Intravaginal ring eluting tenofovir disoproxil fumarate completely protects macaques from multiple vaginal simian-HIV challenges

James M. Smith^{a,1}, Rachna Rastogi^{b,1}, Ryan S. Teller^b, Priya Srinivasan^a, Pedro M. M. Mesquita^c, Umadevi Nagaraja^b, Janet M. McNicholl^a, R. Michael Hendry^a, Chuong T. Dinh^a, Amy Martin^a, Betsy C. Herold^c, and Patrick F. Kiser^{b,d,2}

^aLaboratory Branch, Division of HIV/AIDS Prevention, National Center for HIV/AIDS, Viral Hepatitis, Sexually Transmitted Disease, and Tuberculosis Prevention, Coordinating Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333; ^bDepartment of Bioengineering, University of Utah, Salt Lake City, UT 84112; ^cDepartment of Pediatrics and Microbiology-Immunology, Albert Einstein College of Medicine, Bronx, NY 10461; and ^dDepartment of Biomedical Engineering, Northwestern University, Evanston, IL 60208

Edited by Malcolm A. Martin, National Institute of Allergy and Infectious Diseases, Bethesda, MD, and approved August 19, 2013 (received for review June 25, 2013)

Topical preexposure prophylaxis interrupts HIV transmission at the site of mucosal exposure. Intermittently dosed vaginal gels containing the HIV-1 reverse transcriptase inhibitor tenofovir protected pigtailed macaques depending on the timing of viral challenge relative to gel application. However, modest or no protection was observed in clinical trials. Intravaginal rings (IVRs) may improve efficacy by providing long-term sustained drug delivery leading to constant mucosal antiretroviral concentrations and enhancing adherence. Although a few IVRs have entered the clinical pipeline, 100% efficacy in a repeated macaque vaginal challenge model has not been achieved. Here we describe a reservoir IVR technology that delivers the tenofovir prodrug tenofovir disoproxil fumarate (TDF) continuously over 28 d. With four monthly ring changes in this repeated challenge model, TDF IVRs generated reproducible and protective drug levels. All TDF IVR-treated macaques ($n = 6$) remained seronegative and simian-HIV RNA negative after 16 weekly vaginal exposures to 50 tissue culture infectious dose SHIV162p3. In contrast, 11/12 control macaques became infected, with a median of four exposures assuming an eclipse of 7 d from infection to virus RNA detection. Protection was associated with tenofovir levels in vaginal fluid [mean 1.8×10^5 ng/mL (range 1.1×10^4 to 6.6×10^5 ng/mL)] and ex vivo antiviral activity of cervicovaginal lavage samples. These observations support further advancement of TDF IVRs as well as the concept that extended duration drug delivery devices delivering topical antiretrovirals could be effective tools in preventing the sexual transmission of HIV in humans.

controlled drug delivery | PrEP | nonhuman primate | pharmacokinetics

The prodrug tenofovir disoproxil fumarate (TDF) and its hydrolysis product tenofovir (TFV) have been the major focus of HIV prevention research. TDF is one component of the two-drug mixture Truvada, the first US Food and Drug Administration–approved oral preexposure prophylaxis (PrEP) agent against HIV acquisition in discordant couples and other high-risk populations (1), and TFV is the active pharmaceutical ingredient in the first vaginal gel to show partial HIV protection (2). Both molecules have a long safety record and have been widely used in humans with minimal toxicity. The 1% (wt/wt) TFV vaginal gel formulation administered 30 min before virus exposure provided complete protection (6/6) against multiple simian-HIV (SHIV) challenges (3, 4) and retained partial activity protecting four of six macaques that were treated once weekly with gel and exposed to virus twice weekly; the second exposure being 3 d after gel application (4). Unfortunately, the TFV gel provided only partial protection in clinical trials when women were asked to use the gel before and after coitus and no protection when daily dosing was evaluated (2, 5). There are many factors, both social and biological, that may have contributed to the TFV gel clinical trial outcomes. However, as far as we know the most

important factor in all gel trials to date is the difficulty of sustaining high adherence to frequent dosing (6). Simply put, if the course of protective antiretroviral levels does not match the kinetics of viral exposure, PrEP will not be highly effective. This motivates the development of long duration, drug delivery systems such as intravaginal rings (IVRs) that may overcome these limitations by facilitating adherence and providing less variation in mucosal drug levels (7, 8).

Matrix IVRs delivering the nonnucleoside reverse transcriptase inhibitor (NNRTI) dapivirine and the entry inhibitor maraviroc are being advanced through the clinical pipeline. Neither of these rings, which are currently in phase 3 and phase 1 trials, have been evaluated for efficacy in macaques. Among all antiretroviral IVR efficacy studies conducted to date, only one formulation conferred protection against a single, high-dose viral challenge in nonhuman primates, whereas the remainder yielded partial protection (9–11). None has been evaluated in repeated vaginal challenge models designed to mimic the repeated viral exposure during human sexual transmission (12). Reasons for failure of these device–drug combinations to provide complete protection in macaque models are complex and may reflect differences in the model system [species, use of depo-medroxyprogesterone (DMPA), viral dose, and strain], the pharmacologic properties of the drugs tested,

Significance

Topical prevention of HIV is designed to pharmacologically interrupt sexual transmission at the genital mucosa. Attempts at preventing transmission in women using vaginal gels have yielded disappointing results in part because of poor rates of adherence. Controlled topical drug delivery using intravaginal ring technology should improve efficacy and adherence by providing sustained mucosal delivery of antiretrovirals. In this paper, we describe a reservoir intravaginal ring that delivers tenofovir disoproxil fumarate (TDF) for 1 month. The ring protected pigtailed macaques from weekly vaginal simian–human immunodeficiency virus challenges for 4 mo. The sterilizing performance of this drug delivery system supports the concept that an intravaginal ring delivering TDF could be an effective tool for prevention of HIV sexual transmission in women.

Author contributions: J.M.S., R.R., J.M.M., R.M.H., B.C.H., and P.F.K. designed research; R.R., R.S.T., P.S., P.M.M.M., U.N., C.T.D., and A.M. performed research; J.M.S., R.R., R.S.T., P.S., P.M.M.M., A.M., and P.F.K. analyzed data; and J.M.S., R.R., B.C.H., and P.F.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹J.M.S. and R.R. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: patrick.kiser@northwestern.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1311355110/-DCSupplemental.

Developing IVRs to elute the thermally and hydrolytically unstable TDF at therapeutically relevant rates is a challenge. Although TDF is more hydrophobic than TFV, its polarity is too high for adequate polymer solubility to deliver protective doses of the drug from common elastomers used in IVR technology such as silicone and poly(ethylene-covinyl acetate) (13). Furthermore, TDF is susceptible to hydrolysis, making formulation of this drug in any aqueous topical dosage form impossible (17, 18) and precluding its use in gels. We engineered a class of IVRs capable of achieving high fluxes of hydrophilic antiretrovirals such as TFV and TDF using hollow hydrophilic polyether urethane (HPEU) elastomeric tubes (Fig. 1 *A* and *B*) (19). The hydrophilic–hydrophobic balance of HPEU can be tailored to solubilize and deliver drugs with a range of physical properties (20) and at fluxes that achieve clinically relevant concentrations (21). We assessed the ability of TDF IVR designs to achieve monthly sustainable protective levels of TDF/TFV in vaginal fluid and tissue by performing pharmacokinetic (PK) studies in pigtailed macaques. Pigtailed macaques were chosen for the study because of their similarities in hormonal cycling, vaginal architecture, and microflora to women, and previous studies in this model provide data on intracellular TFV-DP levels that correlate with protection (4). The IVR design that generated high and consistent drug levels in vaginal fluids and tissues was then

Fig. 1. TDF IVR design and in vitro release. (A) Schema depicting mechanism of drug release from TDF reservoir IVR. Vaginal fluid hydrates the swellable HPEU tubing (Left) and water is driven into the osmotically active drug-NaCl core along a gradient, resulting in TDF dissolution and elution from the IVR (Right). NaCl aids in establishing soluble drug in the core and achieving equilibrium drug release (Fig. 2B). (B) Photograph of macaque (left) and human (right) TDF IVR. (Scale bar: 1 cm.) (C) Comparison of 28-d in vitro TDF release rates from matrix ($n = 3$) and reservoir ($n = 6$) IVRs under simulated vaginal conditions (2). Data represented as mean $\pm$ SD. The SDs of some of the collected data are too small to be visualized.

evaluated in a more extensive terminal PK study to determine the levels of TFV-DP in the female reproductive tract and lymph nodes. Finally, we tested the ability of the IVR to provide continuous protection against infection by repeated weekly vaginal 50 tissue culture infectious dose (TCID₅₀) SHIV162p3 challenges over multiple monthly IVR changes coupled to ex vivo viral inhibition in cervicovaginal lavage (CVL) samples.

Results and Discussion

TDF IVR Design. IVRs are divided into two well-known controlled release device categories: matrix and reservoir. Matrix devices exhibit drug release rates that decrease with time, whereas reservoir devices have a rate controlling membrane that allows for more consistent drug flux (Fig. 14) (8). Because matrix devices are simple and more easily fabricated, we originally explored the same matrix design strategy as the dapivirine ring (22, 23). Polyether urethane (PEU) matrix TDF devices containing 10 wt% TDF showed a 20-fold reduction in drug release rates in vitro from day 1 to day 28 (Fig. 1C). A similar effect was seen in vivo in rhesus macaques ($n = 3$) in which more than a 2-log reduction was noted in mean TDF vaginal fluid concentrations from 1.5×10^5 ng/mL (range 2.3×10^4 to 3.3×10^5) on day 3 to 6.3×10^2 ng/mL (range 0 to 2.2×10^3) on day 21 (Fig. 24) and no detectable drug on day 28 (13). The chemical stability of TDF in this device was also unacceptable. Although high levels of TFV were still detected in vaginal fluid at day 28, TDF levels were undetectable.

Given the poor performance of the TDF matrix device, we investigated reservoir IVR designs composed of HPEU tubing with a drug-bearing core sealed by induction-melt welding into a torus (19). The tubing wall acts as rate-controlling membrane for drug release while providing the necessary mechanical support for ring retention in the vaginal canal. Simply filling the device with TDF alone resulted in a long drug-release lag time of more than 20 d (Fig. 2B). Therefore, we included osmotic agents to attract vaginal fluid into the core to solubilize TDF and rapidly establish a concentration gradient of soluble and diffusible drug to drive release (Fig. 1A). A reservoir IVR designed to deliver a TDF formulation with NaCl (14 wt%) as an osmotic excipient was selected by scanning a series of osmoattractants in vitro (Fig. S14). Fig. 1B shows human and macaque reservoir IVRs with outer diameters of 55 and 25 mm, respectively. This IVR design delivers in vitro 0.4–4 mg/day TDF over 28 d, with an average release rate of 2.3 ± 0.3 mg/d [macaque reservoir IVR (mean $\pm$ SD; $n = 3$); Fig. 1C]. This HPEU-reservoir IVR design eliminated the need for exposing TDF to elevated temperatures required for creation of thermoplastic matrix IVRs. Functionally, we found that the maximum rate of NaCl release preceded the large increase in TDF release (Fig. 2C), implying that core hydration was rate-limiting in achieving sufficient drug release. Finally, we found that we could load the wall of IVRs with TDF after ring fabrication by exposing the loaded device to elevated temperature, which decreased the lag time of drug elution (Fig. 2D), potentially providing a loading dose of TDF. We were able to obtain release rates of milligram per day quantities of TDF in vitro from the reservoir device and thereby achieve drug levels in vaginal fluid at all time points, exceeding the macaque (4) and clinical correlate of protection (24) of $\sim 1,000$ ng/mL of TFV in vaginal fluid. This was not the case with the inadequate release observed over time from the matrix TDF IVR (Fig. 2A) (13). Although the in vitro release rate from the reservoir design was not constant over 28 d, the IVRs generated reproducible TFV levels (Fig. 3A) over the first 15 d when the in vitro release rate changed the most, potentially implying early saturation of the system with drug or differences between the in vitro and in vivo TDF release rates.

Drug PK Study in Pigtailed Macaques. We investigated the TDF reservoir IVRs in pigtailed macaques ($n = 6$) in a “28 + 2” day study (28 d of IVR exposure and 2 d after removal) for drug

Fig. 2. Matrix release and design evaluation of reservoir TDF IVR. (A) Comparison of TDF released from matrix PEU IVR in vitro on days 3 and 21 to drug concentrations in vaginal fluid of Chinese rhesus macaques ($n = 3$) in a 28 + 3-d study. Each data point represents a single sample and the bar corresponds to the mean for that dataset. (B) In vitro TDF release rate from HPEU reservoir IVR filled with TDF and TDF-NaCl formulation under simulated vaginal conditions ($n = 3$). Data represented as mean $\pm$ SD. (C) In vitro TDF and NaCl release rates from HPEU reservoir IVR under simulated vaginal conditions ($n = 3$). Data represented as mean $\pm$ SD. (D) Comparison of initial in vitro TDF release (up to day 3) from heat-treated and control-unheated TDF IVR ($n = 3$). Heat treatment of the TDF reservoir IVR at 65 °C for 5 d increased TDF release in the first 2 d. Data represented as mean $\pm$ SD.

distribution and concentration in vaginal fluid and tissue (Fig. 3 A and B and Fig. S2). The TDF IVR provided high TFV (Fig. 3A, Upper) and TDF (Fig. 3A, Lower) mean vaginal fluid concentrations of 7.2×10^4 ng/mL (range 7.1×10^3 to 3.5×10^5) and 1.0×10^2 ng/mL (range 5 to 6.1×10^3), respectively. The former consistently exceeded the TFV concentration of 1,000 ng/mL recovered in cervicovaginal aspirates that correlated with protection in women receiving 1% TFV gel (24). In addition, we detected comparable but more variable concentrations of the more potent TDF. Levels of both drugs appear stable in vaginal fluid from days 3 to 28 and levels seemed similar proximally and distally (Fig. 3A). TFV levels also appeared stable over time in proximal and distal tissues (Fig. 3B), whereas TDF levels were more variable (Fig. S2). This suggests that despite the variable drug release in vitro, the IVRs exhibited a minimal lag time to reach high antiretroviral concentrations in the macaque vaginal vault.

Because TDF hydrolytically converts into TFV, we found variable levels of TDF in swabs and there was no quantifiable TDF detected in vaginal swabs 2 d after ring removal. However, mean TFV levels in swabs were 4.4×10^3 ng/mL (range 2.2×10^2 to 6.9×10^4) and in tissue were 2.9×10^3 ng/g (range 5.4×10^2 to 2.5×10^4) 2 d after ring removal (Fig. 3A and B), exceeding the TFV levels detected after TFV gel application in clinical studies (2, 25). In tissues, the TFV concentrations exceeded the in vitro IC_{50} by ~ 80 times. These high and sustained vaginal fluid and tissue levels of TFV likely reflect the diffusion of luminal pro-drug through the vaginal tissue and its hydrolysis to TFV (8). We do not know the instantaneous in vivo TDF release rate in the macaque vagina. However, as determined by the amount of recovered TDF from the IVRs after use in this PK study, the time-averaged TDF release rate was similar under both in vitro and vivo conditions (Fig. S1B). It has been difficult for others in practice to obtain and sustain these levels of drug in vivo; our

IVR delivered ~ 50 -fold more TDF in vivo compared with the one other TDF IVR in the literature (26). These data and the prolonged half-life of intracellular TFV-DP strongly suggest that this drug–device combination would pharmacologically tolerate removal of the device for hours without significant diminution of drug levels. Furthermore, the high TFV levels in vaginal fluid and undetectable TDF levels 2 d after ring removal suggest the presence of a tissue and/or cellular reservoir of drug that is continuously exchanging with vaginal fluid.

Terminal PK Study in Rhesus Macaques. To evaluate intracellular TFV-DP concentrations, we administered TDF IVRs to rhesus macaques that were scheduled to be euthanized because they had been previously infected with SHIV in other studies ($n = 3$). In this 14-d study, we had the opportunity to evaluate levels of the bioactive metabolite TFV-DP in lymphocytes from vaginal, cervical, and rectal tissue as well as lymph nodes, which are sites where HIV transmission and dissemination is presumed to occur. Previous challenge studies in pigtailed macaques with 1% TFV vaginal gel suggested protection from SHIV infection correlated when TFV-DP levels in vaginal lymphocytes exceeded the IC_{95} of 1.4×10^3 fmol/ 10^6 cells (4). Mean TFV-DP levels after 14 d of IVR application were highest in vaginal and cervical lymphocytes, 3.3×10^3 fmol/ 10^6 cells (range 1.5×10^3 to 7.5×10^3) and 1.7×10^3 fmol/ 10^6 cells (range 8.4×10^2 to 3.2×10^3), respectively. The mean of the intracellular levels exceeds the TFV-DP IC_{95} of 1.4×10^3 fmol/ 10^6 cells and is comparable to levels that showed complete protection in macaques (4) (Fig. 3C). Relative to vaginal and cervical lymphocytes, lower drug concentrations were detected in rectal and inguinal lymphocytes, 13 fmol/ 10^6 cells (range 6 to 1.3×10^2) and 81 fmol/ 10^6 cells (range 17 to 1.3×10^2), respectively (Fig. 3C). Additionally, we observed similar TDF and TFV levels in vaginal fluid and tissue to those observed in pigtailed macaques (Fig. 3A and B). These data indicate that the IVRs provide TFV-DP concentrations that exceed protective levels observed previously in macaques, suggesting that the ring could confer protection against vaginal SHIV challenge.

Repeat SHIV Challenge Study. Based on achieving the TFV concentrations in vivo described previously, we initiated a weekly challenge study in sexually mature, normal cycling pigtailed macaques (12). Six TDF IVR-treated macaques received weekly 50 TCID₅₀ SHIV162p3 vaginal inoculations starting 6 d after IVR insertion (Fig. 4A). Control macaques ($n = 6$ real time and $n = 6$ historical controls) were challenged similarly, of which 11/12 became infected after a median of four exposures to infection, assuming a 7-d eclipse period from time of infection to detection of viral RNA in plasma; peak viral RNA levels were $3.4 \times 10^6 \pm 1.9 \times 10^7$ copies/mL, median $\pm$ SD (Fig. 4B and C). In contrast, all TDF IVR-treated macaques (6/6) remained SHIV viral RNA-negative and -seronegative after 16 weekly exposures spanning 4 mo involving monthly IVR changes. A nonparametric log-rank test was used to compare survival probabilities ($P = 0.0007$, Fig. 4B). Differences in infection probabilities between control and treated animals were statistically significant (Fisher's exact test, $P < 0.0001$). The median survival time among control animals was four exposures, 95% CL (2, 10). All TDF treated macaques remained uninfected after 4 additional weeks of follow-up with the IVRs in place. The infection probability per exposure among control animals was 0.162, 95% CL (0.084–0.271); the infection probability among treated animals was 0.0, 95% CL (0.0–0.038). Estimated efficacy was 100%, 95% CL (80.31–100).

The complete protection observed in the TDF IVR-treated macaques is consistent with the high TFV levels in vaginal fluid samples taken at the time of each ring change [Fig. 5A; 1.8×10^5 ng/mL (mean, range 1.1×10^4 to 6.6×10^5)]. With each monthly IVR change, TDF and TFV levels in vaginal fluid samples remained high (Fig. 5A). Plasma TDF levels were below

Fig. 3. Drug PK in pigtailed (28 + 2-d) and rhesus (14-d) macaques. Each data point represents a single sample and the bar corresponds to the mean for that dataset. (A) TFV (Upper) and TDF (Lower) concentrations in pigtailed macaque vaginal fluid with 28-d TDF IVR administration ($n = 6$). Samples were collected proximal (open symbols) and distal (closed symbols) to IVR placement for the indicated time points. (B) TFV concentrations in vaginal biopsies from 28-d TDF IVR administration. Samples were collected proximal (open symbols) and distal (closed symbols) to IVR placement for the indicated time points. (C) TFV-DP levels in lymphocytes isolated from the indicated tissues of rhesus macaques after 14-d IVR administration ($n = 3$).

detection limit [$n = 102$, lower limit of quantification (LLOQ) = 1 ng/mL] throughout the efficacy study. Detectable TFV levels (median 8 ng/mL, range 7–19 ng/mL; $n = 102$, LLOQ = 5 ng/mL) were observed in five of 102 blood samples collected with 4 sequential months of TDF IVR administration. The protection is also consistent with ex vivo antiviral activity of CVL samples from two additional TDF IVR-treated pigtailed macaques not exposed to SHIV in parallel to the challenge study. CVL collected from these two macaques over the course of 28 + 1 d (28-d IVR exposure and 1 d after removal) displayed high antiviral activity against HIV-1 in vitro (range 73–100%) even after a 1:10 dilution (Fig. 5B and C). Importantly, and consistent with the persistence of TFV in vaginal fluid, CVL collected 1 d following removal of the IVR inhibited HIV infection by 86% (Fig. 5B). The anti-HIV activity correlated with both TDF and TFV levels in the CVL (Spearman PK/pharmacodynamic (PD) correlation; TDF: $r = 0.57$, $P = 0.04$; TFV: $r = 0.61$, $P = 0.02$, Fig. 5C).

Potential Behavioral, Pharmacological, and Biological Implications. The choice of TDF over the less potent TFV, combined with the HPEU reservoir IVR delivery system, may overcome several of the behavioral and biological limitations observed to date with vaginal gels and other drug-IVR combinations studied preclinically and clinically. The major reason for embarking on the more

complex drug delivery technology for topical antiretrovirals is to facilitate PrEP use, increase adherence, and thereby improve clinical outcomes. Many studies have shown a general increase in adherence as device duration increases (27, 28). Thus, it is reasonable to assume that it will be easier for women to adhere to long-duration IVR delivery systems compared with daily, episodic, or coitally dependent gels. This is supported by the fact that IVRs have seen excellent product demand and commercial success as a form of birth control in high-income countries and are gaining acceptance in low-income countries (7, 29).

Pharmacologically, sustained drug delivery from reservoir-type devices should provide tissue drug concentrations that are consistently above the level required to protect immune cells resident in and trafficking through the mucosa and submucosa of the genital tract over the time course of mucosal exposure to virus. Furthermore, behaviorally we need to expect and plan for the fact that women will likely periodically remove IVRs either during sex or around the time of menses. This TDF IVR may meet all of these requirements, whereas other drug-ring combination devices have limitations. First, TDF requires significantly lower doses than TFV because of its increased potency, resulting from its capability to more efficiently permeate cell membranes. This property combined with the long TFV-DP intracellular half-life (14) and corresponding low tissue elimination rate may allow for sustained activity for many hours (possibly days) following ring removal. With this IVR, we observed an approximate 0.5-log drop in TFV concentration in vaginal fluid 2 d after ring removal (Fig. 3A). This differs from an NNRTI such as dapivirine or MIV-150, in which the drug can more freely diffuse in and out of cells and can display, in the case of dapivirine matrix IVRs, approximately a 3-log drop in drug concentration from maximum levels to 2 d following IVR removal in humans (16, 22). It is therefore possible that NNRTI matrix rings that

Fig. 4. TDF IVR protects macaques from repeated vaginal viral challenge. (A) Six TDF IVR-treated cycling female macaques received weekly 50 TCID₅₀ SHIV162p3 inoculations starting 6 d after the first IVR insertion. Control macaques ($n = 6$ real time and $n = 6$ historical controls) were challenged similarly. The ring was replaced as shown in red (every 28 d starting 2 d after the fourth virus exposure). Macaques were monitored weekly (until week 20) for presence of SHIV by RT-PCR and confirmed by Western blot. Macaques were defined as infected and exposures discontinued if vRNA was detected in plasma for 2 consecutive weeks. (B) Kaplan-Meier plot showing time to infection for TDF IVR ($n = 6$; red) and control ($n = 6$ real time and 6 historical naïve; black) groups (nonparametric log-rank test; $P = 0.0007$). The median number of exposures to infection in the untreated group was four. (C) Plasma viral load kinetics in infected macaques aligned at peak. The red line is the median for all infected macaques (11/12). vRNA, viral RNA.

previously described (21, 38). TDF and TFV levels in vaginal fluid collected using Weck-Cels (Beaver Visitec), CVL (wash of genital tract with 5 mL PBS), and vaginal tissue (days 7, 21, and 30) were determined using liquid chromatography (LC)-MS/MS as described (21, 38). Procedures involving euthanasia and evaluation of intracellular TFV-DP were performed as described previously (4).

TDF, TFV, and TFV-DP levels were measured in blood, vaginal fluid, CVL, tissue and lymphocytes by LC-MS/MS methods as described previously (38, 39). The LLOQ for TDF was 1 ng/mL (tissue and blood) and 0.5 ng/mL (vaginal fluid), LLOQ for TFV was 5 ng/mL, and LLOQ for intracellular TFV-DP was 10 ng/mL, which is equivalent to ~ 13 fmol/ 10^6 cells (40). The average fluid and tissue mass was 0.04 g and 0.01 g, respectively. The concentration of drug in vaginal fluid was determined by converting the change in the swab mass to volume, assuming the density of vaginal fluid was 1.0 g/mL. Samples below LLOQ were assigned values midway between zero and LLOQ and then dividing by the mass or volume of the sample.

Efficacy Studies. TDF IVRs were administered to normal cycling, non-synchronized female pigtailed macaques ($n = 6$) followed by weekly inoculation (12) vaginally with 50 TCID₅₀ SHIV162p3 in six TDF-treated and 12 untreated controls (six real time and six historical controls) (39). The first virus exposure was started 6 d after IVR insertion; thereafter, the macaques were inoculated on a weekly basis. The first TDF IVR was replaced on day 30; subsequent IVR changes were done every 28 d, which corresponded to 2 d after the fourth, eighth, and 12th viral inoculation resulting in a total of four

IVR changes in the study period (Fig. 4A). Vaginal swabs for determining drug concentration were collected with every IVR change. Infection status was monitored by RT-PCR and confirmed by serology (ZeptoMetrix) (3, 4). The detection limit of the assay was 50 copies/mL. Positive macaques were defined as having two consecutive positive PCR results above detection limit. Macaques were monitored for 28 d after the last viral inoculation. The antiviral activity of CVL samples diluted 1:10 in PBS was assessed using HIV-1_{BAL} in the TZM-bl assay as previously detailed (41).

Statistical Methods. Fisher's exact test was used to compare the treated and control groups for number of infections per total number of virus exposures. A nonparametric log-rank test was used to compare survival probability curves. Spearman rank-order correlation coefficients were calculated to assess associations between antiviral activity of CVL and drug levels.

ACKNOWLEDGMENTS. We thank David Garber, James Mitchell, Frank Deyoungs, Shanon Ellis, and Leecresia Jenkins for all animal procedures; Chou-Pong Pao for analysis of drug levels; and Gerardo Garcia-Lerma and Jessica Radzio for providing the historical macaque control data. We acknowledge Gilead Sciences for providing tenofovir disoproxil fumarate. This work was supported by the National Institutes of Health Grant U19 AI076980. The findings and conclusions in this paper are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

- Holmes D (2012) FDA paves the way for pre-exposure HIV prophylaxis. *Lancet* 380(9839):325.
- Abdool Karim Q, et al.; CAPRISA 004 Trial Group (2010) Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329(5996):1168–1174.
- Parikh UM, et al. (2009) Complete protection from repeated vaginal simian-human immunodeficiency virus exposures in macaques by a topical gel containing tenofovir alone or with emtricitabine. *J Virol* 83(20):10358–10365.
- Dobard C, et al. (2012) Durable protection from vaginal simian-human immunodeficiency virus infection in macaques by tenofovir gel and its relationship to drug levels in tissue. *J Virol* 86(2):718–725.
- Hendrix CW, et al. (2013) MTN-001: Randomized pharmacokinetic cross-over study comparing tenofovir vaginal gel and oral tablets in vaginal tissue and other compartments. *PLoS ONE* 8(1):e55013.
- Amico KR, Mansoor LE, Corneli A, Torjesen K, van der Straten A (2013) Adherence support approaches in biomedical HIV prevention trials: Experiences, insights and future directions from four multisite prevention trials. *AIDS Behav* 17(6):2143–2155.
- Montgomery ET, et al. (2012) Vaginal ring adherence in sub-Saharan Africa: Expulsion, removal, and perfect use. *AIDS Behav* 16(7):1787–1798.
- Kiser PF, Johnson TJ, Clark JT (2012) State of the art in intravaginal ring technology for topical prophylaxis of HIV infection. *AIDS Rev* 14(1):62–77.
- Singer R, et al. (2012) An intravaginal ring that releases the NNRTI MIV-150 reduces SHIV transmission in macaques. *Sci Transl Med* 4(150):150ra123.
- Aravantinou M, et al. (2012) The nonnucleoside reverse transcription inhibitor MIV-160 delivered from an intravaginal ring, but not from a carrageenan gel, protects against simian/human immunodeficiency virus-RT infection. *AIDS Res Hum Retroviruses* 28(11):1467–1475.
- Fetherston SM, et al. (2013) Partial protection against multiple RT-SHIV162P3 vaginal challenge of rhesus macaques by a silicone elastomer vaginal ring releasing the NNRTI MC1220. *J Antimicrob Chemother* 68(2):394–403.
- Otten RA, et al. (2005) Multiple vaginal exposures to low doses of R5 simian-human immunodeficiency virus: Strategy to study HIV preclinical interventions in nonhuman primates. *J Infect Dis* 191(2):164–173.
- Mesquita PM, et al. (2012) Intravaginal ring delivery of tenofovir disoproxil fumarate for prevention of HIV and herpes simplex virus infection. *J Antimicrob Chemother* 67(7):1730–1738.
- Robbins BL, Srinivas RV, Kim C, Bischofberger N, Fridland A (1998) Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-R-(2-phosphonomethoxypropyl)adenine (PMPA), Bis(isopropylloxymethylcarbonyl)PMPA. *Antimicrob Agents Chemother* 42(3):612–617.
- Chen J, et al. (2012) Biphasic elimination of tenofovir diphosphate and nonlinear pharmacokinetics of zidovudine triphosphate in a microdosing study. *J Acquir Immune Defic Syndr* 61(5):593–599.
- Mesquita P, Kay M, Herold B (2013) Differential intracellular retention of drugs: A tool for rational design of pre-exposure prophylaxis combinations. *20th Conference on Retroviruses and Opportunistic Infections* (CROI Foundation, Atlanta), p 987.
- Yuan LC, Dahl TC, Oliyai R (2001) Degradation kinetics of oxycarbonyloxymethyl prodrugs of phosphonates in solution. *Pharm Res* 18(2):234–237.
- Fardis M, Oliyai R (2007) Case study: Tenofovir disoproxil fumarate: An oral prodrug of tenofovir prodrugs. *Biotechnology: Pharmaceutical Aspects*, eds Stella VJ, et al. (Springer, New York), pp 1347–1357.
- Johnson TJ, et al. (2012) A 90-day tenofovir reservoir intravaginal ring for mucosal HIV prophylaxis. *Antimicrob Agents Chemother* 56(12):6272–6283.
- Johnson TJ, Gupta KM, Fabian J, Albright TH, Kiser PF (2010) Segmented polyurethane intravaginal rings for the sustained combined delivery of antiretroviral agents dapivirine and tenofovir. *Eur J Pharm Sci* 39(4):203–212.
- Johnson TJ, et al. (2012) Safe and sustained vaginal delivery of pyrimidinone HIV-1 inhibitors from polyurethane intravaginal rings. *Antimicrob Agents Chemother* 56(3):1291–1299.
- Nel A, et al. (2009) Safety and pharmacokinetics of dapivirine delivery from matrix and reservoir intravaginal rings to HIV-negative women. *J Acquir Immune Defic Syndr* 51(4):416–423.
- Gupta KM, et al. (2008) Polyurethane intravaginal ring for controlled delivery of dapivirine, a nonnucleoside reverse transcriptase inhibitor of HIV-1. *J Pharm Sci* 97(10):4228–4239.
- Karim SS, Kashuba AD, Werner L, Karim QA (2011) Drug concentrations after topical and oral antiretroviral pre-exposure prophylaxis: Implications for HIV prevention in women. *Lancet* 378(9787):279–281.
- Schwartz JL, et al. (2011) A multi-compartment, single and multiple dose pharmacokinetic study of the vaginal candidate microbicide 1% tenofovir gel. *PLoS ONE* 6(10):e25974.
- Moss JA, et al. (2012) Tenofovir and tenofovir disoproxil fumarate pharmacokinetics from intravaginal rings. *AIDS* 26(6):707–710.
- Osterberg L, Blaschke T (2005) Adherence to medication. *N Engl J Med* 353(5):487–497.
- Kruk ME, Schwalbe N (2006) The relation between intermittent dosing and adherence: Preliminary insights. *Clin Ther* 28(12):1989–1995.
- van der Straten A, et al. (2012) High acceptability of a vaginal ring intended as a microbicide delivery method for HIV prevention in African women. *AIDS Behav* 16(7):1775–1786.
- Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA (2007) Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Mol Hum Reprod* 13(7):491–501.
- Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA (2012) Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *J Immunol* 188(5):2445–2454.
- Rebbapragada A, Kaul R (2007) More than their sum in your parts: The mechanisms that underpin the mutually advantageous relationship between HIV and sexually transmitted infections. *Drug Discov Today Dis Mech* 4(4):237–246.
- Valley-Omar Z, et al. (2012) CAPRISA 004 tenofovir microbicide trial: No impact of tenofovir gel on the HIV transmission bottleneck. *J Infect Dis* 206(1):35–40.
- Chakraborty H, et al. (2001) Viral burden in genital secretions determines male-to-female sexual transmission of HIV-1: A probabilistic empiric model. *AIDS* 15(5):621–627.
- Henning T, et al. (2011) Development of a pigtail macaque model of sexually transmitted infection/HIV coinfection using *Chlamydia trachomatis*, *Trichomonas vaginalis*, and SHIV(SF162P3). *J Med Primatol* 40(4):214–223.
- Radzio J, et al. (2012) Prevention of vaginal SHIV transmission in macaques by a co-italy-dependent Truvada regimen. *PLoS ONE* 7(12):e50632.
- Promadej-Lanier N, et al. (2009) Development and evaluation of a vaginal ring device for sustained delivery of HIV microbicides to non-human primates. *J Med Primatol* 38(4):263–271.
- Moss JA, et al. (2012) Safety and pharmacokinetics of intravaginal rings delivering tenofovir in pig-tailed macaques. *Antimicrob Agents Chemother* 56(11):5952–5960.
- Kuklenyik Z, et al. (2009) Effect of mobile phase pH and organic content on LC-MS analysis of nucleoside and nucleotide HIV reverse transcriptase inhibitors. *J Chromatogr Sci* 47(5):365–372.
- Kuklenyik Z, et al. (2009) On-line coupling of anion exchange and ion-pair chromatography for measurement of intracellular triphosphate metabolites of reverse transcriptase inhibitors. *J Chromatogr B Analyt Technol Biomed Life Sci* 877(29):3659–3666.
- Keller MJ, et al. (2010) Postcoital bioavailability and antiviral activity of 0.5% PRO 2000 gel: implications for future microbicide clinical trials. *PLoS ONE* 5(1):e8781.