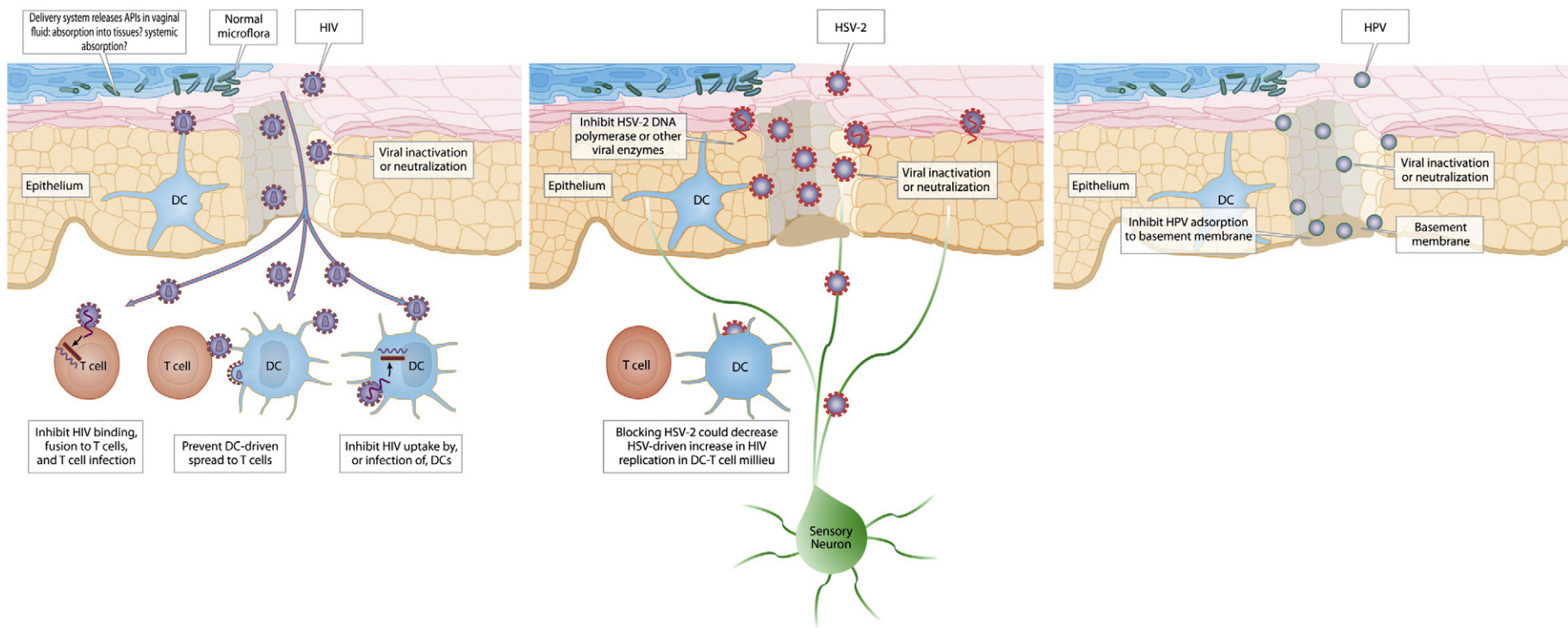


Fig. 1. HIV, HSV-2 and HPV initial site of replication and possible strategies to prevent infection with microbicides. The three viruses share the same route of infection when sexually transmitted, but with differences in tropism and pathogenesis. The targets to prevent viral infection include virus neutralization, blocking adsorption/entry and inhibiting specific viral enzymes. Modified from DS Nikolic and V Piguet [127].

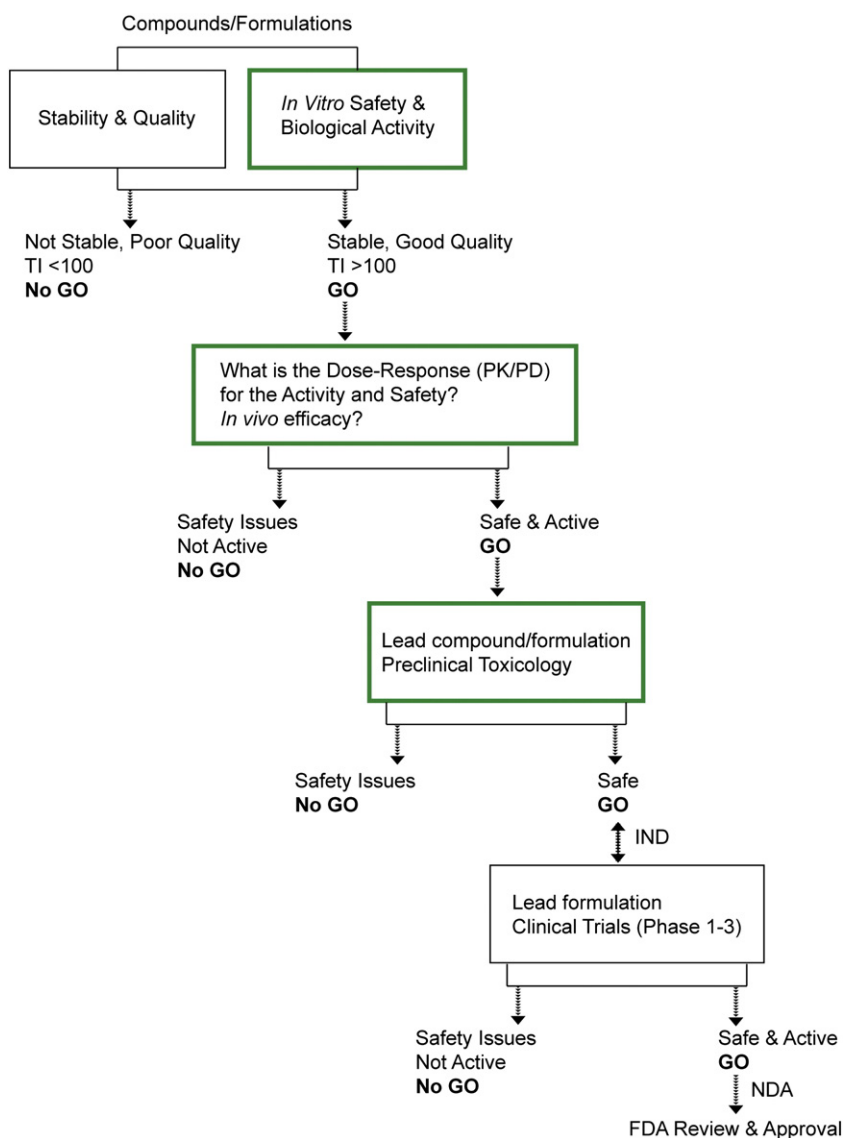


Fig. 2. Possible GO/NO-GO algorithm in microbicide product development. The green boxes are the topics described in this review.

Although outside the scope of this review, but still critically important to microbicide development, stability, quality and acceptability studies need to be incorporated early in the evaluation of a microbicide candidate. These studies start by extensively evaluating the physical and chemical properties of the unformulated and formulated API, combinations of APIs (when multiple APIs are present in the formulation), as well as API compatibility with formulation excipients and chemical stability. Additional tests and specifications on formulations include pH, osmolality, rheological properties in the case of a gel, appearance, odor, and content uniformity of drugs, impurities, dose volume, and product dimensions [44].

Numerous classes of APIs have been tested as vaginal microbicides, including virucidal agents (detergents [45]), molecules that block virus adsorption and cell fusion (polyanions [45]), CCR5 co-receptor antagonists [46,47], lectins [30,33,48] and neutralizing monoclonal antibodies [49], nucleoside reverse transcriptase inhibitors (NRTIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Tenofvir (TFV) [16,27], Dapivirine (DPV) [50], MIV-150 [25,28,29,34,36,51–53], TDF [37,54] and UC781 [55]), and the viral integrase inhibitors (L-870812, raltegravir) [56]. Table 1 summarizes the current state of the art of vaginal microbicides and microbicide/contraceptives.

4. *In vitro* safety and biological activity

4.1. Cytotoxicity and antiviral activity in cell-based assays

Many cell-based assays have been developed for the *in vitro* evaluation of potential antiviral compounds. These assays yield the median effective concentration (EC_{50}), the average cytotoxic concentration (CC_{50}), and the therapeutic index (TI, or CC_{50}/EC_{50}). The EC_{90} (90% inhibition) is also calculated to inform a rational dose selection when formulating the potential microbicide and as an important value to consider in pharmacokinetic (PK) and pharmacodynamic (PD) studies. A minimum acceptable TI value typically ranges from 10 to 100. It is essential to have a cell line or primary cells in which the virus replicates or at least mimics some of the target steps, and a method to quantify this replication like cytopathic effects, detection of viral proteins or nucleic acids, reporter genes.

Fluorescent or colorimetric assays such as CyQuant, Vibrant Assay, Presto Blue, Neutral Red, MTT and XTT are used to determine the CC_{50} and EC_{50} , offering great advantages in speed, simplicity and cost [57]. Additionally, the use of differentiated monolayers to measure transepithelial electrical resistance (TEER), as a measure of

Table 1
State of the art of vaginal microbicide candidates.

Microbicide APIs	Target	Delivery system	Study name	Current status
DPV	HIV	IVR	Aspire	Phase 3
TFV	HIV, HSV-2	1% TFV gel	The Ring Study FACTS 001 VOICE ^a	Phase 3 Phase 3 Phase 3
DPV	HIV	Film, gel	CAPRISA004	Completed Phase 2b
DPV, Maraviroc	HIV	IVR	FAME-02	Completed Phase 1
TFV	HIV, HSV-2	Film, gel	FAME-04	Phase 1
TDF	HIV, HSV-2	IVR	2013-329	Phase 1
MIV-150, Zinc Acetate (ZA), Carrageenan (CG)	HIV, HSV-2, HPV	Gel	Population Council #558	Phase 1
TFV, IQP-0528	HIV	IVR	Preclinical	Preclinical
MIV-150, ZA, CG, Levonorgestrel (LNG)	HIV, HSV-2, HPV, unintended pregnancy	IVR	Preclinical	Preclinical
TFV, LNG	HIV, HSV-2, unintended pregnancy	IVR	CONRAD 128	Phase 1
TFV or Truvada	HIV, HSV-2	Vaginal rapidly disintegrating tablets (RDTs)	CONRAD 117	Phase 1
DPV, LNG	HIV, unintended pregnancy	IVR	Preclinical	Preclinical
Griffithsin	HIV, HSV-2, HPV	Gel, IVR or nanofiber	Preclinical	Preclinical
Neutralizing Antibodies against HIV and HSV-2	HIV, HSV-2	Mapp 66 gel	Preclinical	Preclinical

^a This study included a daily use of a tablet (TFV or Truvada) or daily use of a vaginal gel (1% TFV gel).

monolayer integrity, has been reported in the microbicide field [24, 29,35,58–60]. The assay is performed with differentiated vaginal, ectocervical, endocervical or rectal epithelial monolayers. A modified TEER assay, which measures TEER changes in the epithelial monolayer after exposure to candidate microbicides followed by challenge with HIV and measuring the ability of the virus to cross the epithelium and infect target cells in the lower chamber, has also been used [61]. Using this system, it was suggested that epithelial barrier disruption might have contributed to the increased risk of HIV acquisition observed in Phase 3 trials of N-9 and CS [61]. Additionally, *in vitro* permeability studies in a Franz cell model using excised ectocervical tissue have been described as a tool to determine potential toxicity due systemic absorption and/or accumulation, [62]. Another set of toxicity assays includes the assessment of metabolic stability and potential interactions with CYP450 isoenzymes [63]. Finally, genetic toxicology assays should be performed once a lead API has been identified. Genetic toxicology includes assays like the bacterial reverse mutation assay and *in vitro* chromosome aberration assay in CHO cells with population doubling [63].

It is critical to minimize variables that may affect the *in vitro* evaluation outcome so that objective comparisons can be performed between laboratories and on different antiviral agents. These variables could be the use of different laboratory virus strains or primary virus isolates in different cell lines or primary cells, the use of different multiplicities of infection (m.o.i., the number of infectious particles per cell) and the antiviral assay per se.

In the specific case of HIV, the FDA recently issued guidance for the preclinical evaluation of microbicides, which recommends the use of certain assays in an attempt to standardize procedures and to be able to compare results between different antimicrobial agents that are being developed [64]. The tests and criteria to consider include obtaining the dose–response curves in peripheral blood mononuclear cells (PBMCs) against a wide range of primary isolates (geographically and phenotypically different) but also include at least one well-characterized laboratory strain [29]; determining the antiviral activity in cervicovaginal and colorectal explants [34,65] (Section 4.2); considering only those agents with TI > 10; using an appropriate range of m.o.i.; determining antiviral activity against cell-free and cell-associated virus and the possible interference of biological fluids (semen and vaginal fluid) and pH transition in the antiviral activity of the potential microbicide [66].

A method traditionally used to test antiviral activity against lytic viruses is the viral plaque assay, which despite being a laborious assay, is reliable and reproducible. In the case of HSV-2, the plaque assay in cell lines like Vero cells could be accepted as the “gold standard” for determining the susceptibility to antiviral agents [57]. Other methods, including dye-uptake assays, can be also employed to evaluate anti-HSV-2 activity *in vitro* [57].

HPV relies on the process of epithelial differentiation to complete the viral replication, starting in the basal keratinocytes and finishing with the shedding of new viral progeny in the stratum corneum. *In vitro* organotypic raft culture has been established to recreate this complex process and allows the production of infectious virus to then infect primary human keratinocytes [41,67,68]. The system results in an efficient infection of primary human keratinocytes but the full virus life cycle can be only achieved at very high m.o.i. Alternatively, a cell line-based assay, using different types of HPV pseudovirus (PsV) has been described [69]. The testing of different HPV PsV types is essential to corroborate a broad-spectrum anti-HPV activity. The assay can be performed using flow cytometry or luminescence in microplate formats [29,69,70]. It relies on the self-assembly of the structural HPV proteins L1 or L1/L2, which can encapsulate a reporter genome and mimic the early steps of viral adsorption and entry into the epithelial cells. An important limitation of this particular assay is that only compounds that target these early steps in the HPV cycle of replication can be identified and tested. Additionally, different susceptibility of primary human keratinocytes to HPV (produced in organotypic raft culture versus HPV PsVs produced in cell lines like 293 T cells) needs further investigation [41].

4.2. Toxicity and antiviral activity in explant models

The evaluation of microbicide candidates in explant models is performed later in the preclinical evaluation timeline, typically after a lead API/formulation has been identified. Explants represent biologically relevant systems with STI target cells present in the context of the correct environment and architecture [65]. Several explants models have been described: (i) agarose or matrigel sealed polarized tissue challenge/culture (HIV infection) [71,72]; (ii) tissue challenge by direct application of viruses on the explant or immersion in virus/culture media and subsequent culture on gel-foam rafts (HIV, HSV-2) [73]; (iii) non-polarized tissue challenge/culture (HIV, SHIV-RT, HSV-2) [17, 74–76].

The polarized explant cultures are used to assess product safety (histopathological examination, MTT assay) [18,24,72]. API permeability through tissues can be measured using a Franz diffusion cell [35]. Microbicide activity testing has been reported in both polarized and non-polarized culture models. Several HIV and HSV-2 explant infection models have demonstrated the anti-HIV activity of entry inhibitors, NRTI, and NNRTIs, as well as the anti-HSV-2 activity of TFV [17,18,20,24,26,35,72,77–79]. The advantage of antiviral activity testing in the non-polarized model is that it allows assessment of multiple replicates using readily obtained tissue samples, as the model typically uses 2–3 mm blocks of tissue per assessment [74]. The tissues are incubated with APIs or appropriately diluted formulated products (e.g., gel). While tissue supply can be limiting, this model allows for the calculation of EC₅₀ and EC₉₀ values for the APIs and the analysis of the effects of drug combinations [26,77]. In addition, the impact of a product on virus transmission by cells that migrate from the mucosal tissues can also be determined using this model [20,26,77,80]. In the polarized tissue model larger tissue blocks are used and undiluted/minimally diluted products are applied only on the epithelial surface of the tissue, which mimics the *in vivo* scenario [72]. Furthermore, polarized tissue cultures were recently utilized to mimic systemic exposure to the product, when API was added in contact with explant basolaterally [35].

HIV efficacy models in non-human primates use chimeric virus prepared from the simian immunodeficiency virus (SIV) and genetic information of HIV. The HIV genetic information incorporated in the SIV genome is selected to match the mode of action of the antiretroviral drug being tested. For example, when the compound blocks HIV reverse transcription, a chimeric virus carrying the HIV RT is used. We recently established models of cell-free and cell-associated SHIV-RT infection in rhesus macaque vaginal and cervical explants [17,18,34] (Barnable et al., in preparation). The Population Council's lead microbicide gel containing the NNRTI MIV-150 and zinc acetate in carrageenan (MZC) significantly inhibited cell-free and cell-associated SHIV-RT infection in tissues [18] (Barnable et al., in preparation). HIV/HSV-2 co-infection models of human cervical mucosa [79] (Villegas et al., in preparation) and SHIV-RT/HSV-2 co-infection of macaque vaginal mucosa (Calenda et al., in preparation) also have been established and used for microbicide activity testing. MZC provided anti-HIV, anti-SHIV-RT and anti-HSV-2 activity in these models (Villegas et al., in preparation; Calenda

et al., in preparation). Antiviral activity of the products in explants is measured by ELISA, PCR assays on supernatants, and PCR assays on tissues and immunohistochemistry [72,74] (Villegas et al., in preparation; Calenda et al., in preparation).

Overall, API/product testing in explants provides important information that cannot be acquired with any other models. The system has been used to test antiviral activity of APIs in the presence of biological fluids like vaginal fluid and seminal plasma [17,18,34] (Villegas et al., in preparation; Calenda et al., in preparation). Additionally, safety and antiviral activity in explants demonstrated some predictability of results in the clinic [65]. However, there are several disadvantages of the explant models: inability of the tissue to regenerate/repair, lack of dynamic cell exchange, and the lack of hormonal control [81]. Moreover, the explant assays are cumbersome and costly. Table 2 summarizes the advantages and disadvantages of *in vitro* and *ex vivo* assays to measure safety and efficacy (HIV, HSV-2 and HPV) of potential microbicide formulations.

4.3. *In vitro* toxicity on normal microflora

In vitro assays to assess the impact of microbicide candidates on bacterial flora in the vagina should be performed in the early stage of microbicide development [29,35,58,66,82]. This involves incubating various lactobacilli with the microbicide. Candidates that perturb the bacterial balance in the vagina should be either reformulated or discarded.

5. Selection and characterization of resistant viruses

This topic is especially relevant in the case of microbicides targeting HIV. Antiviral resistance is defined as a reduction of viral susceptibility to an antiviral agent. A moderate resistance is when the EC₅₀ value increases from 5 to 10 fold while more than 10 fold increase results in highly resistant viruses. This result may be confirmed through genetic analysis of the virus and the biochemical study of altered viral protein.

HIV persists in the host as viral quasispecies where the predominant viral sequence is constantly changing as a selective pressure (especially during ARV treatment) is exerted on the virus. This facilitates, under certain circumstances, a rapid selection of resistant virus and treatment

Table 2
Advantages and disadvantages of *in vitro* and *ex vivo* models to test safety and efficacy of potential microbicide formulations.

	Advantages	Disadvantages
Cell-based assays		
Safety	<ul style="list-style-type: none"> • First-line safety screen. • Cells of human origin. • Fast and inexpensive. 	<ul style="list-style-type: none"> • Single cell-type models • Innate mediators: may be difficult to interpret due to variability and absence of validated biomarkers of microbicide safety.
Anti-HIV activity	<ul style="list-style-type: none"> • EC₅₀, EC₉₀, TI calculation. • Testing in the presence of biological fluids. • Fast, inexpensive in the case of assays performed in cell lines. • Antiviral activity in primary cells (PBMCs) allows the testing of a broad range of virus isolates, clones or lab adapted strains. 	<ul style="list-style-type: none"> • Not all viruses, especially primary isolates, replicate well in assays performed in cell lines. • PBMC assay: donor-to-donor variation, not user-friendly and long time to generate an end point.
Anti-HSV-2 activity	<ul style="list-style-type: none"> • Plaque reduction assay is considered a “gold standard” for anti-HSV testing. • EC₅₀, EC₉₀, TI calculation. • Dye-uptake assays are fast and inexpensive. 	<ul style="list-style-type: none"> • Plaque reduction assay is tedious and consume more reagents, appropriate for small-scale testing. • Precipitation in dye-uptake assays may interfere with the results.
Anti-HPV activity	<ul style="list-style-type: none"> • EC₅₀, EC₉₀, TI calculation. • Full cycle of HPV replication in keratinocytes or adsorption/entry with PsVs in cell lines. 	<ul style="list-style-type: none"> • Producing virus stocks is laborious and expensive. • Requires high m.o.i. to achieve full life cycle (keratinocytes). • Only early steps of HPV replication cycle can be assessed with PsVs in cell lines. • Potentially different susceptibility to HPV in primary cells vs. cell lines.
Tissue Explants (human, macaques)		
Safety	<ul style="list-style-type: none"> • Relevant HIV target cells/architectural context. 	<ul style="list-style-type: none"> • No tissue regeneration/repair.
Anti-HIV and anti-HSV-2 activities	<ul style="list-style-type: none"> • Relevant HIV target cells/architectural context. • Choice of polarized or non-polarized viral challenge and culture. • EC₅₀/EC₉₀ calculation (surgical tissue, non-polarized model). • Allows for assessing effect on HIV transmission by migratory cells. • Testing in the presence of seminal plasma. 	<ul style="list-style-type: none"> • No systemic effects (e.g., cell migration, hormonal influence). • Survival cervical/vaginal biopsies are limiting in size. • Cumbersome to obtain. • Costly, labor intensive.

failure manifested as an increased plasma viral load. The best strategy to minimize viral resistance is to combine several drugs with different mechanisms of action, as it would require multiple mutations in the same viral genome for the virus to acquire resistance. This antiviral combination therapy is known as highly active ARV therapy (HAART). However, if treatment is not strictly followed and/or there is lack of monitoring, multidrug resistant viruses can arise [83].

In an ideal scenario, microbicides would be used by HIV-negative individuals to prevent infection. However, individuals who may or may not know their HIV-positive status may use a microbicide or infected people may choose to use a microbicide/contraceptive to avoid becoming pregnant. This could start the selection of mutant viruses with reduced susceptibility to the antiretroviral drug. It is not clear whether the topical, non-systemic use of ARVs is able to exert the necessary pressure to select viral resistance. It is worth noting that many of the potential microbicides are not efficiently absorbed after vaginal application, which may lead to very low concentrations of the APIs in the blood stream [28,29,32,36,84,85]. Yet the viral population in the genital mucosa of infected individuals may be prone to select resistant virus since API levels might be higher there than in the systemic circulation. Recent studies in macaques showed that while intramuscular administration of the NNRTI MIV-150 results in selection of NNRTI-associated mutations in SHIV-RT infected macaques, topical exposure of infected animals to MIV-150-containing IVRs resulted in limited emergence of NNRTI-associated mutations [52] (Kenney et al. submitted).

In vitro selection of resistant viruses against the microbicide candidate is performed during preclinical development. The assay can be run in cell lines or PBMCs using different susceptible HIV clades [19, 52,86]. The selected viruses must be characterized genetically and phenotypically and cross-resistance with APIs used for HIV treatment should be assessed. Selection of resistant viruses would help to predict a possible reduction in the susceptibility of the virus to the microbicide and/or other products (especially those being used in the treatment of HIV infection) and indicate mutations that should be monitored in clinical trials. Similarly, these experiments allow mechanism of action studies on the microbicides since mutations in the target viral proteins may help to better understand the API's mode of action.

6. *In vivo* exploratory studies

6.1. *In vivo* safety studies

Preclinical safety evaluation of microbicides starts with *in vitro* assays that measure cytotoxicity in cell cultures or cervicovaginal and colorectal tissue explants. However, these *in vitro* systems do not allow a prolonged or repeated exposure to a microbicide candidate and do not assess inflammatory responses that can only be seen in intact mucosal tissues.

A series of *in vivo* models have been described in the literature to assess preclinical safety in exploratory studies but require further validation in order to standardize preclinical models. The mucosal delivery of vaginal microbicides demands a close look at the integrity of cervicovaginal mucosa and the possible development of inflammatory processes after microbicide application. Breaks in the integrity of the mucosa and inflammatory conditions will create an ideal environment for the establishment of STIs.

Murine models include the increased susceptibility to infection by HSV-2 in mice (*Mus musculus*), which could be used as a biomarker to predict increased risk for HIV infection [29,58,60,87]. The model consists on successive vaginal applications of a product (often a gel), before infecting the mice with a suboptimal dose of HSV-2. If damage occurs and impairs the integrity of the mice vaginal mucosa increased HSV-2 infection can be observed. Another model incorporates monitoring the tight junctions in the epithelium, transcription factors in nuclear extracts from vaginal tissues, and cytokine and chemokine analyses in vaginal washes [88]. Histological analysis can also be performed

in cervicovaginal tissues after vaginal application of microbicide candidates in mice. This model looks into the architecture of the cervicovaginal tissues and the presence of inflammatory infiltrates within the lamina propria [29,58,60,89]. These murine models have contributed to understanding the failure of some microbicide candidates to show efficacy by suggesting increased susceptibility to HSV-2 probably due to alterations in epithelial architecture [90].

Non-human primate models are also informative, where vaginal gels can be repeatedly applied or IVRs inserted for extended periods of time in pigtailed (*Macaca nemestrina*) or rhesus macaques (*Macaca mulatta*). These models monitor changes in pH, microflora, inflammatory infiltrates on tissues, cytokine and chemokine levels in vaginal swabs, measuring the overall health of the vaginal mucosa [53,91–93] (Kenney et al. submitted).

6.2. PK/PD of microbicide formulations

PK and PD studies can investigate the whole process of a drug from the time it is applied intravaginally until elimination or removal. They also give information on the biochemical and physiological effects, the mechanism of action and the relationship between drug concentration and effectiveness. The ideal microbicide should be distributed in tissues susceptible to STIs, must be metabolized (if necessary) efficiently in these tissues and achieve adequate and sustained concentrations at the necessary sites. Additionally, as discussed earlier, avoiding systemic circulation may minimize the development of viral resistance and/or toxic effects.

Performing PK and PD studies of potential microbicides in animal models has some notable challenges. The interpretation of the results in animal models and the correlation to humans are complicated due to physiological and biological differences. Additionally, we still need to understand better the relevance of an API's presence in specific compartments or subcompartments. Most PK and PD preclinical studies of microbicides have been carried out using macaque, murine, rabbit, and sheep models. These studies have compared different doses after single or repeated application of a microbicide formulation or after using sustained release device, monitoring levels in vaginal fluids, cervical and vaginal biopsies, as well as in plasma [21,27–29,31,32,34,36,37, 53,70,94,95]. In general, there is a need to improve and standardize PK and PD animal models in the microbicide field, especially the delineation of PK relationships between different animal models and between human and animal models [96].

There is a significant amount of data regarding TFV and Truvada (tenofovir disoproxil fumarate/emtricitabine) PK in animal and human models after oral administration [97]. However, changing the route to topical administration, like the case of vaginal microbicides, requires a new analysis of the PK and PD of the novel formulations. Several studies have been performed in macaques or rabbits with TFV or with Truvada. Vaginal gels or vaginal rapidly disintegrating tablets containing TFV alone or Truvada have produced sustained and biologically relevant vaginal tissue levels of APIs that persisted up to 24 h after administration in pigtailed macaques [94,95]. Similar results were also achieved in PK studies performed in rhesus macaques and rabbits [21, 31]. PK studies in macaques and rabbits have also informed the presence of biologically relevant DPV concentrations in vaginal and cervical tissues for more than 24 h after a single gel application [32]. Similarly, PK studies in macaques with MZC gel, containing another NNRTI (MIV-150) but at a lower dose, have shown biologically relevant MIV-150 concentrations for more than 8 h [28,29]. This observation parallels the full protection seen after application of the MZC gel 8 h before vaginal SHIV-RT challenge in macaques [28]. Interestingly, novel PD preclinical models are suggesting the need of sustained released of some microbicide candidates like NNRTIs [98]. In fact, a DPV IVR is currently in Phase 3 trial, and PK/PD studies in animal models and humans have shown successful distribution of DPV throughout the lower genital tract and biologically relevant DPV concentrations in vaginal fluids and

tissues [23,99]. Delivery of the NNRTI MIV-150 in an IVR has also proven successful [36] (Kenney et al. submitted). *Ex vivo* macaque explant challenge models demonstrated the antiviral activity of MIV-150 in tissue and vaginal fluid from macaques treated with MIV-150-containing IVRs [34] (Ugaonkar et al., in preparation). Notably, a prototype IVR carrying MZC and LNG (MZCL IVR) was found to significantly reduce SHIV-RT infection and HSV-2 vaginal shedding after repeated SHIV-RT/HSV-2 co-challenge, and deliver target blood levels of LNG. Finally, a recent report described that a TDF IVR provided full protection of macaques against repeated SHIV-RT challenge, which was associated with TFV levels in vaginal fluid and *ex vivo* antiviral activity of cervicovaginal lavage samples [37].

6.3. *In vivo* evaluation of microbicide formulation efficacy

The regulatory agencies still do not require the preclinical testing of efficacy of microbicide candidates in order to approve them for clinical trials. This is due to the lack of extensive validation of the animal models that are currently used. However, strong efficacy data from animal studies may help to select a lead formulation/device, although recognizing that the relevance of the animal model to the human system needs further investigation.

6.3.1. *Monkey and murine models to test efficacy against HIV*

These models in macaques can be performed with a single higher dose of SHIV typically in DepoProvera-treated animals [17,28,29,36,38,51,53,58,100–102] or repeated lower doses of SHIV on non-DepoProvera-treated animals [37,56,103–106] to more closely mimic the real scenario of human exposure to HIV. Both models have their advantages and disadvantages. The higher single dose model animals are pretreated with DepoProvera to thin the vaginal epithelium and increase susceptibility to SHIV infection while the product is applied before or after the single challenge. This treatment, together with the high dose of the virus used, could be too demanding to screen for potential microbicides. However, if the microbicide blocks transmission under these conditions, it may be considered a microbicide with robust activity against HIV. The second model involves repeatedly exposing macaques to lower doses of SHIV with the microbicide being applied repeatedly at set times relative to each challenge. The repeated challenge model more readily facilitates the evaluation of the effectiveness of the candidate microbicide under controlled conditions that are more akin to real life settings, estimates intervention efficacy, can study natural resistance to infection and also demonstrates product safety after prolonged use. The disadvantages include a longer follow up time (a single experiment may take about one year) and uncertainty regarding point of infection. In general, both models (single or repeated viral challenges) need further validation and have the disadvantages of (i) being expensive, (ii) not being able to use cell-associated virus in the inoculum, and (iii) not simulating the effects of intercourse on product efficacy.

The chimeric bone marrow–liver–thymus humanized mice model (BLT mice model) has been also widely used in the microbicide field [107]. In this model, human fetal bone marrow cells, liver and thymus are transplanted into immunodeficient mouse strains resulting in long-term systemic repopulation with human T cells, B cells, monocytes/macrophages, and DCs. The activity of 1% TFV gel in an experimental design mimicking CAPRISA 004, showed a similar level of protection when compared to the Phase 2b trial results [22]. The disadvantages of this model include the limited availability of human fetal liver tissues and the high cost of the surgical procedure to produce the BLT mouse.

Another murine model has been recently described [108], and explores transmission of chimeric HIV by mating in conventional mice. The model consists in mating EcoHIV/NDK-infected male mice with conventional female mice allowing transmission of the virus during coitus. The EcoHIV/NDK chimeric HIV expresses all HIV structural and regulatory genes but contains a rodent-tropic envelope. The

model showed prevention of HIV infection if female mice were pericoitally treated with NRTIs and also showed reduced susceptibility to infection during estrus. One disadvantage is that microbicide candidates targeting HIV entry cannot be assayed using this particular model due to the absence of HIV glycoproteins in the viral envelope.

6.3.2. *Murine and monkey models to test efficacy against HSV-2*

The vaginal and rectal mouse models to study the efficacy of microbicides with antiviral activity against HSV-2 are the most widely used [29,58,60,109–113]. Mice are examined and scored daily for approximately 20 days, starting on days 3–4 after vaginal or rectal inoculation. Any mice with symptoms of infection including swelling, hair loss, redness, hind limb paralysis, and/or lesions in the vaginal or rectal area are scored as infected and euthanized. The model has been used to challenge mice intravaginally with HSV-2 in the presence of human seminal plasma and explore the possible interference of this biological fluid on the anti-herpes activity of potential microbicides [111,114]. The mouse models have the advantage of low cost and the use of inbred mouse line but the model lacks spontaneous reactivation of virus as seen in humans.

In order to test the activity of putative microbicides against both immunodeficiency virus and HSV-2 (infection and shedding), we established a rhesus macaque co-infection model (single or repeated exposure) [51,115] (Kenney et al. submitted). Similar to humans, infection of macaques with HSV-2 resulted in sporadic HSV-2 shedding, detection of genital lesions in ~10% of the animals, and reactivation of HSV-2 shedding upon mucosal trauma. Most recently, we demonstrated that the levels and frequencies of HSV-2 were significantly greater in animals after repeated SHIV-RT/HSV-2 co-challenge compared to those infected by a single higher dose SHIV-RT/HSV-2 co-challenge (Kenney et al. submitted). This model allowed us to demonstrate the ability of MZC-containing IVRs to significantly block SHIV-RT infection, reduce (although not significantly) HSV-2 infection, and significantly lower HSV-2 shedding (Kenney et al. submitted). While the HSV-2 dosing still might need optimization (*i.e.*, lowering so as not to overpower a product, since the inoculum used is still approximately a log higher than the levels detected in seminal fluids [116–118]), these data underscore the potential for this sort of model in evaluating promising microbicide candidates.

Other models include guinea pigs (*Cavia porcellus*) [119] or cotton rat (*Sigmodon hispidus*) [120] that have the advantage to reproduce the spontaneous reactivation of virus seen in humans.

6.3.3. *Murine and monkey models to test efficacy against HPV*

Two animal models (in mice and macaques) have been described for HPV [121,122]. These models involve the use of HPV PsVs, which, as described before, contain a plasmid with reporter genes within the viral capsid composed of the L1 and L2 proteins. The HPV PsVs have the ability to mimic the early steps of infection from viral adsorption to entry into a basal keratinocyte where the reporter gene (luciferase) is expressed and quantified through *in vivo* imaging techniques. Similar to what was discussed in the HSV-2 murine model, seminal plasma can be used with the viral inoculum to test any possible detrimental effects on the potential microbicide [70]. Using these models carrageenan has been identified as a promising microbicide candidate to prevent HPV acquisition [29,70,121,122]. The model is a good tool to test formulations that may block viral adsorption and/or entry but not any of the subsequent steps in the viral replication cycle. Table 3 summarizes advantages and disadvantages of *in vivo* assays to measure safety and efficacy (HIV, HSV-2 and HPV) of potential microbicide formulations.

7. Core investigational new drug (IND)-enabling studies

Once the lead microbicide formulation is selected, *in vivo* Good Laboratory Practices (GLP)-compliant single and repeated dose vaginal

Table 3Advantages and disadvantages of *in vivo* models to test safety and efficacy of potential microbicide formulations.

	Advantages	Disadvantages
Toxicological studies in mammals	<ul style="list-style-type: none"> Rabbit vaginal irritation (RVI) model is the most commonly used. Most sensitive indicator of irritation/inflammation. RVI determines epithelial ulceration, leucocyte infiltration, edema and vascular congestion. The sheep model allows colposcopy and Optical coherence tomography (OCT). 	<ul style="list-style-type: none"> Anatomical and physiological differences with humans. Test material enters urethra and bladder in rabbits what may induce cystitis. Potential for exaggerated response to product exposure in RVI (vs. exposure in humans). Labor intensive, costly.
Anti-HIV activity in mice EcoHIV/NDK chimeric HIV	<ul style="list-style-type: none"> Reproduces physiological conditions of vaginal intercourse As suggested for HIV transmission in humans, HIV transmission in mice is affected by hormonal environment 	<ul style="list-style-type: none"> Reproductive tract epithelium and function (estrous cycle vs. menstrual cycle) are different from humans Products targeting HIV envelope cannot be assessed
Humanized mice (BLT)	<ul style="list-style-type: none"> HIV infects human cells Allows testing efficacy against cell-free and cell-associated virus Important for initial screening of safety and anti-HIV activity of vaginal microbicides 	<ul style="list-style-type: none"> Reproductive tract epithelium and function (estrous cycle vs. menstrual cycle) are different from humans Mice cannot be bred and must be generated de novo Requires artificially sterile environment Dysregulation of innate immune responses Labor intensive, costly
Anti-HSV-2 activity in small animal models	<ul style="list-style-type: none"> Fast, robust and inexpensive compared to other animal models Guinea pigs and cotton rats show spontaneous HSV-2 reactivation Effect of seminal plasma on microbicides efficacy can be explored 	<ul style="list-style-type: none"> Lack of spontaneous HSV-2 reactivation in mice as seen in humans The virus challenge does not simulate effect of intercourse
Anti-SIV/SHIV/HSV-2 activity in NHP	<ul style="list-style-type: none"> Reproductive mucosa is broadly comparable to humans Macaques have similar, but variable, reproductive cycles compared to humans As suggested for HIV transmission, sex hormones are suggested to influence vaginal SIV/SHIV transmission Single vaginal high cell-free SIV/SHIV dose challenge in Depo-treated animals Repeated more limited dose vaginal challenge on non-Depo-treated animals Single and repeated co-challenge with SHIV and HSV-2 to assess activity against infection and shedding Effect of seminal plasma on microbicides efficacy can be explored 	<ul style="list-style-type: none"> Genetic differences between SIV and HIV SIV/SHIV vs. HIV disease course High challenge dose is stringent Immunosuppressive effect of Depo treatment Repeated low-dose challenge requires long follow up The virus challenge does not simulate effect of intercourse Availability issue, cost Limited data on SHIV cell-associated challenge Lower HSV-2 dose might be more physiological
Anti-HPV activity in mice and macaques	<ul style="list-style-type: none"> Effect of seminal plasma on microbicides efficacy can be explored 	<ul style="list-style-type: none"> Products targeting steps subsequent to viral adsorption/entry cannot be assessed The virus challenge does not simulate effect of intercourse

irritation/toxicity studies with toxicokinetics are performed. FDA approval requires performing toxicological studies in two different animal species.

Animal models in rabbits, pigs, mice and monkeys are used to assess the inflammatory and potential irritability of formulations designed for vaginal use. The rabbit vaginal irritation (RVI) model [123] is perhaps the most used and the only one the FDA approves for the evaluation of vaginal irritation. The model involves the repeated application of a formulation in the vagina of rabbits and monitors the presence of erythema, edema, and vaginal secretions. The study also includes obtaining histological sections of tissues exposed to the formulation to assess the presence of epithelial exfoliation, leukocyte infiltration, vascular congestion and edema.

The female rabbit reproductive system has several anatomical/histological characteristics that are particularly different from humans. Rabbits have cuboidal/columnar tissue in anterior to mid vaginal epithelia and stratified squamous in the posterior vaginal, persistent estrus and a vaginal pH between 7.0–8.0. Guinea pigs and rats can be more similar to humans in these terms but RVI is a more sensitive indicator of vaginal irritation. An alternative to the RVI model is the enhanced RVI model that in addition to the outcomes mentioned above looks at immunotoxicity [124].

The RVI or enhanced RVI models are probably the primary assays when testing gel formulations but in the case of IVR a better approach may be the sheep vaginal irritation model [125]. The sheep model may include pre- and post-treatment colposcopy and optical coherence

tomography (OCT) images that are graded based on an objective scoring system. Biopsies are also collected to investigate the degree of epithelial injury through histology and OCT. However, in order to get data in two different animal species, segments of the IVR can be also tested intravaginally in rabbits [126].

As mentioned earlier, the vaginal irritation/toxicity studies with toxicokinetics must be performed in two different animal species. This may include acute rabbit and rat models in which a single dose of different formulations containing increasing amounts of the APIs is administered. If the single dose study does not reveal any safety concerns, then vaginal chronic (14-day through 90-day) toxicity and PK studies with toxicokinetics in two animal species are performed. The duration of chronic studies is determined by the duration of studies to be conducted in humans during the clinical trials. For example if a 14 day daily application is proposed in human studies, a similar number of days should be tested in the animal models to access vaginal irritation/toxicity.

The vaginal irritation/toxicity assays mentioned above are the key GLP-compliant tests of an optimized microbicide formulation for a successful IND application. However, when looking at a new API with no human experience or at other classes of APIs, for example proteins, an additional set of GLP toxicological testing in animals may be needed/recommended by the regulatory agency. These may include intravenous single dose studies, subacute immunogenicity testing, sensitization study, cardiovascular telemetry study and central nervous system safety [63].

8. Conclusions

The overarching goal of preclinical safety and efficacy testing is to inform selection of formulations for Phase 1 testing by identifying microbicide candidates with the best risk/benefit ratios. Preclinical safety and efficacy testing of microbicide candidates involves a large number of *in vitro*, *ex vivo*, and *in vivo* assays and models. Some are dictated by the regulatory agencies; others are not. In many cases the assays and models are not harmonized, which makes it difficult to objectively compare data on candidates from different microbicide developers. Pre-IND meetings with the US FDA, for example, can be used to get early feedback on the suitability of a preclinical testing plan.

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