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Distinct Pharmacodynamic Activity of Rilpivirine in Ectocervical and Colonic Explant Tissue

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A long-acting injectable form of rilpivirine (RPV) is being evaluated in clinical trials for the prevention of HIV infection. Preclinical testing was undertaken to define RPV pharmacokinetic (PK) and pharmacodynamic (PD) activities in ectocervical and colonic tissue treated *in vitro*. Tenfold dilutions of RPV were added to the basolateral medium of polarized ectocervical and colonic explant tissues. To half the explants, HIV-1_{BaL} was applied to the apical tissue surface. After culture overnight, all the explants were washed and the RPV in the explants not exposed to HIV was quantified using a validated liquid chromatography-mass spectrometry assay. For efficacy, explants exposed to HIV remained in culture, and supernatants were collected to assess viral replication using a p24 enzyme-linked immunosorbent assay. The data were $\log_{10}$ transformed, and PK/PD correlations were determined using GraphPad Prism and SigmaPlot software. The application of RPV to the basolateral medium at 10 μM and 1 μM was effective in protecting ectocervical and colonic tissues, respectively, from HIV infection. When the RPV in paired ectocervical and colonic explant tissues was quantified, significant inverse linear correlations ($P < 0.001$) between p24 and RPV concentrations were obtained; more viral replication was noted at lower drug levels. Using a maximum effect model, RPV concentrations of 271 nM in ectocervical tissue and 45 nM in colonic tissue were needed to achieve a 90% effective concentration (EC_{90}). These data demonstrate that RPV can suppress HIV infection in mucosal tissue but that higher levels of RPV are needed in female genital tract tissue than in gastrointestinal tract tissue for protection.

Rilpivirine (RPV) is a nonnucleoside reverse transcriptase inhibitor (NNRTI) in the diarylpyrimidine family (1), which includes dapivirine (DPV; TMC120) and etravirine (TMC125). These NNRTIs have shown better activity against efavirenz- and/or nevirapine-resistant HIV clinical isolates, and in particular, >60% of isolates resistant to first-line NNRTIs are sensitive to RPV (2). RPV was approved for treatment by the U.S. FDA in 2011. Because of the improved safety profile of RPV compared to that of efavirenz (3), there was interest to create a long-acting (LA) formulation. A nanosuspension of an LA formulation of RPV (RPV LA) was subsequently developed for parenteral delivery and demonstrated good pharmacokinetic (PK) profiles in animals (4) and humans (5, 6). Blood plasma RPV levels were sustained through 60 days in persons receiving the 1,200-mg dose. The LA formulation could improve treatment adherence, as monthly (or possibly less frequent) injections rather than daily pills would be needed. With the extended dosing, there is now interest to investigate RPV LA for use as an HIV preventative.

The clinical trials evaluating topical and oral tenofovir (TFV)-based regimens for prevention have had discrepant results, which were attributed to differential rates of adherence to the study product (7–12). Ongoing analysis of these trial PK results has shown that the detection of drug in blood plasma or cervicovaginal fluid (adherence to study product) correlates with protection from seroconversion (13–16). The use of blood plasma and mucosal fluid for developing PK/pharmacodynamic (PD) correlates is important, but drug levels in tissue can provide more accurate PK/PD correlations (17, 18). RPV quantification has shown that it penetrates rectal tissue better than cervical and vaginal tissue (5, 6). To begin to address the concentration of RPV needed to prevent HIV acquisition, we utilized our polarized ectocervical and colonic explant tissue models to define PK/PD correlates *in vitro*. Our assumption is that the RPV concentrations needed to demonstrate HIV suppression would be higher in colonic tissue than ectocervical tissue due to the differences in HIV immune targets between the two mucosal tissues (19–21). These data should help to provide linkages between preclinical/clinical PK associations with PD results ultimately to improve our preclinical models with the aim of informing clinical outcomes.

MATERIALS AND METHODS

Products. The RPV drug substance was kindly provided by Janssen Pharmaceutica, Belgium. Stocks were made in dimethyl sulfoxide, and aliquots were stored at −20°C in the dark.

Virus. HIV-1_{BaL} (Advanced Biotechnologies Inc., Eldersburg, MD) was used for the efficacy testing, as it represents a virus that uses CCR5 and it readily infects the mucosal tissue utilized for preclinical testing algorithms (22–24) as well as for the *ex vivo* challenge assay (6, 25, 26). The 50% tissue culture infectious dose ($TCID_{50}$) in peripheral blood mononuclear cells was determined using the Reed and Muench method (27). Virus aliquots were made and stored at −80°C until use.

Human tissue. Surgically resected normal human ectocervical and colonic tissues were collected through institutional review board-approved protocols (PRO 0503103 and PRO 0602024, respectively) at the University of Pittsburgh. Tissue was placed in transport medium on wet ice and brought to the laboratory for use. Ectocervical tissue was also

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purchased from the National Disease Research Interchange (NDRI; http://ndriresource.org/) and transported overnight on wet ice.

Drug quantification. The tissue concentrations of RPV were determined by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method as recently reported by Else and colleagues (28). The lower limit of quantification for tissue was 0.05 ng/sample.

Efficacy testing. TZM-bl cells (29) (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were used to determine the 90% effective concentrations (EC_{90}s) and 90% cytotoxic concentrations (CC_{90}s) of RPV using the 4-parameter maximum effect (E_{max}) model $\{y = \min + (\max \times \min)/[1 + (x/EC_{90})^{-\text{Hill slope}}]\}$, where y is the effective concentration, max is the maximum concentration, min is the minimum concentration, and x is the input drug concentration (SigmaPlot11; Systat Software, Inc., San Jose, CA). TZM-bl cells were plated, and 100 μl of 10-fold serially diluted RPV was applied. For cytotoxicity testing, 100 μl of medium was added to each well. On the next day, 100 μl of medium was removed and replaced with 100 μl of the CellTiter-Glo reagent (Promega Corp., Madison, WI) and the luminescence was measured. Cytotoxicity was determined on the basis of deviations from the results for the controls, consisting of cells not treated with RPV, and is presented as the percent cytotoxicity ± standard error of the mean (SEM). For efficacy testing, 100 μl of medium containing 3,000 $TCID_{50}$s of HIV-1_{BaL} was added to each well. After 48 h, 100 μl of medium was removed and replaced with 100 μl of the Bright-Glo reagent (Promega Corp.) and the luminescence was measured. Efficacy was determined on the basis of deviations from the results for the untreated controls infected with HIV-1 and is presented as the percent reduction ± SEM.

Polarized ectocervical and colonic explant tissue cultures were assembled as previously described (30). Briefly, muscle tissue was removed, leaving the lamina propria and epithelium; 5-mm explants were made using a dermal biopsy specimen punch. Explants were maintained at the air-liquid interface with the luminal side up in a transwell, and the edges were sealed with Matrigel matrix (BD Biosciences, San Jose, CA). The lamina propria was immersed in medium for ectocervical explant tissues or was allowed to rest on medium-soaked gel foam for colonic explant tissues and maintained at 37°C in a 5% CO_2 atmosphere. RPV dilutions (10 to 0.01 μM) were added to the basolateral medium on the day of setup. In some tissues, half of the explants were used for PK analysis and the other half were used for PD analysis. For the explants used for PD analysis, HIV-1_{BaL} (5×10^4 $TCID_{50}$s for ectocervical tissue or 1×10^4 $TCID_{50}$s for colonic tissue) was added to the apical tissue surface 24 h after the application of RPV. All explants were cultured for an additional 24 h before being washed. For the PD explants, fresh culture medium without RPV was added to the basolateral compartment. Every 3 to 4 days for 21 days, the culture supernatant was collected, replenished, and stored at −80°C. Viral replication was monitored by enzyme-linked immunosorbent assay (ELISA) of the culture supernatants for HIV-1 p24gag (Alliance; Perkin-Elmer Life Sciences Inc., Boston, MA). The PK explants treated with RPV only were weighed after they were washed and were frozen for RPV quantification. For explants with drug levels below the level of quantification (BLQ), the RPV concentrations were imputed as half the lower limit of detection (LLOD).

Correlations between the $\log_{10}$-transformed p24 levels at the end of culture (day 21) and the $\log_{10}$-transformed drug levels were defined by the use of GraphPad Prism (v5.02) software (La Jolla, CA) using a linear, least-squares regression, where the probability value of the slope indicated a relationship that was significantly different from the zero slope ($P < 0.05$). To define the tissue effective dose, percent inhibition was calculated for each explant on the basis of the value for the untreated p24 control on day 21 of culture, which was considered to be 100% infected. Using the 4-parameter E_{max} model (SigmaPlot11), nonlinear associations were defined and the EC_{90} was calculated on the basis of the significant ($P < 0.05$) linear dose-response relationships.

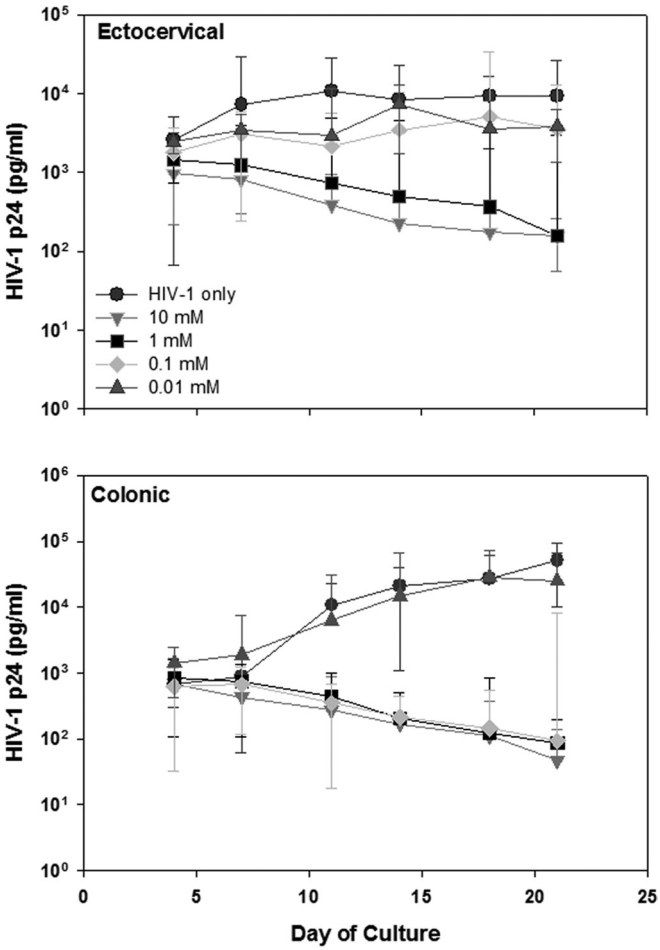

FIG 1 Efficacy of rilpivirine in polarized ectocervical and colonic explant tissues. Ectocervical and colonic explant tissues were placed in a polarized configuration, and different dilutions of rilpivirine were applied to the basolateral medium on the day before HIV-1_{BaL} was added to the apical surface. After an overnight culture, the explants were washed and fresh basolateral medium without rilpivirine was added. Every 3 to 4 days, the basolateral supernatant was collected, stored, and replenished. HIV replication in the basolateral supernatant was monitored by p24 ELISA. The data are presented as the median ± 95% confidence interval for four to seven independent tissue specimens tested in duplicate.

RESULTS

The TZM-bl cell assay showed that RPV had activity against HIV-1_{BaL}, which was used in our mucosal explant tissues, at a nanomolar concentration (EC_{90}, 1.67 nM), which is consistent with its activity against other group M viruses (2). The CC_{90} was 5,827 nM, indicating that RPV has minimal toxicity. With RPV being evaluated for use for the prevention of HIV infection, our interest was to determine its potency in mucosal explant tissue *in vitro*. Polarized ectocervical and colonic tissues were treated with 10-fold dilutions of RPV in the basolateral medium. HIV-1_{BaL} replicated well in ectocervical and colonic explant tissues throughout the culture period, as noted in the control explants infected with HIV-1 only (Fig. 1). Viral replication was suppressed in all of the ectocervical explant tissues dosed with 10 μM RPV and in colonic explant tissues dosed with 1 μM RPV (Fig. 1; Table 1). Addition of lower concentrations of RPV to the basolateral medium resulted

TABLE 1 Inhibition of HIV in ectocervical and colonic explant tissues treated with RPV

RPV concn (μM)[a]	Ectocervical explants		Colonic explants	
	No. protected/no. tested	% inhibition[b]	No. protected/no. tested	% inhibition
10	10/10	98	9/9	99.8
1	7/12	98	15/15	99.8
0.1	2/10	62	11/15	99.7
0.01	0/6	59	2/8	31.0

[a] Concentration of RPV added to the basolateral supernatant.
[b] Percent inhibition was calculated using p24 values from day 21 of culture.

TABLE 2 RPV concentration in ectocervical and colonic explant tissues

RPV concn (μM) added to culture	Ectocervical explants		Colonic explants	
	Mean RPV concn ± SD (ng/ml)	% RPV in tissue[a]	Mean RPV concn ± SD (ng/ml)	% RPV in tissue
10	282 ± 116	8	4,624 ± 1,745	126
1	20 ± 11	6	467 ± 103	128
0.1	BLQ[b]	ND	47 ± 10	127
0.01	BLQ	ND	3.4 ± 1.2	100

[a] Tissue concentration as a percentage of the total amount of RPV added to the culture. ND, not determined.
[b] BLQ, below the limit of quantification.

in partial inhibition, with loss of viral replication being observed at 0.01 μM for both tissue types (Fig. 1; Table 1).

To determine how much RPV was present in the tissue, drug was quantified using a sensitive LC-MS/MS assay, which was used to quantify RPV in the previous clinical trials (5, 6). Interestingly, colonic tissue contained 16- to 23-fold more RPV than ectocervical tissue (Table 2). Of the total amount of RPV added to the basolateral cultures, 8% was quantified in the ectocervical tissue. Conversely, the RPV concentration in colonic tissue appeared to be in equilibrium with that in the culture medium, suggesting better penetration of the NNRTI into colonic tissue than ectocervical tissue in these *in vitro* cultures.

To define the RPV concentrations that were sufficient for inhibiting HIV infection of mucosal tissue, PK/PD correlations were determined. Ectocervical explant tissues showed a significant dose-response according to the amount of RPV added to the culture and HIV suppression (Fig. 2a). RPV at >99 ng/ml (EC_{90}, 271 nM) prevented HIV infection in the ectocervical explant tissues; this EC_{90} was approximately 162-fold greater than the EC_{90} obtained in the TZM-bl cell assay (Fig. 2b). Colonic tissue also demonstrated a significant dose-response according to the amount of RPV added to the culture and HIV suppression (Fig. 2c). RPV at >16.33 ng/ml (EC_{90}, 45 nM) prevented HIV infection in colonic tissue (Fig. 2d); this EC_{90} was 27-fold greater than the EC_{90} obtained in the TZM-bl cell assay.

DISCUSSION

RPV was developed in the early 2000s with the desire to improve the treatment options for HIV-infected persons (1) and as a potential agent for the prevention of HIV infection. Responding to concerns about adherence to oral and topical antiretroviral drugs, an LA formulation was created with the aim of obtaining a single dose that would provide therapeutic levels of RPV for a month or more (4). Two dose-ranging studies of RPV LA have been completed in healthy, HIV-uninfected persons, and both found a dose-response and high levels of RPV in plasma, mucosal fluid, and mucosal tissue through a month postinjection (5, 6). While PK testing has provided a wealth of information, efficacy correlates have yet to be definitively determined. Preclinical evaluation of candidate drugs can develop PK/PD correlates with the intention of informing the doses to be used in clinical trials and, it is hoped, the potential for efficacy. Our interest was to define drug activity and correlate it to drug levels in the mucosal tissue used in explant tissue cultures *in vitro*. The results showed higher RPV concentrations in colonic explant tissues than ectocervical explant tissues and suggest that >6-fold more RPV is required for protection of ectocervical tissue than protection of colonic tissue.

The SSAT040 study evaluated several doses of RPV-LA (300 mg, 600 mg, and 1,200 mg) for safety and PK measures across several body matrices, which included blood plasma, mucosal fluid, and vaginal and rectal tissue (5). This is one of the first studies to compare drug levels in the two mucosal tissue compartments, albeit in two cohorts. All doses were safe, and a dose-response was noted with the 1,200-mg dose, which achieved the highest RPV concentrations in all matrices through 12 weeks of follow-up. The RPV concentrations reached by 1 week postinjection were approximately twice as high for rectal tissue (93 ng/ml, 254 nM) as for vaginal tissue (39 ng/ml, 106 nM) for those participants receiving the 600-mg dose. The difference in RPV concentrations between the two mucosal compartments may reflect differences in circulation, drug penetration, or drug retention. It is interesting to note that the concentration of RPV attained in the rectal tissue exceeded what was needed for protection against HIV infection by 5.6-fold, on the basis of our data for colonic explant tissues. However, the vaginal tissue RPV concentration was 2.6-fold below the concentration needed to prevent HIV infection, on the basis of our data for ectocervical explant tissue. While higher RPV concentrations were attained in the vaginal tissue with the 1,200-mg dose, few subjects had tissue concentrations above 271 nM, which suggests that a single dose of RPV LA may not fully prevent vaginal HIV acquisition. In a second intensive PK study, the MWRI-01 study, HIV-uninfected women and men were dosed with 600 and 1,200 mg of RPV and followed for up to 6 months (6). Blood plasma, mucosal fluid, and mucosal tissue were collected monthly from all participants. The results from the MWRI-01 study were consistent with those from the SSAT040 study, with the concentrations of RPV in rectal tissue (78 ng/ml, 213 nM) being greater than those in cervical tissue (45 ng/ml, 123 nM) or vaginal tissue (38 ng/ml, 104 nM) on day 28 postinjection; the concentrations steadily decreased thereafter.

Importantly, the MWRI-01 study also implemented the *ex vivo* challenge assay (25, 26), which collected paired mucosal tissue specimens for immediate exposure to HIV in the laboratory. Significant suppression of HIV infection was noted at 1 month postinjection for rectal tissue but not for cervical or vaginal tissue in participants receiving the 600- and 1,200-mg doses. Our explant data suggest that >2-fold more RPV than the amount achieved in female genital tissue in the MWRI-01 study would be required to observe a protective effect. This concentration may have been reached soon after injection, but this was not determined. Importantly, rectal tissue from participants receiving the 1,200-mg dose continued to demonstrate HIV suppression *ex vivo*

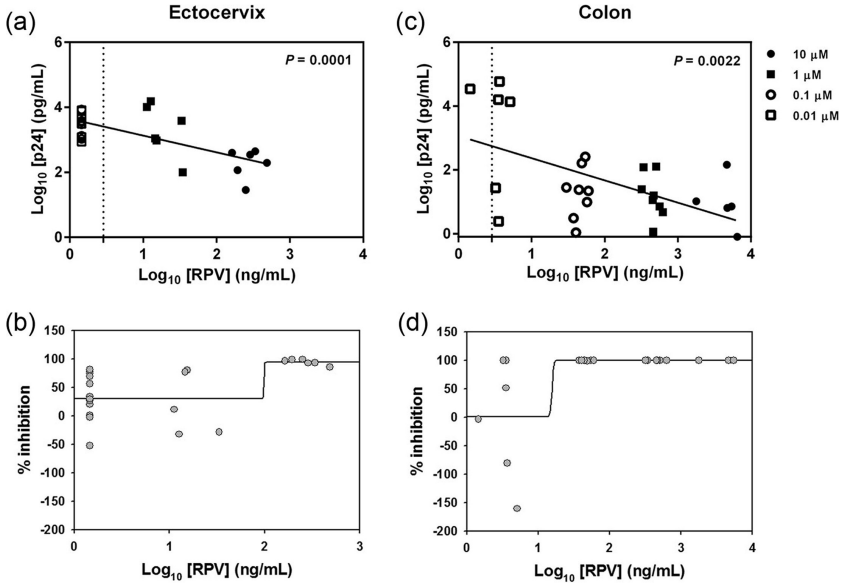

FIG 2 PK and PD activity of RPV in ectocervical and colonic tissue. Three independent ectocervical tissue specimens (a and b) and four independent colonic tissue specimens (c and d) were placed in a polarized configuration in quadruplicate for each treatment, and different dilutions of RPV were applied to the basolateral supernatant on the day before HIV-1_{BaL} was added to the apical surface of two of the four explants. After an overnight culture, the tissues were washed and the two explants not infected with HIV were frozen at −80°C for RPV quantification; for the other two explants, fresh basolateral medium without RPV was added and the explants remained in culture through 21 days. (a and c) Tissue RPV concentrations ($\log_{10}$ nanograms per milliliter) are plotted against the p24 concentration (in $\log_{10}$ picograms per milliliter) on day 21, with significant differences being noted for ectocervical explant tissues (a) and colonic explant tissues (c). The PK (RPV) and PD (p24) data were fit with an inverse, linear least-squares regression model. Data below the limit of quantification were imputed as half the lower limit of quantification (0.6 $\log_{10}$ ng/ml) and are indicated with a vertical dotted line. (b and d) The dose-response relationship between the RPV concentration and percent inhibition [(p24 concentration for treated culture/p24 concentration for control culture) × 100] was determined using a nonlinear E_{max} model (black line) for ectocervical explant tissues (b) and colonic explant tissues (d) to define the EC_{90} for each tissue.

until month 4, when HIV replication was noted in some of the biopsy specimen tissues and the RPV concentration was found to be nearly 20 ng/ml (55 nM), slightly above the colonic explant tissue EC_{90} of 16 ng/ml (45 nM). These explant PK/PD correlates help to interpret the discrepant *ex vivo* challenge data from the MWRI-01 trial and suggest that higher RPV concentrations may be needed for protection against HIV acquisition through vaginal intercourse.

An interesting finding in our work was the discrepant RPV concentrations in the ectocervical and colonic tissues, despite the addition of the same concentrations to both tissues. This was reflected in the inhibition of HIV, where larger amounts of RPV needed to be added to the culture to suppress HIV infection in ectocervical explant tissue than colonic explant tissue. Similar results have been observed *in vitro* with DPV, higher concentrations of which were required to inhibit HIV infection in ectocervical explant tissues than colonic explant tissues (24). The NNRTI drug class has only a limited affinity for transporters that would influence the cell influx or efflux of RPV. However, NNRTI tissue penetration is generally low (31), and the tissue RPV concentration/blood plasma RPV concentration ratio was previously found to be less than 1 but to be higher for rectal tissue than vaginal tissue (5, 6), indicating the better penetration of RPV into colonic tissue than female genital tissue.

Cytochrome P450 (CYP) isoforms metabolize RPV as well as DPV into similar minor oxidative metabolites (1, 32), and UDP-glucuronosyltransferases (UGTs) create glucuronide metabolites (32). CYP expression has been documented in vaginal and colonic tissues, and UGT expression was found only in colonic tissue and contributed to the metabolism of topically applied DPV *in vivo* (33). It remains unknown if these enzymes are active in tissue *ex vivo* and if metabolites are differentially retained in colonic tissue and ectocervical tissue. However, other classes of drugs also show differential penetration/retention in mucosal tissue. For example, TFV, a nucleotide reverse transcriptase inhibitor, has a differential distribution in mucosal tissues, with two studies showing higher concentrations of the active form, TFV-diphosphate, in colonic tissue than vaginal tissue (34, 35). As more drugs are being evaluated for use for the prevention of HIV infection, it will be important to assess their penetration into the mucosal compartments of men and women (36) to define the levels sufficient for protection against HIV acquisition.

Preclinical testing of drugs for the prevention of HIV infection has incorporated mucosal tissue for over 2 decades (22, 37). Preclinical testing with mucosal tissue has centered on product safety and efficacy. However, cross-validation is ongoing to compare and relate preclinical models for various drugs. Nicol and colleagues (38) recently showed that 10- to 1,000-fold higher concentrations of TFV and maraviroc, respectively, were needed to suppress HIV in vaginal tissue than in a TZM-bl cell assay. Using a similar TZM-bl cell assay, we demonstrated that approximately 27- to 162-fold more RPV was needed to suppress HIV in colonic and ectocervical explant tissues, respectively, than in TZM-bl cells. While there are differences between laboratories in the methodologies used for the explant cultures (nonpolarized versus polarized explants) and the detection of HIV (quantitative PCR for spliced viral RNA versus p24 quantification), similar trends were observed for three different classes of antiretroviral drugs.

Cell-based *in vitro* assays are typically composed of homogeneous cell lines and do not fully represent mucosal tissue, which consists of a complex organization of cell types with a diverse array of immune cells. However, understanding these differences between assays will help researchers who do not have access to mucosal tissue to better define target product profiles earlier in the development process.

Our results help to explain the discrepant findings of the *ex vivo* challenge results in the MWRI-01 study, which showed HIV suppression in rectal tissue but not cervical or vaginal tissue from participants receiving parenteral injections of RPV LA (6). However, there are limitations to our findings. The steady-state RPV concentration in the mucosal explant tissue cultures was not determined, and a longer preincubation period may have been required to demonstrate its full effect. However, once tissue is removed from the person, there is a finite time before the architecture is lost (22, 23). Therefore, the experiments were designed to complete treatments within 48 h. While the RPV concentrations in the ectocervical and colonic explant tissues can help to explain the findings of the *ex vivo* challenge in the MWRI-01 study, they may be an overestimate of the *in vivo* therapeutic dose due to the high titer of HIV required for infection of mucosal tissue in the laboratory.

RPV is a potent NNRTI that suppressed HIV infection in mucosal tissue, but >6-fold more RPV was needed in ectocervical explant tissue than colonic explant tissue for protection from HIV infection. On the basis of the PK data from the clinical trials, our data suggest that after parenteral dosing, sufficient levels of RPV appear to be present in the colon, but higher concentrations may be needed in the vagina/cervix for protection against HIV acquisition.

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