MIV-150-Containing Intravaginal Rings Protect Macaque Vaginal Explants against SHIV-RT Infection

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Recent studies demonstrated that intravaginal rings (IVRs) containing 100 mg of the nonnucleoside reverse transcriptase inhibitor (NNRTI) MIV-150 significantly protect macaques against a chimeric simian-human immunodeficiency virus that expresses the HIV-1 HxB2 reverse transcriptase (SHIV-RT) when present before and after vaginal challenge. The objectives of this study were to (i) evaluate the pharmacodynamics (PD) of MIV-150 in vaginal fluids (VF) and in ectocervical and vaginal tissues following 100-mg MIV-150 IVR exposure and to (ii) gain more insight whether pharmacokinetics (PK) of MIV-150 can predict PD. MIV-150 in VF collected at 1 day and 14 days post-MIV-150 IVR insertion inhibited *ex vivo* SHIV-RT infection in vaginal biopsy specimens from untreated animals (not carrying IVRs) in a dose-dependent manner. Previous PK studies demonstrated a significant increase of ectocervical and vaginal tissue MIV-150 concentrations 14 days versus 1 day post-IVR insertion, with the highest increase in vaginal tissue. Therefore, we tested PD of MIV-150 in tissues 14 days post-MIV-150 IVR insertion. *Ex vivo* SHIV-RT infection of vaginal, but not ectocervical, tissues collected 14 days post-MIV-150 IVR insertion was significantly inhibited compared to infection at the baseline (prior to MIV-150 IVR exposure). No changes in vaginal and ectocervical tissue infection were observed after placebo IVR exposure. Overall, these data underscore the use of the *ex vivo* macaque explant challenge models to evaluate tissue and VF PK/PD of candidate microbicides before *in vivo* animal efficacy studies. The data support further development of MIV-150-containing IVRs.

Intravaginal rings (IVRs) represent a promising approach to deliver anti-HIV microbicides with the premise to provide optimal drug concentrations for protection over a month or longer and to potentially improve adherence (1–7). Several IVRs are currently in different stages of development (5, 8). The nucleoside reverse transcriptase inhibitor (NRTI) tenofovir (TFV; 1.6 g) IVR provided a sustained drug level in vaginal fluids (VF), vaginal tissue, and plasma for 90 days in sheep (9). The VF TFV concentrations exceeded concentrations associated with protection against HIV in women using the TFV 1% gel (9, 10). Similarly, tenofovir disoproxil fumarate (TDF; 130 ± 10 mg) IVRs inserted for 28 days provided high TFV levels in VF and tissues in macaques and allowed complete protection against repeated vaginal simian-human immunodeficiency virus 162p3 (SHIV_{162p3}) challenge (11). The nonnucleoside reverse transcriptase inhibitor (NNRTI) dapivirine (25 mg) IVR is in phase 3 clinical trials (8). It was shown to safely deliver dapivirine over 7 days and provide tissue and VF drug concentrations in humans of >1,000-fold the 50% effective concentration (EC₅₀) against wild-type HIV in cell-based assays (12, 13). The Population Council colleagues conducted proof-of-concept pharmacokinetics (PK) and efficacy studies in macaques using NNRTI MIV-150 (100 mg) IVRs (14). MIV-150 IVRs provided >80% protection against SHIV-RT *in vivo* in medroxyprogesterone (Depo-Provera [Depo])-treated animals when IVRs were inserted for 1 day (5 weeks post-Depo) or 14 days (3 weeks post-Depo) before challenge, but the protection was lost if the IVRs did not remain in place postchallenge (14). More MIV-150 was detected in the vaginal and ectocervical tissues, with the highest increase in vaginal tissue, when the IVRs were inserted for 14 days versus 1 day (5 weeks post-Depo) (14). The level of MIV-150 detected in VF 14 days after IVR insertion (5 weeks post-Depo)

was ~100-fold higher than the 50% inhibitory concentration (IC₅₀) against SHIV-RT in TZM_{bl} cells (14, 15).

There is a need to define reliable biomarkers to predict *in vivo* efficacy of candidate microbicides and to help prioritize IVR products before clinical trials. Several lines of evidence in macaques (1, 11, 14, 16, 17) and humans (10) suggest that PK and pharmacodynamics (PD) may serve as candidate biomarkers of efficacy. The present study was designed to determine PD of MIV-150 in VF and in ectocervical and vaginal tissues after insertion of MIV-150 (100 mg) IVRs and to gain more insight into whether MIV-150 PK can predict PD.

MATERIALS AND METHODS

Macaques. Adult female Chinese and/or Indian rhesus macaques (*Macaca mulatta*) were utilized. The characteristics of the animals included in specific experiments are listed below. Naive macaques 4 to 10 years old with weights ranging from 5.3 to 8.85 kg were utilized for *in vivo* IVR studies (PD of MIV-150 in VF and tissues and VF PK studies). Naive and SHIV-RT-exposed uninfected/infected recycled macaques (not carrying IVRs) 5 to 14 years old with weights ranging from 4.05 to 8.7 kg were utilized for collection of vaginal biopsy specimens, which were used in experiments

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FIG 1 Study design schematic. EVA-40 IVRs containing 100 mg MIV-150 or no MIV-150 (placebo) were inserted (5 weeks after Depo treatment) for 1 day or 14 days. VF was collected 1 week before IVR insertion (baseline) and just before IVR removal to assess PK/PD of MIV-150 in VF and the cytokine profile. Vaginal and ectocervical biopsy specimens were collected in the 14-day study only at the baseline (5 weeks before Depo treatment) and just after IVR removal to determine PD of MIV-150 in tissues.

determining PD of MIV-150 in VF. Naive macaques (not carrying IVRs) 6 to 14 years old with weights ranging from 5.2 to 7 kg were utilized for collection of VF for the experiments testing tissue viability in the presence of VF. Animals were housed at the Tulane National Primate Research Center (TNPRC; Covington, LA), accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC no. 000594). The use of macaques was approved by the Animal Care and Use Committee of the TNPRC (OLAW assurance no. A4499-01), and animal care complied with the regulations in the Animal Welfare Act (18) and the *Guide for the Care and Use of Laboratory Animals* (19). Anesthesia was administered prior to and during all procedures, and analgesics were provided afterwards as previously described (17, 20).

In vivo IVR study design and sample collection for PD and PK studies. The *in vivo* IVR study design and sample collection (VF, ectocervical, and vaginal biopsy specimens) are detailed in Fig. 1. To mirror the conditions of the efficacy and PK studies (14), animals received a single 30-mg dose of Depo by intramuscular injection. Five weeks later, 100-mg MIV-150 ($n = 5$) or placebo ($n = 5$) IVRs were inserted for 1 day or 14 days. Two animals (one each in the 1-day and 14-day MIV-150 groups) lost their rings and were excluded from the analyses.

PD of MIV-150 in VF. Anti-SHIV-RT activity of MIV-150 released in VF from MIV-150 IVRs 1 day and 14 days postinsertion was tested in vaginal biopsy specimens derived from untreated animals (not carrying IVRs). VF was collected right before IVR removal and at the baseline (Fig. 1), 1 week before IVR insertion, using foam swabs as described in reference 21. Briefly, the swabs were placed in 1 ml of phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS) and 100 U/ml penicillin–100 μ g/ml streptomycin and transported to our laboratories at the Population Council on ice overnight for processing (within 24 h) (21). The swabs were pressed against the walls of the tube to collect VF. The recovered volume varied across the samples and was typically ~600 to 800 μ l. Cell-free aliquots of VF were stored at -80°C until use.

Vaginal biopsy specimens ($n = 2$) were collected from untreated animals using 3- by 5-mm biopsy forceps, placed in L-15 medium (HyClone Laboratories, Inc., Logan, UT) supplemented with 10% FBS (Gibco, Life Technologies, Grand Island, NY) and 100 U/ml penicillin–100 μ g/ml streptomycin (Cellgro/Mediatech, Manassas, VA) (cL15), and transported to our laboratories at the Council on ice overnight. The biopsy specimens were processed as described previously (22). Briefly, ~3- by 3-mm explants were stimulated with 5 μ g/ml phytohemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO) and 100 U/ml interleukin-2 (IL-2) (Roche Applied Science, Indianapolis, IN, or NCI BRB Preclinical Repository, Frederick, MD) for 2 days. Explants were then challenged with SHIV-RT (10^4 tissue culture infective doses [TCID_{50}] per piece) for ~18 h in the presence of 100 U/ml IL-2 and VF (1:5 final dilution in cDMEM, which is Dulbecco's modification of Eagle's medium [Cellgro/Mediatech]

containing 10% FBS [Gibco, Life Technologies], 100 U/ml penicillin, and 100 μ g/ml streptomycin [Cellgro/Mediatech], with 100 μ M MEM non-essential amino acids [Irvine Scientific, Santa Ana, CA]) that had been collected at the baseline before IVR insertion and right before IVR removal. Then explants were washed and cultured in cDMEM containing 100 U/ml IL-2 for 14 days. Supernatants were collected at 0 (after washes), 3, 7, 11, and 14 days of culture. Infection (individual replicate wells) was monitored with the RETROtek SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix, Buffalo, NY) with a detection limit of 62.5 pg/ml. p27 cumulative (CUM) and soft (SOFT) endpoint analyses (23, 24) were performed from 3 days to 14 days of culture because day 0 data represent carryover input virus.

PD of tissue-associated MIV-150. Anti-SHIV-RT activity of tissue-associated MIV-150 was determined in biopsy specimens collected 14 days post-MIV-150 and post-placebo IVR insertion (versus biopsy specimens collected at the baseline 5 weeks before Depo treatment) (Fig. 1). Biopsy specimens ($n = 2$ vaginal and ectocervical) were collected using 3- by 5-mm and 3- by 4.5-mm forceps for vaginal and ectocervical tissue, respectively. Tissues were processed for SHIV-RT challenge as described previously (22) within an hour of collection. Nonstimulated explants were challenged with 10^5 TCID_{50} SHIV-RT per explant in the presence of 100 U/ml IL-2. This challenge dose was found to reproducibly infect tissues that were not preactivated with PHA–IL-2. Approximately 18 h after infection, tissues were washed and cultured in cDMEM containing IL-2 for 13 or 14 days. Supernatants were collected at 0 (after washes), 3, 7, 10/11, and 13/14 days of culture. Infection was monitored and analyzed as described above.

Tissue viability in the presence of VF and MIV-150. Tissue viability in the presence of VF was tested by thiazolyl blue tetrazolium bromide (MTT) assay (22). Briefly, ~3- by 3-mm explants were immersed for ~18 h in pooled diluted VF alone or spiked with MIV-150. Then tissues were washed and cultured in cDMEM containing 0.5 mg/ml of MTT (Sigma-Aldrich) for 2 h at 37°C . After incubation, each explant was transferred to 1 ml of methanol overnight in the dark. Then 200 μ l of the extractant solution (in triplicate) was measured spectrophotometrically at 570 nm. The tissues were dried for at least 24 h. Tissue viability was determined by normalizing the optical density at 570 nm (OD_{570}) of the formazan product by the dry weight of the explants.

RIA for MIV-150 detection in VF. MIV-150 was detected in VF by radioimmunoassay (RIA) (17) at the Council in the laboratory of J. A. Fernández-Romero, adapting the indirect extraction methodology developed by Kumar et al. (25). The assay was optimized and validated for the detection of MIV-150 in VF, with a sensitivity level of 1 ng/ml (2.7 nM) and a coefficient of variation range of 1.8 to 2.7% at concentrations between 2.7 and 270 nM. The MIV-150 concentration in the samples was calculated by performing a curve-fitting procedure (logistic 4-parameter

model) using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA).

Cytokine levels in VF (Invitrogen cytokine monkey magnetic 28-plex panel). Cytokine levels in VF collected at the baseline and post IVR insertion were measured using the Invitrogen cytokine monkey magnetic 28-plex kit (Invitrogen, Frederick, MD). The expression of epidermal growth factor (EGF), CCL11, fibroblast growth factor (FGF-basic), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF), gamma interferon (IFN- γ), IL-1 β , IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, CXCL8, IL-10, IL-12, IL-15, IL-17, CXCL11, CCL2, CCL22, macrophage migration inhibitory factor (MIF), CXCL9, CCL3, CCL4, CCL5, tumor necrosis factor alpha (TNF- α), and vascular endothelial growth factor (VEGF) was monitored by using the filter plate method. The Luminex 200 (Luminex, Austin, TX) and StarStation softwares (Applied Cytometry Systems, Sacramento, CA) were used to analyze samples.

IVRs. IVRs were prepared at the Council in the laboratory of T. M. Zydowsky as described in reference 14. Ethylene vinyl acetate 40 (EVA-40) IVRs contained 100 mg of MIV-150 or no MIV-150 (placebo IVRs).

Viral stocks. SHIV-RT is a chimeric virus that expresses the HIV-1 HxB2 reverse transcriptase (26), offering a convenient tool for the evaluation of reverse transcriptase inhibitors in nonhuman primates *ex vivo* and *in vivo* (27–30). SHIV-RT was generated from the original stock provided by Disa Böttiger (Medivir AB, Sweden) (15, 22) using PHA/IL-2-activated macaque peripheral blood mononuclear cells (PBMCs). The titer was determined in CEMx174 cells before use (20). A single SHIV-RT stock was used for studies determining PD of tissue-associated MIV-150.

Statistics. (i) PK/PD of VF MIV-150. Comparison between MIV-150 concentrations in VF collected 1 day and 14 days post-MIV-150 IVR exposure was done using a repeated measures analysis of variance (ANOVA) test on log-transformed values. As macaque age spanned 6 years, the age was included as a predictor in this test.

For CUM and SOFT endpoint analyses (23, 24), p27 concentrations of individual replicate values that were $\geq$ the lower limit of quantification (LLOQ) were assumed to be log normal. Any value that was $<$ LLOQ (62.5 pg/ml) at 3 to 14 days of culture was set to $62.5^{1/\sqrt{2}} = 18.616$, a common substitution for log-normal data. A CUM endpoint from 3 to 14 days for replicates below LLOQ corresponds to 74.46 pg/ml.

Correlations between VF MIV-150 concentrations and protection against *ex vivo* infection in tissues derived from untreated animals (not carrying IVRs) were analyzed using Spearman's rank correlation test.

(ii) PD of tissue-associated MIV-150. p27 CUM and SOFT endpoint analyses were done as described above. Comparisons of CUM and SOFT endpoints were performed using a log-normal generalized linear mixed model with the individual replicate data. Because baseline infection was monitored over 13 days of culture and post-IVR exposure tissue infection was monitored over 14 days of culture, a linear interpolation was used to predict p27 concentrations at 13 days of culture. The treatment (MIV-150 versus placebo), biopsy tissue type (ectocervical versus vaginal), biopsy time (baseline versus post-IVR exposure), macaque age, and all interactions were used as predictors. Time was included as a random effect with a random subject effect of tissue type interacted with animal identity nested within treatment. Overall significance was determined by the Type 3 *F*-test, and pairwise comparisons were made with Tukey-adjusted *t* tests. Significant *P* values of <0.05 (*) and <0.001 (***) are indicated in Table 1 and Fig. 5.

(iii) Tissue viability analysis. Analysis of tissue viability (MTT assay) in the presence of VF and MIV-150 was done using a log-normal generalized linear mixed model predicting the weight-normalized OD₅₇₀ of each replicate. A random intercept was included by animal identification. The significance was determined by the Type 3 *F*-test for overall treatment effect.

FIG 2 MIV-150 concentration in VF. The MIV-150 concentrations in VF were measured by RIA 1 day and 14 days post-IVR insertion. Each symbol indicates an individual VF donor, and the means $\pm$ SEM are shown.

(iv) Analysis of cytokine levels. Comparisons of the cytokine levels (MIV-150 versus placebo, pre- and post-IVR) were performed using a log-normal generalized linear mixed model. A significant *P* value of <0.01 (**) is indicated in Fig. S1 in the supplemental material.

All analyses were performed with SAS V9.2, SAS/STAT V9.22, and $\alpha = 0.05$.

RESULTS

MIV-150 in VF inhibits *ex vivo* SHIV-RT infection of vaginal explants in a dose-dependent manner. To determine PK/PD of MIV-150 in VF, vaginal explants derived from untreated animals (not carrying IVRs) were stimulated with PHA/IL-2 and challenged with SHIV-RT (10^4 TCID₅₀) in the presence of paired VF samples collected at the baseline and just before MIV-150 IVR removal 1 day or 14 days post-IVR insertion (Fig. 1). The *ex vivo* inoculum dose was based on studies demonstrating reproducible infection of overnight-transported, PHA/IL-2-stimulated tissues (22). Separate experiments demonstrated the lack of basal p27 production in PHA/IL-2-stimulated vaginal tissues from SHIV-RT-exposed uninfected/infected animals over the 14 days of culture (P. Barnable and N. Teleshova, unpublished data). This suggests that there was no contribution of basal viral replication in the *ex vivo* challenge assays. The baseline VF samples were included to control for endogenous antiviral activity of cervicovaginal secretions as reported in humans (31–33).

Overall, not statistically significant higher concentrations of VF MIV-150 were detected at 14 days (94 to 845 nM [31 to 313 ng/ml]) versus 1 day (11 to 97 nM [4 to 36 ng/ml]) post-IVR insertion (Fig. 2). Since we used animals ranging in age from 4 to 10 years, age was included as a predictor in the repeated measures ANOVA test. There was no effect of macaque age on MIV-150 concentrations in VF. Before testing PD of MIV-150 in VF, we determined that immersion of vaginal explants in diluted pooled VF (alone or spiked with up to 1 μ M MIV-150) for 18 h does not affect tissue viability ($P = 0.1756$) (Fig. 3A). *Ex vivo* SHIV-RT challenge of vaginal explants in the presence of >19 nM MIV-150 in the diluted VF collected post-MIV-150 IVR exposure strongly inhibited tissue infection (relative to infection in the presence of baseline VF) as determined by p27 CUM (~ 78 to 99% inhibition) and SOFT (~ 70 to 99%) endpoints (Fig. 3B and C). A correlation between MIV-150 concentration in the VF and antiviral activity against *ex vivo* SHIV-RT infection in explants was observed (Spearman's correlation test, $r = -0.8105$ and $P = 0.0008$ for CUM and $r = -0.8050$ and $P = 0.0009$ for SOFT).

Tissue-associated MIV-150 inhibits *ex vivo* SHIV-RT infection of vaginal explants. To determine PD of tissue-associated MIV-150, vaginal and ectocervical biopsy specimens were collected 14 days post-MIV-150 IVR (versus placebo IVR) insertion and at the baseline (Fig. 1) and challenged with SHIV-RT (10^5

FIG 3 MIV-150 in VF inhibits *ex vivo* SHIV-RT infection of vaginal explants in a dose-dependent manner. (A) Macaque vaginal explants from untreated animals were immersed in culture medium containing 1:5 diluted pooled VF from untreated animals (alone or spiked with MIV-150) for ~18 h. Tissue viability was determined using the MTT assay. (OD₅₇₀ values of the formazan product were measured in triplicate and normalized to the dry weight of the explants.) Each symbol indicates an individual tissue donor, and the means $\pm$ SEM are shown. (B and C) PHA/IL-2-stimulated explants from untreated animals were challenged with SHIV-RT (10^4 TCID₅₀/explant; duplicates to quadruplicates) in the presence of diluted VF (1:5 dilution in culture media) collected at the baseline (pre-IVR) and at 1 day (solid symbols) and 14 days (open symbols) after MIV-150 IVR insertion (post-IVR). The explants were cultured for 14 days in the presence of IL-2. Infection was monitored with the RETROtek SIV p27 antigen ELISA kit. The activity of each pre-IVR and post-IVR VF pair was tested in tissues from 1 or 2 different donors. Changes in p27 CUM (B) and SOFT (C) endpoints are shown relative to the VF MIV-150 concentration. Each symbol represents an individual tissue donor.

TCID₅₀ of SHIV-RT) *ex vivo* (without prior activation with PHA/IL-2). The study design was based on the previous PK study demonstrating significantly increased tissue concentrations of MIV-150 after a 14-day versus 1-day administration regimen (14). Nonstimulated ectocervical and vaginal biopsy specimens were processed for viral challenge within 1 h of collection to avoid loss of MIV-150 in culture during stimulation (before viral challenge). A high challenge dose was chosen to ensure reproducible infection.

Variable viral growth kinetics were detected in vaginal and ectocervical tissues in the placebo IVR group (Fig. 4, open symbols). Infection of vaginal tissues before MIV-150 IVR insertion was higher than post-MIV-150 exposure (Fig. 4, solid symbols). Summaries of log-transformed p27 CUM and SOFT endpoints of these

experiments are shown in Fig. 5. The details of statistical analyses (infection ratios and *P* values) are presented in Table 1. As macaque age spanned 6 years, age was included as a predictor together with treatment, biopsy tissue type, and biopsy time in the log-normal generalized linear mixed model. No significant interactions of age with other predictors were found for either p27 CUM or SOFT endpoints (CUM and SOFT *P* values = 0.2181 and 0.2403, respectively), indicating that there was no effect of age on tissue infection at the baseline or on the activity of tissue-associated MIV-150 against SHIV-RT infection. There was no evidence that age confounded any of the other predictors. Based on this analysis, macaque age as a predictor was excluded from the final models.

A significant interaction between treatment and time (p27 CUM, SOFT), regardless of tissue type, was detected (Table 1). This effect was due to significant decrease in tissue infection from MIV-150 IVR-carrying animals, but not in the placebo IVR group (Table 1). Although interactions of treatment, tissue type, and time were not significant overall, pairwise comparisons revealed extreme decreases in p27 CUM (91%) and SOFT (95%) endpoints in the vaginal tissues exposed to MIV-150, while there were none seen in vaginal tissues exposed to placebo (Fig. 5 and Table 1). These results suggest that it is the MIV-150-exposed vaginal replicates that drive the significant tissue type-time interaction (Table 1). No significant changes in infection of ectocervical tissues post-IVR insertion were detected for either MIV-150 or placebo (Fig. 5 and Table 1). The PK/PD relationships of tissue-associated MIV-150 were not determined as multiple explant replicates were prioritized for the *ex vivo* challenge assays and tissue MIV-150 was not measured.

The vaginal tissue infection level was not affected by Depo treatment (compare pre- and postplacebo IVR data in Fig. 5 and Table 1). In contrast, although not significant, ectocervical tissue infection measured by CUM and SOFT endpoint analyses was higher (3.14 and 4.90 times, respectively) post-Depo treatment versus the baseline (Fig. 5 and Table 1).

VF cytokine levels post-IVR exposure. As a measure of safety, VF cytokine levels were determined 1 day and 14 days post-IVR exposure and at the baseline. Eleven of 28 cytokines were within the detection range of the assay. Seventeen cytokines were below the LLOQ. In line with the data by Singer et al. reporting that the 100-mg MIV-150 IVRs caused no distress or vaginal irritation (14), there was no evidence that detected cytokine levels were different between MIV-150 and placebo IVR treatment groups 1 day and 14 days post-IVR insertion (see Fig. S1 in the supplemental material). However, there was a significant increase of IL-6 level 14 days post-placebo IVR exposure versus baseline (*P* = 0.0099). No changes in IL-6 levels 1 day and 14 days post-MIV-150 IVR exposure versus the baselines were detected (see Fig. S1). Other cytokine levels remained unchanged post-IVR exposure relative to the baselines (see Fig. S1).

DISCUSSION

Our data demonstrate that MIV-150 released from 100-mg MIV-150 IVRs *in vivo* is active in the presence of VF and provides dose-dependent inhibition of *ex vivo* SHIV-RT infection in vaginal tissues from untreated macaques. The data indicate that PK of MIV-150 in VF can predict PD. In agreement with previous PK studies (14), MIV-150 concentrations in VF after 14 days of exposure to the MIV-150 IVRs tended to be higher than after 1 day of

FIG 4 *Ex vivo* SHIV-RT infection kinetics in tissues from animals carrying MIV-150 and placebo IVRs. MIV-150 or placebo IVRs were inserted 5 weeks post-Depo treatment for 14 days. Ectocervical and vaginal biopsy specimens were collected at the baseline (pre-IVR) and 14 days post-IVR insertion (post-IVR). Five or six (vaginal) and 4 to 6 (ectocervical) nonstimulated explants were challenged with 10^5 TCID₅₀ of SHIV-RT/explant for ~18 h, washed, and cultured for 13 or 14 days with IL-2. Infection in individual explants was monitored with the RETROtek SIV p27 antigen ELISA kit. Each symbol represents an individual animal ($n = 5$ for the placebo group [open symbols] and $n = 4$ for the MIV-150 group [solid symbols]). Shown are the means of replicates (p27 values) for each animal.

exposure. Seventy to 99% inhibition of *ex vivo* tissue infection was observed when viral challenge of tissues from untreated animals was done in the presence of diluted VF containing ≥ 19 nM MIV-150 (≥ 10 -fold IC₉₀ in cell-based assays) (15) pointing to a higher

MIV-150 concentration being needed for infection inhibition in tissues versus in cell-based assays.

The data demonstrate that tissue-associated MIV-150 released from MIV-150 IVRs *in vivo* provides protection against *ex vivo*

FIG 5 Tissue-associated MIV-150 inhibits *ex vivo* SHIV-RT infection of vaginal explants. p27 CUM and SOFT endpoint analyses of the data ($\log_{10}$ transformed) presented in Fig. 4 are shown (means $\pm$ SEM). Each symbol represents an individual animal in the placebo IVR ($n = 5$ animals [open symbols]) and MIV-150 IVR ($n = 4$ animals [solid symbols]) groups. The comparisons were performed using a log-normal generalized linear mixed model. Significant P values of < 0.001 (***) are indicated.

TABLE 1 PD of tissue-associated MIV-150

Pairwise comparison (post- vs. pre-IVR)	Cumulative analysis ^a		Soft endpoint analysis ^a	
	Infection ratio (95% CI)	P value	Infection ratio (95% CI)	P value
Treatment-time	Overall	0.0236*	Overall	0.0200*
MIV-150	0.30 (0.09–0.98)	0.0450*	0.23 (0.05–0.99)	0.0471*
Placebo	1.72 (0.35–8.44)	0.7891	2.06 (0.29–14.37)	0.7429
Tissue type-time	Overall	0.0159*	Overall	0.0107*
Ectocervical	1.77 (0.53–5.84)	0.5702	2.28 (0.54–9.71)	0.4174
Vaginal	0.27 (0.08–1.00)	0.0507	0.20 (0.04–0.98)	0.0463*
Tissue type-treatment-time	Overall	0.3751	Overall	0.4398
Ectocervical				
MIV-150	1.07 (0.11–10.74)	1.0000	1.10 (0.06–19.80)	1.0000
Placebo	3.14 (0.42–23.83)	0.5934	4.90 (0.43–56.46)	0.4234
Vaginal				
MIV-150	0.09 (0.02–0.43)	0.0006***	0.05 (0.01–0.34)	0.0005***
Placebo	0.94 (0.04–23.89)	1.0000	0.86 (0.02–45.72)	1.0000

^a Included are the *P* value for overall Type 3 effect and the Tukey-Kramer adjusted 95% confidence interval (95% CI) and *P* value for multiple pairwise comparisons. Significant *P* values of <0.05 (*) and <0.001 (***) are indicated in boldface.

tissue SHIV-RT infection. MIV-150 IVRs inserted for 14 days significantly reduced vaginal tissue infection compared to the baseline infection (>90% inhibition as detected by p27 CUM and SOFT endpoint analyses). Although tissue MIV-150 PK data were not collected in the present study, previous PK studies using identical IVRs and administration regimens detected 0.616 ± 0.221 ng/mg (mean $\pm$ standard error of the mean [SEM]) of MIV-150 in vaginal tissue 14 days after IVR insertion (14). This tissue PK data and our 14-day IVR tissue PD studies suggest that ~ 0.6 ng/mg of MIV-150 associated with the vaginal mucosa affords significant protection against mucosal SHIV-RT infection. We cannot exclude that lower tissue MIV-150 concentrations also provide protection against infection. Our VF PK studies suggest that ≥ 94 nM/31 ng/ml MIV-150 in VF at day 14 post-MIV-150 IVR insertion (the lowest concentration detected) could be associated with protective level of MIV-150 in the corresponding vaginal tissues. However, VF MIV-150 concentrations may underestimate or overestimate MIV-150 levels in the corresponding tissues. No activity against *ex vivo* SHIV-RT challenge was observed in the ectocervix post-MIV-150 IVR exposure. Overall, lower concentrations of MIV-150 were reported in the ectocervix versus vagina 14 days post-IVR insertion (0.28 ± 0.075 ng/mg) (14), which could contribute to the lack of antiviral activity in the ectocervix in our study.

A previous report demonstrated higher frequency of SIV acquisition in progesterone-treated animals (34). While a statistically significant increase in infection post-Depo treatment was not seen in vaginal and ectocervical tissues in our study, adjusted mean levels of infection in ectocervical tissue were 3 to 5 times higher post-Depo treatment, which could potentially mask an inhibitory effect of MIV-150. The low power due to fewer ectocervical explant replicates may be the reason for the lack of significant difference between infection of ectocervical tissue pre- and post-Depo treatment.

Supporting earlier work (14), the safety assessment demonstrated that there was no adverse effect of the high-dose MIV-150

IVR on immune factors in the VF compared to placebo IVR. However, the increase in proinflammatory IL-6 14 days post-placebo IVR (but not post-MIV-150 IVR) relative to the baseline warrants further evaluation to determine IVR effects on long-term safety in larger groups of animals.

The tissue explant models have limitations (35). However, testing of several products in these models demonstrated some predictability for results in the clinic. In line with lack of efficacy or lack of efficacy and safety concerns in phase 2/3 clinical trials (36–38), nonoxynol-9 was toxic and Carraguard was safe but only partially effective in cervical explants (39–41). The 2% PRO 2000 gel arm, but not 0.5% PRO 2000, was halted due to futility in the MDP301 trial (42). In cervical explants, 4% PRO 2000 gel demonstrated toxicity (39). In contrast, 0.5% PRO 2000 gel showed no toxicity and was effective at preventing HIV-1 infection of tissues (39). However, the activity of PRO 2000 is decreased in the presence of semen (43) and may explain the lack of efficacy in the HPTN035 clinical trial (44). We have shown separately that seminal fluid does not impact the *in vitro* antiviral activity of MIV-150 in vaginal tissues (P. Barnable and N. Teleshova, unpublished data). Further supporting the potential predictive value of product testing in explants, the TFV gel inhibited HIV in the tissues (45, 46) and was shown to reduce HIV incidence by 39% overall (47). TFV gel was not effective in the VOICE trial (48), possibly due to lack of adherence as determined by PK studies (7). The tissue PK/PD assessment in clinical trials represents a challenge as only 1 to 2 ectocervical and vaginal biopsy specimens can be collected from participants (3). A recent study defined female genital tract tissue collection procedures for use in the *ex vivo* challenge assays (49). These approaches are being evaluated in ongoing clinical trials (41). Our data demonstrate that vaginal and cervical tissue PD studies are feasible in macaques.

Overall, these data underscore the use of the *ex vivo* macaque explant challenge models to evaluate tissue and VF PK/PD of candidate microbicides before *in vivo* animal efficacy studies. The data support further development of MIV-150-containing IVRs.

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