Ex Vivo Models of HIV Sexual Transmission and Microbicide Development

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Abstract: Here, we review the armamentarium on *in vitro*/*ex vivo* models of HIV sexual transmission and discuss how these models can be applied to study candidate microbicides.

Keywords: HIV, transmission, microbicides, vagina, cervix, *in vitro*, modeling.

Recently, several clinical trials have shown proof-of-concept for promising antiretroviral (ARV) prevention strategies including microbicides and pre-exposure prophylaxis (PrEP). In parallel with treatment, the use of HIV-specific drugs as prevention tool has been a focus from the beginning of the ARV era, with an undisputable success in the prevention of mother-to-child transmission of HIV. Microbicides are intravaginal or intrarectal formulations that can protect against sexually transmitted infections (STI's), including HIV. They can be formulated as gels, creams, films, or in solid rings and should be able to protect both men and women upon vaginal or rectal exposure. This review will focus mainly on microbicides in women (and men). For expert discussion on PrEP and PEP (post-exposure prophylaxis) we refer to excellent reviews by Kelisidis [1], Garcia-Lerma [2], Rey [3] and Barber [4].

Just recently, the HIV Prevention Trials Network (HPTN) 052 study showed that ARV treatment reduced the rate of HIV-1 sexual transmission significantly in a large number of discordant couples [5]. Drugs to prevent HIV-1 transmission are being investigated in seropositive and uninfected individuals, both men and women. In this regard, the use of 1% tenofovir intravaginal gel as microbicide in women [6] and oral intake of the combination therapy with tenofovir and emtricitabine (TRUVADA) in men who have sex with men [7] provided encouraging evidence for protection.

However, the road towards success has been bumpy. Several non-specific microbicide candidates, among which surfactants, polyanions and acid buffers, have previously been tested and failed in clinical trials [8-13 and reviewed by 15, 16]. The field has learned from these failures and more stringent selection and development criteria are now handled to qualify candidate products. Antiretrovirals that specifically target the virus are currently at the forefront of microbicide and PrEP development [reviewed by 15]. Many of these products are extremely potent *in vitro* and some have already demonstrated their success in the clinic in combination therapy for the treatment of HIV infected individuals (e.g. HAART). However, there are concerns about low bioavailability upon topical application of these products and the risk of developing resistance that would jeopardize the product for therapeutical usage [17-19].

Even after the successes of TRUVADA and tenofovir as PrEP, the need remains to expand the pipeline of topical microbicide candidates. We should focus our search towards more potent and more specific molecules, with high local (i.e. mucosal) bio-availability, but low systemic penetration and with a high barrier to resistance or a resistance profile that is different from existing therapeutics. This implies that candidate microbicides should preferably not be in use as a therapeutic [17].

Furthermore, significant effort should be put in developing new and appropriate formulations, since this will impact not only the bioavailability and hence the biological activity, but also the acceptability (i.e. cultural and behavioral factors). Most common formulations include vaginal/rectal gels and solid rings. Both types of formulations are already in use for various other clinical and pharmaceutical applications. For recent and expert reviews on vaginal drug delivery systems, we refer to Yu *et al*. [20], Malcolm *et al*. [21] and Turpin [22].

A comprehensive evaluation algorithm is required to rationally select the best microbicide candidates in a preclinical development stage. This evaluation algorithm includes assays to assess antiviral activity, toxicity, physicochemical properties, pharmacokinetics and –dynamics, formulations, release rates and ultimately efficacy.

Many of these assays are built upon current understanding of HIV sexual transmission across the female genital mucosa and can help to better understand the microbicide-tissue interaction, in terms of efficacy and toxicity.

IN VITRO ASSAYS TO EVALUATE ACTIVITY AND SAFETY OF MICROBICIDE CANDIDATES

Antiretroviral products and formulations are sequentially evaluated in cell-based (*in vitro*), tissue-based (*ex vivo*) and animal-based (*in vivo*) models.

Straightforward Assays with Cell Lines

It all starts with the identification of products with anti-HIV activity. Most often this is done in a high throughput assay by screening compound libraries on cell lines that are

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susceptible to HIV infection. Frequently used cell lines are CEM, H9, PM-1, MOLT-4, Jurkat, MT4, GHOST and TZM-bl. These tests include evaluating activity against CCR5- and CXCR4-tropic, lab-adapted (e.g. NL4.3, HxB2, IIIB, Ba-L) and patient isolates, cell-free and cell-associated viruses, in short compound-cell-virus incubation assays [17, 23-26]. Although these cell lines are useful tools to assess initial activity (and safety) of candidate microbicides, most are derived from carcinomas and the results obtained with these cell lines might not be fully representative of the normal human physiological conditions. Nevertheless, these systems enable selecting hits and lead molecules, preferably with IC50 values in low nanomolar to picomolar range and with a selectivity index (SI= toxicity/activity) surpassing 10^5. The effective compound concentration required to inhibit infection *in vitro* and in animal models and the dose required for complete inhibition in humans may differ by several orders of magnitude [27]. As a consequence, the toxicity profile of a candidate microbicide should be extremely well documented and this requires *in vitro* toxicity assays that reliably predict the *in vivo* reality (see paragraphs on toxicity).

Primary HIV Target Cells

Further activity testing involves primary cells, such as peripheral blood mononuclear cells, dendritic cells, T-lymphocytes and macrophages, to evaluate the potency against HIV-1 clades, with specific emphasis on those dominant in HIV prevalent areas. These primary cells present a physiologically more relevant model of the immune target cells underlying the genital epithelial barrier. CD4+ T cells, interstitial dendritic cells, Langerhans cells and tissue-resident macrophages are all involved in the early stages of HIV infection in the human genital mucosa [28, 29], either as passive conveyers or as sources of active viral replication and dissemination and thus need to be safeguarded upon sexual HIV transmission. Equally important is to test the activity against mutant viruses that show clinically relevant resistance. This is even more important when the same product or a close chemical relative is evaluated for systemic treatment of HIV infection and as a topical application for prevention [17, 18]. Exposure of uninfected and HIV+ individuals to antiretrovirals (in a microbicide or PrEP strategy), raises concerns about potential resistance development. Because NRTI and NNRTI constitute the backbone of HAART, their use in HIV prevention raises two major concerns. First, to guarantee maximal accessibility microbicides (and oral PrEP) need to be available over the counter without prior HIV testing. Substantial absorption of product in the mucosa could select for resistant viruses in the FGT, which may find their way to more peripheral tissues. An exchange of viruses between the genital tract compartment and the periphery has been shown before [30]. Resistance development may as such compromise first-line ART [17-19, 31, 32]. Second, it is not known whether ARV-based microbicides will protect against ARV-resistant viruses, which, as a consequence of therapy, are already circulating in the HIV+ population. The large-scale introduction of ARV-based microbicides could potentially promote the selective transmission of resistant viruses in the population. Because only few successful clinical trials have been done to date, there is no convincing data yet on the probability of emergence and spreading of drug resistance with the use of PrEP and ARV-based microbicides. It is however clear that lack of adherence is much more of an issue in prevention trials compared to HIV+ individuals under treatment.

Epithelium Cells from the Genital and Anorectal Cavity

Safety of candidate microbicides should not only be assessed on blood-derived HIV target cells (such as PBMC, T-cells, etc.) but also on epithelial cells lining the cervico-vaginal cavity. Since this epithelial layer forms a natural barrier against HIV transmission, candidate microbicides should be devoid of any cytotoxicity towards genital epithelia, not to compromise the epithelial layer integrity nor induce pro-inflammatory responses that would create a HIV-friendly environment.

Various epithelial cell lines from carcinoma origin have been used for safety assessment, such as the endometrial HEC-1A cell line, the cervical ME-180 and the HPV-infected CaSki cell lines. Other cell lines used include the Papillomavirus oncogen-immortalized primary endocervical cells End1/E6E7, the vaginal VK2/E6E7 cells and the ectocervical Ect1/E6E7 cells [33]. In a classical approach, cells are exposed to increasing doses of compound, formulation or formulated product and epithelial layer integrity and morphology are scored using microscopic evaluation, whereas cell viability is determined using viability stains to visualize membrane integrity and colorimetric assays to quantify viability and proliferative capacity (e.g. WST-1, MTT, XTT and LDH-assays). Similarly, colorectal cells (i.e. Caco-2) have been used to study safety of candidate microbicides with intended rectal application [34].

Primary genital epithelial cells (PGEC's) can be isolated from cervico-vaginal hysterectomy tissue from premenopausal women by enzymatic digestion and present a more relevant model than cell lines [35-38]. An alternative approach using cotton swabs to collect epithelial cells from the vaginal walls has also been described [39]. The technical procedures associated with PGEC isolation are labor-intensive and time consuming. The success of PGEC isolation and subsequent *in vitro* survival and proliferation, strongly depends on the quality of cervico-vaginal tissue that is available.

Dual Chamber Models of the Female Genital Mucosa

Another *in vitro* model that incorporates some more complexity in simulating the female genital mucosa is the dual chamber transmission model. Several of these models have been developed, each with their own specificities. In general, they all grow epithelial cells on a semi-permeable support in the apical compartment and HIV target cells in the basal compartment of a multi well tissue culture plate [40-45]. These models have been used to study the basics of HIV sexual transmission but can also be applied to evaluate the activity or toxicity of candidate microbicides.

The commercially available EpiVaginal^{TM} model (MatTek Corp., Ashland, MA, US) offers a multilayered tissue consisting of an organized basal layer and multiple

non-cornified layers analogous to native healthy human vaginal-ectocervical tissue, with or without dendritic cells, grown on plastic cell culture inserts. This model has been used to test *in vitro* toxicology of raw materials and final product formulations for feminine hygiene, personal care, and pharmaceutical products, including microbicides. A recent report validated the EpiVaginal™ assay as an *in vitro* alternative method to assess vaginal irritation [46], an assay that is otherwise done in rabbits.

Similar models have been described by Van Herrewege *et al*. [45], Gali *et al*. [42], Ariën *et al*. [43] and Mesquita *et al*. [44] using epithelial cell lines instead of PGEC's grown on plastic insert wells and HIV target cells (cell lines [44] or primary cells [42, 43]) in the basal compartment.

Using this model, it was shown that nonoxynol-9 (N-9) and cellulose sulfate (CS), two products that failed as microbicide in clinical trials, induced rapid and sustained disruption of the otherwise protective epithelial barrier and dramatically enhanced HIV replication in the basal compartment [44]. Gali *et al*. [42] established that transmigration of HIV-infected cells (i.e. cell-associated virus) across intact and confluent epithelia occurred and caused productive infection of target cells in the subepithelial basal compartment. Next, the model was applied as a preclinical screening tool for toxicity of various microbicide candidates and formulation excipients [42, 47]. It was shown that cell viability and epithelial layer integrity were compromised by most excipients at concentrations near the typically used concentration in vaginal products. The proinflammatory cytokine IL-8 was induced by several excipients at sub-toxic concentrations, suggesting that an inflammatory and thus HIV-friendly environment was created. Dapivirine, PRO2000, glycerol monolaurate (GML) and N-9 were found to damage epithelial integrity and induce IL-8 secretion at increasing drug concentration. The lack of selectivity of N-9 was evidenced by epithelial disruption and induced inflammation at doses previously used in clinical trials [47, 48]. The picture is different for PRO2000 and GML (SI <250) and dapivirine (SI $>10^3$). The RTI's tenofovir and UC-781 were found to be safe at concentrations 10^3-10^4 fold above their IC50.

Influence of the Genital Microenvironment

In addition to the physical barrier formed by a healthy and intact epithelium, non-specific defense mechanisms are present in the female and male genital tract.

Vaginal lactobacilli maintain an acidic pH by producing lactic acid and H_2O_2. Together with a mucus layer inclosing bactericidal and virucidal factors (e.g. secretory IgA, β-defensins, mucins, SLPI) and covering the epithelium, a HIV hostile environment is kept in place.

During heterosexual transmission, a mixture of seminal plasma and vaginal secretions create favorable conditions for HIV transmission. Seminal plasma contains alkaline amines that protect virions from acidic inactivation and factors that increase transmissibility (e.g. amyloid fibrils). However, much like cervico-vaginal secretions, seminal plasma also contains various factors with direct or indirect antimicrobial activity (SLPI, lactoferrin, β-defensins). The complexity of interactions of the various factors in male and female genital secretions, that can both increase and decrease HIV transmissibility, are not well understood [49-52].

For a microbicide to be effective, it is essential that the activity is not impaired by genital or rectal conditions such as acidic pH, seminal plasma, cervico-vaginal and ano-genital secretions and microflora. From a toxicity perspective it is equally important that a candidate microbicide does not interfere with the normal physiology in the vaginal and rectal cavity.

In vitro assays have been developed to study candidate microbicides under these physiological conditions. Compound activity can be assessed in the presence of semen and vaginal fluid simulants [53, 54] or in the presence of donor seminal plasma and cervico-vaginal lavage fluid (CVL) [55]. The intrinsic cellular toxicity of CVL and seminal plasma requires that these fluids are diluted out in an antiviral assay, limiting the power to measure the true attribute to potential changes in compound activity.

A candidate microbicide should not interfere with normal growth of the vaginal (and colorectal) microflora. Bacterial vaginosis (BV) is a condition where the normal vaginal flora is disrupted and overgrown by non-lactobacilli species, such a *Atopobium vaginae* and *Gardnerella vaginalis*. Several studies have shown that BV favors HIV transmission *in vivo* [56-58]. Lackman-Smith *et al*. [24] have published an algorithm for preclinical testing of topical microbicides that included a *Lactobacillus* growth inhibition assay. Dose-dependent growth inhibition of the common probiotic vaginal species *L. jensenii* and *L. crispatus* was determined, but newer algorithms include *L. vaginalis*, *L. gasseri*, *L. acidophilus*, *L. iners* and the BV-associated species *A. vaginae* and *G. vaginalis* [34].

PHYSICOCHEMICAL PROPERTIES OF MICROBICIDE CANDIDATES

From a development point of view it is important to know the physicochemical properties of a microbicide candidate as early as possible, because unfavorable physicochemical properties may exclude a candidate molecule at an early phase in development.

Physicochemical characterization includes (1) preformulation testing, encompassing studies of solubility and stability of dry, dispersed or dissolved drug(s) across a range of pHs, invoked degradation studies under various extreme conditions (heat, acid/base, UV, etc.), and compatibility of dosage forms and drug combinations, and (2) formulation testing, including the evaluation of the drug in the dosage form and the dosage form itself. Gathering this information at an early phase in development will help identify the ideal dosage form for a new microbicide candidate and may guide acceptability of a product [59-61].

EX VIVO ASSAYS TO EVALUATE ACTIVITY AND SAFETY OF MICROBICIDE CANDIDATES

Tissue explants from cervico-vaginal and colorectal origin are often used to assess anti-HIV compounds and formulations, as a step just prior to animal testing [47, 62-67]. In general, cervical tissue is obtained from HIV negative women undergoing hysterectomy for medical conditions that

are not related to cervical pathologies. Several explant systems have been described in scientific literature, with non-polarized or polarized exposure of product and/or challenge virus [65, 68, 69].

However, tissue explants have several limitations, such as unknown clinical history, presurgical exposure to hormone therapy, lack of physiological hormonal modulation, lack of immune cell recruitment, tissue deterioration after surgical excision, and inter-donor variability that all may affect the interpretation of results [70, 71]. Consequently, tissue explants are best regarded as a "worst-case scenario" model, where a microbicide candidate is evaluated under conditions that reflect extreme *in vivo* conditions, e.g. damaged genital epithelium related to a concomitant sexually transmitted infection.

Because many transmission events occur across the colorectal epithelial lining upon anal receptive intercourse in the context of both homosexual and heterosexual contacts, colorectal explants are used as a model in the study to prevent for this type of transmission [62, 64, 72]. The tissue is obtained from patients undergoing rectocele repair and colectomy for colorectal cancer. Many of the problems encountered with cervico-vaginal explants are also applicable to colorectal explants.

Current microbicides are mainly designed to prevent HIV acquisition through vaginal and rectal receptive sex, assuming bi-directional protection of both the receptive and insertive partner. As a result, the efficacy of microbicide candidates against penile infection is often not tested. Few studies to date have used male genital tissue to evaluate efficacy and safety of candidate microbicides [73]. Several randomized controlled trials concluded that circumcision reduced HIV transmission from infected women to men by at least 60% [74-76]. The presence of Langerhans cells in the inner foreskin and the lower degree of keratinization correlate with increased HIV acquisition [77]. Together, these data point out the importance of penile HIV acquisition and warrant for good *ex vivo* models of the male genital tract.

Just recently, Ganor *et al*. [78] reported on a new model of the adult male foreskin epithelium that allowed for polarized infection with cell-free and cell-associated HIV. Unfortunately, this report is one of few describing *ex vivo* modeling of the male genital tract, and much more effort is needed to develop penile models to guide microbicide development.

In summary, we reviewed the *in vitro* and *ex vivo* models currently applied to evaluate candidate HIV microbicides for vaginal and rectal application. Although the field of microbicides and prevention already disposes of a broad armamentarium of models and assays to examine the various aspects of activity, safety, physicochemical characteristics, formulation and acceptability of a potentially successful microbicide, there is still need for reproducible and more predictive *ex vivo* models using human tissue. Moreover, the macaque model as 'gatekeeper' to predict clinical outcome of candidate microbicide efficacy in humans is disputable, because of inherent differences between macaque and human vaginal and rectal mucosae, conditions of microbicide application and/or source of challenge inoculum (HIV *vs* SHIV). This warrants for a more thorough science on *in vitro*/*in vivo* toxicity of candidate microbicides.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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