Ex vivo comparison of microbicide efficacy for preventing HIV-1 genomic integration in intraepithelial vaginal cells

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ABSTRACT

Vaginally applied microbicides hold promise as a strategy to prevent sexual HIV transmission. Several non-specific microbicides, including the polyanion cellulose sulfate, have been evaluated in large-scale clinical trials but failed to show significant efficacy. These findings have prompted a renewed search for preclinical testing systems that can predict negative outcomes of microbicide trials. Moreover, the pipeline of potential topical microbicides has been expanded to include anti-retroviral agents such as reverse transcriptase, fusion, and integrase inhibitors. Using a novel ex vivo model of vaginal HIV-1 infection, we compared the prophylactic potential of two forms of the fusion inhibitor T-20, the CCR5 antagonist TAK-778, the integrase inhibitor 118-D-24 and cellulose sulfate (Ushercell®). The T-20 peptide with free N- and C-terminal amino acids was the most efficacious compound, causing significantly greater inhibition of viral genomic integration in intraepithelial vaginal leukocytes, measured by an optimized real-time PCR assay, than the more water soluble N-acetylated T-20 peptide (Fuzeon®) (IC$_{50}$ 0.153 µM vs. 51.2 µM [0.687 ng/ml vs. 230 ng/ml], respectively; p<0.0001). In contrast, no significant difference in IC$_{50}$ values was noted in peripheral blood cells (IC$_{50}$ 13.58 µM vs. 7.57 µM [61 ng/ml vs. 34 ng/ml], respectively; p=0.0614). Cellulose sulfate was the least effective among all compounds tested (IC$_{50}$ 1.8 µg/ml). These results highlight the merit of our model to screen the mucosal efficacy of novel microbicides and their formulations, and potentially rank order candidates for clinical evaluation.
INTRODUCTION

The lower genital tract of women constitutes a major site for HIV invasion. Therefore, prevention of vaginal infection would be a major milestone toward curbing the global AIDS epidemic. While male condoms are very effective at preventing vaginal HIV transmission, women are frequently not empowered to negotiate their use by their sexual partners or may object to their use due to sociocultural norms or the desire to conceive. The most effective long-term method of HIV prevention is a vaccine, because it would confer immunological protection, but a successful HIV-1 vaccine is unlikely to emerge in the near future. Thus, alternative strategies are urgently needed to slow the spread of HIV.

Systemic antiviral pre-exposure prophylaxis and vaginally or rectally delivered topical microbicides are receiving increased attention as preventive tools. To date, only detergent, pH-buffering and polyanion topical microbicides have completed testing in large-scale clinical trials. With one recently reported potential exception (6), none of these non-specific microbicides have demonstrated a protective effect against vaginal HIV transmission (23, 24, 45, 47). An increased risk of HIV transmission was indeed documented for the detergent nonoxynol-9 (46). These disappointing findings have had two major effects on the microbicide field. First, efforts have intensified to develop and standardize preclinical and animal testing models with high predictive power of clinical microbicide efficacy. The application of these models in appropriate testing algorithms should have the capacity to screen out compounds such as cellulose sulfate before they
enter phase II and III clinical trials (21). Second, the focus has shifted to compounds with specific antiretroviral activity. For example, the reverse transcriptase inhibitors (RTIs) tenofovir, which is already used to treat HIV infection, and dapivirine are currently entering testing as prophylactic vaginal gel formulations in phase IIb and phase III efficacy trials (10). There are concerns, however, that the use of RTIs as microbicides might increase the spread of HIV-1 resistance (21, 35). Therefore, other classes of antiretroviral drugs that attack HIV-1 at different points in the viral replication cycle would be valuable additions to the microbicide development pipeline. This includes more HIV-specific fusion and entry inhibitors, some of which have already demonstrated protection in macaques from vaginal transmission of simian human immunodeficiency virus (SHIV) (31, 49), as well as integrase inhibitors (37).

Recently, improved animal models in macaques and humanized mice have been developed that could become part of a standardized go/no-go decision algorithm for candidate microbicides under development (3, 8, 12, 20). However, these animal models have drawbacks. Apart from simian immunodeficiency virus (SIV) or SHIV not being fully representative of HIV-1 (42), study designs in macaques that are informative require large sample sizes and are expensive. Likewise, the two humanized mouse models have limited throughput because human fetal tissues are required to be transplanted into each individual mouse (3, 12). Consequently, human explant organ cultures of the genital or rectal mucosa are being explored as higher throughput and less expensive preclinical testing models (9, 18). Ex vivo microbicide screening in explant organ cultures could be used to narrow down the number of agents that are
subjected to further evaluation in animals. Conceivably, an optimal explant model of HIV infection may even obviate the need for animal models to be used for efficacy testing.

An effective preclinical testing model should simulate mucosal HIV transmission *in vivo* as closely as possible. We have demonstrated that CD4+ T lymphocytes and Langerhans cells (LC) residing in the outer epithelial layer of the human vagina are the initial targets infected by HIV-1 (26). A successful topical microbicide must block or abort infection of these first-line intraepithelial leukocytes. Thus, we view that the gold standard for a microbicide efficacy read-out in a pre-clinical model is the quantitative and sensitive measurement of productive infection of these intraepithelial leukocytes. Here, we present an *ex vivo* vaginal HIV infection model that uniquely combines these necessary features.

We have utilized our model to compare the efficacy of the polyanion microbicide cellulose sulfate with three classes of antiretrovirals, the fusion inhibitor T-20 (52), the CCR5 antagonist TAK-779 (2, 15) and the viral integrase inhibitor 118-D-24, a diketo-acid derivative (43, 53). Furthermore, an advantage of the *ex vivo* organ culture over the *in vitro* cell line culture is the ability to evaluate tissue bioavailability, including chemical modifications of the same agent and the effect of drug delivery vehicles. Local tissue bioavailability is a crucial factor for microbicide efficacy. Thus, we compared the FDA-approved T-20 peptide (Fuzeon®) with the T-20 peptide lacking N-acetylation, a chemical modification that increases T-20 lipid solubility (28).
METHODS AND MATERIALS

Vaginal epithelial sheets. Using a protocol that was approved by the Institutional Review Board (IRB) of the Fred Hutchinson Cancer Research Center in Seattle, we harvested routinely discarded tissues from vaginal repair surgeries performed in adult women at three medical centers in Seattle. No personal identities or demographic information were collected from the patients. For this reason, a waiver of consent was granted by the IRB. Tissues were placed in ice-cooled phosphate-buffered saline (PBS) and transported to the laboratory within one hour of removal from the donor. The deep submucosa was removed with surgical scissors and the remaining mucosa was treated with EDTA to separate the epithelial layer from the underlying stroma. EDTA interferes with the divalent cation-mediated epithelial-stromal union at the basal membrane (40). We achieved the most consistent results by cutting the vaginal mucosa in 3 mm–wide strips and incubating the strips with agitation in a 5 mM EDTA solution overnight at 4 °C. After this treatment, the intact epithelium could be dissected from the vaginal stroma under magnification using a Zeiss KL1500 stereoscope and two surgical microforceps. Cells within the epithelial sheets, including resident intraepithelial leukocytes, remained viable after this procedure; isolated sheets stained with the live cell marker calcein AM showed almost 100% staining, while virtually no staining was observed in sheets treated with the dead cell marker ethidium homidimer-1 (26). Higher EDTA concentrations and higher incubation temperatures yielded faster epithelial-stromal separation, but at the expense of decreased cell viability. The isolated intact epithelial sheets were placed in
Hanks buffered salt solution containing 5 mM calcium chloride for 1 h on ice, washed in PBS, and placed in culture medium (HEPES-buffered RPMI containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). A small amount of the stromal tissue was retained for CCR5 genotyping.

**Viruses.** Molecular clones of HIV-1$_{JRCSF}$ Env (CCR5-tropic) and HIV-1 $\Delta$Env were produced by calcium phosphate transfection of 293T cells with the proviral construct pLAI-JR (JRCSF Env in the pLAI backbone) and p-LAI-$\Delta$Env, respectively, as previously described (26, 51). To label virions with the green-fluorescent protein (GFP), 293T cells were co-transfected with the proviral construct and the peGFPC3 plasmid (Clontech, Mountain View, CA). The peGFPC3 plasmid contains the entire Vpr-coding region fused to the COOH terminus of eGFP (GFP-Vpr) (36). Cells were washed 18 h post-transfection, the culture medium was replaced 40 h post-transfection, and supernatants containing labeled virus were collected two to three times at 2 to 4 h intervals thereafter. Harvested viruses were concentrated 10-100 fold with Centricon Plus-80 100K centrifugal filter units (Millipore, Billerica, MA) and stored at -70°C. A primary mucosal HIV-1 isolate (HIV-1$_{M1}$) was isolated from mucosal mononuclear cells derived from the ectocervix of an HIV-1-infected woman, expanded in phytohemagglutinin (PHA)-activated lymphoblasts, and stored at -70°C. The peripheral blood mononuclear cells (PBMC) used for the generation of PHA-activated lymphoblasts were obtained from volunteers attending the research clinic of the HIV Vaccine Trials Unit (HVTU) in Seattle. All volunteers signed informed consent for these blood draws. HIV-1$_{M1}$ was typed CCR5-tropic by infection of MAGI indicator cell lines.
(50). All virus preparations were assayed for infectivity in MAGI cells (50) or PHA-activated lymphoblasts, and the Gag p24 concentration of the viral stocks was determined by an enzyme-linked immunosorbent assay (HIV-1 p24 Antigen Capture Assay Kit, NCI-Frederick, Frederick, MD).

**Virus inoculations and pre-exposure treatments with candidate microbicides.** For confocal fluorescence microscopy, epithelial sheets were cut into ~1.5 x 1.5 mm pieces and placed into round-bottom 96-well plates containing 50 µl of culture medium per well. Epithelial sheets were spinoculated with viruses (200 ng/ml Gag p24 of GFP-tagged HIV-1JRCSF or HIV-1 ΔEnv) at room temperature for 2 h at 1200 x g (38), washed in staining buffer (SB; PBS containing 1% bovine serum albumin and 0.05% sodium azide), immunostained, and examined by confocal microscopy.

To detect productive infection in LC and T cells emigrating from HIV-1-exposed vaginal epithelium, we placed several strips of epithelial sheets in 6-well plates containing 2 ml of culture medium per well. Prior to viral challenge, we treated some sheets with the fusion inhibitor T-20 (enfuvirtide) or its N-acetylated T-20 derivative Fuzeon® (manufactured by Roche), the CCR5 antagonist TAK-779, the integrase inhibitor 118-D-24, the CXCR4 antagonist AMD-3100 (bicyclam JM-2987) (all obtained from the NIH AIDS Research and Reference Reagent Program, Germantown, MD), or cellulose sulfate (Ushercell; kindly provided by George Usher, Polydex Pharmaceuticals, Toronto, Canada) for 1 h at room temperature. Sheets were spinoculated with 100 ng/ml Gag p24 of HIV-1JRCSF of HIV-1M1 for 2 h at 1200 x g (38), washed at least six times with PBS, and cultured in culture medium for 2-3 days at 37°C
and 5% CO₂. For HIV-1 Gag p24/p55 detection, all cells that had emigrated from the epithelium into the culture medium after 2-3 days were harvested and immunostained for flow cytometric analysis. To detect HIV-1 DNA integration by PCR, emigrated cells and epithelial sheets were harvested 2-3 days after viral infection and combined for DNA isolation.

For in vitro HIV-1 infection of single cell suspension cells, we obtained PBMC from two blood donors and activated the cells for 2 days with 0.4 µg/ml PHA (Remel, KS) in cell culture medium. 100,000 activated lymphoblasts per well were then distributed into a 96-well plate, treated in duplicates with various concentrations of the T-20 peptide with free N- and C-terminal amino acids, its N-acetylated T-20 derivative Fuzeon®, or cellulose sulfate for 1 h at room temperature, infected with 200 ng/ml Gag 24 of HIV-1JRCSF for 2 h, washed, cultured for 48 h at 37°C and 5% CO₂, and collected for DNA isolation. Microbicide treatment, infection, and cell culture were performed in culture medium containing 50 U/ml interleukin-2.

**Immunostaining for confocal microscopy.** Virus-challenged epithelial sheets were incubated in SB for 1 h at RT with 10 µg/ml anti-CD1a antibody (NA1/34; Dako, Carpinteria, CA). Sheets were washed in SB, incubated for 30 min with Alexa Fluor 568-conjugated F(ab)2 goat-anti-mouse in SB (5 µg/ml; Molecular Probes, Eugene, OR), washed again, and fixed at 4°C overnight in 4% buffered para-formaldehyde. Nuclei were counterstained with TOPRO3 (1 µM; Molecular Probes), and the sheets were embedded in Mowiol 40-88 containing 2.5% w/v DABCO (Sigma-Aldrich, Milwaukee, WI). Cellular staining was visualized with a Leica TCS SP spectral confocal
microscope equipped with Argon 488, Krypton 568, and Helium/Neon 633 lasers. We analyzed the acquired image stacks using Imaris software (Bitplane AG, Zurich, Switzerland).

**Flow cytometry.** We used flow cytometry to detect HIV-1 Gag p24/p55 expression in T lymphocytes that had emigrated from virus-exposed sheets. Emigrated cells harvested from virus-exposed sheets were incubated in SB for 30 min on ice with 10 µg/ml PE-conjugated anti-CD3 mAb (UCHT1). For the detection of non-viable cells, we used a previously described method that employed 7-amino-actinomycin D (7-AAD) (Sigma-Aldrich) to stain dead cells before subsequent fixation (41). Next, the cells were fixed, permeabilized, and incubated with FITC-conjugated anti-HIV-1 Gag p55/p24 mAb (KC57; Coulter, Fullerton, CA) according to the manufacturer’s protocol. Finally, the cells were again fixed in 2% paraformaldehyde for at least 12 h, acquired on a Calibur flow cytometer (BD Biosciences), and analyzed using CellQuest 3.3 (BD Biosciences) with gates set to isolate single CD3+ HLA-DRlow T cells and to exclude 7-AAD+ dead cells.

**PCR assays for HIV-1 proviral and integrated genomic DNA.** We isolated DNA from emigrated cells using the QiaAmp Blood Mini Kit (Qiagen, Valencia, CA) and performed an Alu-LTR-based nested PCR assay, which amplifies viral DNA that has been integrated into the host cell genome; unintegrated viral DNA is not amplified. We introduced the following modifications to the previously published method (5). Platinum Taq SuperMix (Invitrogen) was used for the first round amplification in an ABI 7900HT...
thermal cycler (Applied Biosystems), starting with a denaturation step of 2 min at 96°C and then 12 cycles of amplification (96°C for 15 s, 55°C for 60 s, and 70°C for 60 s). One-tenth the volume of first round amplicons were then amplified a second round in 1X ABsolute Blue QPCR ROX Mix (ABgene USA, Rochester, NY), starting with a denaturation step of 15 min at 96°C and then 40 cycles of amplification (96°C for 15 s and 60°C for 60 s). A 6-FAM-labeled LTR hybridization probe was used to detect the product in the second round. Reactions were carried out in an ABI 7900HT thermal cycler (Applied Biosystems, Foster City, CA). We generated a standard curve using DNA isolated from serially diluted ACH-2 cells that are latently infected with HIV-1 LAV (NIH AIDS Research and Reference Reagent Program). Alu-LTR copy numbers were calculated in reference to this standard curve.

In the singleplex PCR assay, each sample was tested in parallel wells with a human β-actin primer/probe set (TaqMan B-actin Detection Reagent; Applied Biosystems). For multiplexing the detection of the Alu-LTR and the β-actin sequences in the same wells, we used the forward and reverse β-actin primers (82 mM) from the kit as outer primers during the first PCR round. For the second PCR round, 305 nM of inner β-actin primers (Hu β-actin Forward 5’-ACCCACACTGTGCCCATCTACGA-3’ and Hu β-actin Reverse 5’-CGGAACCGCTCATTGCCAATGG-3’) were used. The β-actin hybridization probe that was added to the second round of PCR was the same oligonucleotide provided in the B-actin kit, but labeled with JOE (200 nM) instead of 6-FAM. A standard curve for β-actin was created using DNA isolated from serially diluted ACH-2 cells. β-actin copy numbers were calculated in reference to this standard curve. The Alu-LTR PCR copy numbers were normalized to the corresponding β-actin copy
numbers to yield Alu-LTR copies per cell. Absolute Alu-LTR copies per cell varied widely between individuals, for example for the HIV-1JRCSF infections without pre-exposure prophylaxis had between 0.129 and 1875 copies per cell. However, the method does not employ precisely quantified Alu-LTR and β-actin DNA standards and does not allow reliable determination of absolute copy numbers. Thus, the magnitude of viral integration in the various experimental conditions is reported relative to the virus infection performed, without pre-exposure prophylaxis as the calibrator. Quadruplicate PCR reactions were run for each experimental set-up. Using ACH-2 cells, the lower detection limit of integrated HIV-1 DNA was ~50 copies for the singleplex PCR assay and ~5 copies for the multiplex PCR assay (not shown).

**CCR5 genotyping.** PCR and DNA restriction fragment length polymorphism analyses were performed with DNA isolated from vaginal tissues as previously described (25).

**Statistics.** Differences in the levels of relative viral integration between different experimental conditions were determined by two-sided paired (Figure 2) or unpaired (Figure 3A and B) t tests. The standard deviations calculated from the raw cycle threshold values of quadruplicate PCR reactions were compared between singleplex and multiplex assays by a two-sided unpaired t test. Dose response curves for drug titrations were fitted by nonlinear regression (Figure 3C and D) and IC\textsubscript{50} values were compared between different compounds by an F test using Prism 4.0 (GraphPad, San Diego, CA).
RESULTS

A simplified ex vivo model of intraepithelial HIV-1 infection in the human vagina

To enable larger-scale screening of candidate microbicides for their antiviral efficacy in the vaginal mucosa, we simplified and optimized our previous explant model designed to investigate the initial events of vaginal HIV transmission (26). In this earlier model, epithelial sheets were isolated from the underlying vaginal stroma by suction blistering. This method was not efficient, yielding epithelial sheets from only ~20% of each vaginal tissue sample. For the present study, we therefore optimized the treatment with the calcium and magnesium chelator ethylene diamine tetraacetic acid (EDTA) as an alternative to suction blistering or using proteolytic enzymes, which have the undesirable effect of cleaving off HIV-1 surface receptors (1, 19). HIV-1 co-receptor expression remained intact after EDTA treatment. We have previously reported high frequencies of CCR5 expression on resident CD3+ T cells and CD1a+ LC in EDTA-separated viable sheets (26). This allowed us to isolate and utilize 100% of the epithelium from each tissue sample for our experiments.

Next, we determined the optimal conditions for HIV-1 infection in the EDTA-separated sheets. We first confirmed that GFP-tagged HIV-1 virions localized to intraepithelial leukocytes (Figure 1A and B) in similar distribution to previous localization experiments with suction blister sheets (26). By confocal microscopy, we observed that HIV-1 virions bound to intraepithelial lymphocytes in a characteristic circular pattern.
while also localizing to a subset of CD1a+ LC (Figure 1A and B). Because a previous study had demonstrated that EDTA treatment interferes with HIV-1 envelope-mediated fusion after CD4 binding (14), we determined if calcium replenishment following EDTA treatment increased the level of productive infection in our model. After 1 h incubation in Hanks buffered salt solution with or without 5 mM calcium chloride, EDTA-isolated vaginal epithelial sheets were exposed to HIV-1JR-CSF. Intracellular HIV-1 Gag expression as determined by flow cytometry was used to detect the productive infection of T cells that had migrated over 48 h from the epithelium into the culture supernatant. One representative sample is depicted in Figure 1C showing that calcium replenishment of the epithelial sheets after EDTA treatment increased the percentage of infected CD3+ T cells. Without calcium treatment, 1.5% of the emigrant CD3+ lymphocytes expressed HIV-1 Gag (Figure 1C, middle panel) in EDTA-treated sheets. By contrast, a 7.2-fold increase was observed after calcium treatment of EDTA-treated sheets, with 10.8% of CD3+ lymphocytes expressing HIV-1 Gag (Figure 1C, right panel). A second donated tissue produced concordant results, with a 4.6-fold increase in infected CD3+ T cells when calcium was replenished after EDTA treatment (not shown). Thus, for all subsequent infection experiments, the EDTA-treated epithelial sheets were routinely replenished with 5 mM calcium chloride for 1 h.

**Ex vivo pre-exposure prophylaxis of HIV-1 chromosomal integration in vaginal intraepithelial leukocytes**

To evaluate the feasibility of our vaginal infection model in screening potential microbicides for antiviral efficacy, we determined the ability of three model compounds,
representing three different mechanisms of HIV-specific antiviral activity, to inhibit HIV-1 infection. We isolated vaginal epithelial sheets from various tissue donors, treated the sheets for 1 h with the fusion inhibitor T-20 (peptide version with free N- and C-terminal amino acids), the CCR5 antagonist TAK-779, or the integrase inhibitor 118-D-24, and then exposed the sheets to CCR5-tropic HIV-1\textsubscript{JR-CSF}. To detect infection, we harvested the epithelial sheets and the supernatants containing the emigrated cells after a 48 h culture period and measured HIV-1 genomic DNA integration by a sensitive nested real-time PCR assay (5). This method requires less cellular material than flow cytometric methods and is specific for post-entry events that signify the initiation of a productive viral life cycle.

In initial experiments, epithelial sheets from two donors were exposed for 2 h to HIV-1\textsubscript{JR-CSF} at a relatively low virus concentration (10 ng/ml Gag p24). No viral integration was detected with this infection protocol. However, increasing the viral dosage to 100 ng/ml Gag p24 and including a spinoculation step (38) as used in our previous study (26) permitted HIV-1\textsubscript{JRCSF} infection of intraepithelial vaginal cells in five of six donor tissues. The one donor tissue lacking detectable integrated HIV-1 provirus tested heterozygous for the CCR5-\textDelta32 mutation (CCR5\textsubscript{wt/\textDelta32}), consistent with this tissue’s reduced susceptibility to a CCR5-dependent HIV-1 infection (32). In the five CCR5\textsubscript{wt/wt} donor tissues, all three compounds (T-20, TAK-779, and 118-D-24) strongly suppressed genomic integration of HIV-1\textsubscript{JRCSF} (Figure 2A). To avoid potential operator bias, we repeated the PCR assays four times, with three experimental replicates conducted by three different operators who were blinded to the sample
treatment. Using these assays, we found that T-20 (0.223 µM) decreased viral integration to 8% (mean, range 0.01 – 15.5%; p<0.0001) of the level detected when infection was performed without pre-exposure prophylaxis. Similarly, TAK-779 (30 µM) decreased viral integration to 10% (mean, range 1.8 – 17.9%; p<0.0001) and 118-D-24 (1 µM) decreased integration to 8.4% (mean, range 0.24 – 19%; p<0.0001), relative to the samples with no pre-exposure prophylaxis. The CXCR4-antagonist AMD-3100 (1 µM), which does not inhibit infection by HIV-1 variants using the co-receptor CCR5, was used as a positive control treatment and exhibited a tendency to increase viral integration to 177% (mean, range 113.7 – 265.2 %; p =0.066) relative to samples with no pre-exposure treatment. These data show that the HIV-1 inhibitory activity of three tested microbicides can be measured by viral integration in our ex vivo model, indicating the potential of the model for pre-clinical microbicide screening.

**Advantages of measuring viral integration with a multiplexed PCR assay**

The comparative screening of microbicides requires a highly reliable assay read-out. Therefore, we analyzed our PCR results for consistency between operators and PCR reaction replicates. The three operators in our study consistently produced real-time PCR amplifications with raw cycle threshold values for positive samples between 15 and 25 cycles and only rare outliers (Figure S1). The quadruplicate cycle threshold values for each sample were generally tight, with small standard deviations (note error bars in Figure S1). However, the variability in raw cycle threshold values between different operators was still of concern, in particular with the singleplex assay in which the Alu-LTR and the control β-actin gene PCR amplifications are performed in separate
wells. In this assay format, pipetting inconsistencies between the Alu-LTR PCR wells and the β-actin PCR wells adversely affect the accuracy of calculating the Alu-LTR copy number per cell. We therefore attuned the PCR conditions of the Alu-LTR and the β-actin sequences to each other so that both amplifications could be carried out efficiently within the same wells (Figure 2B). In this multiplexed PCR assay, each Alu-LTR amplification is related to its internal β-actin control.

We then compared the multiplex and singleplex PCR assays by measuring HIV-1 integration in the same DNA samples that were derived from screening a panel of microbicides ex vivo in five vaginal tissue donors (Figure 2A and B). Two independent multiplex assays confirmed the biological results of the singleplex assay. In the multiplex assay, T-20 decreased viral integration to 6% (mean, range 1.8 – 18.7%; p<0.0001), TAK-779 to 8.6% (mean, range 0.1 – 19.4%; p<0.0001), and 118-D-24 to 6.5% (mean, range 0.5 – 20.3%; p<0.0001) of the level detected when infection was performed without pre-exposure prophylaxis. Less enhancement of viral integration after treatment with AMD-3100 was noted with the multiplex assay (mean 121%, range 94.6 – 172.2%; p=0.195) than with the singleplex assay (mean 177%).

The overall variability between the quadruplicate PCR amplifications of each DNA sample was lower for the multiplex than for the singleplex assays. The individual standard deviations calculated from the raw cycle threshold values of each of the quadruplicate PCR reactions averaged 0.99 for the singleplex and 0.46 for the multiplex Alu-LTR amplifications (p<0.0001). For the β-actin amplifications, these averages were
2.03 and 0.78 for the singleplex and multiplex reactions, respectively (p<0.00001). In summary, the multiplex assay yielded the same biological results as the singleplex assay and displayed lower variability between identical replicates. Moreover, the multiplex assay required only half the DNA material. Thus, we adopted the multiplex protocol for our subsequent studies.

Prophylaxis of vaginal chromosomal integration of a mucosal HIV-1 isolate

Effective microbicides need to prevent infection with HIV-1 wild-type strains that are adapted to the mucosal environment. We were therefore interested to determine if the candidate microbicides could inhibit intraepithelial cell integration of a CCR5-tropic HIV-1 isolate (HIV-1\textsubscript{M1}) derived from the ectocervical mucosa of an HIV-1-infected woman. We obtained vaginal epithelial sheets from two additional donors and pre-incubated the tissues with T-20 (0.223 µM), TAK-779 (1 µM), or AMD-3100 (1µM) before infecting with HIV-1\textsubscript{M1}. After a 48 h culture period, we detected chromosomal integration of HIV-1\textsubscript{M1} using the multiplex PCR assay. Both T-20 and TAK-779 strongly suppressed genomic integration of HIV-1\textsubscript{M1} to less than 2% of the level detected when infection was performed without pre-exposure prophylaxis (Figure 2C). The control CXCR4-antagonist AMD-3100 increased viral integration of HIV-1\textsubscript{M1} in the two tissues donors to 296% and 117%, respectively (Figure 2C). These data lend support that our ex vivo vaginal infection model is suitable to test the antiviral efficacy of candidate microbicides against wild-type HIV-1 variants adapted to the mucosal environment.
N-acetylated T-20 is less effective than free T-20 in preventing vaginal HIV-1 infection

We further validated the feasibility of our vaginal infection model for antiviral drug screening by performing a dose-dependency analysis with titrations of the three inhibitory compounds, T-20, TAK-778, and 118-D-24. In addition, we wanted to determine if the model discriminates between pharmacological versions of the same drug that differ in water and lipid solubility. For this purpose, we included two distinct peptides of T-20 in the titration: 1) the N-acetylated peptide, which is present in Fuzeon® (produced and distributed by Trimeris/Roche), and 2) a peptide with free N- and C-terminal amino acids (produced by the Division of AIDS, NIAID). Both T-20 versions exhibited a clear dose-dependent inhibitory effect on viral integration (Figure 3A and C). The concentration at which the more lipid-soluble T-20 peptide from DAIDS induced a 50% inhibition of HIV-1$_{JRCSF}$ genomic integration in leukocytes residing within the vaginal epithelium (IC$_{50}$) was 0.153 µM (0.687 ng/ml; 95% confidence interval, 0.563 – 0.84 ng/ml; n=7 independent experiments with 4 donor tissues). In contrast, the more water soluble Fuzeon® product exhibited an IC$_{50}$ of 51.2 µM (230 ng/ml; 95% confidence interval, 198 – 267 ng/ml; n=8 independent experiments with 4 donor tissues) and was thus markedly less effective than the T-20 peptide from DAIDS (p<0.0001) (Figure 3A and C). Of note, this marked difference in efficacy between the two chemical forms of T-20 was not observed for inhibition of HIV-1 integration in phytohemagglutinin (PHA)-activated peripheral blood lymphocytes infected with HIV-1$_{JRCSF}$ in single cell suspension (Figure 3D). The IC$_{50}$ values of inhibiting HIV-1...
integration with T-20 from DAIDS and T-20 from Roche in PHA-activated lymphocytes were 7.57 and 13.58 µM (34 and 61 ng/ml), respectively, and were not significantly different from each other (p=0.0614).

A dose-dependent inhibition of HIV-1\textsubscript{JRCSF} integration in the vaginal epithelium was also observed for 118-D-24 and TAK-778 (Figure S2). The IC\textsubscript{50} values of 118-D-24 and TAK-779 were 190.13 µM (47 ng/ml; n=3 donor tissues) and 5.84 µM (3.1 ng/ml; n=2 donor tissues), respectively. In the seven donor tissues used in the titration studies, pre-treatment with the control CXCR4 antagonist AMD-3100 increased viral integration to an average of 126% relative to samples with no pre-exposure treatment (range 97 – 180%; Figure S3).

In summary, we observed a clear dose-dependent inhibitory effect on viral integration in intraepithelial vaginal leukocytes by all four tested compounds. In multiple titrations of the two T-20 peptides, we found that the titration curves were highly reproducible between independently performed experiments, both within the same and across different donor tissues (Figure 3A and C). Moreover, the distinct properties of lipid solubility and water solubility between the two T-20 peptides had a strong impact on the efficacy of T-20 to inhibit HIV-1 infection of leukocytes residing within the vaginal epithelium, but not on its efficacy to inhibit infection of peripheral blood leukocytes in single cell suspension.

**Efficacy of cellulose sulfate in preventing vaginal HIV-1 infection**
In a large clinical trial, cellulose sulfate, a non-specific HIV entry inhibitor, did not prevent HIV infection and may have increased the risk of HIV acquisition (45). Moreover, a previous analysis of in vitro data suggested a biphasic effect of cellulose sulfate on HIV-1 infection (44). Cellulose sulfate inhibited infectivity at concentrations of ≥10 µg/ml, but was minimally effective and at times enhanced infectivity at lower concentrations (≤ 0.5 µg/ml). To test if cellulose sulfate exhibits a similar biphasic effect on HIV-1 infectivity in our vaginal tissue model, we performed seven independent cellulose sulfate titrations with tissues from four different donors (Figure 3B and C). We observed a clear titration effect of cellulose sulfate yielding an IC50 of 1.8 µg/ml (95% confidence interval, 1.116 – 2.92 µg/ml). However, no enhancement of infection was present at any of the concentrations, except for an increase of viral integration to 132% relative to no pre-exposure treatment when cellulose sulfate was used at a concentration of 0.1 µg/ml in a single experiment. Likewise, no enhancement of infection by cellulose sulfate was observed in two titration experiments performed with PHA-activated peripheral blood lymphocytes from two separate donors (Figure 3D). In contrast, 1 µM (0.7944 µg/ml) of the control CXCR4 antagonist AMD-3100 increased viral integration of HIV-1JRCSF in the vaginal epithelium to an average of 125% relative to samples with no pre-exposure treatment (range 94.6 – 180%; p<0.01) across all 12 tested donor tissues in our study (Figures 2B and S3).

Of all the tested compounds, cellulose sulfate was the least effective in inhibiting the infection of vaginal intraepithelial leukocytes with R5-tropic HIV-1. Comparing the tissue IC50 value of cellulose sulfate to the tissue IC50 values of the two T-20 peptides,
cellulose sulfate was approximately ~1 log less effective than the Fuzeon® product and >3 logs less effective than the T-20 peptide from DAIDS (p<0.0001 for both IC\textsubscript{50} comparisons) (Figure 3C). At the specific concentration of 0.5 \( \mu \text{g/ml} \), cellulose sulfate decreased viral integration in intraepithelial leukocytes only slightly to 81.4% (mean, range 65.8 – 102.9%) of uninhibited infection, compared to a reduction to 30.4% (mean, range 28.2 – 38.4%) after treatment with 0.5 \( \mu \text{g/ml} \) Fuzeon® (p<0.0001) and to 1.92% (mean, range 0.75 – 4.21%) after treatment with 0.1 \( \mu \text{g/ml} \) T-20 DAIDS (p<0.0001).

Thus, our vaginal model reproducibly identified cellulose sulfate as a compound with a relatively weak efficacy for preventing HIV-1 infection of leukocytes residing in the outer vaginal epithelium. On the other hand, cellulose sulfate also did not enhance infection in our model.

**DISCUSSION**

We consistently detected integrated HIV-1 provirus in intact, stroma-free epithelial sheets from the human vagina within two days of HIV-1 exposure, demonstrating that cells residing within the outer vaginal epithelium are highly susceptible to infection by HIV-1. A microbicide that fails to block this initial step of infection is unlikely to be successful in preventing sexual HIV transmission. Thus, pre-screening novel microbicides for HIV-1 inhibitory activities using \textit{ex vivo} vaginal intraepithelial cells can permit rational choices of which candidates may hold promise in larger scale \textit{in vivo} preclinical and clinical studies.
To build a feasible platform for systematic microbicide evaluation, we adapted our *ex vivo* vaginal HIV transmission model (26) to quantify alterations in infectivity with pre-exposure prophylaxis. First, we improved the efficiency of epithelial-stromal separation, allowing us to harvest 100% of the epithelium from each vaginal tissue sample, and thereby decreasing the total number of samples needed for testing. Second, we adopted a read-out of productive infection based on real-time PCR amplification of HIV-1 proviral DNA sequences that have integrated into the genome of infected cells. This method of detecting HIV-1 infection of vaginal intraepithelial cells offers three major advantages: high sensitivity, an indication for an advanced step in the productive viral life cycle, and its ability to reliably quantify the relative antiviral efficacy of a given panel of microbicides. The high sensitivity of the PCR assay can detect a newly occurring HIV-1 infection of cells within the vaginal epithelium as early as two days following the viral challenge. During this short interval, contamination of the *ex vivo* cultures with bacteria or fungi rarely occurs, even if tissue processing is performed under clean, but not sterile conditions. Previously reported *ex vivo* human explant studies of microbicide efficacy have employed whole mucosal organ cultures (7, 9, 18, 22, 27, 34), which usually require longer culture periods for detection of HIV infection, potentially increasing the risk of tissue degradation and pathogen contamination. The greater sensitivity of the real-time PCR assay also ensures that a number of different microbicides with wide titration ranges can be tested in pair-wise comparisons within the same donor tissues, as each experimental condition requires only a relatively small amount of epithelium.
The real-time format of the PCR assay allows quantification of the amount of integrated viral copies per cell. To create a standard curve for the calculation of integrated HIV-1 copies, we titrated latently infected ACH-2 cells in parallel to each experimental PCR assay. Because the precise number of proviral copies in a given number of ACH-2 cells is not known, we used the ACH-2 cell standard curve to calculate the relative amount of integrated provirus under different experimental conditions rather than the absolute number of integrated viral copies. For the purpose of our study, which was to determine whether a given microbicide inhibits viral integration in vaginal cells relative to viral integration in the absence of the microbicide, relative quantification is sufficient. Our comparative dose response studies clearly demonstrate the power of relative quantification by our PCR assay to discriminate the efficacy of different microbicides to inhibit viral integration in vaginal target cells.

Of note, the measurement of viral integration is not specific for a particular cell type. Thus, unless mucosal cells are sorted into subpopulations before DNA isolation, the PCR assay does not identify which cells are infected. The specific cell type(s) infected with HIV-1 within the vaginal epithelium could be determined by flow cytometric analysis of isolated cells or by in situ microscopy techniques, as we have performed previously (26). Compared to real-time PCR, flow cytometry relies on the analysis of a relatively high number of isolated cells in single cell suspension and therefore, for enumerating infected cells, requires a much larger amount of vaginal tissue for each experimental condition. Microscopy techniques, on the other hand, are labor intensive and harder to accurately quantify than real-time PCR results.
While the PCR assay does not specify the cell type infected with HIV-1, our model ensures that cells within the outer vaginal epithelium, which are the first encountered by HIV during viral penetration \textit{in vivo}, are the sole source of the integrated HIV provirus. The vaginal epithelial sheets were completely stroma-free and did not contain any microvasculature, which focuses the analysis on T lymphocytes and LCs, the sole two leukocyte subtypes consistently residing within the outer vaginal epithelium (17, 26). Our prior studies have demonstrated that CD4$^+$ T lymphocytes are the main cell type within the vaginal epithelium that is productively infected by HIV-1 (26). Thus, we presume that integrated provirus detected in our present study is derived mostly or entirely from infected intraepithelial CD4$^+$ T cells.

Utilizing our vaginal intraepithelial infection model to compare the HIV-1 inhibitory efficacy of several potential microbicides yielded some relevant findings about the potential activities of microbicides in future studies. The differential potency of these microbicides to prevent HIV-1 integration in intraepithelial target cells, which was consistent in experiments with several donor tissues, demonstrates the potential utility of the model for preclinical microbicide screening. Importantly, we observed a pronounced difference in efficacy between the two different pharmacological versions of the fusion inhibitor T-20 in the tissue model but not in single target cell suspensions. This underscores two crucial points: (1) Microbicides that show promise after initial testing using PBMCs or indicator cell lines require testing in tissue infection models; \textit{in vitro} testing alone is not sufficient. (2) Drugs that are efficacious systemically may be
less so when applied as a topical microbicide. The T-20 peptide with free terminal ends likely exhibits higher lipid solubility than the Roche-manufactured N-acetylated T-20 peptide (28) and may thus penetrate the vaginal epithelium more readily. Compared to our IC_{50} determination for the Roche-manufactured T-20 (230 ng/ml in tissue; 61 ng/ml in PHA-activated T cells) or the IC_{50} ranges that have been previously reported for this agent (0.4–480 ng/ml for CCR5-tropic viruses in various in vitro systems (30)), the T-20 peptide lacking N-acetylation was highly protective against HIV-1 chromosomal integration in the vaginal epithelium (0.687 ng/ml).

Notably, both T-20 versions inhibited infection of vaginal intraepithelial cells in our model more effectively than cellulose sulfate (Ushercell®). Likewise, the CCR5 antagonist TAK-779 and the integrase inhibitor 118-D-24 were markedly more efficacious than cellulose sulfate. Going forward, clear results criteria for comparative efficacy screening in a relevant ex vivo model like the one presented here need to be formulated to determine whether a product may proceed to further evaluation in vivo. These criteria will have to incorporate toxicity in the form of a therapeutic index that puts efficacy in relationship to the compound’s potential toxicity for the vaginal epithelium. Moreover, screening criteria cannot focus solely on comparing equal dosages of microbicidal agents but will have to consider what concentrations are actually achievable in vivo and at what cost. Our findings indicate that the T-20 DAIDS peptide with free N- and C-terminal amino acids may be topically effective in the vagina at a much lower dosage than Fuzeon®. At 10 ng/ml, >80% inhibition of viral integration in the mucosa was achieved. Extrapolating the amount of tissue we treated in each
titration step to the complete surface of the vaginal cavity (39), we estimate that a total
dose of 10 mg T-20 DAIDS could be effective as a vaginal microbicide, costing in
between two and three US dollars. While even less expensive topical microbicides are
desirable, this nevertheless indicates that due to the potent protective efficacy of some
fusion inhibitors, as is exemplified both in our study for T-20 DAIDS and in previous
work with other compounds (16, 48, 49), fusion inhibitors could potentially be efficacious
in humans as topical microbicides at concentrations that are not prohibitively expensive.

Enhancement of HIV infection of peripheral blood mononuclear cells by cellulose
sulfate at low concentrations of around 0.3 µg/ml was suggested by a recently published
study (44). The authors concluded that this may explain why cellulose sulfate appeared
to increase the risk of HIV infection in one of two large clinical trials (45). However, the
final statistical analysis comparing the HIV transmission risk between the cellulose
sulfate and the placebo groups of the two trials was not significant (23, 45). Indeed, one
of the two studies explicitly concluded that a 6% cellulose sulfate vaginal gel was safe
(23). These clinical observations are consistent with the monophasic dose response
curve observed for cellulose sulfate in our vaginal infection model: while cellulose
sulfate was less efficacious than the other four compounds tested, it also did not
enhance infection at any concentration.

In conclusion, we developed and validated an ex vivo tissue model that uniquely
quantifies the sum of the initial events whereby HIV-1 establishes infection of cells
embedded in the outer epithelial layer of the human vagina. Mucosal tissues for this
model can be easily obtained on a weekly basis from one University Medical Center as a discarded by-product of vaginal repair surgeries (4, 29, 33). We demonstrate that our vaginal infection model can be utilized to screen topical microbicide candidates for their efficacy to block chromosomal integration of HIV-1, measured by a sensitive real-time PCR assay, in intraepithelial vaginal cells. The relative inefficiency of cellulose sulfate to prevent infection of intraepithelial leukocytes, as well as the superior efficacy of a lipid-soluble over a water-soluble version of T-20 in our model, underscores our model's potential as a screening tool for microbicides in the development pipeline. We propose that this preclinical testing system can be used to narrow down the number of microbicide candidates that progress to human trials.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.
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FIGURE LEGENDS

Figure 1. HIV-1 infection of leukocytes residing in vaginal epithelial sheets obtained by ETDA treatment. (A and B) Confocal microscopy of HIV-1 binding to intraepithelial lymphocytes and Langerhans cells. Epithelial sheets were separated from the underlying vaginal stroma by overnight incubation in 5 mM EDTA at 4 °C, placed in Hanks buffered salt solution containing 5 mM calcium chloride for 1 h on ice, washed in PBS, and spinoculated for 2 h with either GFP-Vpr tagged HIV-1JRCSF or HIV-1ΔEnv at room temperature. Subsequently, sheets were stained with anti-CD1a antibody and analyzed by confocal microscopy. Virus is shown in green, and the round staining pattern is typical for virus that is bound to intraepithelial T lymphocytes (white arrows) (26). White arrowheads point to virus that localizes to CD1a+ Langerhans cells. The overlay between green fluorescent labeled virus and red fluorescent CD1a staining
is in yellow. The blue staining shows the TOPRO-3 nuclear counterstain. A and B depict tissues from two different individuals. Exposure to HIV-1 ΔEnv exhibited no specific binding (not shown). (C) Calcium replenishment after EDTA treatment of vaginal epithelial sheets improves HIV-1 infectivity. EDTA-separated vaginal epithelial sheets were incubated with or without the addition of 5 mM calcium chloride for 1 h, spinoculated with HIV-1 JRCSF for 2 h, and cultured for 48 h. Cells that had emigrated from the epithelium into the culture supernatant were harvested, stained for HIV-1 Gag and CD3 using anti-HIV-1 Gag p55/p24 and anti-CD3 antibodies, and analyzed by flow cytometry. The gating in CellQuest was set to exclude 7-AAD\(^+\) dead cells, and HIV-1 Gag p24/p55 expression is plotted on the x-axis versus CD3 on the y-axis. The percentages of Gag positive CD3\(^+\) T cells are given in the right upper quadrants. One tissue donor out of two donors examined is shown.

**Figure 2.** *Ex vivo* pre-exposure prophylaxis inhibits HIV-1 genomic integration in vaginal intraepithelial cells. Vaginal epithelial sheets from six tissue donors were incubated with the indicated compounds for 1 h at 37°C, spinoculated with 100 ng/ml HIV-1 Gag p24 of the CCR5-tropic laboratory clone HIV-1 JRCSF (A, B) or the primary mucosal isolate HIV-1 M1 (C) for 2 h, washed extensively, and cultured for 2 days. Total DNA was isolated from the sheets and emigrated cells, and the amount of integrated HIV-1 DNA in tissues treated with microbicides before infection was determined relative to the amount of integrated HIV-1 DNA in tissue not treated with microbicides before infection (=100%). (A) In initial experiments, the PCR reaction for the housekeeping gene $\beta$-actin, which is used to normalize variability in DNA input, was performed in
separate wells (singleplex PCR assay).  (B, C) Subsequently, conditions were optimized to run the Alu-LTR and the β-actin PCR reaction in the same wells (multiplex PCR assay). Each PCR assay was performed independently, either by four operators for the singleplex assay (A) or two operators for the multiplex assay (B, C). Individual PCR reactions were performed in quadruplicates and averaged. Depicted values represent means and error bars depict the standard deviations across individual operators. The concentrations of the compounds used to treat the tissues were based on previously published in vitro studies (11, 13, 15, 43) and were as follows: CXCR4 antagonist AMD-3100, 0.794 µg/ml (1 µM); fusion inhibitor T-20 (manufactured by the Division of AIDS, NIAID), 1 µg/ml (0.223 µM); CCR5 antagonist TAK-779, 0.531 µg/ml (1 µM); and integrase inhibitor 118-D-24, 7.416 µg/ml (30 µM). The viral inoculum contained negligible amounts of Alu-LTR DNA.

Figure 3. Titration and comparison of the HIV-inhibitory effect of N-acetylated T-20 (Fuzeon®), free T-20, and cellulose sulfate in the vaginal epithelium.  (A) T-20 titrations. Vaginal epithelial sheets from 6 tissue donors were incubated either with the T-20 peptide manufactured by Roche (Fuzeon®) (T-20 Roche) or the T-20 peptide produced by the Division of AIDS (NIAID) (T-20 DAIDS) at the indicated concentrations and then infected with R5-tropic HIV-1JRCSF (100 ng/ml HIV-1 Gag p24).  (B) Cellulose sulfate titration. Vaginal epithelial sheets from 4 tissue donors were incubated with cellulose sulfate (Ushercell®) at the indicated concentrations and then infected with R5-tropic HIV-1JRCSF (100 ng/ml HIV-1 Gag p24). In (A) and (B), infections and measurement of integrated HIV-1 DNA were performed as outlined in the legend to
Figure 2. Individual PCR reactions were performed in quadruplicate and averaged. Colors represent measurements derived from different donor tissues. Solid and dashed lines of similar color represent two independent experiments performed with the same donor tissue. (C) IC_{50} determination for T-20 Roche, T-20 DAIDS, and cellulose sulfate. Dose-response curves were fitted on the data in (A) and (B) by nonlinear regression and IC_{50} concentrations were determined using Prism 4.0. Mean values of the individual data points in (A) and (B) are depicted; error bars represent the standard deviations. Concentrations were log10 transformed and relative viral integration values were normalized to percent of maximum response. (D) IC_{50} determination for T-20 Roche, T-20 DAIDS and cellulose sulfate in PHA-activated T cells pre-treated in vitro with indicated microbicides and infected with R5-tropic HIV-1_{JRCSF} similarly to the vaginal epithelial sheets (n=2 blood donors; means and standard deviations are shown).
Figure 1

A

B

C

Calcium, no virus

No calcium, virus

Calcium, virus

0.5%

1.5%

10.8%
Figure 2

A) Relative viral integration (%).

B) Relative viral integration (%).

C) Relative viral integration (%).
Figure 3

A

B

C

D

Concentration (µg/ml)

Relative viral integration (%)

T-20 Roche

Fuzeon®

T-20 DAIDS

Concentration (µg/ml)

Relative viral integration (%)

Cellulose sulfate

(IC50: 6.1)

Fuzeon®

(IC50: 0.061)

T-20 DAIDS

(IC50: 0.034)

T-20 Roche

Donor 8

Donor 9

Donor 10

Donor 11

Donor 12

Donor 13

Concentration (µg/ml)

Relative viral integration (%)

Cellulose sulfate

(IC50: 1.81)

T-20 Roche

Fuzeon®

T-20 DAIDS

Concentration (µg/ml)

Relative viral integration (%)

Cellulose sulfate

(IC50: 0.23)

T-20 DAIDS

(IC50: 0.0007)