Pharmacodynamic correlations using fresh and cryopreserved tissue following use of vaginal rings containing dapivirine and/or maraviroc in a randomized, placebo controlled trial

Charlene S. Dezzutti, PhD, Nicola Richardson-Harman, PhD, Lisa C. Rohan, PhD, Mark A. Marzinke, PhD, Craig J. Hoesley, MD, Lori Panther, MD, MPH, Sherri Johnson, MPH, Jeremy P. Nuttall, MS, Annalene Nel, MD, PhD, Beatrice A. Chen, MD, MPH, On behalf of the Microbicide Trials Network, MTN-013/IPM 026 Protocol Team

Abstract

Background: The ex vivo challenge assay is a bio-indicator of drug efficacy and was utilized in this randomized, placebo controlled trial as one of the exploratory endpoints. Fresh and cryopreserved tissues were evaluated for human immunodeficiency virus (HIV) infection and pharmacokinetic (PK)/pharmacodynamic (PD) relationships.

Methods: HIV-negative women used vaginal rings containing 25 mg dapivirine (DPV)/100 mg maraviroc (MVC) (n = 12), DPV only (n = 12), MVC only (n = 12), or placebo (n = 12) for 28 days. Blood plasma, cervicovaginal fluid (CVF), and cervical biopsies were collected for drug quantification and the ex vivo challenge assay; half (fresh) were exposed immediately to HIV while the other half were cryopreserved, thawed, then exposed to HIV. HIV replication was monitored by p24 enzyme-linked immunosorbent assay from culture supernatant. Data were log-transformed and analyzed by linear least squared regression, nonlinear $E_{\text{max}}$ dose–response model and Satterthwaite t test.

Results: HIV replication was greater in fresh compared to cryopreserved tissue ($P = 0.04$). DPV was detected in all compartments, while MVC was consistently detected only in CVF. Significant negative correlations between p24 and DPV levels were observed in fresh cervical tissue ($P = 0.01$) and CVF ($P = 0.03$), but not plasma. CVF MVC levels showed a significant negative correlation with p24 levels ($P = 0.03$); drug levels in plasma and tissue were not correlated with HIV suppression. p24 levels from cryopreserved tissue did not correlate to either drug from any compartment.

Conclusion: Fresh tissue replicated HIV to greater levels and defined PK/PD relationships while cryopreserved tissue did not. The ex vivo challenge assay using fresh tissue could prioritize drugs being considered for HIV prevention.

Abbreviations: BLQ = below limit of quantification, CPA = cryopreserving agent, CVF = cervicovaginal fluid, DMSO = dimethyl sulfoxide, DPV = dapivirine, $E_{\text{50}}$ = 50% effective concentration, HIV = human immunodeficiency virus, IPM = International Partnership for Microbicides, MTN = Microbicide Trials Network, MVC = maraviroc, PD = pharmacodynamic, PK = pharmacokinetic, VR = vaginal ring.

Keywords: bio-indicator, ex vivo challenge assay, HIV prevention, microbicide, mucosal tissue
1. Introduction

Many drug candidates have been considered for microbicidal development. Preclinical testing has shown most are potent against human immunodeficiency virus (HIV) in vitro. Likewise, mucosal tissue and animal models have evaluated unformulated and formulated drugs, with most candidates showing promise for human clinical testing.\[1\] These data provide proof-of-concept that most candidate microbicidal products, when tested using in vitro assays and animal models, are effective against HIV. However, with the exception of 2 trials,\[17,18\] numerous formulated drugs evaluated in phase 2b/3 clinical trials failed to demonstrate efficacy and in some cases have increased HIV acquisition.\[19–24\] Limitations exist for all of the preclinical testing models as none can fully recapitulate product use by a participant. Consequently, new models are needed.

The ex vivo challenge assay is the next development in testing product efficacy. This assay entails participants using a study product for a period of time followed by mucosal tissue collection via biopsies that are transported to a laboratory and exposed to HIV.\[25–27,28,29\] Using this method, participants applied aqueous-based gels containing UC781,\[26\] tenofovir,\[25\] or their respective vehicle controls rectally, followed by colonic biopsy extraction and exposure of the biopsy tissue to HIV in the laboratory. For both drugs, there was a dose–response where higher tissue drug levels correlated to reduced viral replication.\[25,30\] The use of the ex vivo challenge assay with female genital tract tissue has been more challenging due to the restriction on the number and frequency of mucosal sampling. Nevertheless, cervical and vaginal tissues have been assessed for HIV replication\[27\] and used to evaluate 3 dosage forms (aqueous gel, film, and vaginal ring [VR]) of 2 drugs (dapivirine [DPV] and maraviroc [MVC]).\[28,29\] Findings from these studies have again identified dose–responses between higher tissue drug levels and reduced viral replication.

Expanding the utility of this assay to multiple sites in a clinical trial is a priority as it will streamline testing and increase diversity of populations for pharmacogenomic considerations.\[31,32\] To accomplish site expansion, capacity must be built or tissue must be cryopreserved. Because of the complexity of developing the infrastructure needed for exposing tissue to HIV, cryopreserving tissue at the clinical sites is a more feasible option. Multiple sites could participate in the clinical study; collect, cryopreserve and ship the mucosal tissue to a central laboratory for processing. Using this approach, Microbicide Trials Network (MTN)-013/International Partnership for Microbicides (IPM) 026 was designed as a multisite clinical study evaluating the safety and pharmacokinetics (PK) of VRs containing DPV/MVC, DPV only, MVC only, or no drug (placebo).\[31,32\] VRs were fitted and after 28 days the rings were removed and specimens were collected including blood plasma, cervicovaginal fluid, and cervical tissue biopsies for PK and pharmacodynamic (PD) testing. To compare the feasibility of cryopreserving tissue, 1 site immediately used the cervical tissues (fresh) for the ex vivo challenge assay, while 2 other sites cryopreserved and shipped the cervical tissue to a central laboratory. Here, we characterize the PK/PD relationships using fresh and cryopreserved tissue to determine their utility for use in the ex vivo challenge assay.

2. Materials and methods

2.1. Study design

MTN-013/IPM 026 (NCT01363037) was a multisite, double-blind, randomized, placebo-controlled trial in 48 HIV-negative sexually abstinent women randomized 1:1:1:1 to 1 of the 4 study arms (25 mg DPV/100 mg MVC, 25 mg DPV only, 100 mg MVC only, or placebo) at 3 clinical sites (Birmingham, AL; Boston, MA; and Pittsburgh, PA).\[29\] The sites received IRB approval prior to initiation of the protocol. This sample size was based upon the size of similar phase 1 studies of vaginal microbicidal products. Study arm randomization was stratified by site to ensure balanced assignment of products at each site and was generated and maintained by the statisticians. The statisticians provided each study site with 1 set of randomization envelopes to be stored and used in the study clinic. A sufficient number of envelopes were assigned to each site so that they could enroll up to 24 participants. Clinic staff assigned these envelopes in sequential order, by envelope number, to eligible participants. Each randomization envelope included study product arm randomization assignment as well as end of study period PK/PD sampling time assignment. The complete protocol and supporting materials can be found at http://www.mtnstopshiv.org/studies/2241. The primary and secondary objectives were previously reported.\[29\] The capacity of drugs to protect against HIV infection of mucosal tissue was part of the exploratory objective of this trial and presented here.

2.2. Specimen collection and processing

VRs were used for 28 days then removed prior to blood, CVF, and ectocervical tissue collection. Plasma was separated from whole blood and aliquots were frozen at –20°C. CVF was collected at the surface of the cervix using Tegaderm™ strips which were weighed and frozen at –20°C. Last, 3 cervical biopsies were collected without the use of topical antiseptic and anesthetic.\[27\] At all sites, 2 cervical biopsies were weighed and snap frozen for drug quantification. At Birmingham and Boston, the 3rd cervical biopsy was weighed, placed in cryopreserving agent (CPA) (10% dimethyl sulfoxide [DMSO]/90% fetal bovine serum), and cooled overnight to –80°C. Biopsies were stored at –80°C until shipped for the ex vivo challenge assay. At Pittsburgh, the 3rd cervical biopsy was placed in transport medium on wet ice, brought to the laboratory within 30 minutes of collection, and immediately used for the ex vivo challenge assay.\[27\] All testing was performed blinded to study arm with analysis done after the database was locked.

2.3. In vitro vaginal ring analysis

To define drug release from the MVC only, DPV only, and MVC/DPV VRs, in vitro release studies were carried out using a USP method II (paddle method) apparatus, (Distek, North Brunswick, NJ). A 1% w/v cremorphor solution was used as the dissolution medium and maintained at 37°C using a circulation water bath. In triplicate, aliquots were taken daily (except weekends) for drug content analysis through 14 days and fresh medium was replenished. Aliquots were analyzed using a high pressure liquid chromatography method. Separation was achieved by gradient elution using a Waters high performance liquid chromatography system (Millvale, MA) with a SunFire C8 2.5 μ 50 × 4.6 mm high performance liquid chromatography column (Waters, Millvale, MA). The wavelengths for detection of DPV and MVC were 290 and 210 nm, respectively. The limit of detections for DPV and MVC were 0.4 and 1.0 ng/ml, respectively.

2.4. Drug quantification for clinical samples

DPV and MVC concentrations from plasma and CVF were quantified via validated ultraperformance liquid chromatographic-tandem mass spectrometric methods.\[29,33–35\] The limits of
quantification for DPV in plasma and CVF were 0.02 ng/mL and 0.05 ng/tear strip (2 ng/mL), respectively. The limits of quantification for MVC in plasma and CVF were 0.5 ng/mL and 0.025 ng/tear strip (1 ng/mL), respectively. Following homogenization and protein precipitation, tissue DPV and MVC concentrations were determined via ultraperformance liquid chromatographic-tandem mass spectrometry with limits of quantification of 0.05 ng/sample (50 ng/mL) and 0.20 ng/sample (200 ng/mL), respectively.

2.5. Ex vivo challenge assay

Fresh cervical biopsies (N = 24) were immediately used for the ex vivo challenge assay. Briefly, biopsies were exposed to HIV-1BaL, washed, and placed in fresh culture medium. Every 3 to 4 days, culture medium was collected, stored at -80°C, and replenished. At study end, the tissue was weighed. HIV replication was measured in the stored culture medium using a p24 enzyme-linked immunosorbent assay (Alliance; Perkin-Elmer Life Sciences Inc., Boston, MA).

Frozen cervical biopsies (N = 22) were quickly thawed to retain a small piece of ice and then transferred to 1 mL of cold, fresh culture medium in a well of a 48-well plate, covered and rocked for 5 minutes at room temperature. Over the next 20 minutes, the cervical biopsies were transferred 4 more times to wells containing fresh culture medium to slowly remove the DMSO from the tissue. After the 5th wash, the cervical biopsies were treated with HIV and cultured as described above.

2.6. Statistical analysis

Cumulative p24 was corrected for weight of the tissue remaining at culture end. PK and PD data were log10 transformed after below limit of quantification (BLQ) values had been imputed at 1/2 the lower limit of quantification. A log–log plot was used to linearize the PK/PD relationship that was then tested using a linear, least squares regression where the probability value of the slope indicated a relationship that was significantly different to zero slope. A nonlinear Emax model with variable slope (Equation 1) was used to further evaluate statistically significant (P ≤ 0.01) linear dose–response relationships.

\[ E = \frac{E_0 + (E_{\text{Max}} - E_0)}{1 + 10^{(\log EC_{50}-\text{conc})/H}} \] (1)

A Satterthwaite t test to compare groups with unequal variance was used to compare cumulative p24 data at day 11 between fresh and cryopreserved biopsy tissues for placebo subjects.

3. Results

3.1. Capacity of cryopreserved tissue to replicate HIV

The capacity of cryopreserved tissue to support HIV replication was analyzed from the participants using the placebo VR. Only 4 cryopreserved samples were available for analysis since 2 participants randomly assigned to the placebo ring arrived at the Birmingham clinical site for their day 28 visit with their VRs already removed, thus cervical biopsies were not taken. Using cumulative p24 through day 11 of the culture, fresh tissue replicated HIV to 2.64 log10 p24 pg/mg (n = 6) compared to cryopreserved tissue that replicated HIV to 1.16 log10 p24 pg/mg (n = 4; Fig. 1). The 1.48 log10 difference in p24 was significant (P = 0.04).

3.2. Drug release from the vaginal rings

In vitro release of DPV and MVC was performed to determine drug release rates from the VRs (Table 1), which helps define drug availability. DPV had a linear release profile from the single and combination VR with similar release rates (229.6 μg/hour1/2 and 241.12 μg/hour1/2, respectively), which was approximately 776 and 814 nM in the 1st hour, more than 350-fold above the in vitro 50% effective concentration (EC50). Although both VRs had linear release kinetics, twice as much MVC was released from the MVC only VR as compared to the combination VR (120.51 μg/hour1/2 and 55.49 μg/hour1/2, respectively), which was 260 and 119 nM in the 1st hour, 100- and 50-fold above the in vitro EC50, respectively.

3.3. PK/PD relationships using fresh cervical tissue

DPV was quantified in all compartments tested; DPV concentrations ranked CVF > cervical tissue > plasma. Conversely, MVC was quantified from all CVF, 4 cervical tissue (2 detectable but BLQ), and no plasma samples. Significant negative correlations between DPV and HIV p24 levels were found with cervical tissue (P = 0.01; Fig. 2A; left panel) and CVF (P = 0.03; Fig. 2B; left panel). There was no correlation between plasma and protein precipitation, tissue DPV and MVC concentrations were determined via ultraperformance liquid chromatographic-tandem mass spectrometric with limits of quantification of 0.05 ng/sample (50 ng/mL) and 0.20 ng/sample (200 ng/mL), respectively.

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Table 1

| Product                  | Drug       | Release rate (μg/hour1/2) | Regression coefficient (R²) |
|-------------------------|------------|---------------------------|----------------------------|
| DPV single entity ring  | DPV        | 229.60 ± 6.86             | 0.99 ± 0.01                |
| DPV single entity ring  | MVC        | 120.51 ± 0.07             | 0.99 ± 0.01                |
| DPV/MVC combination ring| DPV        | 241.12 ± 101.4            | 0.96 ± 0.05                |
| DPV/MVC combination ring| MVC        | 55.49 ± 5.96              | 0.95 ± 0.03                |

DPV = dapivirine, MVC = maraviroc.

*Regression was done for the linear portion of the release data from day 1 to day 14 (n = 3).
DPV and HIV p24 levels ($P=0.65$; Fig. 2C; left panel). There was no correlation between MVC tissue levels and ex vivo tissue p24 levels (Fig. 2A; right panel). High drug levels in CVF (Fig. 2B; left and right panel) resulted in significant ($P=0.03$) dose–response correlations for DPV and MVC levels in CVF and HIV suppression in ex vivo cervical tissues from the same subjects. MVC concentrations in plasma were BLQ and thus correlations could not be defined (Fig. 2C; right panel).

A nonlinear dose–response model was fit to the dose–response relationship between fresh tissue HIV growth in the ex vivo challenge model and DPV levels in tissue, using the placebo HIV levels as the virus control. The nonlinear $E_{max}$ model was fit to the more robust linear relationship between DPV levels in fresh tissue and suppression of HIV in the ex vivo challenge assay (Fig. 3). Percent virus control was calculated as the % change in p24 compared to the upper 95% confidence interval of the placebo levels at day 11 to provide a comparison to vigorous virus growth. An EC$_{50}$ of approximately 100 ng/mL of DPV was found where concentrations higher than 100 ng/mL were predicted to be necessary to suppress HIV to less than 50% of the placebo control levels.

### 3.4. PK/PD relationships using cryopreserved cervical tissue

Lack of viral replication (p24 < 3 log$_{10}$ pg/mg; Fig. 4) was noted for the majority of cryopreserved tissues regardless of study arm. Consequently, no significant dose–response correlations were defined between ex vivo tissue p24 levels and tissue, CVF, or plasma DPV/MVC levels (Fig. 4A–C). However, a trend ($P=0.06$) for a positive correlation between p24 and MVC CVF levels was observed (Fig. 4B; right panel).
Tenofovir gel. Tenofovir is a prodrug and needs to be metabolized to be effective as a DNA chain terminator and inhibit HIV replication. One trial evaluating tenofovir gel for rectal use utilized the ex vivo challenge assay to define PD/PK correlates. Strong negative correlations, lower HIV cumulative p24 levels associated with higher tenofovir drug levels, were found in isolated mucosal immune cell populations and mucosal tissue, while a weak negative correlation with the luminal fluid and no correlation with blood plasma were determined. These data show the best correlations were derived using the PK matrix, similar to the PD matrix (tissue/mucosal immune cells) and are similar to the findings presented here using fresh tissue with different antiretroviral drugs. Collectively, these data suggest the PK/PD relationships should be developed using the same matrices or those closest to each other.

DPV concentrations in fresh tissue were associated with significantly reduced cumulative p24 levels. A nonlinear dose–response model for the relationship between fresh tissue p24 and tissue DPV drug levels was tested where DPV concentrations above 100 ng/mL (>300 nM) would be needed to suppress 50% or more of HIV growth found in placebo, untreated, subject biopsy tissue. The single agent and combination rings demonstrated similar in vitro DPV release and suppression of HIV. DPV levels in the cervical tissue ranged from 30 to 14,170 ng/mL, which equates to 0.125 nM to 430 μM, with a median DPV level of >1800 nM. These tissue levels are consistent with another clinical trial evaluating a similar DPV single agent VR with tissue DPV levels ranging between 140 nM and 14 μM. Tissue drug transporters are not known for DPV, but drug is lost quickly after removal of the ring. Thus, higher tissue DPV concentrations may be achieved while the VR is in use and should exceed the amount needed to block HIV infection in the majority of ring users. Indeed, it was recently shown that use of a DPV VR by more than 2600 women in a phase 3 clinical trial demonstrated a significant reduction of HIV acquisition with higher adherence demonstrating better efficacy.

Unfortunately, MVC failed to achieve measureable drug levels in the majority of fresh cervical tissues and in plasma, thus no PK/PD correlations could be made. It is currently unclear why MVC levels in these matrices were BLQ as drug was released from the ring as shown here (Table 1) and with analysis of the used VRs from the participants showing ~5 mg of the 100 mg of MVC was released over 28 days of use. Higher MVC levels in CVF were associated with reduced cumulative p24 in fresh tissue, similar to that achieved with DPV. Of interest, the MVC levels obtained in the CVF samples were greater for the single agent as compared to the combination ring. Indeed, tissues with detectable MVC were from the MVC only VR group. This trend was not unexpected based on the varied release rates obtained for MVC from the in vitro studies. Lack of quantifiable MVC could be partially due to active transport out of the tissue by the P-glycoprotein/MDR1 transporter which is highly expressed in the female genital tract and impacted MVC accumulation. The amount of drug released from the VR may not have been sufficient to overcome this efflux.

Cryobiological techniques were first used in 1949 with the discovery of the cryoprotective properties of glycerol to preserve sperm. Since then, cryopreservation of cells, including peripheral blood mononuclear cells, are routinely performed using DMSO with a constant cooling rate, typically 1°C/minute and storage ≤−150°C. DMSO and glycerol decrease ice crystal formation inside the cells, reducing cell damage. However, these agents can be toxic so care is taken to minimize exposure of cells/tissue at room temperature. At Birmingham and Boston, cervical tissue was cryopreserved using a standard peripheral blood
mononuclear cells cryopreservation protocol. At study end, the biopsies were sent to the central laboratory to be thawed and washed to ensure removal of the DMSO. The cryopreserved tissue did not show robust HIV replication as compared to the fresh tissue and thus PK/PD correlations with DPV or MVC could not be reliably assessed. Although tissue viability was not determined due to the limited number of biopsies collected, a previous report demonstrated good retention of cervical tissue viability and equivalence of HIV replication between fresh and “snap-frozen” tissue (frozen without a CPA). This is in contrast to another study demonstrating cryopreserved colonic tissue was not reproducibly infected with HIV and showed moderate to severe histological damage despite retention of metabolic activity. The paucity and inconsistency of published studies indicate that the various potential methods for cryopreservation of mucosal tissues have not been systematically evaluated. This is important because optimal cryopreservation will likely allow for more robust and reproducible viral replication, comparable to infection of fresh tissues. Biobanks have been cryopreserving skin to help with wound healing, in particular for burn victims, for several decades. The use of DMSO is common in these applications. Recently the inclusion of trehalose with DMSO has shown improved tissue viability and decreased wound healing time when tissues were engrafted onto recipients as compared to tissues cryopreserved with DMSO alone. Trehalose is a carbohydrate found in prokaryotes and invertebrates and is thought to stabilize proteins and membranes when the organism undergoes stress such as heat, cold, oxidation, and desiccation. The use of a nonpenetrating CPA (trehalose) with a penetrating CPA (DMSO) may benefit mucosal tissue viability for use in the ex vivo challenge assay. Independent of the cryopreservation method, it is conceivable that penetration of the chosen CPA into the tissue/cells could modify the drug content by solubilizing it. As the cells are warmed, the CPA leaves and water enters the cell, which could flush the solubilized drug out of the tissue/cells, which is then

Figure 4. DPV and MVC concentration and cryopreserved cervical tissue cumulative p24 dose–response relationships. DPV and MVC were quantified from paired cervical tissue (A), CVF (B), and plasma (C) on day 28 of VR use. The ex vivo challenge assay was performed on tissue collected on day 28 of VR use and cumulative p24 from day 11 are shown. The data shown are for 12 participants (n = 6 DPV only VR [red circle], n = 6 DPV/MVC VR [blue circle], left panels; and n = 6 MVC only VR [green circle], n = 6 DPV/MVC VR [blue circle], right panels). The vertical dotted line represents the limit of quantification for each matrix. CVF = cervicovaginal fluid, DPV = dapivirine, MVC = maraviroc, VR = vaginal ring.
washed away. This could affect the drug’s impact on HIV infectivity ex vivo. The use of trehalose may offset the osmotic changes by increasing the osmolality of the extracellular solution creating an “osmotic buffer,” but this remains to be tested. How osmotic changes that occur during freezing and thawing affect drug concentration in tissues collected after product use is not known and more work is needed to understand this issue. In summary, HIV challenge of fresh cervical tissue and tissue DPV levels provided the best PK/PD correlations. The further removed the PK matrix (tissue CVF plasma) was from the tissue the weaker was the DPV-associated HIV suppression. Lack of sufficient MVC in the tissue and plasma precluded testing of PK/PD dose–response relationships in these compartments. However, a PK/PD dose–response was found with MVC in the CVF which was consistent with DPV-mediated virus suppression in the fresh tissue for the ex vivo challenge assay. Cryopreserved tissue did not support HIV replication and thus did not allow us to define PK/PD dose–responses. Although optimizing a cryopreservation protocol for tissue to allow for reproducible HIV replication is needed, the impact cryopreservation may have on tissue drug content needs to be defined. Expanding the clinical sites to include those that can collect, cryopreserve the tissues, and ship to a central laboratory remains elusive at this time. Although these data provide additional support for the incorporation of the ex vivo challenge assay into early clinical trials, the implementation of the ex vivo challenge assay should be in laboratories with adequate resources and in close proximity to the clinical site.

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