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Next-generation 3D printed multipurpose prevention intravaginal ring for prevention of HIV, HSV-2, and unintended pregnancy

Denali K. Dahl^{a,1}, Priya Srinivasan^{b,1}, Rima Januszewicz^a, Jasmine L. King^a, Roopali Shrivastava^a, Jining Zhang^b, Dawn Little^c, Shanon Bachman^d, Kristen Kelley^e, Mackenzie L. Cottrell^f, Amanda P. Schauer^f, Craig Sykes^f, Angela D.M. Kashuba^f, James Smith^{b,2,**}, S. Rahima Benhabbour^{a,g,*}

^aJoint Department of Biomedical Engineering, North Carolina State University and The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

^bLaboratory Branch, Division of HIV Prevention, National Center for HIV, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, GA, USA

^cKatmai Government Services (KGS), Anchorage, AK, USA

^d4ES Corporation, San Antonio, TX, USA

^eHonnête, LLC, Atlanta, GA, United States of America

^fDivision of Pharmacotherapy and Experimental Therapeutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

^gDivision of Pharmacoengineering and Molecular Pharmaceutics, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract

Globally, nearly half of all pregnancies are unintended, ~1.3 million new human immunodeficiency virus (HIV) infections are reported every year, and more than 500 million people are estimated to have a genital herpes simplex virus (HSV-2) infection. Here we report the

*Corresponding author at: Joint Department of Biomedical Engineering, North Carolina State University and The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. benhabs@email.unc.edu (S.R. Benhabbour). **Corresponding author at: Centers for Disease Control and Prevention, Atlanta, GA, USA. ajo9@cdc.gov (J. Smith).

¹These authors contributed equally.

²These authors jointly supervised this work.

CRedit authorship contribution statement

Denali K. Dahl: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Priya Srinivasan:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Rima Januszewicz: Writing – review & editing, Validation, Software, Methodology, Formal analysis. **Jasmine L. King:** Writing – review & editing, Validation, Formal analysis. **Roopali Shrivastava:** Visualization, Validation, Methodology, Formal analysis, Data curation. **Jining Zhang:** Visualization, Methodology, Investigation, Data curation. **Dawn Little:** Methodology, Formal analysis, Data curation. **Shanon Bachman:** Visualization, Validation, Data curation. **Kristen Kelley:** Formal analysis, Data curation.

Mackenzie L. Cottrell: Writing – review & editing, Investigation, Funding acquisition, Formal analysis, Data curation. **Amanda P. Schauer:** Validation, Formal analysis. **Craig Sykes:** Writing – original draft, Validation, Methodology, Formal analysis.

Angela D.M. Kashuba: Supervision, Resources. **James Smith:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **S. Rahima Benhabbour:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Appendix A. Supplementary data

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first 3D printed multipurpose prevention technology (MPT) intravaginal ring (IVR) for prevention of HIV, HSV-2, and unintended pregnancy. The IVRs were fabricated using state-of-the-art Continuous Liquid Interface Production (CLIP™) 3D printing technology using a biocompatible silicone-urethane based resin. Anti-HIV drug (Dapivirine, DPV), anti-herpes drug (Pritelivir, PTV) and a contraceptive drug (Levonorgestrel, LNG) were loaded in a macaque size IVR (25 mm outer diameter, OD; 6.0 mm cross-section, CS) allometrically scaled from the human size (54 mm OD; 7.6 mm CS) IVR analogue. All three active pharmaceutical ingredients (APIs) were loaded in the IVR using a single-step drug loading process driven by absorption. DPV, PTV, and LNG elicited zero-order release kinetics in vitro in simulated vaginal fluid (SVF) at pH 4 and pH 8 relevant to human and macaque vaginal pH respectively. CLIP 3D printed MPT IVRs remained stable after 6 months of storage at 4 °C with no change in physical, dimensional, or mechanical properties and no change in drug concentration and absence of drug degradation byproducts. The MPT IVRs elicited sustained release of all three APIs in macaques for 28 days with median plasma concentrations of 138 pg/mL (DPV), 18,700 pg/mL (PTV), and 335 pg/mL (LNG). Safety studies demonstrated that the MPT IVRs were safe and well tolerated in the macaques with no observed change or abnormalities in vaginal pH and no significant changes in any of the 22 mucosal cytokines and chemokines tested including pro-inflammatory (IL-1 β , IL-6, IL-8, IFN- γ , TNF- α , IL-17, IL-18) and anti-inflammatory (IL-10, IL-12) cytokines while the MPT IVR was in place or after its removal. Additionally, MPT IVRs elicited no observed alterations in systemic CD4⁺ and CD8⁺ T cells during the entire study. Collectively, the proposed MPT IVR has potential to expand preventative choices for young women and girls against unintended pregnancy and two highly prevalent sexually transmitted infections (STIs).

Keywords

Multipurpose prevention; Intravaginal rings; 3D printing; HIV; HSV-2; Contraception; macaques

1. Introduction

Globally, an estimated 121 million unintended pregnancies [1], ~1.3 million new human immunodeficiency virus (HIV) infections [2], and 374 million other sexually transmitted infections (STIs) [3] were reported in 2020. Despite increased use of modern contraceptives and condoms [4,5], distinct sexual and reproductive health challenges remain, particularly for young women (15–24 years old). Sub-Saharan Africa has seen the greatest improvement in reducing the burden of HIV over the past twenty years, however adolescent girls and young women (15–24 years old) remain at the highest risk and are twice as likely to be living with HIV than men [2]. Herpes simplex virus (HSV-2) is sexually transmitted and causes genital herpes. In 2023, 491 million people were living with HSV-2, which is equivalent to 13 % of the global population of reproductive age (15–49 years old) [6]. Furthermore, 30 % of new sexually acquired HIV infections in 2022 were attributable to established and recent HSV-2 infection [7–9], and women have a higher risk than cisgender heterosexual men to HIV with established and recently acquired HSV-2 infection [9]. The highest unmet need for contraception overlaps with the highest unmet need for prevention of sexually transmitted diseases in adolescents and young women [10], highlighting the need

for improved multipurpose prevention technologies (MPTs). MPTs address multiple sexual and reproductive health unmet needs with one product [11].

The condom is an on-demand form of STI protection and contraception and the only MPT currently available. The use of condoms is limited by sociocultural factors involved with perceptions of condom use and partner negotiations, particularly in cases of unequal power dynamics [12–14]. As such, there is a critical need for a female controlled MPT. Research into user preferences has shown that an overwhelming majority of women of reproductive age in various geographic locations and demographic groups prefer MPTs [14], especially long-acting products administered once a month or longer [14]. A great need exists for a variety of MPT products to meet the diversity of user preferences and lifestyles and enhance adherence and efficacy outcomes. The intravaginal ring (IVR) is uniquely positioned to provide a long-acting MPT platform by offering a discrete, female-controlled platform for sustained topical delivery. We set out to develop an MPT IVR to deliver dapivirine (DPV, anti-HIV), levonorgestrel (LNG, contraceptive hormone), and pritelivir (PTV, anti-HSV-2) over 30 days or longer. Dapivirine, a nonnucleoside reverse transcriptase inhibitor (NNRTI), is one of the lead microbicides for HIV prevention. The dapivirine (DPV) ring demonstrated a 35 % reduction in risk of HIV infection in the Ring Study [15] and a 27 % reduction in risk in the ASPIRE study [16]. Since 2021, the WHO recommends the 30-day DPV ring for HIV prevention for women at high risk of infection to be used in combination with other prevention methods [17,18]. Levonorgestrel (LNG) is a well-established contraceptive with known safety and efficacy in daily oral and long-acting forms (Norplant[®], Norplant II[®], Jadelle[®], Mirena[®], Skyla[®]) [19]. The WHO sponsored the development of the first LNG IVR in 1972 using a reservoir-type IVR that released 20 µg LNG/day [20,21]. Contraceptive efficacy with LNG IVRs releasing 20 µg LNG/day was demonstrated in several clinical trials [22–25]; however, no studies have been conducted to assess contraceptive efficacy with a lower dose of LNG delivered topically using an IVR [19]. Pritelivir (PTV) is in phase 3 clinical trials and is a highly potent (EC₉₀ 64 ng/mL) inhibitor of HSV-2 and a member of a new class of HSV inhibitors that act via a novel mechanism involving inhibition of the viral helicase-primase enzyme complex [26,27]. Several MPT IVRs are currently in development, including matrix IVRs, segmental IVRs, and pod IVRs [19]. These IVRs rely on traditional manufacturing processes, injection molding (IM) and hot-melt extrusion, which produce rings with a solid cross-section and limited ability to fine-tune ring design, size, and compression, which are key attributes of end-user preferences. Moreover, injection molded IVRs have limited ability to control drug release kinetics, which can be critical to enhancing efficacy outcomes. Leveraging state-of-the-art 3D printing technology known as continuous liquid interface production (CLIP[™]) we report on the development of uniquely designed, geometrically complex IVRs to deliver a triple-drug combination for prevention of HIV, HSV-2, and unintended pregnancy. Implementation of geometric complexity within the IVR allows precise fine-tuning and control over drug loading and drug release kinetics. The honeycomb (HC) design was selected based on its high specific surface area (surface area/volume; SSA 6.3 mm⁻¹) relative to a solid IVR (SSA 1 mm⁻¹) [28]. Additionally, the HC design offers a unit cell strut thickness of 250 µm compared to 7.6 mm (solid IVR cross section) allowing a much smaller diffusion distance. We have previously demonstrated the role of diffusion distance in drug delivery duration and the role of SSA in the prediction of

drug release rate. As such, the HC design was selected for the development of this MPT IVR to achieve optimized release rates and duration for the selected APIs [28,29]. With this clinically translational drug combination regimen, we assessed the in vivo safety and pharmacokinetics of the DPV/PTV/LNG MPT IVR in pigtailed macaques as an established animal model for IVRs [30–41]. Female pigtailed macaques have vaginal physiology and a continuous lunar menstrual cycle similar to women [34,42]. Co-infection models have been developed to study STIs and simian-human immunodeficiency virus (SHIV) in pigtailed macaques [35] and as such, these models provide a critical step in the development and translation of MPTs by providing an experimentally controlled platform to evaluate the safety, pharmacokinetics, and efficacy of MPTs prior to human clinical trials. We also assessed the long-term stability of MPT IVRs to assess potential drug/material or drug/drug interactions, and post-storage in vitro release kinetics. Collectively, we demonstrated the ability to develop a uniquely designed 3D printed MPT IVR, incorporate three APIs using a single-step drug loading process, and sustain the release of all three APIs over 30 days in vitro and in vivo with no signs of toxicity in pigtailed macaques.

2. Materials and methods

2.1. Materials

Sodium acetate, Kolliphor HS 15 (Solutol), isopropanol alcohol, and levonorgestrel (LNG) were purchased from Sigma-Aldrich (Saint Louis, MO). 0.1 % trifluoroacetic acid (TFA) in water and 0.1 % TFA in Acetonitrile were purchased from Fisher Scientific (Hampton, NH). Silicone-based polyurethane resin (SIL 30 resin) was generously supplied by Carbon, Inc. Dapivirine (DPV) was purchased from Selleckchem (Houston, TX), and Pritelivir (PTV) was purchased from MedChemExpress (Monmouth Junction, NJ).

2.2. Methods

2.2.1. Design of macaque-size IVRs—Geometrically complex intravaginal rings (IVRs) were generated with CAD software in a multi-step process as previously described in detail [43]. Briefly, a template ring of given outer and cross-sectional diameter was generated in SolidWorks (Dassault Systèmes), and the template was subsequently converted into standard tessellation language (.STL, binary). The Honeycomb unit cell was generated in Solidworks, and then imported into the unit cell collection as a .STL file. Theoretical values of volume and surface area were noted. The human-size IVRs have an outer diameter (OD) of 54 mm and a cross-section (CS) of 7.6 mm with an internal honeycomb geometry arrayed at 2.53 mm (Fig. 1A). An optimal IVR size for pigtailed macaques has been previously reported in the literature to ensure IVRs were not displaced and did not induce physical discomfort or show signs of vaginal irritation or inflammation [40]. As such, we designed our macaque-size IVRs to have a 25 mm OD and a 6.0 mm CS. The macaque-size IVR is not proportionally smaller than the human-size IVR and as a result not all dimensions could be kept constant; however the IVR specific surface area (SSA) and internal honeycomb geometry (strut thickness), which influences drug diffusion rate [29], were kept constant to ensure optimal comparison between macaque and human IVR studies (Fig. 1, Supplemental Table 1).

2.2.2. Fabrication of CLIP 3D printed intravaginal rings—Intravaginal rings (IVRs) designs were generated using CAD software with methods previously described [43]. A solid torus was generated in SolidWorks (Dassault Systemes). The solid torus design was saved as a binary .STL and exported for further processing. Geometric complexity was introduced by arraying a unit cell into the solid torus. The honeycomb (HC) unit cell was designed in SolidWorks (Dassault Systemes) and exported as a .STL. The solid torus and unit cell were imported into Magics (Materialise). Using the Structures Module, the imported HC unit cell was given specific X, Y, Z dimensions, denoting the unit cell size. All unit cells were sized to have uniform dimensions at intervals of the cross-sectional diameter of the solid torus. No other parameters in the Structures Module were altered. The resulting geometrically complex IVR was exported as a .STL. These rings (denoted as unbanded rings) were imported into MeshMixer (AutoDesk) for further processing.

A separate band was generated in SolidWorks (Dassault Systemes) that mirrored the initial torus dimensions. The band was exported as a .STL and imported into MeshMixer with the geometrically complex IVR (unbanded rings). Using the combine feature in MeshMixer, the IVR and band were aligned, the tessellations merged, and exported as a .STL to the Carbon user interface (UI).

Rings were fabricated on an M1 digital light synthesis (DLS) (Carbon, Inc.) 3D-printer in a silicone-based polyurethane resin (SIL 30, Carbon). The IVRs CAD file was uploaded to the Carbon UI and orientated to print vertically on the build platform of the 3D printer. This is a two-part resin that was dispensed immediately prior to printing using the Carbon-supplied static mixer. For human sized IVRs, each print consisted of 16 rings and used approximately 150 mL of SIL 30. For macaque sized IVRs, each print consisted of 34 rings and used approximately 130 mL of SIL 30. Carbon's pre-optimized and standardized UV exposure parameters for SIL 30 photopolymerization were used and yielded a fabrication time of 3 h 9 min for human-sized IVRs, and 1 h 30 min fabrication time for macaque-size IVRs. This equates to a print time of ~12 min per human-sized IVR and ~ 3 min per macaque-size IVR. IVRs were removed from the build platform and supports were removed with a razor following fabrication. IVRs were exposed to two 30 s cycles of approximately 200 mL of isopropyl alcohol (IPA) per ring using a previously optimized cleaning process [43]. IVRs were subsequently air dried for 45 min and then placed in a programmable oven for 8 h. at 120 °C, as prescribed by Carbon.

2.2.3. High-performance liquid chromatography (HPLC) analysis—A reverse-phase HPLC method was previously developed and validated to quantify the concentrations of dapivirine, pritelivir, and levonorgestrel concurrently. A Finnigan Surveyor HPLC system (Thermo Finnigan, San Jose, California, USA) with a Photodiode Array (PDA) Plus Detector, auto-sampler, and LC Pump Plus was used. The stationary phase utilized for the analysis was an Inertsil ODS-3 C18 column (5 μ m, 4.6 Å ~ 150 mm, GL Sciences, Torrance, CA) maintained at 40 °C. Chromatographic separation was achieved by gradient elution using a mobile phase consisting of 0.1 % trifluoroacetic acid in water and acetonitrile (ACN) (H₂O/ACN 95:5 v/v). The flow rate was 1.0 mL/min and the total run time was 20 min for each 25 μ L injection. Three distinct peaks were observed at different retention times, and an optimal wavelength was determined to assess each API. Dapivirine had a retention time of

13.1 min, pritelivir had a retention time of 10.3 min, and levonorgestrel had a retention time of 16.3 min.

2.2.4. Drug loading—CLIP 3D printed honeycomb (HC) IVRs (macaque dimensions) were fabricated as described above, and a solvent swelling method was used to load APIs individually or in combination. APIs were dissolved in acetone to create a drug loading solution, and placebo IVRs were placed in the drug loading solution for an optimized loading duration of 24 h. IVRs swell in acetone thereby incorporating the APIs homogenously into the SIL 30 HC matrix. The IVRs were subsequently removed from the drug-loading solution and dried at ambient conditions for 24 h to fully remove the acetone. The resulting drug loaded IVR has APIs physically entrapped and homogenously distributed throughout the polymer matrix.

As described in previous work, weight-based drug loading equations were derived for each API to achieve target loading amounts in the final IVR [29,44]. The loading equation correlates linearly the target weight-percent of the API in the final IVR with the concentration of the loading solution. These equations and calculations are summarized in Supplemental Table 2.

2.2.5. In vitro drug release—Drug release kinetics were evaluated in vitro under static conditions (no shaking) or dynamic conditions (120 rpm) at 37 °C in simulated vaginal fluid (SVF). The SVF was adapted from Owen and Katz [45] and consisted of 25 mM sodium acetate buffer adjusted to pH 4.2 (human vaginal pH) or 8 (macaque vaginal pH) and 2 % Solutol was added to maintain sink conditions. Macaque sized IVRs were incubated in SVF (100 mL) at 37 °C and sample aliquots (1 mL) were collected and replaced with fresh media daily for the first week, and then weekly until all APIs reached 100 % release. The entire release media was replaced each week to maintain sink conditions [44,46]. Release samples were analyzed by HPLC to quantify drug concentration and determine cumulative drug release overtime. All experiments were performed in triplicate.

2.2.6. IVR segment stability study—Human-sized HC IVRs were prepared for drug loading and cut into 300–400 mg segments. Previous work demonstrated the ability to use CAD to determine the number of segments that can represent a whole HC IVR [28,29]. Using this method, HC IVRs were segmented into 12 equal parts and segments ($n = 3$) were loaded with each API individually or in combination. Drug loaded segments were subsequently stored at 4 °C or in an accelerated humidity chamber (40 °C, 75 % RH). After 1, 3, and 6 months of storage, IVR segments were removed from storage and incubated in 10 mL of acetone for 24 h to fully extract APIs. Samples of the extract were analyzed by HPLC to quantify each API concentration and identify potential degradation peaks. For each IVR segment, a weight-percent of API loaded was calculated by dividing the analytical amount of API (mg) determined by HPLC over the weight of the IVR segment (mg). The change in weight-percent of APIs loaded individually and in combination were evaluated over time.

2.2.7. Liquid chromatography mass spectrometry (LC-MS)—Liquid Chromatography with tandem Mass spectrometry (LC-MS) analyses were carried out to identify degradation peaks that were present in HPLC chromatograms when APIs were

extracted from IVRs post storage. Given that degradation peaks were present only in IVRs formulated with PTV, either alone or in combination with another API, it was hypothesized that the unknown degradation peaks observed by HPLC from the stability studies were a result of PTV degradation. To test this hypothesis, extracted samples from the stability study of PTV IVRs and PTV/LNG IVRs post 3 and 6-months of storage were analyzed by LCMS to determine the proposed structure of the degradation products.

Samples were analyzed with a ThermoFisher Q Exactive HF-X (ThermoFisher, Bremen, Germany) mass spectrometer coupled with a Waters Acquity H-class liquid chromatograph system. Samples were introduced via a heated electrospray source (HESI) at a flow rate of 0.3 mL/min. Electrospray source conditions were set as: spray voltage 3.0 kV, sheath gas (nitrogen) 60 arb, auxiliary gas (nitrogen) 20 arb, sweep gas (nitrogen) 0 arb, nebulizer temperature 375 °C, capillary temperature 380 °C, RF funnel 45 V. The mass range was set to 150–2000 *m/z*. All measurements were recorded at a resolution setting of 120,000.

Separations were conducted on a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μ m particle size). LC conditions were set at 95 % water with 0.1 % formic acid (A) ramped linearly over 5.0 mins to 100 % acetonitrile with 0.1 % formic acid (B) and held until 6.0 mins. At 7.0 mins the gradient was switched back to 95 % A and allowed to re-equilibrate until 9.0 mins. Injection volume for all samples was 3 μ L.

Xcalibur (ThermoFisher, Bremen, Germany) was used to analyze the data. Solutions were analyzed at 0.1 mg/mL or less based on responsiveness to the ESI mechanism. Molecular formula assignments were determined with Molecular Formula Calculator (v 1.2.3). All observed species were singly charged, as verified by unit *m/z* separation between mass spectral peaks corresponding to the ¹²C and ¹³C¹²Cc-1 isotope for each elemental composition.

2.2.8. In vivo biological sample collection—The pharmacokinetic and safety study was performed in three sexually mature female pigtailed macaques (*Macaca nemestrana*) that were housed at the Centers for Disease Control and Prevention (CDC), an AAALAC approved facility (AAALAC #00052 and PHS assurance #A4365–01) in accordance with protocols approved by the CDC Institutional Animal Care and Use Committee and the recommendations of the Guide for the Care and Use of Laboratory Animals [47]. The sample collections were performed as shown in Fig. 8 according to published protocols [37,38,48].

The DPV/PTV/LNG MPT IVRs were inserted into the macaques' posterior vagina on day 0 and the rings remained in place for 28 days. Blood collected throughout the study was processed to obtain plasma and PBMCs. Weekly plasma progesterone levels were performed as described previously [49]. Vaginal fluids were obtained proximal and distal to the ring with merocel spears (BVI, Waltham, USA) at baseline (day 0) and following ring insertion on days 3, 7, 14, 21 and 28 and post ring removal on day 30. Vaginal pinch biopsies were obtained both proximal and distal to the ring on days 7, 21 and 30 using a Miltex Townsend model 30–1445 minibite cervical biopsy punch forceps for drug content analysis.

2.2.9. Safety analysis—Visual abnormalities in the cervix and the lateral vaginal walls were monitored by colposcopic observations on days 0 and 14 and the findings were recorded [50]. The anesthetized animal's vaginal cavity was opened with the help of a pediatric speculum and a Rebel Tli/EOS 500D digital camera (15 megapixels; Canon, Melville, NY) was used [36]. Changes in vaginal pH were determined in vaginal fluid samples collected using a Dacron swab throughout the study. The secretions were rolled onto a pH colorimetric indicator strip (Millipore, Burlington, USA). The vaginal pH was not possible to be measured in animals that were undergoing menstruation due to the bleeding and exudation. Macaque-specific multiplexed Milliplex™ MAP (Millipore, Burlington, USA) bead-based fluorescent assays were used to determine the induction of any mucosal inflammation in vaginal fluid samples collected throughout the study [39,40].

Multicolor flow cytometry analysis was performed on peripheral blood mononuclear cells (PBMCs). The PBMCs were incubated with the following monoclonal antibodies [clone numbers in parenthesis]: CD20-FITC [2H7], CD3- Alexa 700 [SP34–2], CD8-V450 [RPA-T8], CD4-V500 [L200] for 20 min at 4 °C (BD Biosciences, San Jose, CA). The cells were then washed and fixed with 1 % paraformaldehyde and 100,000 events per sample acquired on LSRII Flow cytometer (BD BioSciences, San Jose, California). The data was then analyzed on FlowJo software version 10 (Tree Star, San Carlos, CA).

2.2.10. Analytical methods of macaque pharmacokinetic samples—All drug concentrations were quantified at the University of North Carolina Center for AIDS Research Clinical Pharmacology and Analytical Chemistry Core, using liquid chromatography–tandem mass spec-trometry (LC-MS/MS) methods.

2.2.10.1. Drug quantification in blood.: Plasma was separated from sodium citrate treated whole blood by centrifugation. Following a protein precipitation extraction (DPV, PTV) or a liquid-liquid extraction (LNG) with isotopically labeled internal standards (DPV-d₄ (for both PTV and DPV) and LNG-d₆), plasma analytes were separated using reverse-phase chromatography via a Waters Atlantis T3 (2.1×50mm, 3.0 µm particle size) for DPV and PTV or a Phenomenex Synergi Polar-RP (2.0×50mm, 2.5 µm particle size) analytical column for LNG. An AB Sciex API-5000 triple quadrupole mass spectrometer was used to detect analytes and internal standards under positive ion electrospray conditions. Precision and accuracy of the calibration standards and quality control samples were within 20 % for the following dynamic ranges: 0.0500–200 ng/mL of DPV, 2.00–4000 ng/mL of PTV, and 0.250–500 ng/mL of LNG.

2.2.10.2. Drug quantification in vaginal fluids.: Cervicovaginal fluid collected onto Merocel sponges was eluted by adding 2 mL of 70:30 acetonitrile:water to tubes containing a single sponge and vortexed for 1–2 min. Following a protein precipitation extraction with isotopically labeled internal standards (DPV-d₄ (for both PTV and DPV) and LNG-d₆), analytes were separated using reverse-phase chromatography via a Waters Xterra MS C18 (2.1×50mm, 3.5 µm particle size) analytical column. An AB Sciex API-5000 triple quadrupole mass spectrometer was used to detect analytes and internal standards under positive ion electrospray conditions. Precision and accuracy of the calibration standards and quality control samples were within 20 % for the following dynamic range: 1.00–2500

ng/mL in NHP vaginal fluid. Final concentrations were reported in ng/swab and then converted to pg/mL using the volume of sample collected from each individual swab.

2.2.10.3. Drug quantification in vaginal tissues.: Weighed tissues were transferred into Precellys® hard tissue reinforced metal bead kit tubes (Cayman Chemical Company) containing 1 mL of 70:30 acetonitrile:1MM ammonium phosphate, homogenized then centrifuged. A portion of the supernatant was mixed with isotopically labeled internal standards (DPV-d₄ (for both PTV and DPV) and LNG-d₆). Analytes were then separated using reverse-phase chromatography via a Waters Xterra MS C18 (2.1×50mm, 3.5 μm particle size) analytical column. An AB Sciex API-5000 triple quadrupole mass spectrometer was used to detect analytes and internal standards under ion electrospray conditions. Precision and accuracy of the calibration standards and quality control samples were within 20 % for the following dynamic ranges: 0.500–2500 ng/mL (DPV, PTV) and 0.0100–12.5 ng/mL (LNG). Final concentrations were normalized to tissue mass and reported in ng/g units.

2.2.11. Residual drug quantification post-in vivo PK in macaques—Following the completion of the in vivo PK study in pigtailed macaques, IVRs removed at day 28 post administration were extracted to quantify residual drug post in vivo release. Each IVR was weighed and incubated in 50 mL of acetone for 48 h to fully extract residual drug. Extraction samples (1 mL, *n* = 3) were analyzed by HPLC to quantify API concentration. The quantified residual drug in each IVR was subtracted from the known initial drug concentration to determine the amount of drug (DPV, PTV, LNG) that was delivered to each pigtailed macaque over 28 days.

2.2.12. In vitro-in vivo comparison—A comparison between in vitro drug release kinetics and in vivo drug pharmacokinetics (PK) was performed to determine the ability to correlate in vitro results to in vivo PK in a macaque model. For direct comparison, a single batch of MPT IVRs was used to assess in vitro and in vivo drug release to ensure consistent loading of each API across IVRs tested in vitro and in vivo. Given that the macaque MPT IVRs elicited zero-order release profiles in vitro, the in vivo daily release rate for each API was determined using quantified residual drug, and dividing the amount of drug known to be delivered to each macaque by 28 days. Target daily release rates for each API were set based on known benchmarks for efficacy for DPV and LNG in human IVRs (200 μg/day for DPV [51–53] and 20 μg/day for LNG [54–56]). Since PTV is still in development [57–59], its target release rate when delivered topically is not yet known. As such, we set the target in vitro release rate for PTV based off acyclovir, which demonstrated therapeutic efficacy at a daily release rate of 200 μg/day when formulated in an IVR [37,60–62].

To extrapolate the target release rates from human IVRs to the macaque IVRs a ratio of MPT macaque IVR weight (mg) to MPT human IVR weight (was) determined using the following equation: $\left(\frac{1247 \text{ mg macaque IVR}}{4633 \text{ mg human IVR}}\right) = 0.27$. Using the weight-by-weight ratio, the macaque IVRs are expected to achieve about 27 % of the target daily release compared to the human IVRs, which corresponds to target daily release rates for DPV and PTV of 54 μg/day and 5.4 μg/day for LNG. Using these approximations an in vitro-in vivo (IVIV)

comparison was determined by dividing the calculated in vivo release rate by the in vitro release rate. An IVIV ratio of 1 indicates that the in vivo and in vitro release rates are identical. An IVIV ratio > 1 indicates that the in vivo release is greater than the in vitro release, and IVIV ratio < 1 indicates that the in vitro release is greater than the in vivo release.

2.2.13. Statistical analysis—Statistical analysis for mucosal cytokine and chemokine concentrations following ring insertion or upon its removal was performed using two-way ANOVA Tukey's multiple comparisons test with GraphPad Prism Software 10.2.2.

3. Results

3.1. Post-fabrication drug loading

CLIP 3D printing and the SIL 30 matrix provide the unique ability to incorporate APIs post IVR fabrication in a controlled fashion using an absorption process. 3D printed IVRs were loaded with APIs using previously described weight-based linear drug loading equations [44]. The macaque size IVR was allometrically scaled to the human size IVR to elicit equivalent specific surface area (SAA) and equivalent honeycomb unit cell strut thickness to allow for a direct translation of drug loading and release kinetics from the human size MPT IVR to a macaque size MPT IVR. DPV, PTV, and LNG were formulated in macaque size IVRs at equivalent weight percent (wt%) to the human size IVR. A direct comparison of the human and macaque size MPT IVRs is shown in Fig. 2. Dapivirine was loaded at 0.65 % w/w (30.0 mg/human-size IVR; 8.11 mg/macaque-size IVR); pritelivir was loaded at 0.43 % w/w (20.0 mg/human-size IVR; 5.36 mg/macaque-size IVR); and levonorgestrel was loaded at 0.043 % w/w (2.0 mg/human-size IVR; 0.536 mg/macaque-size IVR).

3.2. In vitro drug release of MPT (DPV/PTV/LNG) IVRs

There is no established apparatus or standardized method reported in the literature for testing the release kinetics from IVRs in vitro, and several methods using different dynamic settings and release media have been reported in the literature [63]. Herein, we investigated in vitro release kinetics using both static and dynamic conditions. For the dynamic in vitro release studies, it was determined that shaking at 120 rpm in an orbital shaker elicited similar release kinetics to a USP Dissolution Apparatus (Vision 8 Elite Dissolution Tester, Hanson) set at 60 rpm. As such, in vitro release studies were carried out with human size IVRs in SVF (pH 4.2, 37 °C) under static and dynamic (120 rpm) conditions. As shown in Fig. 3, all three APIs elicited higher initial burst release in the first 24 h under dynamic conditions (4.7 % DPV, 7.4 % PTV, 5.6 % LNG) compared to static conditions (3.6 % DPV, 5.3 % PTV, 4.4 % LNG). However, the daily zero order release rate was not significantly different when comparing static versus dynamic settings. Following the burst release, DPV, PTV, and LNG daily release rates under dynamic setting were 369.9 $\mu\text{g/day}$, 358.4 $\mu\text{g/day}$, and 24.4 $\mu\text{g/day}$ respectively. The daily release rate under static conditions was 441.5 $\mu\text{g/day}$ (DPV), 353.3 $\mu\text{g/day}$ (PTV), and 32.2 $\mu\text{g/day}$ (LNG). The cumulative percent release over 35 days for DPV and PTV was ~40 % and 55 % respectively for both the static and dynamic release studies. Whereas LNG elicited higher cumulative percent release at day 35 (~55 %) under dynamic release compared to static release (~40 %).

In vitro release studies of human and macaque size IVRs were conducted at pH 4 and pH 8 SVF to mimic physiological vaginal pH of humans and macaques respectively. Release studies were conducted under static and dynamic conditions to determine the optimal conditions that better correlate in vitro release kinetics to in vivo pharmacokinetics. Results showed that IVRs exhibited similar release profiles regardless of pH or whether tested under static or dynamic conditions. Following the initial burst release, APIs elicited sustained zero order release kinetics from day 2–35. A linear regression was performed for each API under each testing condition.

In vitro release under dynamic conditions (pH 8) elicited higher initial burst release within the first 24 h for all APIs, with 9.96 % (808 μg) DPV, 10.3 % (551 μg) PTV, and 11.2 % (60 μg) LNG in comparison to lower burst release obtained under static (pH 8) conditions with 4.1 % (333 μg) DPV, 5.9 % (319 μg) PTV, and 5 % (27 μg) LNG (Fig. 4A–B, D–E). Following the initial burst, IVRs elicited similar daily release rates under static and dynamic conditions with (135.8–188.8) $\mu\text{g}/\text{day}$, (90.4–121.4) $\mu\text{g}/\text{day}$, and (10.1–12.7) $\mu\text{g}/\text{day}$ for DPV, PTV, and LNG respectively (Fig. 4G). On the other hand, IVRs incubated at pH 8 elicited higher initial burst release and higher daily release rates for all APIs compared to pH 4 (Fig. 4B–C, 4E–F).

We hypothesized that the difference in drug release between equivalent IVRs at pH 4 and pH 8 was due to the difference in swelling of the SIL 30 IVR matrix as a function of pH. To test this hypothesis, a swelling study was conducted using solid (no design) human size placebo IVRs. IVRs were placed in conditions that mimic the aforementioned static in vitro release study, where $n = 3$ IVRs were incubated individually in SVF (pH 4 or pH 8) at 37 °C. At $t = 0, 1, 7, 14, 21$, and 28 days, IVRs were removed from the media and the mass (mg), outer diameter (OD, mm), and cross section (CS, mm) were recorded to capture the swelling of the SIL 30 matrix in the release media. Results showed that IVRs exhibited higher swelling at pH 4 than pH 8. At $t = 0$ the average mass, OD, and CS of the IVRs tested at both pH conditions were equivalent. Over the 28-day study, IVRs incubated at pH 4 exhibited a 73 % increase in mass, 20 % increase in OD, and 21 % increase in CS. IVRs incubated in SVF at pH 8 demonstrated about 10 $\times$ less swelling as demonstrated by the significantly lower changes in IVR metrics (5 % increase in mass, 1 % increase in OD, and 3 % increase in CS) relative to pH 4 (Fig. 5).

3.3. Stability studies

To assess the long-term stability of the MPT IVRs, we completed 1, 3, and 6 months stability studies under two storage condition: 4 °C and accelerated stability conditions (40 °C/75 % RH). At each time point, $n = 3$ IVRs were used to investigate post-storage in vitro release, and $n = 1$ IVR underwent full extraction in acetone to quantify drug concentration and assess drug stability by HPLC analysis. Results showed that DPV remained stable over 6 months with no change in drug concentration and absence of degradation peaks by HPLC analysis. On the other hand, results showed that the PTV concentration decreased by 50 % after 3 months of storage with no further decrease recorded after 6 months of storage. Similarly, LNG concentration decreased by 50 % after 3 months of storage, with an additional 50 % decrease in concentration recorded after 6 months of storage [64].

Unknown degradation peaks were present in the HPLC chromatogram at 7.7 and 8.7 min (Supplemental Fig. 1). To identify the degradation peaks detected post storage, a series of studies were set up to evaluate whether the degradation was caused by the SIL 30 IVR matrix, individual APIs, or a combination of APIs in the SIL 30 matrix.

First, placebo HC IVRs were stored in a humidity chamber under accelerated stability conditions (40 °C /75 % RH) for 6 months, and subsequently assessed for in vitro release (SVF, pH 4, 37 °C). Sample aliquots (1 mL) were collected on day 1, 7, and 14 based on when the unknown degradation peaks were detected during the in vitro release study post 6-months storage of the MPT IVR. Unlike the MPT IVRs, no unknown degradation peaks were detected with the placebo IVRs in the release samples as determined by HPLC analysis (Supplemental Fig. 1). To assess the APIs' stability under storage, neat APIs were stored in glass vials individually ($n = 3$) or in combination ($n = 3$) and analyzed after 3 and 6-months of storage under accelerated stability conditions (40 °C /75 % RH). At each time point, APIs were dissolved in acetone to mimic the API extraction from an IVR, and samples were analyzed by HPLC. HPLC analysis showed no presence of degradation peaks in these samples (Supplemental Fig. 1). Based on the above results, it was determined that the degradation peaks were a result of API's interaction with the SIL 30 matrix under accelerated storage conditions.

Previous studies showed that the DPV/PTV/LNG combination in the SIL 30 matrix resulted in presence of degradation peaks after storage under accelerated stability conditions (40 °C/75 % RH) [64]. To investigate the nature of degradation peaks, a comprehensive stability study was carried out using IVR segments. Human size HC IVRs were cut into segments (~400 mg each) as representative units of the whole IVR. IVR segments were batch loaded with each API individually (DPV, PTV, or LNG) or in dual combination (DPV/PTV, DPV/LNG, PTV/LNG). IVR segments ($n = 3$) were stored at 40 °C/75 % RH or at 4 °C and evaluated after 1, 3, and 6 months of storage (Supplementary Fig. 2, 3). At each time point, IVR segments were removed from their respective storage conditions and underwent a 24 h extraction in acetone. Samples of the extract were analyzed by HPLC to quantify each API and investigate presence of degradation peaks. The weight percent loading of each API in the segments was kept constant by incubating all segments in the same drug solution. To compare drug loading across segments, the weight percent (wt%) of API loaded was determined using the following equation: $\left(\frac{API\ extracted(mg)}{IVR\ segment(mg)} \right) \times 100\% = wt\% API per segment$.

The wt% of each API loaded individually and in dual combination was compared across the 1, 3, and 6-month time points for both storage conditions (Fig. 6, Table 1, and Table 2).

Results showed that DPV remained stable with absence of degradation peaks when formulated alone or in combination with either PTV or LNG under both storage conditions. However, when formulated in combination with PTV, the weight percent of DPV quantified after 6-months storage under accelerated conditions decreased significantly (0.89 ± 0.03 wt%) compared to 1-month post storage (1.10 ± 0.07 wt %).

PTV did not elicit statistically significant changes in weight percent when stored at 4 °C (Table 1, Supplementary Fig. 3). On the other hand, after 3 months of storage under accelerated stability conditions (40 °C/75 %RH), PTV individually and in dual combination

with DPV and LNG elicited a significant decrease in weight percent when compared to the 1-month storage (Fig. 6, Table 2). After 6 months of storage under accelerated conditions, PTV alone and PTV in dual combination with DPV elicited significantly lower weight percent values compared to both the 1 and 3-month storage.

LNG remained relatively stable when stored at 4 °C when formulated alone or in combination with DPV or PTV (Table 2).

Further analysis of the individual and dual-loaded IVR segments with DPV, PTV, and LNG demonstrated that degradation peaks were detected in the DPV/PTV/LNG combination and were primarily generated from PTV degradation post storage at 40 °C/75 % RH. In addition to the observed changes in API wt%, degradation peaks were also observed when PTV was formulated alone or in combination with DPV or LNG (Supplemental Fig. 2). No degradation peaks were present for DPV or LNG when formulated alone. Furthermore, samples of the PTV solutions were analyzed using mass spectrometry analysis to identify a proposed structure for the degradation peaks. Results from the mass spectrometry analysis showed that the degradation peaks were by-products of PTV, most likely from an amid hydrolysis reaction (Supplemental Fig. 4).

Results from the triple-drug combination IVR segments stored at 4 °C for 1, 3, and 6 months are shown in Fig. 7. All three APIs remained stable over 3 months of storage at 4 °C but elicited a significant decrease in drug wt% after 6 months of storage. No degradation peaks were observed by HPLC analysis (Supplemental Fig. 3).

3.4. Pharmacokinetics of MPT IVR

Macaque size IVRs were loaded with DPV, PTV, and LNG and investigated in vivo in pigtailed macaques ($n = 3$) for safety and pharmacokinetics (PK) over 30 days. Plasma samples, vaginal tissues (proximal and distal to the cervix), and vaginal fluid samples (proximal and distal to the cervix) were collected longitudinally. An illustration of the sampling schema for the PK samples is shown in Fig. 8. At $t = 0$ plasma and vaginal fluid samples were collected, and DPV/PTV/LNG MPT IVRs were subsequently administered vaginally to each macaque. The IVR remained in place for 28 days and then removed, and final samples were collected on day 30, two days post-IVR removal. At $t = 3, 7, 14, 21, 28$, and 30 days, plasma and vaginal fluid samples were collected. At $t = 7, 21$, and 30 vaginal tissue samples were collected.

Results of the PK analysis for each biological sample are summarized in Fig. 9, and the exact weight and API dosing per macaque is shown in Supplemental Table 3. Results showed that the plasma drug concentrations remained constant over the 28 days of IVR use for each API. Median (range) plasma concentrations of API while the rings were present from day 3–day 28 for the group ($n = 3$) were 138 (110–192) pg/mL for DPV, 18,700 (7550–49,000) pg/mL for PTV and 335 (125–1720) pg/mL for LNG (Fig. 9A–C, Supplemental Table 4). At day 0 prior to IVR insertion and at day 30, two days post-IVR removal, the concentrations of DPV, PTV, and LNG each reached concentrations below the limit of quantification (BLOQ). Drug concentrations in both proximal (Fig. 9D–F) and distal (Fig. 9G–H) vaginal fluids likewise remained relatively constant across the 28-day study. Median

(range) API concentrations proximal to the cervix for the group ($n = 3$) while the rings were present from day 3-day 28 were 375,520.7 (6521–1,103,053.2) pg/mL for DPV, 632,327.9 (12,152.7–2,918,559) pg/mL for PTV, and 6642.2 (500–60,648.1) pg/mL for LNG. Median (range) API concentrations distal to the cervix for the group ($n = 3$) while the rings were present from day 3-day 28 were 76,174.6 (38,081.6–330,178.8) pg/mL for DPV, 171,289.3 (22,365.4–800,431.4) pg/mL for PTV, and 2469.9 (500–15,668.1) pg/mL for LNG. The concentration of DPV, PTV, and LNG was about 5, 4, 3-fold higher respectively in the proximal vaginal fluid compared to distal vaginal fluid. DPV had the highest vaginal tissue concentration with a median (range) for the group ($n = 3$) while the ring was present at 808,163 (151,170–1,630,721) pg/g, and all three APIs had steady concentrations from 7 days to 21 days, with the vaginal tissue concentrations falling below the limit of quantification post-IVR removal. Data presented as median with 95 % confidence interval of $n = 3$ macaques/timepoint per dose. Individual replicates shown in Supplemental Fig. 5.

After completion of the in vivo study, the MPT IVRs were extracted to quantify residual drugs remaining in the IVR. IVRs ($n = 3$) were placed in acetone to extract residual drug, and extraction samples were quantified using HPLC (Table 3). The average residual DPV was of 8.82 ± 0.11 mg (94.3 %), corresponding to an average of 0.53 ± 0.11 mg DPV released over 28 days (~ 19 $\mu\text{g/day}$ DPV). Residual PTV was quantified at 2.15 ± 0.49 mg (36.7 %) corresponding to an average of 3.71 ± 0.49 mg released over 28 days of use (~ 133 $\mu\text{g/day}$ PTV). For LNG, the quantified residual drug was 0.08 ± 0.02 mg (15.4 %) corresponding to an average of 0.44 ± 0.02 mg LNG released over 28 days (~ 16 $\mu\text{g/day}$).

A comparison between the in vitro and in vivo release rates was done for all three APIs (Table 4). Results showed that the in vitro and in vivo release rates for PTV and LNG were closely comparable, with correlative values of 1.1 and 1.2 respectively. LNG exceeded the target in vivo macaque daily release rate of 5.4 $\mu\text{g/day}$ and human in vitro daily release rate of 20 $\mu\text{g/day}$. Similarly, PTV elicited release rates above the set target of 200 $\mu\text{g/day}$ in vitro and 54 $\mu\text{g/day}$ release in vivo in macaques. Unexpectedly, DPV elicited daily release rate, based on residual drug, of about 10 $\times$ lower in vivo (~ 19 $\mu\text{g/day}$) compared to the in vitro release rate (~ 189 $\mu\text{g/day}$) resulting in an in vitro-in vivo ratio of 0.1.

In vitro-in vivo comparisons are difficult to do for IVRs due the complexities of the formulations, lack of standardized in vitro release testing methods, and inter-subject variability in pharmacokinetic data [63,65]. The comparison reported here is a level B correlation that compares the cumulative mean API released over 28 days in the translational in vitro release study and the in vivo study in macaques. The in vitro release conditions that most closely matched the in vivo data were the macaque size IVRs tested under pH 8 dynamic conditions. It was found that PTV and LNG had similar cumulative release when tested under the translational in vitro conditions and in vivo. While the in vitro results cannot explain the drug distribution that is observed in vivo, the translational in vitro release conditions can provide an estimate of how much PTV and LNG will be released from the MPT IVR.

3.5. Changes in progesterone production

The measured plasma progesterone correlated with the LNG detected following insertion of the IVR with an average six-fold decrease in the progesterone concentrations on day 3 (Supplemental Fig. 6). A 47-, 45-, and 39-fold decrease in the average progesterone concentrations on days 7, 14, and 21 respectively in comparison to day 0 was detected. Progesterone concentrations on days 28 and 30 were similar to that measured on day 0. This corresponded with the LNG being below the detection limit at those time points. Moreover, the plasma measurement at day 30 was obtained 2 days following ring removal. It could be likely that the low levels detected on days 0–21 in macaque B corresponds to it having a different cycling pattern in comparison to the other two macaques. We did not collect samples in these macaques at time points prior to day 0. Thus, we have provided a representative progesterone pattern in a normally cycling pigtailed macaque with no contraceptive modality (not a part of this study) to exhibit the effects of LNG released in the 3D IVR macaques (Supplemental Fig. 6).

3.6. In vivo safety of MPT IVR

The in vivo safety of the application and the retention of the MPT IVRs were evaluated by colposcopic observations, changes in vaginal pH, mucosal cytokine and chemokine concentrations and systemic T cell subset population frequencies. Routine visual examination during regular mucosal sample collection and colposcopic observations on days 0 and 14 revealed a normal finding with no tissue abrasion or inflammation. The vaginal pH measurements revealed no abnormal variations at any of the time points tested following ring insertion and removal (Fig. 10). The pH range observed during the study is similar to that normally detected in pigtailed macaques [48,66,67].

Mixed-effects model analysis with Tukey test correction for multiple comparisons revealed no statistically significant changes in any of the 22 cytokines and chemokines tested including the pro- (IL-1 β , IL-6, IL-8, IFN- γ , TNF- α , IL-17, IL-18) and anti-inflammatory (IL-10, IL-12) cytokines while the 3D IVR was in place or upon its removal (Fig. 11). To determine the influence, if any, of the three drugs, and specifically the contraceptive LNG on the immune cell population, we measured the frequency of systemic CD4⁺ and CD8⁺ T cells during the entire study. We observed no variation in cell frequencies in all samples collected following ring insertion and post removal on day 30 (Supplemental Fig. 7).

4. Discussion

Long-acting, self-administered, and discrete MPTs can expand preventative options for adolescent girls and young women to prevent against HIV, HSV, and unintended pregnancy with a single administration. Here, we developed a uniquely designed first-in-line 3D printed MPT IVR with DPV, PTV, and LNG as anti-HIV, anti-HSV-2, and contraception. Our results demonstrated the ability to co-formulate all three APIs in a single-step drug loading process administered as a single safe and well-tolerated topical delivery with sustained release of all drugs for 30 days and plasma concentrations above the protective benchmarks for HIV, HSV-2, and contraception.

Building on our prior work with a well-established Islatravir/Etonogestrel/Ethinyl estradiol MPT IVR (ISL/EE/ENG IVR) [44], we developed a new MPT IVR utilizing DPV as the ARV to be combined with PTV (anti-HSV-2), and a clinically proven hormonal contraceptive, LNG. Once feasibility of an MPT IVR was established, we set out to assess the safety and PK in a translational pigtailed macaque model. Overall, a unique 3D printed honeycomb MPT IVR was developed and elicited sustained in vitro release of all three APIs for >30 days. We assessed the presence of drug-drug interactions in vitro by investigating the in vitro release of MPT IVRs and single-drug loaded IVRs at time zero and post storage under different storage conditions and durations.

Notably, DPV and LNG in vitro release was unchanged when co-formulated with either APIs or when formulated alone and whether stored at 4 °C or accelerated conditions (40 °C/75 % RH) for up to 6 months. On the other hand, PTV elicited degradation peaks when stored under accelerated conditions when co-formulated with LNG or alone. All three APIs were stable for up to 6 months when stored at 4 °C and did not elicit any degradation peaks. Furthermore, we assessed post-storage drug content (wt%) and demonstrated that all three APIs were more stable at 4 °C and did not elicit a significant decrease in drug content.

Given the promising in vitro data, the MPT IVR was assessed for safety and PK in female pigtailed macaques. The MPT IVR was safe and well-tolerated without any local reactions or systemic inflammation for 30 days. In addition, MPT IVRs elicited sustained plasma concentrations over 28 days and DPV and LNG plasma concentrations were at or above their established plasma PK benchmark for protection in humans (223–340 pg/mL DPV; 200 pg/mL LNG) [68]. For PTV, the vaginal fluid concentrations were well above the EC₉₀ (64 ng/mL) [26]. Furthermore, all APIs elicited a significant decrease in plasma, vaginal fluid, and vaginal tissue concentrations 2 days post IVR removal demonstrating the ability to terminate treatment, if needed, and reverse contraception.

Notably, our 3D printed MPT IVR contained 8.11 mg DPV, resulting in a DPV dose ranging from 0.81 to 1.42 mg/kg for the pigtailed macaque (6.6–10.3 kg). Pereira et al. previously reported on a DPV/Darunavir (DRV) IVR for HIV prevention [69]. The dual DPV/DRV IVRs were evaluated in cynomolgus macaques (3–7 kg) over 28 days with the macaque size IVR containing 25 or 100 mg of DPV alone, or 100 mg DPV in dual combination with 300 mg DRV. The IVRs containing 25 mg of DPV elicited an in vitro release rate in SVF of 196.8 µg/day, and in vivo it resulted in a C_{max} in vaginal fluid of 7591 ± 2508 ng/g and a C_{max} in serum of 250 ± 150 pg/mL. The dual IVR containing 100 mg DPV 300 mg DRV elicited an in vitro release rate of 361.08 µg/day, and a C_{max} in vaginal fluid of 19,260 ± 5675 ng/g and C_{max} in serum of 206 ± 108 pg/mL [69]. While the DPV dose of the 25 mg DPV and 100 mg in these EVA IVRs were significantly higher than our CLIP 3D printed IVRs (8.11 mg DPV), the resulting DPV PK values were comparable. The 25 mg DPV EVA IVR delivered a DPV dose ranging from 3.57 to 8.3 mg/kg, which is more than 2.5× the dose delivered with our CLIP MPT IVR yet; both IVRs elicited plasma concentrations of DPV on the order of 10² pg/mL. When comparing DPV concentrations in vaginal fluid, the 25 mg DPV EVA IVR resulted in DPV vaginal fluid concentrations on the order of 10³ ng/g, similar to that achieved with our MPT IVR. Similarly, the DPV dose (100 mg) delivered with the DPV/DRV EVA IVR ranged from 14.3 to 33.3 mg/kg, which is about 10× higher

than the DPV dose delivered with our MPT IVR. Despite the higher dose, the DPV/DRV IVR resulted in plasma DPV concentrations that were comparable (10^2 pg/mL) to our MPT IVR. For DPV, the mechanism of action requires a high concentration of API in the target mucosal tissue [70]. Our MPT IVR elicited DPV concentrations in the vaginal tissue in the range of 10^5 – 10^6 pg/g.

Clinical trials have shown moderate efficacy with a monthly 25 mg DPV ring [15,71]. Both phase 3 trials had classified as women being adherent when their plasma DPV concentrations were ≥ 95 pg/mL. No data on plasma drug levels needed for efficacy were reported in those trials. As extended duration rings may improve user adherence, efficacy, and reduce cost, Liu et al. compared the pharmacokinetics of two three month 100 and 200 mg DPV ring to the monthly 25 mg DPV ring in a phase 1 randomized trial [72]. The extended duration rings achieved higher plasma, and cervicovaginal fluid (CVF) DPV concentrations than the 25 mg ring. The plasma concentration of the 25 mg DPV IVR used in this phase 1 trial ($C_{\max}$ geometric mean- 280 pg/mL) was comparable to that seen with our 8 mg macaque MPT IVR ($C_{\max}$ geometric mean 168 pg/mL). However, a higher CVF concentration of DPV was obtained with the 25 mg ring ($C_{\max}$ geometric mean 33 ng/mg or $3.3\text{E}+07$ pg/mL) than with our macaque MPT IVR ($C_{\max}$ geometric mean $7.13\text{E}+05$ pg/mL). These data suggest that a higher loading of DPV in the macaque IVR may be needed to increase plasma and CVF concentrations of DPV and confer high protection against SHIV exposures. Efficacy studies in the macaque model will help define the correlate of protection for DPV and inform DPV dose selection for the clinical development of our DPV MPT IVR.

Ugaonkar et al. reported in vivo LNG efficacy with an injection molded EVA-based MPT IVR with plasma concentrations of 0.19 ng/mL resulting in effective contraception [73]. Our results showed that our MPT IVR (0.536 mg LNG; 0.045–0.079 mg/kg) resulted in plasma concentrations ranging between 0.13 and 1.72 ng/mL in the pigtailed macaques, which are within the reported target levels for contraceptive efficacy [68].

Of note, one limitation of our in vitro and in vivo release rate comparison (Table 4) is the assumption of zero order release kinetics in vivo. We did not directly test this assumption. However, upon visual observation of the concentration time profile, we believe that zero order kinetics were maintained in vivo for DPV. While the degree of change between LNG and PTV $C_{\max}$ and C_{trough} exhibit a declining trend, the slope of this decline for the mean concentration values suggests that LNG and DPV release rates may have ranged from 4 to 60 and 50–200 $\mu\text{g/day}$, respectively.

Collectively, our results demonstrate the development of a first-inline 3D printed MPT IVR that elicited sustained release in vitro and in vivo of three clinically proven APIs (DPV, PTV, LNG) indicated for the prevention of HIV, HSV-2, and unintended pregnancy. Ultimately, using state-of-the-art 3D printing and a unique post-fabrication drug loading process, this discrete and women-controlled MPT IVR has the potential to elicit high end-user acceptability and adherence and expand preventative options for women and girls around the world, particularly in areas where sexually transmitted infections like HIV and HSV-2 are most prevalent, and unintended pregnancies result in significant economic and health burdens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data availability

Data will be made available on request.

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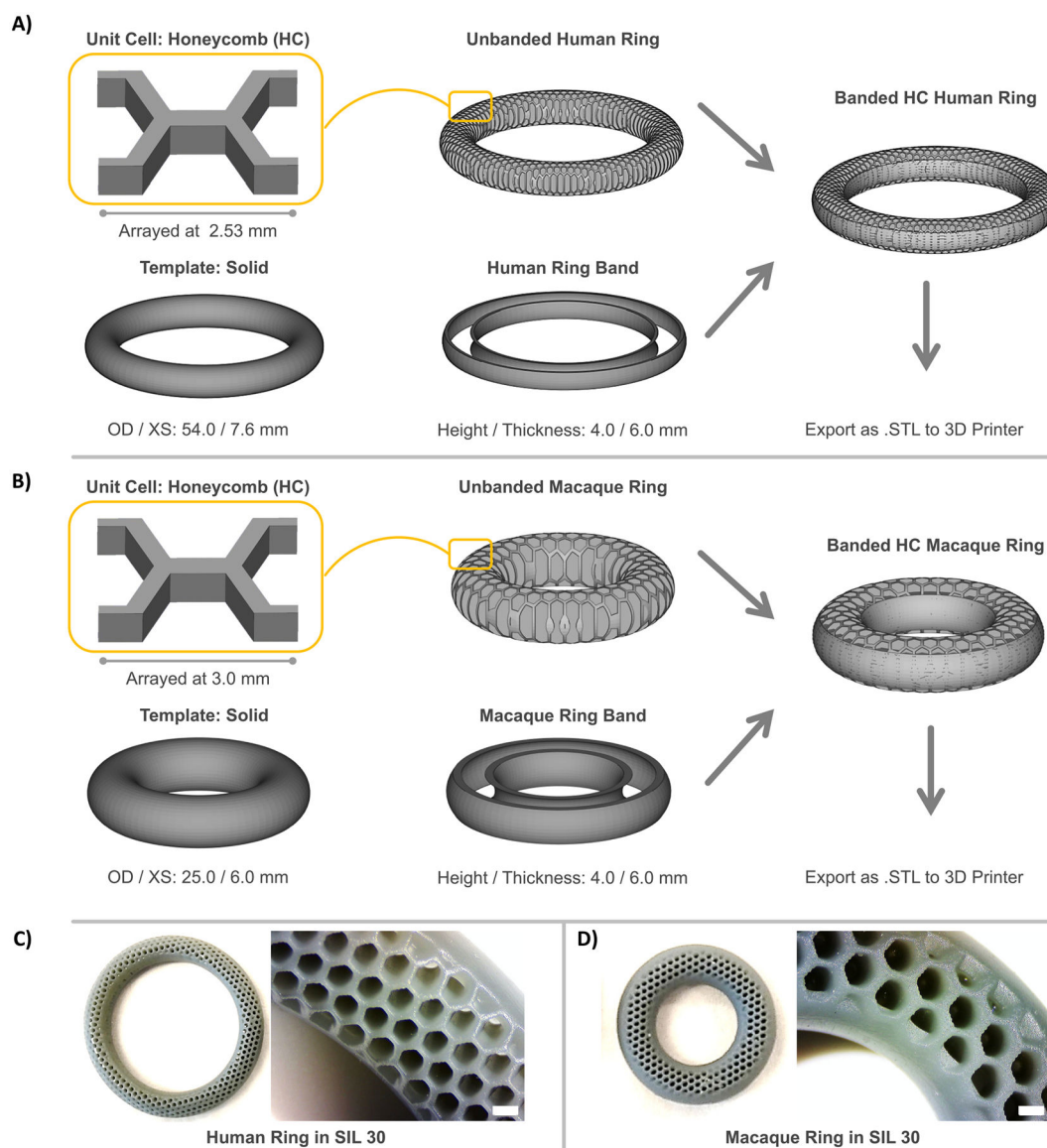
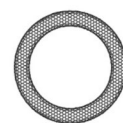


Fig. 1.
Diagram of the internal geometric complexity of the SIL 30 honeycomb IVRs.

API	API Weight % in IVR	Target API per human IVR (mg)	Target API per macaque IVR (mg)
Dapivirine (DPV)	0.65%	30.0 mg	8.11 mg
Pritelivir (PTV)	0.43%	20.0 mg	5.36 mg
Levonorgestrel (LNG)	0.043%	2.0 mg	0.536 mg



Average Mass: 4633.0 mg
Outer Diameter: 54 mm
Cross-Section: 7.6 mm

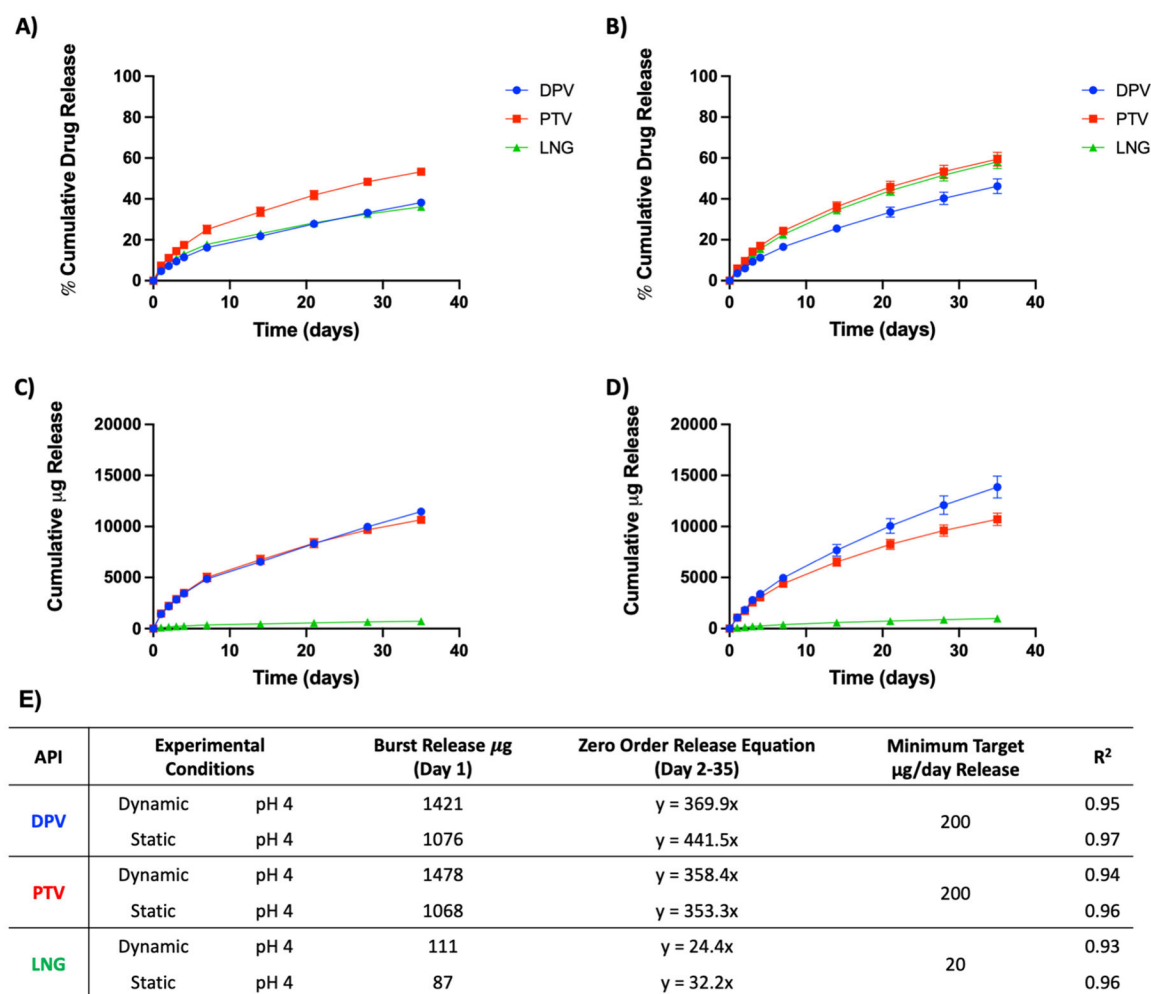


Average Mass: 1247.0 mg
Outer Diameter: 25 mm
Cross-Section: 6.0 mm

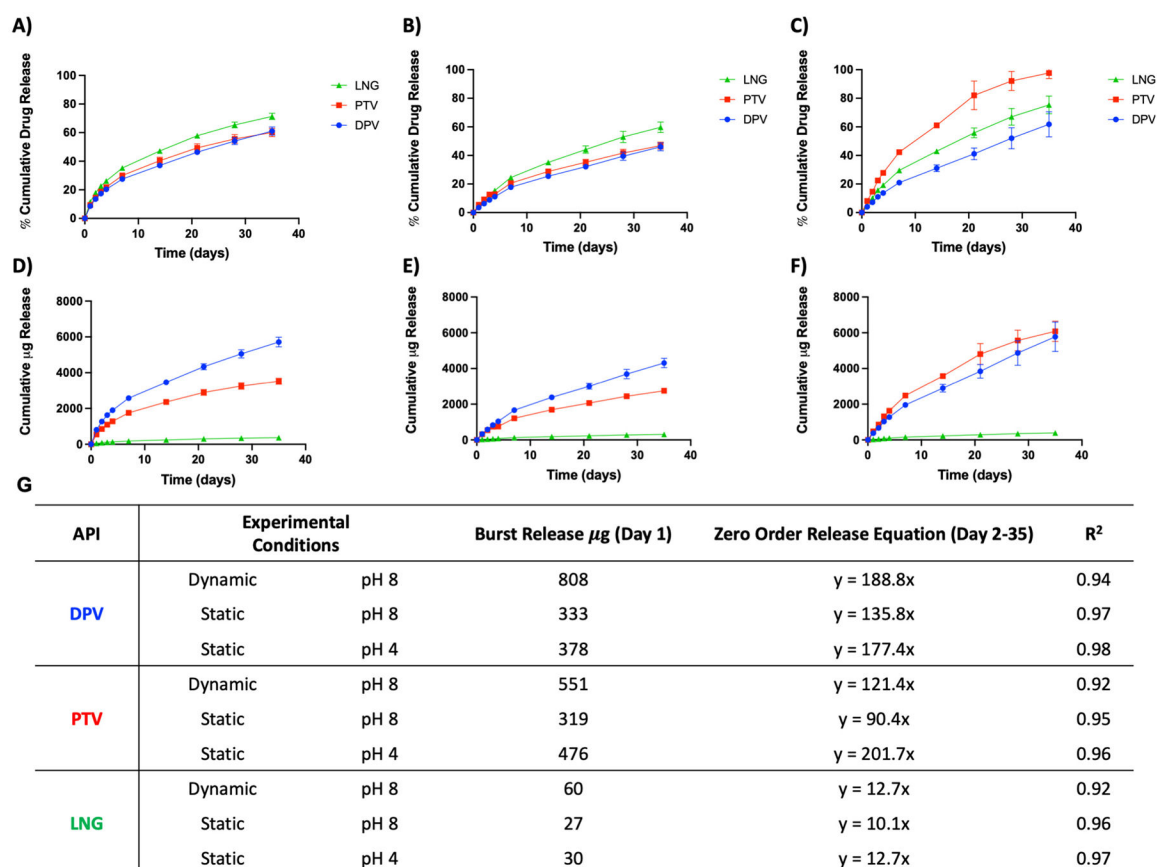


Fig. 2.

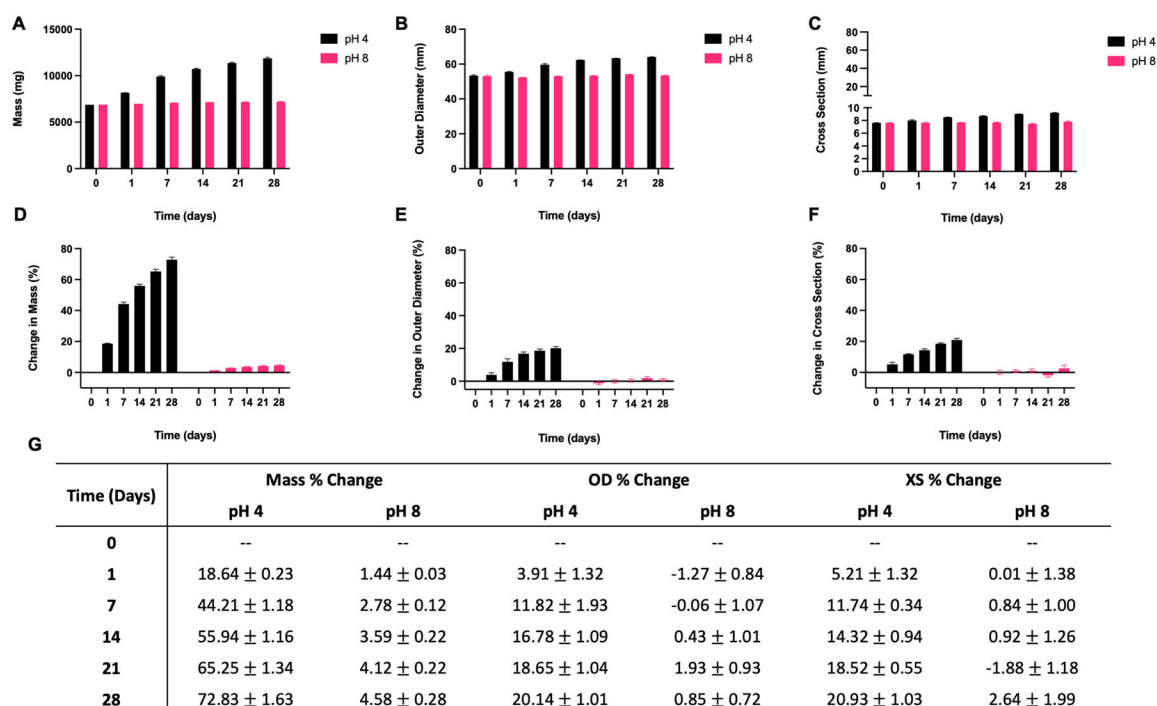
Detailed drug loading (% w/w) for DPV, PTV, and LNG in honeycomb CLIP 3D printed IVRs. Direct comparison of human size and macaque size IVRs summarizing API percent loading and IVR metrics (mass, OD, CS). The weight percent loading for each API was kept constant across human size and macaque size IVRs tested.

**Fig. 3.**

In vitro drug release kinetics for SIL 30 honeycomb human-sized (54 mm OD, 7.6 mm CS) MPT IVR over 35 days. IVRs were incubated in SVF + 2 % Solutol (pH 4) under sink conditions at 37 °C, using dynamic settings (orbital shaker, 120 rpm) or static settings. A) Cumulative % drug release for each API under dynamic release settings. B) Cumulative % drug release for each API under static release settings. C) Cumulative μg drug release for each API under dynamic release settings. D) Cumulative μg drug release for each API under static release settings. E) Summary of in vitro burst (day 1) and daily release rates (day 2–35) comparing static and dynamic release at pH 4.

**Fig. 4.**

In vitro drug release kinetics of SIL 30 honeycomb macaque size (25 mm OD, 6.0 mm CS) MPT IVRs over 35 days. Release studies were performed in SVF + 2 % Solutol at 37 °C under three different experimental conditions: dynamic release study (120 rpm, pH 8) and static release study (pH 4 and pH 8). For each release study the percent (%) and μg drug release were determined. A) Cumulative % drug release for each API in the dynamic, pH 8 study. B) Cumulative % drug release for each API in the static, pH 8 study. C) Cumulative % drug release for each API in the static, pH 4 study. D) Cumulative μg drug release for each API in the dynamic, pH 8 study. E) Cumulative μg drug release for each API in the static, pH 8 study. F) Cumulative μg drug release for each API in the static, pH 4 study. G) Summary of initial burst and daily release rates for each API under various release conditions.

**Fig. 5.**

Placebo solid human size IVRs (54 mm OD, 7.6 CS) were acetone washed for 24 h in accordance with IVR drug-loading preparation methods. A) The mass (mg), B) outer diameter (mm), and C) cross section (mm) dimensions were recorded at $t = 0$. In triplicate ($n = 3$), IVRs were incubated at 37 °C in SVF release media at pH 4 or pH 8. IVRs were removed from media and dimensions were recorded at $t = 1, 7, 14, 21$, and 28 days. D-F) The percent change in swollen dimensions of the IVRs was calculated by comparing to the baseline dimensions for each IVR at $t = 0$. G) Summary of percent (%) change in IVR metrics (mass, OD, CS) at pH 4 and pH 8 over 28 days relative to time 0.

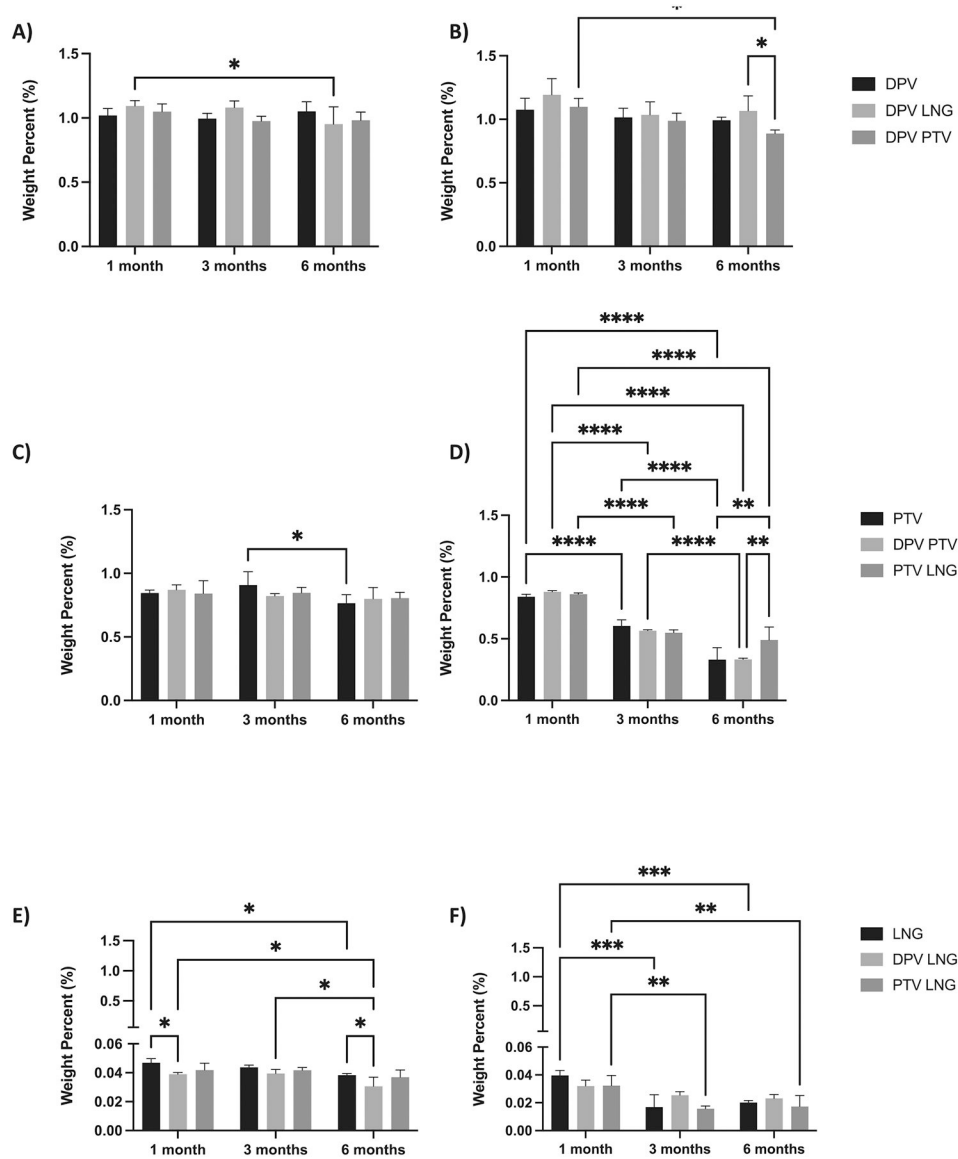
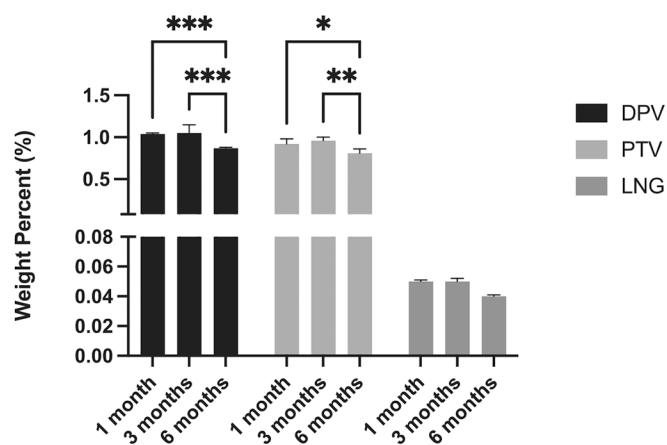


Fig. 6.

API weight percent changes in SIL 30 honeycomb IVR segments stored at 4 °C or at accelerated stability conditions (40 °C/75 % RH). After 1, 3, and 6 months of storage, IVR segments were extracted to determine the remaining API present. A) DPV segments loaded with single or dual-drug combination, stored at 4 °C. B) DPV segments loaded with single or dual-drug combination, stored at 40 °C/75 % RH. C) PTV segments loaded with single or dual-drug combination, stored at 4 °C. D) PTV segments loaded with single or dual-drug combination, stored at 40 °C/75 % RH. E) LNG segments loaded with single or dual-drug combination, stored at 4 °C. F) LNG segments loaded with single or dual-drug combination, stored at 40 °C/75 % RH (* indicates $p < 0.05$; ** indicates $p < 0.005$; *** indicates $p < 0.001$; **** indicates $P < 0.0001$).



	DPV wt% (mg/mg)			PTV wt% (mg/mg)			LNG wt% (mg/mg)		
	1 mo	3 mo	6 mo	1 mo	3 mo	6 mo	1 mo	3 mo	6 mo
Triple Drug	1.04 ± 0.01	1.05 ± 0.10	0.87 ± 0.01	0.92 ± 0.06	0.96 ± 0.04	0.81 ± 0.05	0.05 ± 0.001	0.05 ± 0.002	0.04 ± 0.001

Fig. 7.

Weight percent changes for APIs loaded as a triple-drug combination in IVR segments after storage at 4 °C for 1, 3, and 6 months (*indicates $p < 0.05$; **indicates $p < 0.005$; ***indicates $p < 0.001$).

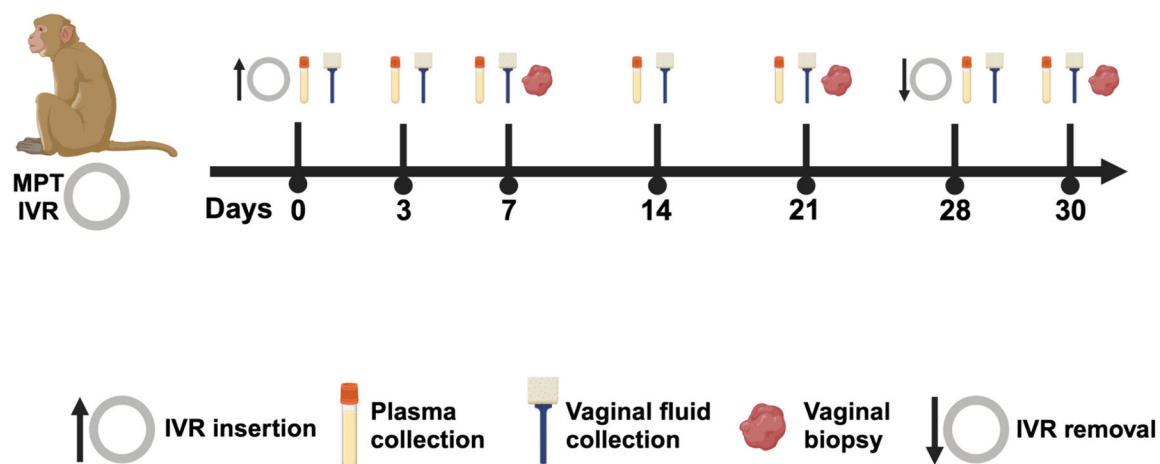
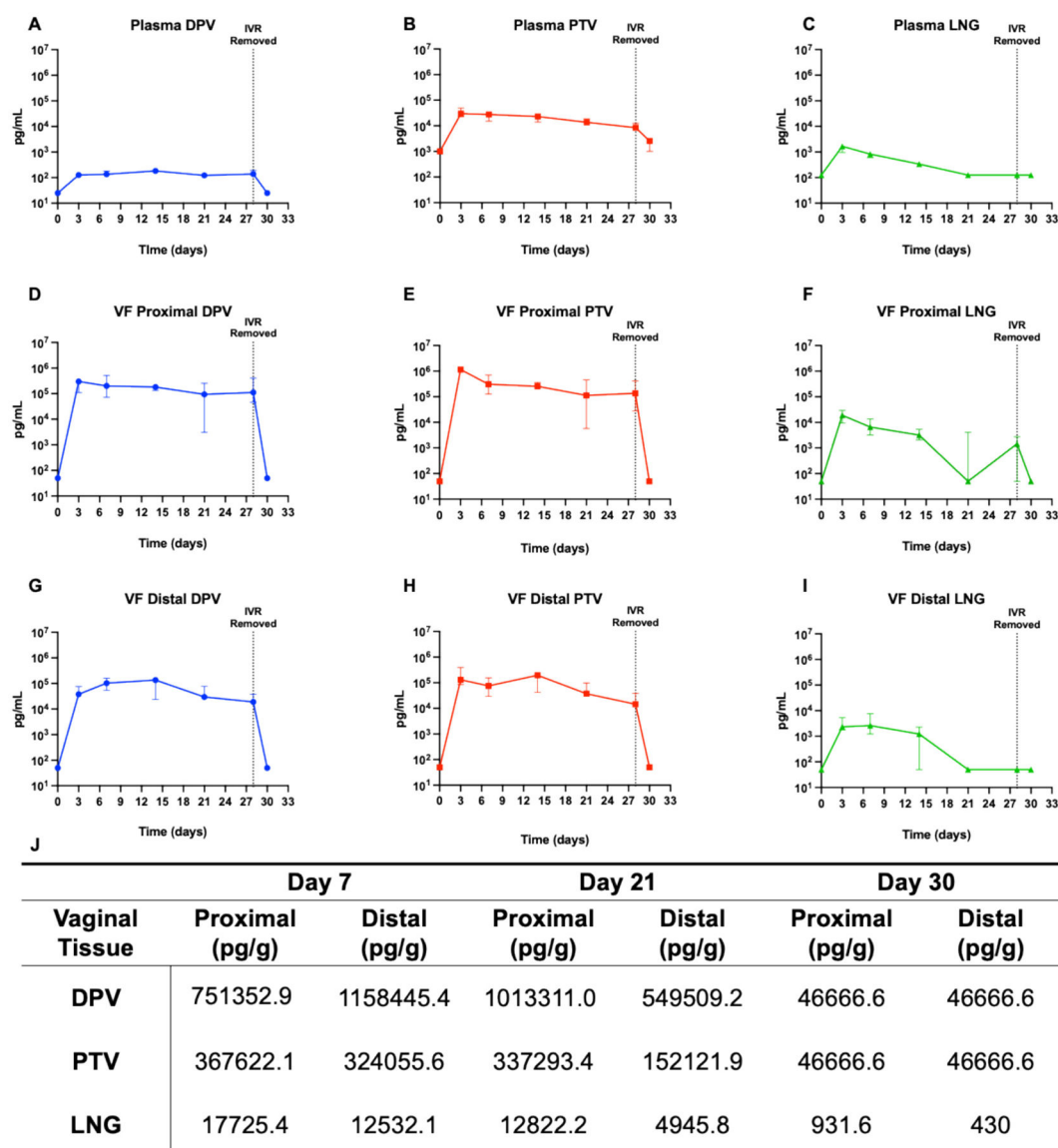


Fig. 8.

Timeline for sampling of biological samples in in vivo study. DPV/PTV/LNG loaded macaque size (25 mm OD, 6.0 mm CS) IVRs were investigated in $n = 3$ female pigtailed macaques. Samples were collected at $t = 0$. IVRs were inserted for 28 days and then removed. Final samples were collected on day 30, two days post-IVR removal.

**Fig. 9.**

Comparing the drug concentration in vivo for DPV, PTV, and LNG. IVRs were inserted in pigtailed macaque for 28 days and biological samples collected over 30 days. A) DPV in plasma (pg/mL). B) PTV in plasma (pg/mL). C) LNG in plasma (pg/mL). D) DPV in vaginal fluid proximal to the cervix (pg/mL). E) PTV in vaginal fluid proximal to the cervix (pg/mL). F) LNG in vaginal fluid from proximal to the cervix (pg/mL). G) DPV in vaginal fluid distal to the cervix (pg/mL). H) PTV in vaginal fluid distal to the cervix (pg/mL). I) LNG in vaginal fluid distal to the cervix (pg/mL). J) DPV, PTV, and LNG in proximal and distal vaginal tissues (pg/g).

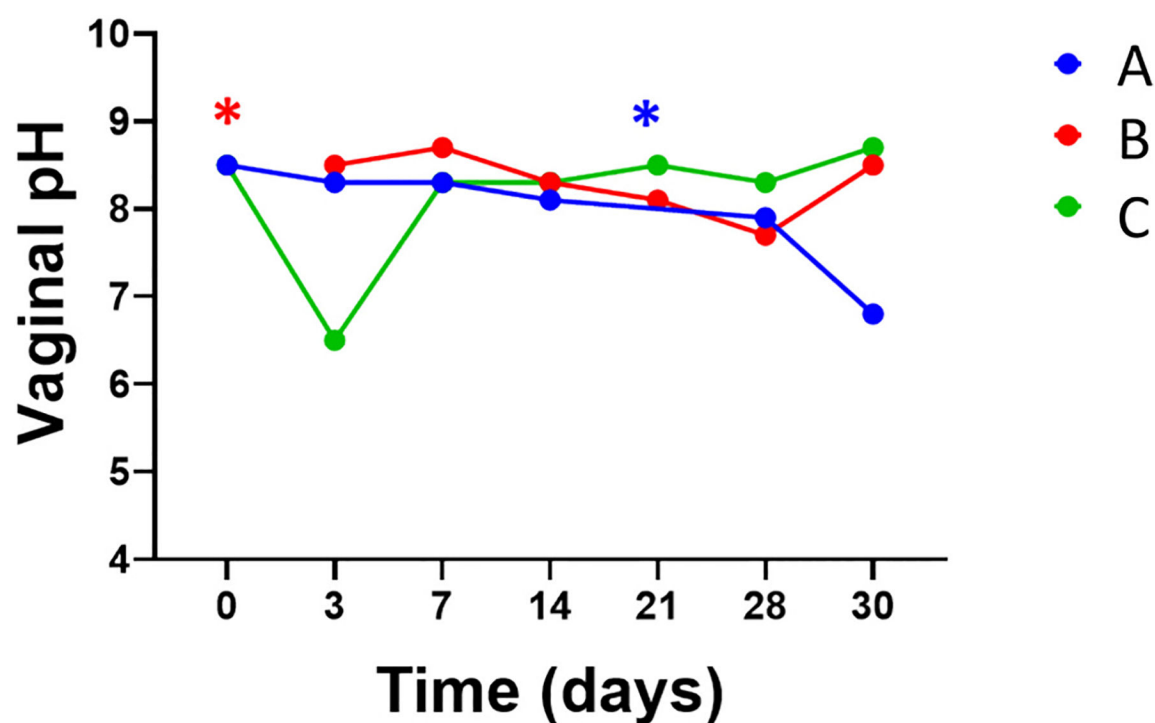


Fig. 10.

Vaginal pH measurements in macaques that received the 3D IVR: The vaginal pH was measured prior to ring insertion (day 0), and on days 3, 7, 10, 14, 21, 28 after ring insertion. A post ring removal measurement was obtained at day 30. The vaginal pH is not measurable when a macaque is undergoing menstruation. The absence of a pH reading for an animal undergoing menstruation is indicated by an *.

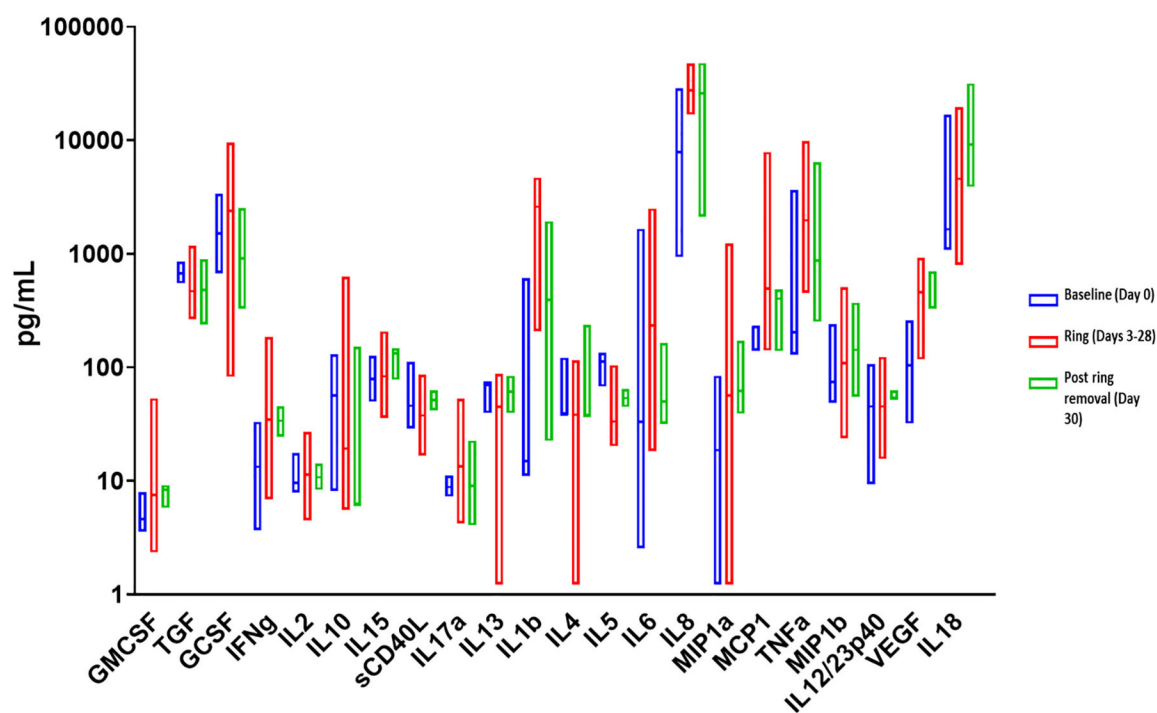


Fig. 11.

Vaginal cytokine and chemokine measurements in female macaques that received the 3D printed MPT IVR: 22 vaginal cytokines and chemokines were measured in the three macaques prior to ring insertion (day 0), and on days 3, 7, 10, 14, 21, 28 after ring insertion. A post ring removal measurement was done at day 30. The measurements were grouped as baseline (day 0, blue columns), while the ring was present (days 3–28, red columns) and post ring removal (day 30, green columns). Minimum to maximum floating bars with line at median of the grouped measurement for the 22 cytokines are shown. No statistically significant changes ($p < 0.05$) were detected while the ring was present or upon its removal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Change in weight percent (wt%) for each API loaded individually and in dual combination with each API after 1, 3, and 6 months storage at 4° C.

	DPV wt% (mg/mg)			PTV wt% (mg/mg)			LNG wt% (mg/mg)		
	1 mo	3 mo	6 mo	1 mo	3 mo	6 mo	1 mo	3 mo	6 mo
APIs Loaded									
DPV	1.02±0.05	0.99±0.04	1.05±0.07						
PTV				0.85±0.02	0.91±0.10	0.77±0.07			
LNG							0.05±0.003	0.04±0.002	0.04±0.001
DPV LNG	1.09±0.04	1.08±0.05	0.95±0.13				0.04±0.001	0.04±0.003	0.03±0.006
DPV PTV	1.05±0.06	0.98±0.04	0.98±0.06	0.87±0.04	0.82±0.02	0.80±0.09			
PTV LNG				0.84±0.10	0.85±0.04	0.81±0.04	0.04±0.005	0.04±0.002	0.04±0.005

Change in weigh percent (wt%) for each API loaded individually and in dual combination with each API after 1, 3, and 6 months storage under accelerated stability conditions (40° C/75 % RH).

Table 2

APIs Loaded	DPV wt% (mg/mg)			PTV wt% (mg/mg)			LNG wt% (mg/mg)		
	1 mo	3 mo	6 mo	1 mo	3 mo	6 mo	1 mo	3 mo	6 mo
DPV	1.08±0.09	1.02±0.07	0.99±0.02						
PTV				0.83±0.05	0.60±0.05	0.33±0.10			
LNG							0.04±0.004	0.02±0.009	0.02±0.001
DPV LNG	1.19±0.13	1.04±0.10	1.07±0.12				0.03±0.004	0.03±0.003	0.02±0.003
DPV PTV	1.10±0.07	0.99±0.06	0.89±0.03	0.88±0.03	0.57±0.01	0.33±0.01			
PTV LNG				0.86±0.03	0.55±0.02	0.49±0.10	0.03±0.010	0.02±0.002	0.02±0.008

Table 3

Residual drug in IVRs removed after 28 days of use in pigtail macaque (n = 3). Table of values showing the initial amount of API per macaque IVR, residual drug (%), and total amount of API released in vivo over 28 days.

	DPV	PTV	LNG
Analytical API loading/IVR (mg)	9.35 ± 0.79	5.86 ± 0.08	0.52 ± 0.01
Residual API (mg)	8.82 ± 0.11	2.15 ± 0.49	0.08 ± 0.02
Residual API (% of total)	94.3 %	36.7 %	15.4 %
Total API (mg) delivered	0.53 ± 0.11	3.71 ± 0.49	0.44 ± 0.02
Total API (%) delivered	5.67 ± 1.16	63.33 ± 8.36	84.33 ± 4.51

Table 4

Comparison of in vitro and in vivo release rates. In vitro release rates of MPT macaque IVRs in SVF pH 8 and dynamic (120 rpm) conditions compared to in vivo release rates determined by this equation ($Total\ drug\ (mg) - residual\ drug\ (mg) \div 28\ days$). The in vitro-in vivo correlation was calculated by dividing in vivo daily release rates by the in vitro daily release rates.

API	Cumulative API delivered over 28 days (mg)		Release Rate ($\mu\text{g/day}$)			
	In vitro	In vivo	In vitro	In vivo	Target ($\mu\text{g/day}$)	IVIVC
DPV	5.06 $\pm$ 0.23	0.53 $\pm$ 0.11	189	19	54	0.1
PTV	3.26 $\pm$ 0.18	3.71 $\pm$ 0.49	121	133	54	1.1
LNG	0.34 $\pm$ 0.01	0.44 $\pm$ 0.02	13	16	5.4	1.2