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A 90-Day Tenofovir Reservoir Intravaginal Ring for Mucosal HIV Prophylaxis

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A vaginal gel containing the antiretroviral tenofovir (TFV) recently demonstrated 39% protection against HIV infection in women. We designed and evaluated a novel reservoir TFV intravaginal ring (IVR) to potentially improve product effectiveness by providing a more controlled and sustained vaginal dose to maintain cervicovaginal concentrations. Polyurethane tubing of various hydrophilicities was filled with a high-density TFV/glycerol/water semisolid paste and then end-sealed to create IVRs. *In vitro*, TFV release increased with polyurethane hydrophilicity, with 35 weight percent water-swelling polyurethane IVRs achieving an approximately 10-mg/day release for 90 days with mechanical stiffness similar to that of the commercially available NuvaRing. This design was evaluated in two 90-day *in vivo* sheep studies for TFV pharmacokinetics and safety. Overall, TFV vaginal tissue, vaginal fluid, and plasma levels were relatively time independent over the 90-day duration at approximately 10^4 ng/g, 10^6 ng/g, and 10^1 ng/ml, respectively, near or exceeding the highest observed concentrations in a TFV 1% gel control group. TFV vaginal fluid concentrations were approximately 1,000-fold greater than levels shown to provide significant protection in women using the TFV 1% gel. There were no toxicological findings following placebo and TFV IVR treatment for 28 or 90 days, although slight to moderate increases in inflammatory infiltrates in the vaginal epithelia were observed in these animals compared to naïve animals. In summary, the controlled release of TFV from this reservoir IVR provided elevated sheep vaginal concentrations for 90 days to merit its further evaluation as an HIV prophylactic.

Recent progress in antiretroviral HIV prevention research has advanced the field from concept toward medical practice (46). The CAPRISA 004 study demonstrated that a vaginal gel containing the reverse transcriptase inhibitor tenofovir (TFV) was partially effective in preventing HIV transmission in women (1), with significant protection observed in women who maintained preventative TFV concentrations of at least 1,000 ng/ml in vaginal fluid (23). However, the overall effectiveness (39%) was likely reduced by poor user adherence to the inconvenient before-and-after-sex dosing regimen. The correlation of adherence and TFV vaginal fluid concentrations to protection was a key finding (23, 24), indicating the need for vaginal drug delivery systems that attain and maintain elevated user adherence and vaginal drug concentrations. More recently, the VOICE trial tested the same TFV 1% gel formulation as CAPRISA 004 but with a once-daily dosage regimen and failed to show any effectiveness in women. Here, as well, low adherence may have contributed to the gel's inability to prevent HIV transmission (54). As a result, we (6, 21) and others (4, 35, 36, 44, 49) aim to develop TFV drug delivery systems to provide sustained protective vaginal tissue concentrations and potentially increase user adherence.

The micromolar anti-HIV activity of TFV motivated selection of the high dose in the CAPRISA 004 trial (up to two 40-mg doses within 24 h). Inter- and intrauser TFV vaginal fluid and tissue concentrations were likely dependent on several poorly understood factors and processes, such as adherence, time between gel applications, vaginal product clearance, menstrual cycle phase, vaginal fluid volume and composition, and frequency of intercourse (i.e., more sex acts over a given time would result in higher vaginal TFV administration since the dosing regimen was coitally dependent). Moreover, episodic dosage forms like gels are intrinsically short acting; the TFV 1% gel formulation attains peak vaginal tissue concentrations in women 2 h postvaginal application and diminishes rapidly thereafter (45). Finally, the anatomical site and kinetics of HIV transmission itself are poorly understood (19). Therefore, a drug delivery system that maintains elevated yet controlled and consistent TFV cervicovaginal tract drug concentrations over a duration longer than HIV transmission and throughout multiple episodic HIV exposures has the potential to increase efficacy over the dynamic drug levels provided by vaginal gels.

The silicone intravaginal ring (IVR), invented circa 1970 (8, 34), was designed to elute hormones for a 30-day duration and provide sustained drug levels in the range of 10 to 100 μg/day. Since then, there has been little innovation in IVR technology. In fact, current IVR technology is inadequate to meet the high topical dose requirements of TFV. Groups have claimed successful TFV formulation and delivery from silicone- and ethylene vinyl acetate-based IVRs (4, 35, 36, 44, 49), yet the *in vitro* and *in vivo* daily delivery rates reported were in micrograms, rather than milligrams, as is required for HIV prophylaxis with TFV. The TFV release from silicone and ethylene vinyl acetate polymers is therapeutically insignificant, primarily due to TFV's hydrophilicity

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and resultant low solubility in these elastomeric polymers commonly used for IVRs.

Although IVRs have a higher per-unit cost than gels, for a global health application, they offer the advantage of distributing the per-unit cost over many days, weeks, or months (15). Therefore, from an economic perspective, a long-lasting and low-manufacturing-cost IVR may be more affordable than a frequently applied gel since hundreds of gel units would be needed per year, as opposed to several rings. However, developing a long-duration IVR to deliver milligrams per day of TFV for at least 90 days requires a design capable of delivering approximately 1 g of TFV from a 3- to 6-g IVR. With such a high weight fraction of drug incorporated in the IVR, maintaining both time-independent TFV release and ring mechanical stiffness for a 90-day duration presents a significant design challenge. We recently reported TFV delivery from hydrophilic polyether urethane (HPU) matrix IVRs formulated with up to 20 weight percent (wt%) TFV (6, 21). Although TFV release rates were greatly improved compared to hydrophobic polymer IVRs, matrix IVRs inherently demonstrate decreased drug release rates with time. Matrix IVRs with a high fraction of undissolved drug similarly display time-dependent decreases in mechanical stiffness as the drug is released. Therefore, we developed and evaluated *in vitro* and *in vivo* the first reservoir IVR using a water-absorbable polyurethane as a rate-controlling membrane capable of delivering 10 to 30 mg of TFV daily for up to 90 days. Herein, we report the ring's *in vitro*/*in vivo* TFV release, mechanics, and vaginal safety and TFV pharmacokinetics in a sheep model.

MATERIALS AND METHODS

Materials. Hydrophilic aliphatic polyether urethanes (HPU) Tecophilic HP-60D-20, HP-60D-35, and HP-60D-60 were purchased from Lubrizol Advanced Materials (Wickliffe, OH), with equilibrium mass increases in water of approximately 20, 35, and 60 wt% of their dry mass, and shore hardnesses of 43D, 42D, and 41D, respectively. A custom-synthesized 35-wt% swelling HPU with 78A shore hardness was provided by DSM Biomedical (Berkeley, CA). Tenofovir monohydrate was supplied by Gilead Sciences (Foster City, CA). TFV 1% gel was supplied by Patheon Pharmaceuticals (Cincinnati, OH). United States Pharmacopeia (USP) grade glycerol and water were purchased from Spectrum Chemical (New Brunswick, NJ). Starch 1500 USP grade partially pregelatinized maize starch was provided at no cost by Colorcon (Harleysville, PA). Unless noted, all solvents and reagents were ACS grade.

Ring fabrication. HPU resins were dried overnight in a compressed-air microdryer (Dri-Air, East Windsor, CT) to less than 0.05-wt% water content, as determined using Karl Fisher titration (Mettler-Toledo, Columbus, OH). The dried pellets were fed into a three-quarter-inch single-screw hot-melt extruder attached to an advanced torque rheometer drive (C.W. Brabender, South Hackensack, NJ) with a tubing crosshead (Guill Tool, West Warwick, RI). The extruder heating zones (1 to 3) were set at 150, 160, and 170°C, and the tubing crosshead tip and die temperatures were 150°C and 130°C, respectively. Upon leaving the crosshead, the extrudate was drawn down using a CPC2-12 combination puller/cutter (Conair, Cranberry Township, PA) to create a final tubing product with 0.7-mm wall thickness and a 5.5-mm cross-sectional diameter. The extruded tubes were cut to 171 mm in length and weighed, and the tubing lumens were filled with either 100% TFV powder or a 65:33:2 wt% TFV/glycerol/water mixture, resulting in 1.6 g of TFV loaded into each IVR lumen. The TFV/glycerol/water semisolid was mixed using a Hobart (Troy, OH) mixer with the "B" beater attachment and back-filled into the tubing lumen using a high-pressure hydraulic filling system (Dymax, Torrington, CT). TFV powder was manually filled into the tubing lumen. The tubing ends were sealed using an induction welder equipped with a stainless steel reverse bonding die, and the resultant plugged ends were welded together using a stainless steel split die induction welder (PlasticWeld Systems, Inc., Newfane, NY) to create an IVR. A custom-machined 12-cavity aluminum mold was used to anneal and shape the IVRs in a circular conformation and minimize tubing lumen kinking. IVRs were placed in the mold, which was first heated via water circulation to 65°C for 15 min, followed by 5 min cooling at 10°C.

***In vitro* TFV release and IVR mechanical testing.** *In vitro* release and ring mechanical compression testing were performed as previously described (6, 21). Briefly, IVRs were immersed in 50 ml of 25 mM sodium acetate buffer (pH 4.2) at 37°C and 80 rpm. Release medium was periodically collected for TFV concentration measurement by high-performance liquid chromatography (HPLC) and replaced every 24 h to maintain sink conditions. Simultaneously, IVRs were periodically subjected to mechanical compression testing. Briefly, a custom-machined probe attached to an Instron 3342 uniaxial mechanical testing system was used to compress the IVR 25% of its initial 55-mm diameter at a rate of 1 mm/sec and record the required force. The force values corresponding to 10% ring compression were compared across all samples.

Drug content analysis for *in vitro* studies. TFV *in vitro* release sample concentrations were determined by HPLC using a method previously described (6). Briefly, 2 μl of sample was injected on an Agilent (Santa Clara, CA) 1200 series HPLC with a diode array detector and a Phenomenex (Torrance, CA) Luna C_{18} 5-μm, 150- by 4.6-mm column. A 15-min gradient method consisted of 100% mobile phase A (potassium phosphate buffer, pH 6.0) switching to 100% mobile phase B (95.5:4.5 [vol/vol] acetonitrile-potassium phosphate buffer, pH 6.0) upon run completion. The flow rate was 1.5 ml/minute with a typical TFV retention time of 7 min and TFV detection at 260 nm. Cumulative percent release was calculated by numerically integrating between collection time points using the trapezoidal rule and subsequently dividing by the original amount of TFV in the IVR.

Sheep pharmacokinetic study (study 1). The 78A-shore-hardness HPU IVR (35-wt% swelling) and the clinically tested TFV 1% gel were comparatively evaluated for pharmacokinetics in 1- to 2-year-old Dorset Crossbred sheep. The study was conducted at MPI Research (Mattawan, MI), which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The study protocol was reviewed and approved according to the standard procedures of the performing laboratory's Institutional Animal Care and Use Committee. The sheep were housed indoors, and fluorescent lighting was provided for approximately 12 h per day. On occasion, the dark cycle was interrupted due to study-related activities. Reproductive cycles were not monitored or synchronized, and the study was performed in September through December. Eight animals in treatment group 1 received a TFV IVR that was inserted in the vagina on day 0 and retained in the vaginal tract for 90 days. The exterior of the vagina was cleaned with chlorhexidine solution, and the IVR was inserted as aseptically as practical using a gloved hand. Following insertion, a speculum was used to confirm correct placement of the ring, and the animals were examined daily for evidence that the ring was still in place. If the ring was expelled or otherwise needed to be replaced, a new ring was inserted and the old ring was recovered for residual drug content analysis (if found). Seven animals in treatment group 2 received once-daily vaginal administration of 4 ml of the TFV 1% gel for 28 days (40 mg/day). Each gel dose was administered using a prefilled, single-dose polypropylene applicator (HTI Plastics, Lincoln, NE) similar to that used in the clinical gel administration regimen. Vaginal tissue, vaginal fluid, and blood plasma periodically were collected for drug content analysis throughout the dosage duration and up to 3 days following the last gel dose or IVR removal, as depicted in Fig. 1. Blood samples (approximately 1 ml) were collected from all animals via the jugular vein, placed in tubes containing K_3EDTA anticoagulant, and centrifuged under refrigeration to isolate the blood plasma. At all specified time points, two Weck-Cel (distributed by Beaver-Visitec International, Inc., Waltham, MA) swabs and biopsy specimens

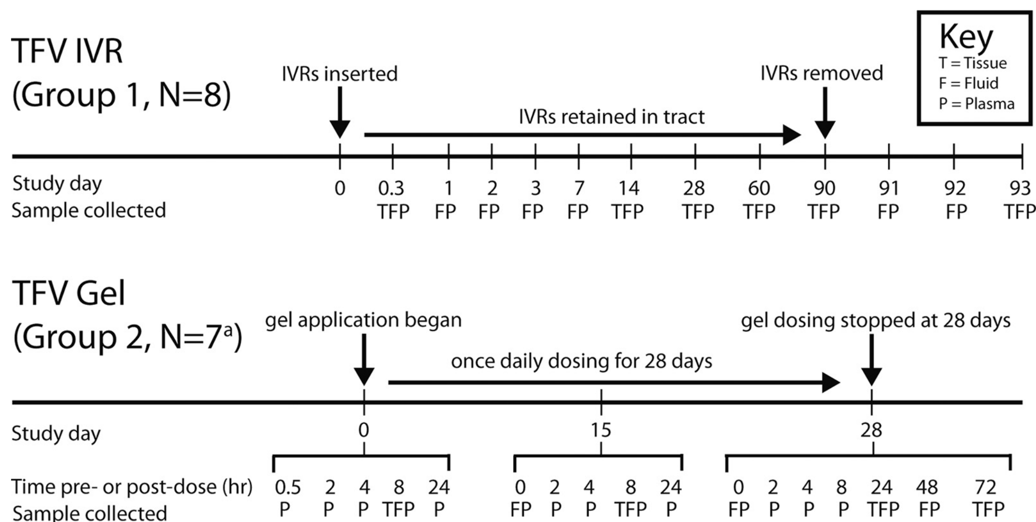

FIG 1 Diagram of the sheep pharmacokinetic study design/dosing protocol for TFV IVR group 1 (top) and TFV gel group 2 (bottom). a, $n = 6$ for the group 2 day 28 time point.

were collected per animal for TFV concentration determination in vaginal fluid and tissue, respectively, with one proximal to the ring/gel site of administration (cranial vagina, 5 to 7.5 cm from the introitus) and one distal to the ring/gel site of administration (caudal vagina, 2 to 4 cm from the introitus). Weck-Cel swabs were preweighed, inserted, allowed to absorb fluid for approximately 1 min, and subsequently removed and reweighed to determine fluid mass uptake. Prior to vaginal tissue biopsy specimen collection, animals were anesthetized and a chlorhexidine solution was used to clean the exterior vagina. Kevorkian-Younge biopsy forceps (Miltex, York, PA) and speculum were then used to acquire approximately 50 mg biopsy specimens. Vaginal tissue biopsy location (left or right side) was alternated with time point to minimize tissue trauma and facilitate healing. If bleeding was observed after biopsy specimen collection, direct pressure with gauze or gauze with thrombin was applied to encourage clotting. Following biopsy specimen collection, a local analgesic was given. Upon completion of the 90-day time period, IVRs were removed and analyzed for remaining drug content as described below. At study termination on days 93 (group 1, 72 h following IVR removal) and 31 (group 2, 72 h after the last gel dose), surviving animals were euthanized by an intravenous overdose of sodium pentobarbital solution followed by exsanguination via severing the femoral or axillary vessels, and vaginal tissues were collected for final drug content determination. All samples (blood plasma, Weck-Cel swabs, and tissues) were snap-frozen and stored at −70°C until bioanalysis was performed, as described below.

TFV measurement in plasma, vaginal fluid, and vaginal tissue. TFV was extracted from vaginal tissue, vaginal fluid, and plasma and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS), similar to methods described elsewhere (7, 36). Briefly, for TFV plasma quantification, 100 μl of standard, quality control sample or study sample was mixed with 50 μl of a working internal standard TFV-d_6 solution at 50 ng/ml. A 500-μl aliquot of 0.5% formic acid in water was added to all plate wells, and the samples were transferred to a preconditioned Oasis MCX solid-phase extraction plate. The plate was then washed with 800 μl of 0.5% formic acid in water and 800 μl of methanol, followed by sample elution with 600 μl of 5:95 ammonium hydroxide/methanol. The eluent was evaporated to dryness, and the residue was reconstituted with 200 μl of 0.1% ammonium hydroxide in water. The samples were vortexed, and an aliquot of each sample was injected onto an LC-MS/MS system for analysis. Similarly, TFV was measured in tissue by mixing 200 μl of standard, quality control sample or study sample with 50 μl of a working internal standard solution containing TFV-d_6 at 50 ng/ml. The samples were vortexed and centrifuged, and an aliquot of each sample was injected onto an LC-MS/MS system for analysis. Lastly, swabs were processed by mixing 50 μl of standard, quality control sample or study sample with 50 μl of a working internal standard TFV-d_6 solution (200 ng/ml) in a tube containing a clean spear or study sample. A 950-μl aliquot of 50:50 methanol/water was added to the standards and quality control samples, whereas a 1,000-μl aliquot of 50:50 methanol/water was added to the study samples. The samples were vortexed and centrifuged, and an aliquot of the supernatant was injected onto an LC-MS/MS system for analysis.

The chromatography varied with sample type: plasma samples were analyzed using a Phenomenex Synergi Polar-RP column that was 75 mm by 2 mm (particle size, 4 μm) with a gradient flow of 0.1% acetic acid in water, 0.1% acetic acid in acetonitrile, and 0.2% ammonium hydroxide in water at a flow rate of 300 to 500 μl/minute. Swabs were analyzed using the same column but with an isocratic flow of water/acetonitrile/acetic acid/ammonium hydroxide (930:70:5:1) (vol/vol/vol/vol) at a flow rate of 200 μl/minute. Tissue samples were analyzed using a BioBasic (Markham, ON) AX column that was 50 by 3.0 mm (particle size, 5 μm) with a gradient flow of acetonitrile/10 mM ammonium acetate in water at pH 6 (30:70) and acetonitrile/1 mM ammonium acetate in water at pH 10.5 (30:70) at a flow rate of 400 to 1,000 μl/minute.

The analyte and internal standard were detected using a Sciex (Framingham, MA) API 5000 triple quadrupole LC-MS/MS system equipped with an electrospray ionization (ESI) (TurboIonSpray) ionization source operated in the positive and negative ion mode. Multiple-reaction-monitoring mode transitions of the respective ions were used to monitor TFV and TFV-d_6 and may have been slightly modified to optimize system performance. For TFV, *m/z* 288 to 176 was monitored with a retention time of 1.15 to 3.67 min. For TFV-d_6, *m/z* 294 to 182 was monitored with a retention time of 1.15 to 3.65 min. The lower limits of quantification (LOQs) for TFV in plasma, Weck-Cel, and vaginal tissue were 1.00 ng/ml, 5.00 ng/spear, and 20.0 ng/g, respectively.

TFV pharmacokinetic analysis. Individual pharmacokinetic parameters were determined by noncompartmental methods using WinNonlin Phoenix software (Pharsight Corporation, Sunnyvale, CA). Area under the curve (AUC) estimates were determined using the linear trapezoidal rule with the linear interpolation calculation method. Values below the respective LOQs were treated as "0" for the analysis. Pharmacokinetic parameters were defined as follows: T_{max}, time of maximum observed concentration; C_{max}, maximum observed concentration occurring at T_{max}; and AUC_{0-t}, area under the curve from the time of dosing to the time of the last observation (IVR group only).

TABLE 1 Sheep safety study design

Group	Treatment	Dose	No. of animals	
			28 days	90 days
1	Control	Sham dose only	5	5
2	Placebo IVR	1 IVR/animal	5	5
3	TFV IVR	1 IVR/animal	5	5

Quantification of IVR residual TFV content. TFV was extracted out of the recovered IVRs to determine the total amount of TFV released. Each IVR was cut into approximately 1-cm segments that were placed together in a 50-ml volumetric flask, and 100 mM phosphate buffer (pH 7.4) was added to dissolve the entirety of the residual TFV. After removing the polymer segments, the flask was filled to volume and an aliquot of the resulting solution was diluted volumetrically and analyzed for TFV content by HPLC as previously described (6). Original semisolid material used for IVR manufacture was kept and extracted in the same manner to calculate percent recovery based on IVR fill mass.

Sheep toxicology study (study 2). The local tolerance and systemic toxicity of the 78A-shore-hardness HPU TFV IVR (35-wt% swelling) were assessed in female Welsh Mule sheep (age, 49 months; weight at the start of study was 59.5 to 83.5 kg) over a 1-month (28-day) or 3-month (90-day) period, in comparison with a placebo IVR or sham control (i.e., underwent restraint and sham insertion, but no ring was inserted). The placebo IVR consisted of 60:38:2 wt% starch/glycerol/water in the tubing lumen, but otherwise, the IVR was manufactured using HPU tubing, processes, and equipment identical to those for the TFV IVR. The study was conducted at Huntingdon Life Sciences (Huntingdon Life Sciences, United Kingdom), which is fully accredited by AAALAC International. The study design, outlined in Table 1, was based on the current International Conference on Harmonisation (ICH) Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (20), and the study protocol was reviewed and approved according to the standard procedures of the performing laboratory's Ethical Review Process Committee. Physical examinations were conducted on all animals pretreatment. The animals were observed at least twice daily throughout the study for any clinical abnormalities or signs of reaction to treatment. More-frequent observations were made on the day of dosing. Body weights were recorded weekly, and food consumption was recorded on a daily basis. Hematology, blood chemistry, and urinalysis were conducted on all animals pretreatment and pretermination (day 28 or day 90). Upon termination of the study, all animals were sacrificed and gross necropsy was conducted. Most major body organs were isolated and weighed. Tissues were then fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at approximately 4- to 5-μm thickness, and stained with hematoxylin and eosin (H&E) for histological examination by light microscopy. Microscopic findings were either reported as present (i.e., no adverse finding) or assigned a severity grade of minimal, slight, moderate, marked, or severe. A certified pathologist undertook a peer review of the microscopic findings.

In addition to the standard analyses described above, H&E-stained slides of the cranial vaginal tissue were used for vaginal irritation scoring in accordance with the rabbit vaginal irritation method of Eckstein et al. (9). Approximately 10 areas were analyzed using a Nikon 600 microscope, and individual scores from 0 to 4 were assigned depending on the extent of congestion, edema, leukocyte infiltration, and epithelial damage. The individual scores were combined to yield a total group score (0 to 16) that in rabbits correlates with human irritation potential.

Similar to the sheep pharmacokinetic study, TFV IVRs were retrieved from the animals prior to termination at 28 and 90 days and analyzed for remaining drug content as described above. In addition, blood plasma, vaginal fluid (Weck-Cel swabs), and vaginal tissue were collected from the TFV IVR-treated animals prior to termination and analyzed for TFV content using the methods described above.

RESULTS

***In vitro* characterization and optimization.** An elastomeric HPU tubular IVR design was utilized, as it maximized the weight ratio of the drug to the total device, was able to be filled with solid or liquefied drug, and provided sufficient time-independent mechanical stiffness and drug release. Previous research beyond the scope of this publication determined that HPU IVRs of 5.5-mm outer cross-sectional diameter, 0.7-mm wall thickness, and 55-mm outer diameter (Fig. 2) would provide ring mechanical properties, lumen volume, and drug release kinetics suitable for the proposed TFV IVR (data not shown). The *in vitro* TFV release rate was evaluated using various core compositions and equilibrium percent swelling HPU. IVRs containing only TFV powder in the lumen showed low yet steadily increasing TFV release rates with time which did not reach steady-state levels by 28 days (Fig. 3A). When the highly water-miscible and HPU-permeable compound glycerol was added into the tubing lumen, the lumen was rapidly hydrated and attained the equilibrium TFV release rate after only a 1-day transient state. The TFV release rate increased with polymer equilibrium swelling for 20-, 35-, and 60-wt% swelling Tecophilic HPUs. The 20-wt% swelling IVRs did not achieve the desired 10-mg/day minimum release rate and were stopped after 28 days, whereas the 35- and 60-wt% swelling IVRs achieved approximately 17- and 25-mg/day steady-state release rates, respectively. However, TFV was depleted from the 60-wt% swelling IVRs before the target 90-day duration. The TFV percent cumulative release profile was linear until greater than 90% of the initial drug load was depleted from the IVRs (Fig. 3B). The softer-shore-hardness 35-wt% swelling HPU IVR released 10 mg/day of TFV for 90 days (Fig. 4A) and demonstrated mechanical stiffness similar to that of NuvaRing (Fig. 4B). Furthermore, the HPU IVR stiffness did not change considerably with time when comparing the dry state, hydrated state, and after a majority of the TFV was

FIG 2 (A) Comparison of the HPU IVR with commercially available IVRs: Estring (left), TFV IVR with a novel HPU reservoir design (center), and NuvaRing (right). (B) Diagram of the HPU IVR with a 55-mm outer diameter (OD), a 0.7-mm wall thickness (W), and a 5.5-mm outer cross-sectional diameter (XD).

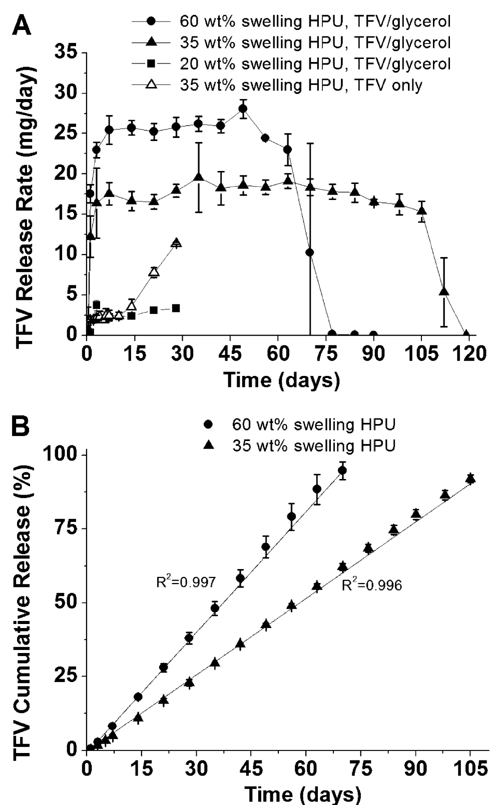

FIG 3 (A) *In vitro* TFV daily release rate as a function of time from prototype IVRs composed from various swelling HPU tubes whose lumen contained 65:33:2 wt% TFV/glycerol/water (solid symbols) or TFV only (open triangles). (B) Percent cumulative release as a function of time for 60 and 35-wt% swelling HPUs with glycerol in the tubing lumen. Data are means ± SD; $n = 3$.

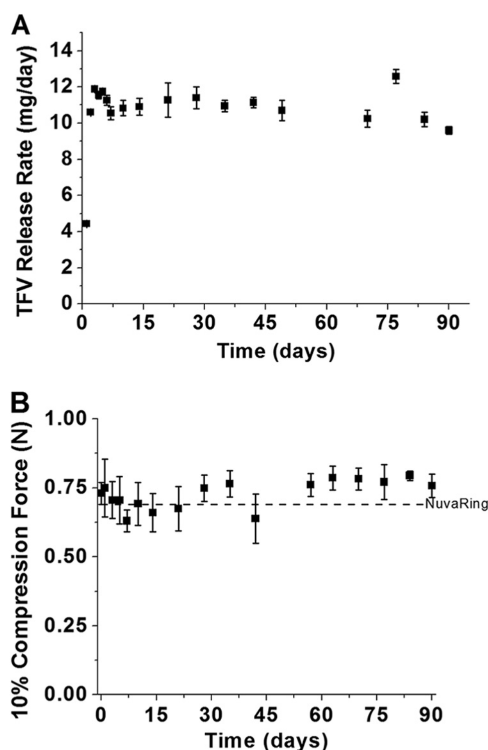

FIG 4 *In vitro* TFV release rate (A) and the force required for 10% compression of the ring diameter compared to that of NuvaRing (B) for the softer-shore-hardness 35-wt% swelling HPU IVRs composed of 65:33:2 wt% TFV/glycerol/water (IVRs were manufactured from the same HPU as tested *in vivo*). Data are means ± SD; $n = 5$.

released (i.e., 0, 1, and 90 days, respectively). Ten-percent ring compression values were compared, as this is approximately the *in vivo* compression of NuvaRing, as determined by magnetic resonance imaging (3).

TFV pharmacokinetics in sheep (study 1). The HPU TFV IVRs were evaluated in a sheep model to compare the TFV pharmacokinetics to those of the clinically tested TFV 1% gel. Vaginal rings were replaced in four animals throughout the 90-day duration, primarily due to expulsions. However, pharmacokinetic data from these sheep did not differ from those of sheep that retained a single IVR for the full 90-day duration, and thus, no data were excluded. One sheep in the TFV gel group expired on day 15, with complications from anesthesia being determined as the cause of death (non-treatment related). The calculated time-averaged *in vivo* TFV release rate from devices that were retained for 90 days *in vivo* was 17.0 ± 1.1 mg/day (mean ± standard deviation [SD]; $n = 5$), as determined by residual TFV extraction, approximately 70% higher than the 10-mg/day *in vitro* release rate.

TFV vaginal fluid concentrations from the IVR group attained their steady-state levels of approximately 10^6 ng/g by day 1, levels which were maintained for the remainder of the 90-day duration (Fig. 5A). Mean TFV concentrations from IVR insertion through 90 days were 3.5-fold higher proximal to the ring than distal. Comparatively, steady-state mean TFV vaginal fluid concentrations from the IVR group (approximately 10^6 ng/g) were similar to mean TFV vaginal fluid concentrations at 8 h after the gel dose (Fig. 5B). Mean TFV concentrations from the gel group were 1.4-fold higher proximal than distal. Up to 3 days beyond IVR removal (Fig. 6) and after the last gel dose, vaginal fluid concentrations for both groups were approximately 10^4 ng/g.

Eight hours after IVR insertion, TFV vaginal tissue concentrations were approximately 10^2 ng/g (Fig. 7A). TFV tissue concentrations at day 14 (the next biopsy time point) through day 90 were approximately 10^4 ng/g, with concentrations trending slightly higher with time. For the full duration of IVR residence in the vaginal tract (0 to 90 days), mean TFV tissue concentrations were similar proximal and distal to the ring. Mean TFV tissue concentrations were 10^4 ng/g 8 h after the 1st gel dose, similar to day 14 TFV concentrations with the IVR group (Fig. 7B). Eight hours after the 15th gel dose and 24 h after the 28th gel dose, mean TFV levels decreased to approximately 10^3 and 10^2 ng/g, respectively. TFV tissue concentrations 3 days after IVR removal and 3 days after the last gel administration were significantly lower, with several biopsy specimens from both groups below the LOQ. Across all gel group time points, mean TFV tissue concentrations were 3.1-fold higher proximal than distal. TFV-diphosphate concentration determination in vaginal tissue was also attempted but was undetectable or highly variable in both IVR and gel groups and thus was excluded from further analysis (data not shown).

Mean TFV plasma concentrations at day 7 through day 90 from the IVR group were steady at approximately 15 ng/ml, with the exception of day 14, when mean concentrations were 28 ng/ml (Fig. 8A). In contrast, the gel group achieved similar TFV concentrations at 2 h but rapidly decayed thereafter (Fig. 8B). Also in the gel group,

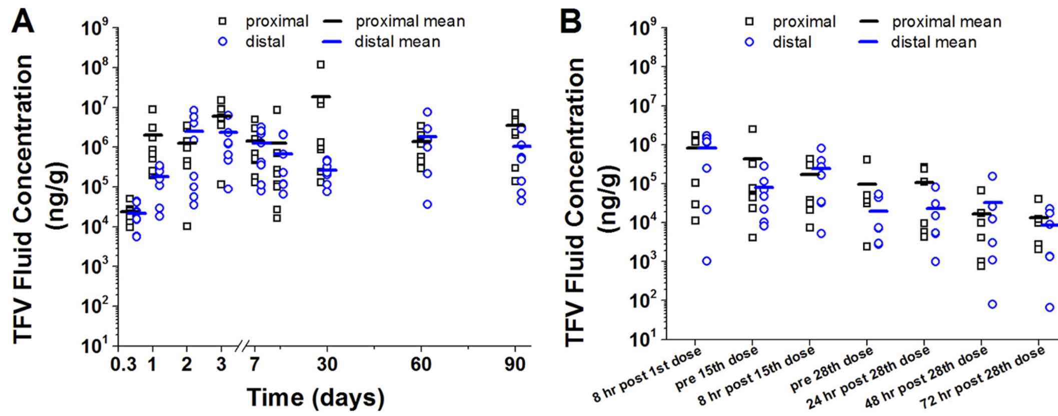

FIG 5 Proximal and distal TFV vaginal fluid concentrations for TFV IVR (A) and TFV gel (B).

mean postdose TFV plasma concentrations decreased with increased dose (mean TFV concentrations from the 1st dose > concentrations after the 15th dose > concentrations after the 28th dose). TFV was undetectable in plasma at 24 h after IVR removal or gel dose. TFV pharmacokinetic parameters for vaginal fluid, vaginal tissue, and plasma (Table 2) were consistent with the data presented above.

Toxicological evaluation. A second sheep study was performed to evaluate the systemic toxicity and local irritation potential of TFV IVRs compared to those of placebo IVRs and a sham control. IVRs were well tolerated and retained, with only one IVR expulsion noted on the day of scheduled removal (day 90). No treatment-related toxicological findings regarding body weight, food consumption, hematology, blood chemistry, urinalysis, organ weight, or gross pathology were observed. One- and 3-month histopathological inspections of vaginal tissue (Fig. 9) and cervical tissue were performed postnecropsy, whereby no epithelial disruption was observed in any group. Leukocytic infiltration in the vagina at 1 month and the uterine cervix at 3 months was greater with ring presence (TFV and placebo), with a grading of slight to moderate (see Table S1 in the supplemental material).

When quantifying the vaginal irritation potential in sheep, slight increases in the mean vaginal irritation scores were observed for both placebo and TFV IVRs compared to those of the sham controls (Fig. 10). Specifically, mean vaginal irritation scores for sham controls, placebo IVRs, and TFV IVRs were 1.8, 2.4, and 3.2, respectively, at 1 month and 2.2, 3.2 and 3.8, respectively, at 3 months. Differences between the TFV IVR and sham control were found to be statistically different at both time points (P values of 0.03 and 0.01 for 1 and 3 months, respectively; one-way analysis of variance [ANOVA] with Dunnett's multiple-comparison test).

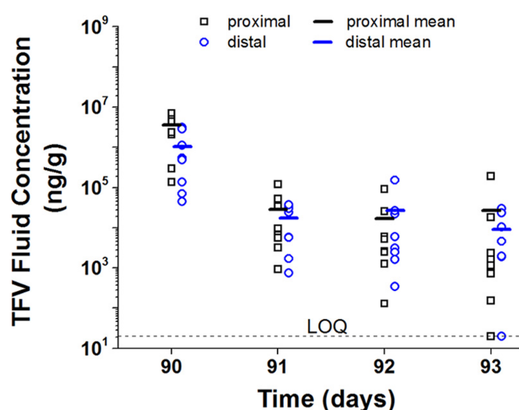

FIG 6 Proximal and distal TFV vaginal fluid washout concentrations following IVR removal on day 90.

Upon animal necropsy at 28 and 90 days, IVRs were recovered for drug content analysis, and plasma, vaginal fluid, and vaginal tissue were analyzed for TFV concentration. The time-averaged TFV release rates of IVRs retrieved from sheep after 28 and 90 days of treatment were 14.0 ± 2.8 and 16.9 ± 1.6 mg/day, respectively (mean ± SD; $n = 5$). TFV vaginal tissue, vaginal fluid, and plasma concentrations at 28 and 90 days were generally similar to those in the pharmacokinetic study (see Table S2 in the supplemental material).

DISCUSSION

We designed and tested a new reservoir intravaginal ring for long-duration TFV vaginal delivery. IVR fabrication incorporated materials and manufacturing methods commonly utilized by the pharmaceutical and medical device industries, making the IVR a scalable and cost-effective product for resource-poor regions in which the HIV pandemic is most prevalent (51). The 35-wt% swelling HPU released at least 10 mg/day of TFV *in vitro* for 90 days with time-independent IVR mechanical stiffness. In sheep receiving IVRs, TFV concentrations in plasma, vaginal fluid, and vaginal tissue were nearly time independent for 90 days at levels similar to peak concentrations in the TFV 1% gel group. Lastly, in the sheep safety study, no significant toxicological effects were observed, although slight to moderate increases in inflammatory infiltrates in the vaginal epithelia were observed with the placebo and TFV IVRs.

***In vitro* studies.** The steady-state *in vitro* TFV release rate increased with HPU equilibrium swelling, thus allowing simple modulation of the release rate by selecting an HPU with appropriate equilibrium swelling. Since TFV is virtually insoluble in the nonswelling polymer phase (6), increased water presence allowed for the water-soluble TFV to dissolve and diffuse through the aqueous phase of the hydrated polymer. The lower-durometer 35-wt% swelling HPU released approximately 10 mg/day TFV for 90 days with time-independent IVR mechanical stiffness similar to that of NuvaRing, which was utilized as a benchmark for mechanical stiffness due to its high user acceptance and extensive safety record in women (43).

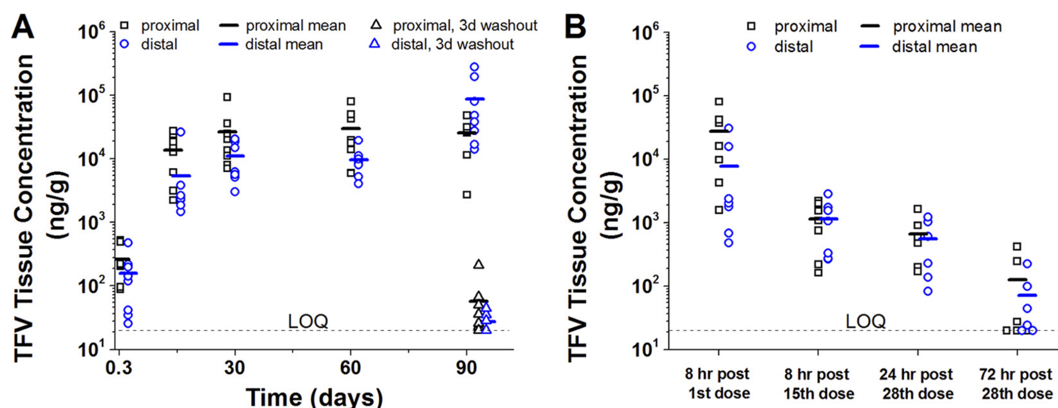

FIG 7 Proximal and distal TFV vaginal tissue concentrations for TFV IVR (A) and TFV gel (B).

Glycerol incorporation in the IVR core formulation yielded several benefits over 100% TFV powder, including easier lumen filling and increased TFV density to maximize TFV loading. Although TFV comprised approximately 36% of the ring's total mass, the ring mechanical stiffness remained unchanged throughout the 90-day duration even after the majority of the TFV was released. This unique performance attribute was due to the semisolid core, whose presence or absence did not significantly impact the overall ring elasticity, in contrast to the solid core TFV matrix IVR that was initially quite stiff but softened upon hydration and as TFV was released (6).

Glycerol also acted as an osmotic agent to rapidly draw water into the TFV-loaded core and nearly eliminated the time to steady-state TFV release. Following initial HPU hydration, we hypothesized that the small amount of water at the inner surface of the HPU lumen mixed with the high concentration of glycerol present, resulting in a large inward osmotic driving force since glycerol and water are infinitely miscible. Following lumen hydration, TFV quickly saturated the aqueous solution to establish a fixed TFV concentration gradient across the tubing wall and allow TFV release by membrane-controlled steady-state diffusion. In the absence of a highly water-miscible molecule in the tubing lumen, TFV steady-state release was not achieved after several weeks since TFV alone is a relatively poor osmotic agent (~1% [wt/vol] solubility in the acidic aqueous medium utilized) (6). The excess of undissolved and highly mobile TFV in the tubing lumen allowed for true time-independent TFV release until virtually the entire load was depleted, thus minimizing drug waste. Conventional solid-core polymeric reservoir IVRs, such as NuvaRing, create environmental and cost concerns since up to 85% of the initial drug load remains in the IVR at the end of its planned duration (60).

Another advantage of the HPU reservoir IVR design is its tunability, with which HPU equilibrium swelling, cross-sectional diameter, wall thickness, and shore hardness (elastic modulus) may be independently varied to achieve the desired TFV loading, TFV release rate, and ring stiffness. Commonly utilized silicone IVRs typically demonstrate a lower elastic modulus than thermoplastic elastomers and therefore require a wider cross-sectional diameter and/or the addition of an inert filler excipient to increase ring stiffness (12). As increasing IVR dimensions may negatively impact user acceptability (48), we minimized the HPU IVR cross-sectional diameter while still ensuring that the target TFV release rate and duration were achieved.

When accounting for the overall societal costs associated with HIV infection, including health care and inability to work, TFV 1% gel is expected to be quite cost-effective by South African standards at a projected cost of approximately $5/month (58, 61). The

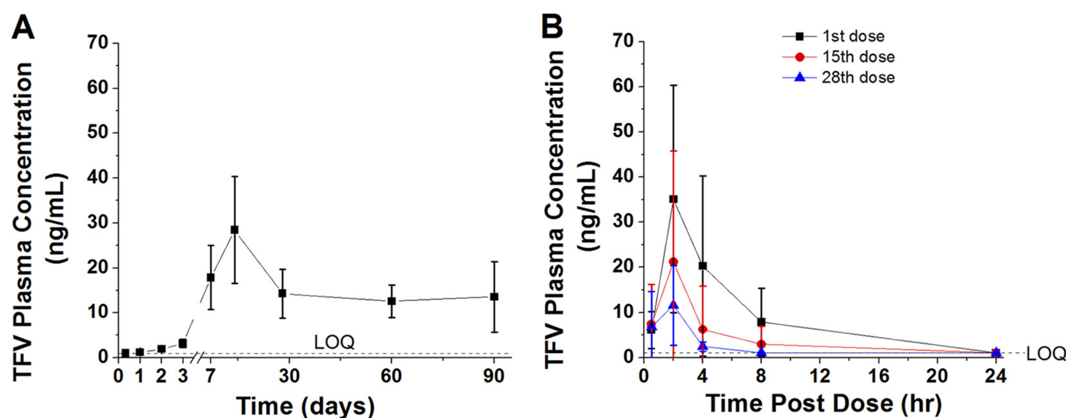

FIG 8 TFV plasma concentrations for TFV IVR (A) and TFV gel (B). TFV IVR and gel plasma levels were less than the LOQ at 24, 48, and 72 h following IVR removal and following the 28th gel dose (only 24 h after the gel dose are shown). Data are means ± SD; *n* = 8 and *n* = 7 for IVR and gel, respectively, except *n* = 6 for the 28th TFV gel dose.

TABLE 2 Mean (standard deviation) pharmacokinetic parameter values for the TFV IVR and TFV gel[a]

	Group 1 (IVR)			Group 2 (gel)		
Parameter	Proximal value	Distal value	Systemic value	Proximal value	Distal value	Systemic value
Vaginal fluid						
T_{max}[b] (h)	504	72		336	8	
C_{max} (ng/g)	2.4E+7 (3.9)	5.1E+6 (2.5)		1.1E+6 (0.8)	9.0E+5 (6.2)	
AUC_{0-t} (ng · h/g)	1.3E+10 (2.2)	2.4E+9 (2.2)		NA	NA	
Vaginal tissue						
T_{max}[b] (h)	1.1E+3	2.2E+3		8	8	
C_{max} (ng/g)	4.7E+4 (2.6)	8.8E+4 (9.7)		2.7E+4 (2.8)	8.1E+3 (11.2)	
AUC_{0-t} (ng · h/g)	5.2E+7 (2.0)	5.0E+7 (3.9)		NA	NA	
Plasma						
T_{max}[b] (h)			336			2
C_{max} (ng/ml)			31.3 (8.3)			46.8 (20.8)
AUC_{0-t} (ng · h/g)			3.2E+4 (0.6)			NA

[a] NA, not applicable.
[b] Median T_{max} reported. For the gel group, T_{max} refers to the time following the 1st dose (336 h = pre-15th gel dose).

90-day reservoir TFV IVR, with an estimated per-unit manufacturing cost of less than $1 (ProMed Pharma LLC, Plymouth, MN, personal communication and estimate), should therefore cost significantly less per month than the gel. Despite its unconventional design, the reported IVR combines existing pharmaceutical and medical device materials, techniques, and equipment to minimize associated manufacturing costs and ensure affordability in resource-poor countries. From a supply chain and distribution perspective, a year's supply of gel would require significant transportation efforts for both the supplier and the end user compared to 4 rings. In addition to lower transportation costs and end-user convenience, the IVR would also minimize waste management issues associated with the number of gel applicators and packaging required for frequent use.

***In vivo* pharmacokinetics.** In the CAPRISA 004 clinical trial evaluating the TFV 1% gel, women with vaginal fluid concentrations greater than 10^3 ng/ml were significantly more protected against HIV infection than women with concentrations less than 10^3 ng/ml (23). In the sheep pharmacokinetic study described in this report, the TFV IVR attained near-steady-state vaginal fluid concentrations at day 1 (10^6 ng/g or ng/ml) which were approximately 1,000 times higher than the clinically protective concentration, and this level was maintained for the remainder of the 90-day duration. Vaginal dosing of the TFV 1% gel both in a controlled clinical pharmacokinetic study previously reported by Schwartz et al. (45) and in our sheep gel group demonstrated peak 10^6-ng/g TFV vaginal fluid concentrations several hours postdose which were equivalent to the observed steady-state TFV IVR concentrations.

Although TFV tissue concentrations were not measured in the CAPRISA 004 trial, the clinical pharmacokinetic study by Schwartz et al. reported approximately 10^5-ng/g peak vaginal tissue concentrations at 2 h postdose which plateaued at around 10^4 ng/g thereafter (45). Our sheep gel group attained approximately

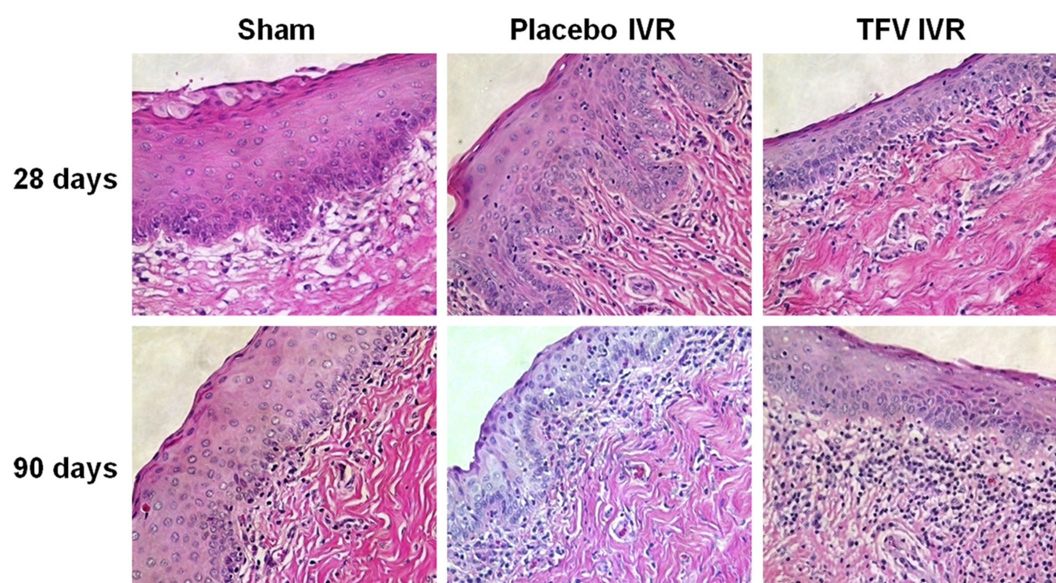

FIG 9 Representative micrographs of H&E-stained vaginal tissue after 1 or 3 months of treatment with sham control, placebo IVR, or TFV IVR.

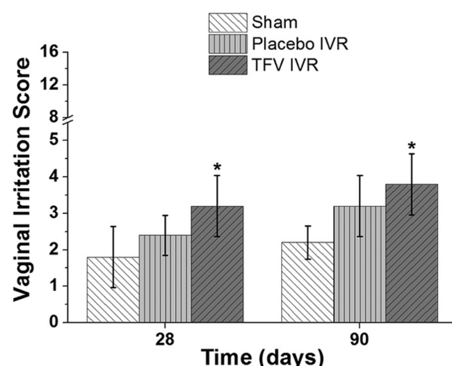

FIG 10 Sheep vaginal irritation scores (on a scale of 0 to 16) after 28 or 90 days of treatment with a TFV or placebo IVR or the sham control. Asterisks indicate that the values for TFV IVRs were significantly different from those for the sham control ($P < 0.05$; one-way ANOVA with Dunnett's multiple-comparison test). Data are means ± SD; $n = 5$.

10^3- to 10^4-ng/g tissue levels 8 h postdose but were lower at 24 h postdose. TFV tissue concentrations at 8 h after IVR insertion were comparatively lower than those at 8 h after the gel dose. However, mean TFV tissue concentrations of approximately 10^4 to 10^5 ng/g were observed at the next sampling time point (day 14) through the remaining dosing duration (day 90), values which were similar to C_{max} gel tissue concentrations. Since tissue biopsy time points between 8 h and 14 days were not collected, it is not known whether similarly high tissue concentrations were achieved earlier than 14 days with the TFV IVR. However, since vaginal fluid and plasma concentrations were near steady-state levels by days 1 and 7, respectively, it seems probable that near-steady-state tissue levels were reached within the first several days, as vaginal tissue acts as the intermediary compartment for drugs between vaginal fluid and the systemic circulation (62). The elevated sheep tissue TFV concentrations reported herein from the TFV HPU IVR were approximately 1,000 times higher than sheep tissue TFV concentrations from an alternative TFV IVR design (35), reflecting the nearly 1,000-fold-greater TFV *in vitro* release rate from the HPU IVR.

The mean TFV concentrations in vaginal fluid, vaginal tissue, and plasma in the IVR group were similar to or greater than the highest observed TFV concentrations in the gel group. This is an interesting result considering that the time-averaged daily TFV release from the IVR, as determined by residual drug extraction, was less than one-half of the daily TFV gel dose. The percentage of dosed TFV absorbed following TFV 1% vaginal gel administration has not been reported, but it is likely that a significant fraction is not absorbed because the gel leaks out of the vaginal tract (2, 33). The gel more rapidly achieved high TFV vaginal and systemic concentrations and therefore may be advantageous for coitally dependent, intermittent use when it is applied just prior to sex (similar to CAPRISA 004 dosing). Conversely, the IVR may be advantageous when women desire a long-lasting, coitally independent, and discrete dosage form.

It is generally assumed that antiretrovirals should be well distributed throughout the vaginal tract to maximize HIV prevention. TFV vaginal fluid and tissue concentrations measured proximal and distal to the IVR's placement were nearly identical over the 90-day period and trended similarly to concentrations for the TFV 1% gel. This finding corroborates previous TFV IVR pharmacokinetic studies in sheep in which TFV levels proximal and distal were indistinguishable (36). Conversely, vaginal biodistribution of hydrophobic antiretrovirals delivered from IVRs has been evaluated in women and macaques, and both studies have shown a higher drug concentration proximal to the ring than distal (22, 40). Therefore, if uniform vaginal biodistribution proves necessary, hydrophilic antiretrovirals such as TFV may be better suited for IVR delivery than hydrophobic antiretrovirals.

Reservoir drug delivery devices typically offer nearly time-independent release of molecules given that sink conditions are satisfied (25, 42). Time-independent TFV release rates were attained by day 2 *in vitro*, and similarly, TFV vaginal fluid concentrations stabilized by day 2 *in vivo*, suggesting that the device was releasing drug in a zero-order fashion as expected. Following a 3- to 7-day lag time, TFV plasma concentrations in the IVR group were steady for the remaining 90 days with the exception of day 14. Hydrophobic compounds are known to more rapidly cross the vaginal epithelium and enter the circulation than hydrophilic compounds (62). However, there have been limited reports of hydrophilic drug plasma pharmacokinetics from IVRs, in part due to IVR formulation limitations of such molecules as previously discussed. The reported TFV IVR did not provide an initial burst of drug, in contrast to conventional matrix and reservoir IVRs. Nonetheless, we observed a plasma concentration-time profile similar to that of the contraceptive NuvaRing, where plasma concentrations of the hydrophobic small molecules ethinyl estradiol and etonogestrel peaked approximately 1 week after IVR insertion in women (50).

A significantly higher release rate was observed *in vivo* than *in vitro*, a result which is atypical for controlled-release devices. Of note is that TFV is an acidic compound, with increased aqueous solubility at higher pH. The drug release rate from reservoir IVRs is proportional to the dissolved concentration of drug in the tubing lumen (55). Therefore, the sheep neutral vaginal pH may increase the dissolved TFV concentration in the IVR lumen, thus demonstrating higher TFV flux than in the *in vitro* pH 4.2 acetate buffer release medium. The pH of the healthy human vagina is acidic due to buffering by lactic acid-secreting microflora (62). Therefore, future studies in women will be needed to determine whether the increased *in vivo* flux in sheep is also observed in women.

The 35-wt% swelling HPU TFV IVR demonstrated similar TFV *in vivo* release rates and TFV vaginal concentrations in both sheep studies, although the second study did not include as many time points, as its primary objective was toxicological evaluation. Nonetheless, the studies at separate institutions utilizing two different sheep breeds confirmed the ability of the HPU TFV IVR to provide elevated and sustained TFV vaginal concentrations for up to 90 days.

***In vivo* toxicology.** An efficacious microbicide product must not compromise vaginal barrier function. Thinning or disruption of the protective epithelium and/or recruitment of susceptible immune cells via an inflammatory response may increase HIV infection, as was observed with early HIV prophylaxis trials using surfactants and anionic polymers (26, 52, 53). Microbicide IVRs are designed to be present in the vagina for a duration of weeks to months and, beyond brief excursions, would likely be reinserted or replaced throughout a woman's sexually active life. The potential long-term vaginal effects of an IVR and its corresponding influence on HIV prophylaxis are therefore a concern. Bounds et al. studied an early version of the hormone replacement therapy ring

Femring that had remarkable mechanical stiffness, and it was concluded that the ring contributed to the creation of ulcerative lesions in the vagina (5). Subsequently, several large clinical safety studies have evaluated an array of medicated and nonmedicated IVRs composed of silicone and ethylene vinyl acetate polymers comprising various dimensions and varied ring stiffness (14, 27, 41, 43, 47, 59). None of these studies found a significant ring contribution in creating epithelial lesions and/or altering vaginal microflora when tested for up to 1 year. Furthermore, several marketed IVRs have now recorded thousands of woman-years of use with favorable safety records (16, 43). Polyurethane IVRs have not been clinically evaluated to date, although evaluation in pigtail macaque monkeys shows no significant alteration in native microflora or mucosal and proinflammatory cytokines compared to those in naïve animals (22) or animals receiving silicone IVRs (J. Smith, Centers for Disease Control and Prevention, Atlanta, GA, personal communication).

Care was taken at the onset of product development to utilize materials that possess strong evidence of safety in humans. Medical-grade polyurethanes have demonstrated long-term biocompatibility in many biomedical and drug delivery applications (28). Of the potential water-miscible excipients, glycerol was utilized, as it is generally recognized as safe by the Food and Drug Administration and is one of the most commonly used excipients in vaginal formulations (17), including in the clinically efficacious TFV 1% gel (1). The TFV IVR contained approximately 825 mg of glycerol, 95% of which was released over the first 3 days in *in vitro* release medium (data not shown). This total amount of glycerol is approximately equivalent to that incorporated in a single dose of the TFV 1% gel (800 mg), which was well tolerated in women when dosed twice daily for 14 days (32) and demonstrated no significant adverse events in women who followed the "BAT24" CAPRISA 004 dosing regimen for up to 30 months (1).

Following 28 or 90 days of treatment with a single IVR, no signs of systemic toxicity were observed. Minimal cellular debris or bacterial adhesion was found on the IVR surface after 28 days in sheep as observed by electron microscopy (data not shown). Slight but notable changes in the microscopic pathology of the cervicovaginal epithelium were found to be associated with the presence of the placebo and TFV IVRs. These histopathological findings also correlated with a mild increase in the vaginal irritation potential of the IVRs. The modest increase in both leukocytic infiltration and vaginal irritation potential of sheep receiving placebo and TFV IVRs warrants further evaluation to determine any ring and/or TFV effect on long-term safety and prophylactic efficacy. Though sheep have been used for the systemic safety evaluation of microbicide IVRs (37) and to evaluate the effects of candidate vaginal microbicides on the epithelium (56, 57), they are yet to be considered a validated model for the testing of vaginal microbicide products. Nonetheless, this is the first time, to the best of the authors' knowledge, in which the vaginal irritation scoring methodology developed by Eckstein et al. (9) has been applied to sheep. As the semiquantitative methodology was relatively simple to perform yet yielded noticeable differences between test groups, additional vaginal irritation scoring of clinically tested microbicide products may help to define its usefulness as a nonclinical vaginal irritation model.

Sheep possess several additional advantages and limitations for HIV prophylactic IVR safety and pharmacokinetic evaluation beyond those mentioned above (for a more detailed discussion of the female sheep reproductive anatomy and physiology, the reader is referred elsewhere [10, 11, 13]). In contrast to the more commonly utilized rhesus and pigtail macaque nonhuman primates (18, 22, 30, 38), most sheep breeds demonstrate similar body mass and vaginal anatomical dimensions to women, allowing for human-sized IVR testing and avoidance of scaling issues when extrapolating ring mechanics, drug dosing, biodistribution, and pharmacokinetics (35, 36). Although sheep are not susceptible to infection by related immunodeficiency viruses, they are more affordable and easier to acquire than nonhuman primates. Similar to humans, the sheep vaginal epithelium is stratified squamous and thus is pertinent for toxicological irritation studies, such as the one described herein. The vaginal microflora species and vaginal fluid pH of sheep and women are generally different, although large variation in both variables exists in both populations (29, 31, 57). Another limitation of sheep is their seasonal breeding and shorter estrous cycle (approximately 17 days) compared to women, a difference which may potentially alter drug pharmacokinetics via changes in vaginal epithelium thickness and permeability (33, 39). The estrous cycle was not monitored or controlled in this study, and future studies may be justified to understand whether or not this variable significantly alters local or systemic drug pharmacokinetics.

The optimal TFV daily dose to deliver from a ring is unknown, as dose-ranging efficacy studies with any vaginal prophylactic TFV dosage form have not been performed. The reported TFV IVR demonstrated approximately 10-mg/day *in vitro* release rates which correspondingly maintained vaginal TFV concentrations similar to or exceeding peak concentrations with the TFV 1% gel. Future nonhuman primate and human toxicity and efficacy studies will aid in determining an optimal TFV daily dose. The reported IVR platform may be easily modulated to attain this optimal TFV daily dose by varying the HPU equilibrium percent swelling and/or tubing cross-sectional dimensions (as reported herein and in unpublished data).

In summary, we believe that the HPU reservoir IVR provided sustained sheep TFV vaginal fluid, vaginal tissue, and plasma concentrations for 90 days which were similar to peak concentrations in women using the TFV 1% gel. The TFV IVR showed no attributable toxicological effects, although a slight to moderate increase in inflammatory infiltration of the vaginal epithelium was observed with the placebo and TFV IVR compared to the sham control. While human studies are needed to confirm the safety, pharmacokinetics, and potential efficacy of this new HPU reservoir IVR design, the IVR achieved what are likely prophylactic concentrations for the 90-day study duration. This IVR design addressed conventional IVR limitations in delivering significant quantities of drug for sustained duration and further demonstrated flexibility in modulating drug release kinetics and ring mechanical stiffness. Altogether, these results support further exploration of the HPU reservoir IVR as a sustained TFV delivery system.

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