

Vaginal Microbicide Gel for Delivery of IQP-0528, a Pyrimidinedione Analog with a Dual Mechanism of Action against HIV-1^{▽†}

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Pyrimidinediones, a novel class of compounds, have previously been shown to possess antiviral activity at nanomolar concentrations. One member of this class of compounds, IQP-0528, was selected as the lead molecule for formulation development owing to its stability at physiologically relevant conditions, wide therapeutic window, and antiviral activity in the nanomolar range. Here, we report the development of two vaginal gels—3.0% hydroxyethyl cellulose (HEC) formulation and a 0.65% Carbopol formulation—for the sustained delivery of IQP-0528. Stability studies under accelerated conditions confirmed the chemical stability of IQP-0528 and mechanical stability of the gel formulation for 3 months. *In vitro* release studies revealed that diffusion-controlled release of IQP-0528 occurred over 6 h, with an initial lag time of approximately 1 h. Based on the drug release profile, the 3.0% HEC gel was selected as the lead formulation for safety and activity evaluations. The *in vitro* and *ex vivo* safety evaluations showed no significant loss in cell viability or significant inflammatory response after treatment with a 3.0% HEC gel containing 0.25% IQP-0528. In an *in vitro* HIV-1 entry inhibition assay, the lead formulation showed a 50% effective concentration of 0.14 $\mu\text{g/ml}$ for gel in culture media, which corresponds to $\sim 0.001 \mu\text{M}$ IQP-0528. The antiviral activity was further confirmed by using polarized cervical explants, in which the formulation showed complete protection against HIV infection. In summary, these results are encouraging and warrant further evaluation of IQP-0528 gel formulations in *in vivo* models, as well as the development of alternative formulations for the delivery of IQP-0528 as a microbicide.

The need for prophylactic strategies to prevent heterosexual transmission of human immunodeficiency virus (HIV) was recognized more than 20 years ago (30). Since then researchers from various fields have actively been developing technologies to combat the rapid spread of HIV. These strategies include pre-exposure prophylactics (28, 41) and microbicides (14, 31) designed to topically deliver single or combination antiviral agents. Currently, microbicide delivery systems under development include gels, rings, films, and suppositories. Of these systems, vaginal gels remain the preferred choice for the first line of microbicide product development. This can be attributed to the ease of vaginal gel development, the extensive work reported in the literature using this dosage form, and the existence of several vaginal semisolid products in the market. Microbicide administration via the vaginal route is advantageous since it allows local, noninvasive delivery of antiviral agents and enhanced biodistribution of drugs facilitated by the rich underlying blood supply (37, 40). Furthermore, vaginal gels can be self-administered, which is critical in the develop-

ment of woman-controlled preventive strategies such as vaginal microbicides (37).

An effective prevention strategy will undoubtedly require a multitargeted approach that can be achieved either by combining multiple antiviral agents or by using drugs that possess multiple mechanisms of inhibiting HIV transmission (20). Pyrimidinedione [PYD; 1-(3-cyclopenten-1-ylmethyl)-5-ethyl-6-(3,5-dimethylbenzoyl)-2,4(1*H*,3*H*)] derivatives constitute a unique class of non-nucleoside reverse transcriptase inhibitors that also inhibit HIV entry through an unknown mechanism thought to target a conformational epitope formed prior to the fusion of the viral envelope with the host cellular membrane (5). The dual mechanisms of action, high potency, minimal toxicity to vaginal cells and natural flora and broad range of activity against wild-type and drug-resistant clinical viruses make these molecules attractive candidates for microbicide development. Previous work by Buckheit et al. reported a detailed exploration of the structure-function relationship of PYD analogs (3, 4). All of the PYD derivatives demonstrated inhibition of both reverse transcriptase and virus entry; however, the effective concentration for the inhibition of cell entry and reverse transcriptase varied depending on the chemical modifications to 2,4(1*H*,3*H*)-pyrimidinedione (3, 4). In the present study, we report a preformulation screen of nine PYDs with reverse transcriptase inhibition activity in the nanomolar range and therapeutic indices of $\geq 10,000$ (see Table S2 in the supplemental material) (4).

This work focuses on the development of an inexpensive

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vaginal gel formulation for the sustained delivery of PYD using GRAS (generally regarded as safe) ingredients—polyacrylic acid (PAA) and a cellulose derivative (15, 27, 34, 38). In particular, hydroxyethyl cellulose (HEC) has been used in several vaginal gel formulations, including the universal placebo gel (32). Chemical stability of the active compound and mechanical stability of the gel formulation was evaluated under accelerated conditions for 3 months. The release kinetics of the active compound from the gel matrices were assessed using *in vitro* and *ex vivo* drug release studies.

In addition to careful selection of the drug delivery vehicle and the active ingredient, a detailed exploration of the safety and efficacy of the end formulation is critical to the development of a microbicide. Particularly after the clinical failures of cellulose sulfate, N-9, Savvy, and several other microbicides, *in vitro* safety and efficacy evaluations have become a major component of topical microbicide product development (6, 16, 24). These studies offer a cost-effective method of evaluating microbicide formulations prior to more complex and expensive *in vivo* studies (36). We evaluated the safety and efficacy of the PYD gel formulation using reconstructed VEC-100 tissue (MatTek Corp.) and human polarized ectocervical tissue. The VEC-100 tissue recapitulates only the morphological characteristics of the stratified squamous epithelial layer, whereas the polarized ectocervical explants contains both epithelium and relevant immune cells (2, 7, 8, 26). In an *in vitro* HIV-1 entry inhibition assay the 3.0% HEC gel with 0.25% IQP-0528 demonstrated a 50% effective concentration (EC_{50}) of 0.14 $\mu\text{g}/\text{ml}$ of gel in culture media, corresponding to $\sim 0.001 \mu\text{M}$ IQP-0528, and showed complete protection of the human polarized ectocervical tissue against HIV infection.

MATERIALS AND METHODS

Materials. The PYD derivatives were provided by ImQuest BioSciences, Inc. (Frederick, MD). HEC 250 HX PHARM was purchased from Ashland, Inc. (Wilmington, DE). Carbopol 974P NF was purchased from Lubrizol (Wickliffe, OH). Glycerin was supplied by Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Hydrogen peroxide (ACS grade), 19-norethindrone (purity > 98%), bovine serum albumin, methylparaben, and propylparaben were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Acetonitrile and isopropyl alcohol (high-pressure liquid chromatography [HPLC] grade) were obtained from Fisher Scientific (Houston, TX). Lecithin and nonoxynol-9 (N-9) were purchased from Spectrum Chemicals (Gardena, CA). Solutol HS-15 was purchased from BASF (Florham Park, NJ). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) kit was obtained from Promega (Madison, WI), and all cell culture medium supplies were purchased from Invitrogen Corp. (Carlsbad, CA). VEC-100 tissues were purchased from MatTek Corp. (Ashland, MA).

Selection of lead PYD analog. We investigated the stability of nine PYD analogs (see Table S2 in the supplemental material) to select a lead candidate for formulation development. The stability studies were conducted under various physiologically relevant conditions including pH 7.0 (neutral pH), pH 4.2 (vaginal pH) (34), and pH 4.2 with a 0.1% hydrogen peroxide solution (simulating vaginal environment). All of the samples were stored at 40°C and 75% relative humidity to evaluate the stability of the PYD analogs under accelerated conditions. Stock solutions of PYD were prepared in methanol. Dilutions were made with 100 mM phosphate buffer (pH 7.0) containing 2% Solutol HS-15 to obtain a final concentration of 30 μM . Similarly, solutions were made in 100 mM acetate buffer (pH 4.2) containing 2% Solutol HS-15 for the pH 4.2 conditions. Solutol HS-15 was added to the buffers to keep the compounds solubilized after dilution. Samples were collected at 0, 1, 2, and 4 weeks and analyzed by using HPLC. All samples were run on a Zorbax ODS 5- μm , 4.6-by-150-mm C_{18} column at 37°C with a flow rate of 1.0 ml/min. A 12-min isocratic method was run at a 65:35 water/acetonitrile ratio. PYD was detected at 267 nm. PYD quantification was performed on an Agilent 1200 Series HPLC equipped with ChemStation32

software. For each sample, the concentration of analyte was determined from a standard curve relating peak area to analyte concentration. Percentage recovery of the PYD at each time point was calculated by normalizing the recovery to time zero. Samples were run in triplicate.

Preparation of the gel formulation. Appropriate amounts of the gelling agent (Carbopol, 0.65%; HEC, 3.0%) were added to 80% 18 M Ω distilled water and stirred for 45 min. To increase the buffering capacity of the gel, acetate buffer (25 mM, pH 5.2) was used instead of distilled water in the preparation of the HEC gel. A paste of the active compound (0.25%) in glycerin (3.5%) was added to the solution containing the gelling agent. The preservatives methylparaben (0.15%) and propylparaben (0.05%) were weighed and mixed with preheated glycerin (1.5%) until all solids had dissolved. The paste obtained was added to the solution containing the gelling agent, with constant stirring using an overhead stirrer equipped with a paddle-shaped propeller. Lastly, the pH of the gel was adjusted to 5.2 ± 0.2 , and the total weight was adjusted to 100%. All gels were allowed to equilibrate overnight and were retested for pH before use.

Chemical stability of the active compound in the gel formulation. Upon identification of the lead PYD molecule, the drug (at a concentration of 0.25%) was incorporated into a gel containing 3.0% HEC gel or a gel containing 0.65% Carbopol 974P. Gel formulations were evaluated for stability under accelerated conditions, which would be predictive of the long-term stability of the formulation at room temperature. Aliquots of the gel formulations were prepared in amber vials with rubber stoppers and aluminum crimps. The vials were stored at 40°C and 75% relative humidity (RH; Caron, Marietta, OH) and at 50°C in a dry convection oven (Thermo Haake, Waltham, MA). Samples were collected at 0, 1, 2, 4, 8, and 12 weeks, and IQP-0528 was extracted for quantification by using HPLC.

IQP-0528 extraction was performed in the presence of an internal standard (19-norethindrone). Required amounts (100 μl) of 19-norethindrone stock in methanol were added to each vial containing the gel. The contents of the vials were transferred into 10-ml volumetric flasks and sonicated for 30 min. Approximately 1 ml of the supernatant was filtered through a 0.2- μm -pore-size PTFE filter and analyzed by HPLC. To determine the extraction efficiency of the above-mentioned method, we created controls consisting of placebo gels spiked with known amounts of IQP-0528. The extraction efficiency of the method was determined using percent recovery of the internal standard and IQP-0528 from the controls. Samples and controls were run in triplicate.

Mechanical stability of the gel formulation. The viscosity of the gel formulations was measured by using a stress-controlled AR 550 rheometer equipped with 20-mm 4° steel cone geometry. A sample of the gel (150 μl) was allowed to equilibrate for 2 min on a Peltier plate at 37°C, after which a steady-state shear was applied to the gel to obtain viscosity profiles. Viscosity was measured in triplicate over the range of 1 to 100 s^{-1} to simulate physiological conditions (25). The mechanical stability of the gel formulations stored at 40°C in 75% RH and at 50°C in a dry atmosphere was evaluated at 0, 1, 2, 4, 8, and 12 weeks. Viscosity measured at each time point was compared to viscosity at time zero.

***In vitro* release study.** A continuous flow in-line Franz cell was used to perform the *in vitro* release study. The gel (200 μl) was placed on the donor compartment by using a positive-displacement pipette. The donor and receptor compartments were separated by a 13-mm-pore-size nylon membrane (Millipore, Billerica, MA) prewetted by soaking in the receptor solution for 1 h. Three different sink conditions were evaluated: 2% Solutol HS-15 in 100 mM acetate buffer (pH 4.2), liposomes (prepared from soy lecithin) (17) in acetate buffer (pH 4.2), and 50:50 isopropyl alcohol-phosphate buffer (IPA-PBS; pH 7.0, 100 mM). The receptor solution was circulated at a flow rate of 0.18 ml/min and collected at 2, 4, 6, 8, and 24 h for IQP-0528 content analysis using HPLC ($n = 5$).

IQP-0528 uptake study. Porcine vaginal tracts were collected immediately after sacrifice, placed in Krebs' buffer, and transported to the laboratory at ambient temperature within 1 h (21). Small sections of the tissue were dissected by using surgical scissors, snap-frozen, and stored at -80°C until testing. To confirm the integrity of the porcine vaginal tissue after a single freeze-thaw cycle, permeability studies were performed with the model compound caffeine. As previously reported, results from the permeability measurements indicated no difference between fresh and frozen vaginal tissue (35). The frozen tissue was thawed prior to use. To obtain consistent tissue thickness, the tissue samples were sliced by using a Thomas-Stadie-Riggs tissue slicer. The epithelium was separated by using blunt tweezers and gently pulled up to generate a final tissue thickness of approximately 500 μm ($500 \pm 200 \mu\text{m}$). A biopsy punch was used to obtain tissues with the desired cross-sectional diameter.

IQP-0528 uptake by the porcine vaginal tissue was determined by using a continuous flow Franz cell. Gel (20 μl) was placed on the donor compartment by using a positive-displacement pipette. Donor and receptor compartments were separated using a 6-mm biopsy specimen of porcine vaginal tissue. Phosphate-

buffered saline (PBS) was circulated continuously through the receptor compartment to keep the tissue hydrated. Tissue samples were collected after 2, 4, and 6 h and washed three times with PBS to remove any residual gel on the tissue before IQP-0528 content analysis.

Determination of IQP-0528 content in porcine vaginal tissue. Porcine vaginal tissue was placed in a 2-ml flat-bottom tube with a lock. To create a calibration curve, 1 to 300 μ l of a 3 mM IQP-0528 stock in methanol was added to blank tissue to create eight spiked samples of known concentrations. To precipitate proteins, 800 μ l of acetonitrile and 200 μ l of a 5% trichloroacetic acid solution were added to each tube, followed by incubation for 5 min. Finally, the samples were homogenized by using a Qiagen TissueLyser for 5 min. Samples were centrifuged at 14,000 \times g for 10 min, and the supernatant was filtered and analyzed for IQP-0528 content by using HPLC. The extraction efficiency for IQP-0528 from tissue samples was determined as 92% \pm 4%, with 19-norethindrone as the internal standard according to the method described above for extraction of the drug from gels. A calibration curve was created using the theoretical and experimental concentrations of IQP-0528 in the tissue sample after extraction. The actual concentration of IQP-0528 in the tissue was calculated by using the calibration curve. All samples were run with $n = 5$.

IQP-0528 permeability studies using human ectocervical tissue. Freshly excised human ectocervical tissue was obtained from the Tissue Procurement Facility at the Magee Women's Hospital, in accordance with institutional review board (IRB) protocol number MWH-98-065. All tissue samples were from premenopausal women undergoing hysterectomy for benign conditions. The tissue was immersed in Dulbecco modified Eagle medium (DMEM; Mediatech Cellgro; Fisher Scientific, Pittsburgh, PA) and used within 30 min of retrieval. Excess stromal tissue from cervical tissue was removed by using a Thomas-Sadie slicer (Thomas Scientific, Swedesboro, NJ). The thickness of the tissue was determined by placing the tissue between two premeasured histology slides, and it was remeasured using calipers.

Permeability studies were conducted using Franz cells (PermeGear, Nazareth, PA). Cervical tissue was placed between the donor and receptor compartments of the apparatus. The test compound was introduced into the donor compartment of the experimental apparatus. DMEM without phenol red (HyClone) was used as the receptor solution. Samples were obtained from the receptor compartment at the predetermined time intervals of 0 min, 15 min, 30 min, and 1 to 6 h. The drug permeability of the formulation across the tissue and the antiviral activity of the receptor solution were examined for both the Carbopol and HEC formulations. Receptor solutions were split; half of each solution was used for quantification of the drug by HPLC and the other half was used for bioactivity testing.

Drug uptake and permeability studies on VEC-100 vaginal tissue. To determine the ability of IQP-0528 to permeate and accumulate in human vaginal tissue, studies were performed on VEC-100 tissue, a stratified, well-studied three-dimensional model of human vaginal tissue. Based on the results from the *in vitro* release study and the permeability studies using human ectocervical tissue, the 3.0% HEC gel with higher IQP-0528 release was selected for the VEC-100 permeability studies. The 3.0% HEC gel was applied to the tissue samples in the inserts and cultured as recommended by the suppliers (2). Tissue samples were washed three times with 100 μ l of PBS, and trans-epithelial electrical resistance (TEER) measurements were recorded to ensure the integrity of the tissue during the study. After a washing step, fresh gel was applied to the tissue. This process was repeated for 3 days to study the effect of the gel on tissue after three repeated exposures. Culture medium was collected every 24 h for 3 days for IQP-0528 quantification ($n = 6$). Drug content in the tissue samples after 3 days was extracted and quantified using the same method as used with the porcine vaginal tissue.

***In vitro* safety and HIV-1 antiviral activity.** A Vk2/E6E7 human vaginal cell line and the VEC-100 tissue model were used to evaluate the safety of the IQP-0528 gel formulation *in vitro* after a single exposure and three repeated exposures. Diluted gel samples were added to the Vk2/E6E7 cells and incubated for 24 h. After exposure, cell viability was assessed by using an MTS assay. N-9 and the universal placebo gel (at a 1:100 dilution) were used as toxic and non-toxic controls, respectively.

To evaluate the safety of the formulation in the VEC-100 tissue, gel was applied to the tissue samples each day for 3 days. After three repeated exposures, tissue samples were analyzed for viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Tissues were also fixed in a 10% formalin solution and processed to evaluate tissue morphology. After paraffin embedding, sections were cut and stained with hematoxylin and eosin. Toxicity was determined based on observations of loss or damage to the epithelial layer. Culture medium collected every 24 h for 3 days was evaluated for the induction of inflammatory cytokines (interleukin-8 [IL-8], IL-1 α , IL-6, and tumor necrosis

factor alpha [TNF- α]) using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). These cytokines were chosen because they have been reported to be consistent indicators of cumulative mucosal toxicity and inflammatory responses (12). All samples were evaluated in triplicate except for the samples undergoing cytokine analysis, which were evaluated with six replicates ($n = 6$). *N*-(2-Hydroxypropyl) methacrylamide polymer (pHPMA) (33) and 1% Triton X-100 were used as the nontoxic and toxic controls, respectively.

We also evaluated the *in vitro* antiviral activity of the 3.0% HEC formulation using a luminescence assay as described previously (6). The HEC placebo gel was used as a negative control and Chicago sky blue was used as a positive control. Gels were serially diluted (625 to 200,000 times) to generate solutions that could be directly added to the cells. MAGI cells were seeded in a 96-well microtiter plate at a density of 10,000 cells/well for 24 h prior to the assay. After the overnight incubation at 37°C in 5% CO₂, diluted test samples were added to the cells in triplicate. HIV-1_{IIIIB} was diluted in the assay medium to generate the desired virus titer and was then added to the cells. Cells were incubated with the test sample for 2 h, after which the cell monolayers were washed three times and incubated for an additional 48 h. The inhibitory activity of the gels was evaluated by chemiluminescence detection with the Gal-Screen system, and toxicity was evaluated by using the tetrazolium dye 2,3-bis-(2-methoxy-4-nitro-5-sulfonyl)-(2H)-tetrazolium-5-carboxanilide (XTT). We also assayed the activity of the API and the gel formulation in the presence of 25% seminal fluid.

In a separate assay, we preincubated the cells with the gel formulation for 1 h, after which the virus was added to the cells, followed by incubation for an additional 2 h. In addition to evaluating the residual activity of the formulation, after preincubation of the formulation with the cells, we washed the cell monolayer to remove the formulation. The virus was then added and incubated for 2 h. The antiviral activity was assayed after an additional 48 h of incubation by measuring the β -galactosidase activity. Antiviral and toxicity results were reported as the EC₅₀ and the 50% toxic concentration (TC₅₀).

Safety and antiviral activity in polarized ectocervical explants. Evaluations of the safety and antiviral activity of the lead formulation, IQP-0528, were performed as previously described (7, 26). Briefly, the explant was placed in a transwell with the luminal side up. The edges around the explant were sealed with Matrigel (BD Biosciences, San Jose, CA). The explants were maintained with the luminal surface at the air-liquid interface. The lamina propria was immersed in culture medium. Cultures were maintained at 37°C in a 5% CO₂ atmosphere.

For the safety evaluation, the explants were prepared in duplicate on the day of surgery. To ensure an even spread of the gels and to allow them to be mixed with HIV-1 for the efficacy evaluation (below), a 1:5 dilution of IQP-0528 or HEC placebo gels was applied to the apical side of the explants for 18 h. Control explants were untreated or treated with an apically applied 1:5 dilution of 2% N-9 gel (Gynol II; purchased over the counter). The following day, the explants were washed and safety was evaluated by using an MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] assay and histology methods (7, 26).

For the evaluation of antiviral activity, a 1:5 dilution of IQP-0528 or HEC placebo gel was mixed with HIV-1_{BaL} and added to the apical side of the explants. At 18 h after application, the explants were washed, and fresh culture medium was added to the basolateral compartment. Every 3 to 4 days over a 3-week period, the supernatant was collected and stored at -80°C for HIV-1 p24 gag analysis, and fresh culture medium was replenished. Immunohistochemistry (IHC) was performed at the study endpoint for HIV-1-infected cells by staining for p24 gag.

Statistical analysis. Decreases in the drug content and viscosity of the gel stored under accelerated conditions were evaluated by comparison to levels at time zero by using a Student *t* test. Viability, TEER, and cytokine levels were compared to the untreated (naive) controls by using a Student *t* test. Differences in antiviral activity between the gel formulation and the control medium were determined by the Mann-Whitney U test. *P* values of <0.05 were considered significant.

RESULTS

Selection of lead PYD analog. As shown in Fig. 1a and b, the majority of compounds stored at pH 4.2 and pH 7.0 at 40°C and 75% RH in the dark showed no significant degradation after 4 weeks compared to their concentrations on day 0. However, the recovery of IQP-0558, -0410, and -1187 was significantly reduced (Student *t* test, $P < 0.05$) (Fig. 1a) compared to day 0 concentrations. After 4 weeks at pH 4.2

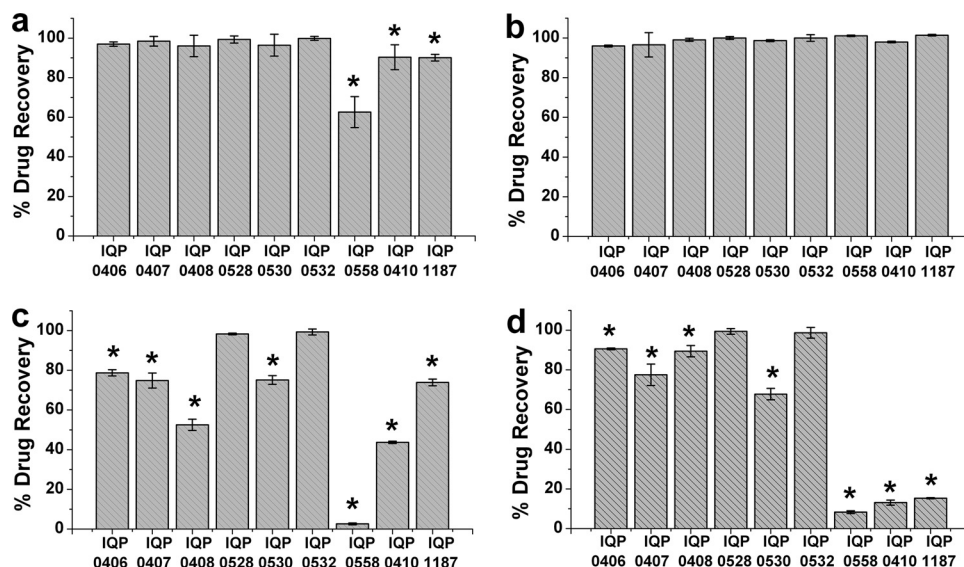


FIG. 1. Percent recovery (normalized to $t = 0$) of nine PYD analogs after 4 weeks under the following conditions: pH 7.0, 40°C, 75% relative humidity (RH), dark (a); pH 4.2, 40°C, 75% RH, dark (b); pH 4.2, room temperature, sunlight (c); and pH 4.2 with 0.1% H₂O₂, 40°C, 75% RH, dark (d). All samples were analyzed using HPLC ($n = 3$) and are expressed as means \pm the standard deviations (SD). *, $P < 0.05$ (Student t test).

in the presence of sunlight, all compounds with the exception of the IQP-0532 and -0528 showed significant degradation (Fig. 1c). Similarly, at pH 4.2 in the presence of 0.1% H₂O₂, all compounds showed significant degradation with the exception of IQP-0528 and -0532 (Fig. 1d). Owing to its higher therapeutic index (see Table S2 in the supplemental material) and greater stability under all conditions tested, IQP-0528 was chosen as the lead candidate for further formulation development.

Chemical and mechanical stability of IQP-0528 and gel formulation. The percent recovery of IQP-0528 at the end of 12 weeks of storage at 50°C in the dark was 101.1% \pm 4.8% in the HEC gel and 102.4% \pm 4.1% in the Carbopol gel (Fig. 2). These results indicate that there was no significant degradation of IQP-0528 in either the HEC or the Carbopol gel after 12 weeks compared to levels on day 0 using a Student t test ($P > 0.05$). Therefore, it can be concluded that the compound's stability in the formulation was not impacted by the elevated temperature.

As shown in Fig. 3, the flow curves for IQP-0528 formulated

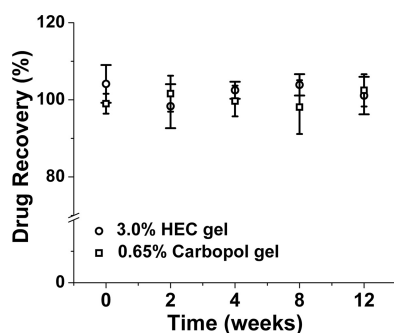


FIG. 2. Percent IQP-0528 recovery from the 3.0% HEC and 0.65% Carbopol gels after 12 weeks of storage at 50°C ($n = 3$, means \pm the SD).

in both HEC and Carbopol gels displayed non-Newtonian shear-thinning behavior. The rheological data on gels stored at 40°C and 75% RH demonstrated no significant loss in viscosity after 12 weeks of storage (Fig. 3). However, HEC gels stored under accelerated conditions (50°C) showed a statistically significant loss in viscosity (Fig. 3b). This loss in viscosity had no statistically significant effect on the drug release profile. Similar losses of viscosity in HEC gels have been reported previously (32) and are considered to be within the product specifications (<15% variation). Carbopol gels showed no loss in viscosity after 12 weeks under accelerated conditions (Fig. 3d). Altogether, these results suggest that the Carbopol and HEC formulations would be mechanically stable after long-term storage.

In vitro release study. After 24 h, ca. 90, 45, and 35% of the IQP-0528 was released in 1:1 IPA-PBS, Solutol, and liposome solutions, respectively (Fig. 4a). A similar trend was observed with 0.65% Carbopol gels, in which ca. 70, 24, and 20% of the IQP-0528 was released in 1:1 IPA-PBS, Solutol, and liposome solutions, respectively (data not shown). To describe the release kinetics of IQP-0528 from the gel formulations, the cumulative amount of IQP-0528 released at each time point was fitted to three mathematical models: Ficks's first and second laws of diffusion and Higuchi's equation (13, 19). Based on the goodness of fit ($r^2 > 0.90$), the IQP-0528 release was fitted to Higuchi's equation. As described by Higuchi's equation, the cumulative IQP-0528 release when plotted as a function of the square root of time was linear, indicating diffusion-controlled release of IQP-0528.

IQP-0528 permeability and uptake studies using porcine vaginal tissue. The amount of IQP-0528 in porcine vaginal tissue samples was quantified after extraction in the presence of an internal standard using HPLC. Exact quantification of IQP-0528 in each layer of the tissue is complex; therefore, in the present study, only the total IQP-0528 content in 500- μ m

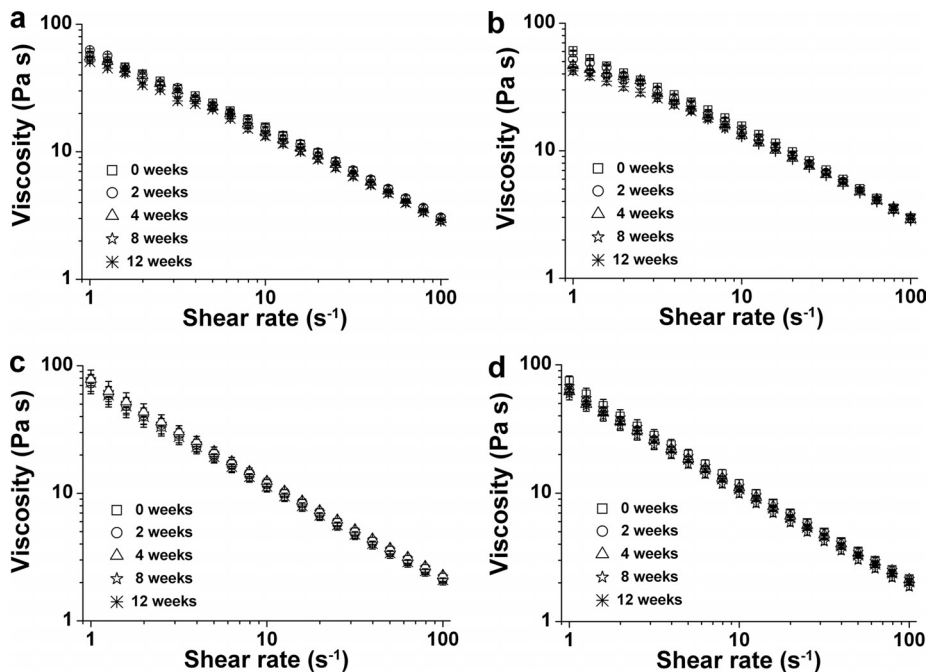


FIG. 3. Steady-state flow curves of 3.0% HEC gel with 0.25% IQP-0528 stored at 40°C and 75% RH (a); 3.0% HEC gel with 0.25% IQP-0528 stored at 50°C (b); 0.65% Carbopol gel with 0.25% IQP-0528 stored at 40°C and 75% RH (c); and 0.65% Carbopol gel with 0.25% IQP-0528 stored at 50°C (d). HEC gels stored at 50°C started to lose viscosity after 2 weeks of storage, but no significant loss in viscosity was observed in HEC gels stored at 40°C ($n = 3$, means \pm the SD, $P > 0.05$ [one-tailed Student t test]).

tissue samples was evaluated. As shown in Fig. 4b, after 6 h, 858 ± 78 nmol of IQP-0528 in the HEC gel was released for each gram of tissue, which corresponds to ca. 30% of the IQP-0528 loaded in the HEC gel. In contrast, with the Car-

bopol gel, 607 ± 160 nmol of IQP-0528 was released per gram of tissue, corresponding to ca. 20% of the IQP-0528 loaded in the Carbopol gel. Sustained release of IQP-0528 was observed from both formulations until 6 h after administration. An ini-

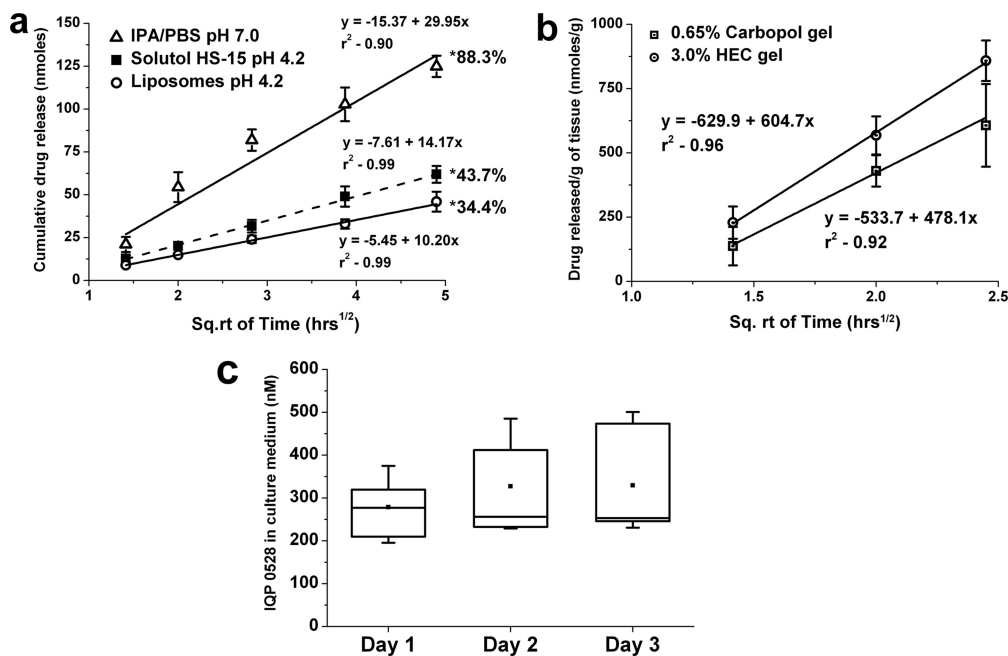


FIG. 4. *In vitro* and *ex vivo* release studies. (a) *In vitro* IQP-0528 release from a 3.0% HEC gel formulation under sink conditions at 37°C ($n = 3$, means \pm the SD). *, Percent drug released at the end of 24 h. (b) IQP-0528 uptake study using porcine vaginal tissue ($n = 5$, means \pm the SD). (c) Box plot of IQP-0528 concentration each day in the VEC-100 culture medium. ($n = 6$).

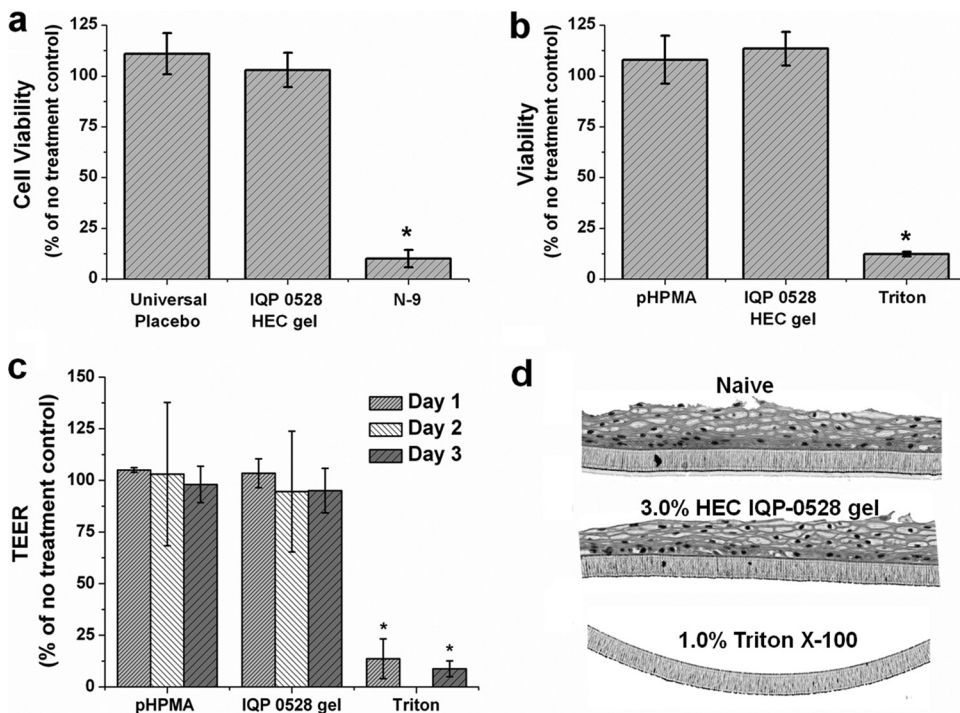


FIG. 5. (a) Vkc2/E6E7 cell viability measured by an MTS assay after 24 h of exposure ($n = 5$, means \pm the SD). The 3.0% HEC IQP-0528 formulation showed no significant loss in cell viability compared to the untreated control. Universal placebo and N-9 at 0.1% were used as the nontoxic and toxic controls, respectively. (b) VEC-100 tissue viability was determined using the MTT assay after three repeated exposures to the 3.0% HEC IQP-0528 formulation ($n = 3$, means \pm the SD). The formulation showed no significant loss in cell viability compared to the untreated control. Triton X-100 (1% solution) was used as the toxic control. *, $P < 0.05$ (one-tailed Student t test). (c) TEER measurements on VEC-100 tissues on days 1, 2, and 3 following treatment. No statistically significant difference was observed in the tissue samples exposed to the 3.0% HEC IQP-0528 formulation compared to the pHPMA samples ($n = 3$; Mean \pm the SD). *, $P < 0.05$ (one-tailed Student t test). (d) Hematoxylin-and-eosin-stained sections of VEC-100 tissue after three repeated exposures to test samples ($n = 3$).

tial lag time of ca. 1 h was calculated using the linear mathematical fit between the amount of drug released and the square root of time.

IQP-0528 permeability studies using human ectocervical tissue. In the permeability study using human ectocervical tissue, no detectable levels of IQP-0528 were found in any of the receptor compartment samples as analyzed by HPLC. However, upon bioactivity testing, the medium in the receptor compartment showed viral inhibition, suggesting the presence of inhibitory concentrations of the drug at 4 h for the HEC formulation and at 6 h for the Carbopol formulation. These data correlate with the observation of increased drug release obtained using the HEC formulation in the *in vitro* and *ex vivo* release studies. Based on the data on accumulation of drug in porcine vaginal tissue and the results from the permeability studies using human ectocervical tissue, the 3.0% HEC IQP-0528 gel was chosen as the lead formulation for further evaluation of toxicity and antiviral activity *in vitro* with cell lines and *ex vivo* with cervical tissue.

Drug uptake and permeability studies using VEC-100 vaginal tissue. As shown in Fig. 4c, the concentrations of IQP-0528 in growth media were measured as 278 ± 67 nM, 329 ± 123 nM, and 360 ± 174 nM on days 1, 2, and 3, respectively, following exposure of vaginal tissue to IQP-0528 gel. In addition, the IQP-0528 that accumulated in the VEC-100 tissue

after 3 days was extracted and the concentration was measured as 115 ± 62 μ M.

In vitro safety evaluation using Vkc2/E6E7 cells and VEC-100 vaginal tissue. As shown in Fig. 5a, the IQP-0528 gel showed no significant toxicity to Vkc2/E6E7 cells after 24 h of exposure compared to the untreated control, whereas the toxic control 0.1% N-9 caused a significant (ca. 90%) loss in cell viability (unpaired Student t test). Cytokine levels in the supernatant culture medium were quantified at 6 h and 24 h. No significant increase in IL-1 α and IL-6 levels were observed at either time points (data not shown). The positive control N-9 showed a statistically significant induction of cytokines compared to the basal levels (two-tailed Student t test).

As shown in Fig. 5b, tissue viability after three repeated exposure was determined to be $113\% \pm 8\%$ compared to the nontoxic control pHPMA. The TEER measurements for the tissue samples exposed to the IQP-0528 3.0% HEC gel formulation were $112\% \pm 19\%$, $105\% \pm 25\%$, and $109\% \pm 28\%$ compared to the nontoxic control on days 1, 2, and 3, respectively, suggesting that the formulation caused no significant loss in tissue barrier properties (Fig. 5c). As shown in Fig. 5d, the IQP-0528 formulation-treated tissues were morphologically similar to the naive tissue. Samples treated with Triton X-100 (1%) showed complete disintegration of the epithelium. These results are in agreement with the TEER measurements,

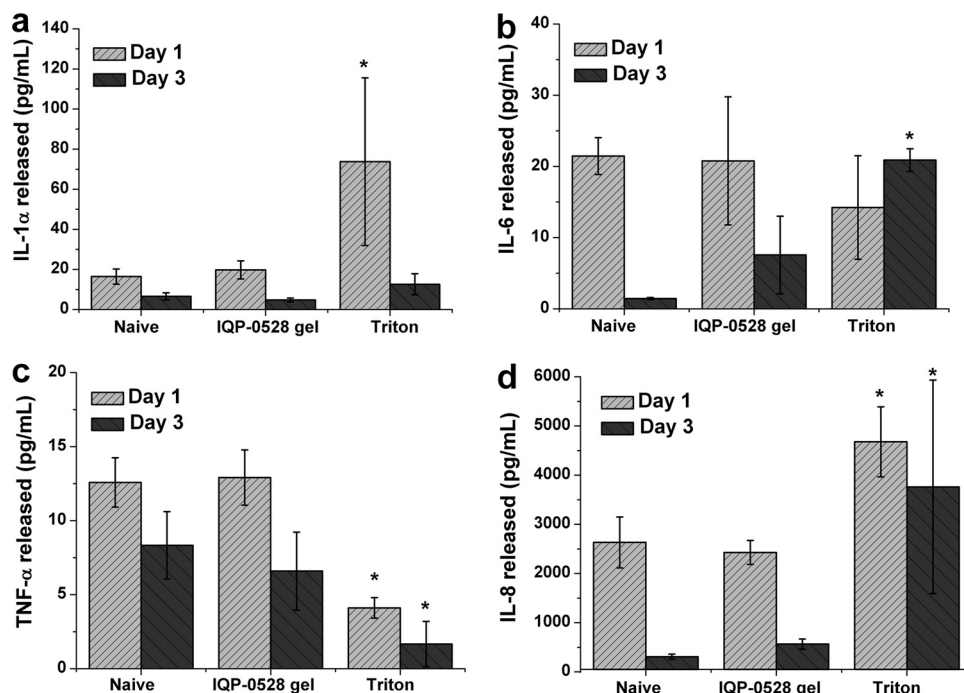


FIG. 6. Cytokine and chemokine release from VEC-100 tissue samples after exposure to a 3.0% HEC IQP-0528 formulation. Naive samples were used as negative controls and 1% Triton X-100-treated samples were used as positive controls. The IQP-0528 formulation showed no significant induction of the cytokines IL-1 α (a), IL-6 (b), and TNF- α (c) and the chemokine IL-8 (d). The positive control 1% Triton X-100 resulted in a significant increase in IL-1 α release on day 1 and in IL-6 release on day 3 compared to the naive samples. Triton treatment resulted in a significant decrease in the release of TNF- α and a significant increase in IL-8 on both day 1 and day 3 compared to the naive samples. *, $P < 0.05$ ($n = 6$, means \pm the SD).

which showed no evidence of structural damage from exposure to the IQP-0528 gel.

Among the four cytokines that were evaluated, there were relatively high basal levels of IL-8 in naive tissues. Similar observations have previously been reported in assays performed using the human vaginal Vk2/E6E7 cell line (11). Compared to the basal cytokine levels detected in the VEC-100 naive tissues, the IQP-0528 3.0% HEC gel formulation showed no significant change in IL-1 α , IL-6, IL-8, or TNF- α levels (Student t test, $P > 0.05$) (Fig. 6).

In vitro antiviral activity. As shown in Fig. 7, the HEC gels with IQP-0528 showed no significant toxicity to cells compared to the naive control (Student t test, $P > 0.05$). To evaluate the effect of the drug delivery vehicle alone, placebo gels were prepared using a composition similar to the IQP-0528 HEC gel formulation, but without the API. The placebo gels did not show any antiviral activity, as indicated by the nearly 100% relative luminescence units (RLU) (Fig. 7a). The 3.0% HEC gel with 0.25% IQP-0528 demonstrated significantly higher antiviral activity compared to the HEC placebo gel (Fig. 7b). The EC₅₀ for the IQP-0528 formulation was calculated using dose-response plots and was found to be 0.14 μ g/ml for the gel in the culture medium, corresponding to \sim 0.001 μ M IQP-0528.

No significant loss in antiviral activity was observed following preincubation of the 3.0% HEC gel containing 0.25% IQP-0528 (Fig. 7c). This assay mimics the *in vivo* situation in which the gel is expected to be applied hours prior to virus exposure. In the assay designed to evaluate the residual antiviral activity

of the IQP-0528 HEC gel formulation we observed a significant reduction (>2 -log scale) in the antiviral activity of IQP-0528 when administered alone. However, the IQP-0528 HEC gel showed only a marginal loss in antiviral activity. After simultaneous exposure to 25% seminal fluid and virus, the API showed severely compromised activity, whereas the IQP-0528 HEC gel showed no significant loss in antiviral activity (Fig. 7d). Taken together, these results reveal enhanced activity of IQP-0528 when formulated in a 3.0% HEC gel, with nanomolar antiviral activity that was retained following preincubation, removal of the formulation and in the presence of seminal fluid. Table 1 summarizes the antiviral activities of the IQP-0528 (API alone), the placebo and the IQP-0528 HEC gel formulation.

Safety evaluation using a polarized ectocervical explant model. To confirm the results obtained with the VEC-100 tissue, human ectocervical tissue was obtained after surgery and placed in culture using a polarized system (7, 26). The HEC IQP-0528 gel and HEC placebo gel preserved tissue viability similarly to control (untreated) explants, as measured by the MTT assay (Fig. 8a). The gel formulations also preserved epithelial/tissue integrity, as shown by histology (Fig. 8b). The N-9-treated explants showed significant reduction in tissue viability as measured by the MTT assay, and epithelial sloughing was observed by histology. The results with human ectocervical tissue confirmed the findings obtained using the reconstructed VEC-100 tissue.

Antiviral activity using a polarized ectocervical explant model. The efficacy of the HEC IQP-0528 gel was evaluated by

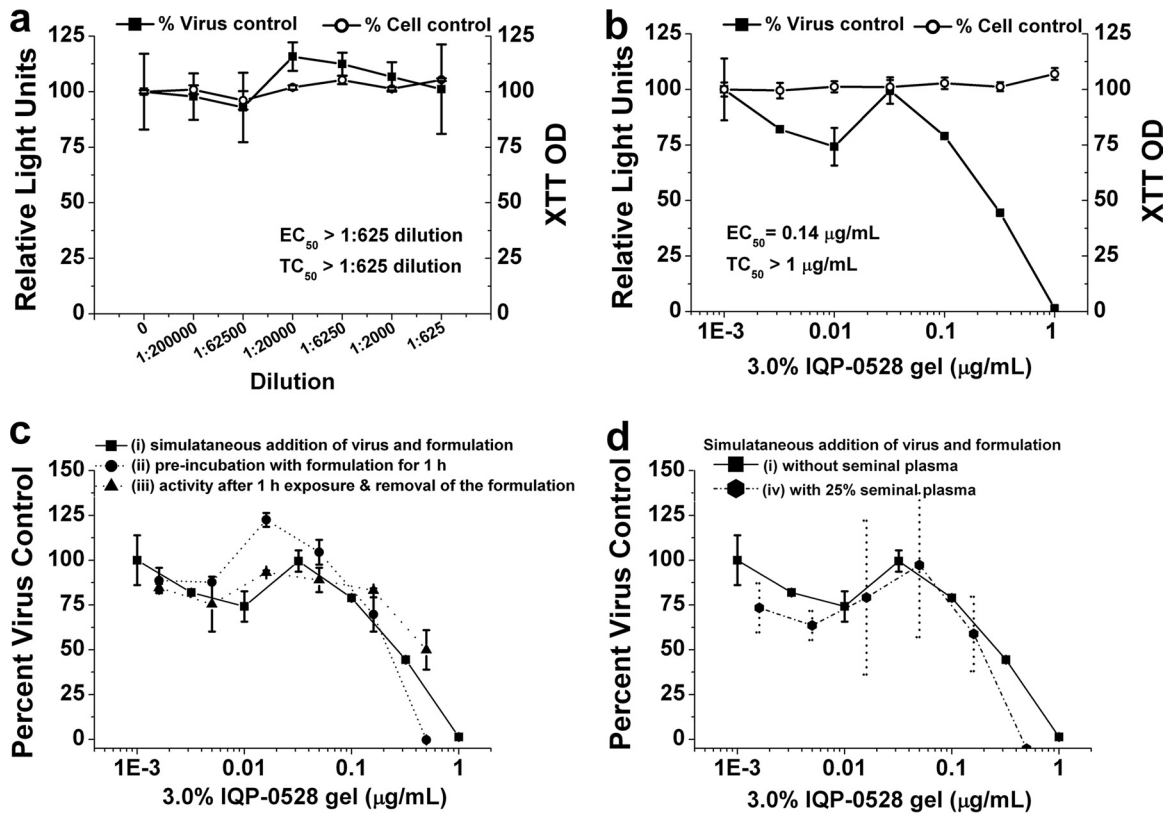


FIG. 7. Assessment of the antiviral activity of the 3.0% HEC gel formulation without IQP-0528 (placebo) (a) or with 0.25% IQP-0528 (b). MAGI cells were incubated with the gel formulation and virus for 2 h. Inhibitory effect was quantified after 48 h of additional incubation using chemiluminescence detection with Gal-Screen. The placebo gel formulation served as the negative control ($n = 3$, means \pm the SD). (c) The antiviral activity of the 3.0% HEC gel with 0.25% IQP-0528 when (i) the formulation was added to cells for 15 min followed by addition of virus and incubation for an additional 2 h, (ii) the formulation was preincubated with cells for 1 h followed by the addition of virus and incubation for an additional 2 h, and (iii) the formulation was added to cells for 1 h and then removed by washing, followed by addition of virus and incubation for an additional 2 h. (d) Comparative antiviral activity of the formulations in the presence of 25% seminal fluid.

mixing a 1:5 dilution of the formulation with HIV-1_{BaL} and applying this mixture to the apical surface of the polarized explants. After an overnight exposure, the explants were washed, and the cultures were observed for 21 days. Unformulated IQP-0528 did not confer any protection in the polarized ectocervical tissue model (data not shown). However, when formulated in a 3.0% HEC gel, IQP-0528 conferred protection

against HIV infection as indicated by a >1 log reduction in HIV-1 p24 antigen levels by day 21 (Fig. 8c). This significant reduction in HIV-1 p24 ($P = 0.007$, Mann-Whitney test) after treatment with the gel formulation emphasizes the role of adequate formulation in enhancing the efficacy of water-insoluble and poorly permeable active pharmaceutical ingredients. The lack of HIV-1 infection in these samples was confirmed by

TABLE 1. Inhibitory activity of IQP-0528 alone and in a 3.0% HEC gel formulation

Assay conditions	Inhibition of HIV-1 _{IIIb}			
	Simultaneous addition of virus and test substance ^a		Preincubation with test substance prior to virus addition ^b	Test substance removed prior to virus addition ^c
	Without seminal fluid	With 25% seminal fluid		
IQP-0528 (API alone) (EC_{50} [$\mu\text{g/ml}$])	<0.0001	0.05	0.05	>0.17
IQP-0528 HEC gel formulation (EC_{50} [$\mu\text{g/ml}$])/ IQP-0528 when formulated (EC_{50} [$\mu\text{g/ml}$]) ^d	0.14/0.0003	0.21/0.0005	0.26/0.0006	0.5/0.0012
HEC placebo (dilution)	>1:625	>1:625	>1:625	>1:625

^a The test substance (API or gel formulation) was added to cells for 15 min before the virus was added. Cells were incubated with virus and the test substance for 2 h. After the incubation, the monolayer was washed, and the activity was assayed after an additional 48 h of incubation by measuring the β -galactosidase activity.
^b The test substance was preincubated with cells for 1 h before the virus was added. Activity was quantified as described in footnote a.
^c The test substance was added to cells for 1 h and then removed by washing. Virus was added, followed by incubation for 2 h. Activity was quantified as described in footnote a.
^d The EC_{50} for IQP-0528 was back-calculated from the EC_{50} for the complete gel formulation, which consists of 0.25% IQP-0528.

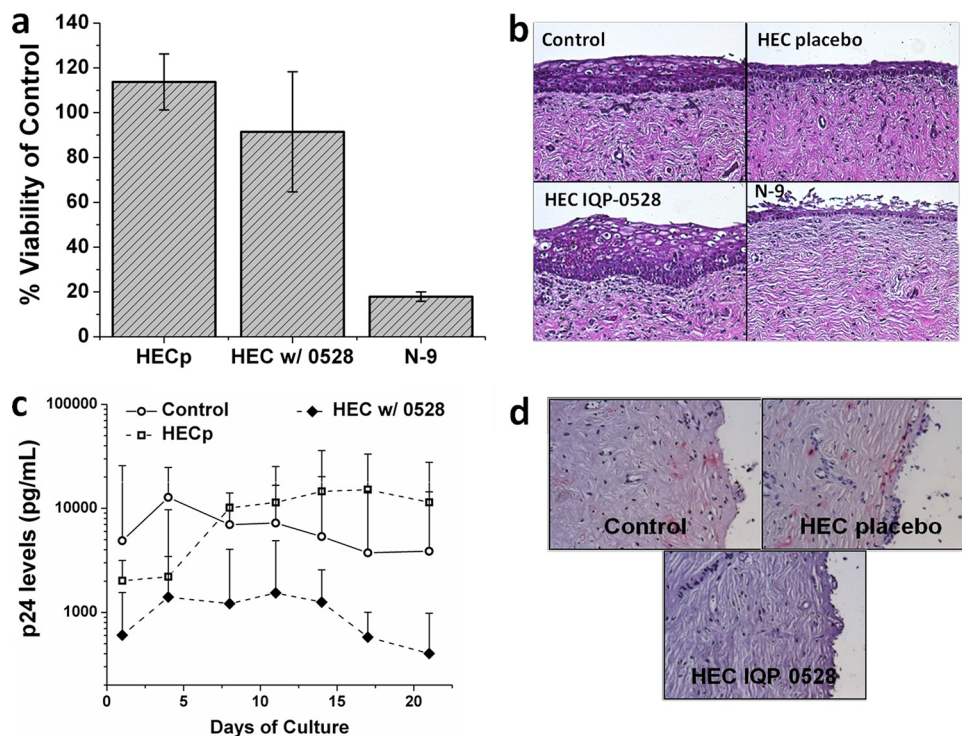


FIG. 8. Evaluation of the safety and efficacy of the 3.0% HEC gel with 0.25% IQP-0528 and placebo gel using ectocervical explant cultures. (a) The safety of the gels was evaluated in duplicate in polarized explants treated with the gels or in untreated controls. After overnight culture, the explants were washed, and one of the duplicates was processed for the MTT assay, and the other was processed for histology. The MTT assay data are based on three independent tissue samples, and the histology image (b) is representative of one of those samples. Tissues were harvested after overnight exposure to the gel. (c) To evaluate the effectiveness of the gel, polarized ectocervical explants were treated with the gels, along with HIV-1. After overnight culture, the explants were washed and then observed for 21 days. Culture medium on the basolateral side was harvested to test for viral replication by p24 gag ELISA. The HEC gel with IQP-0528 showed a significant decrease in p24 levels compared to the placebo gel and the untreated control. (d) Tissues were exposed to the formulation overnight, followed by washing and 21 days of incubation. Postincubation the tissues were stained with p24 monoclonal antibody. Representative tissue samples are shown. The red cells in the untreated control and the tissues treated with placebo gels indicate p24-positive cells, reflecting the presence of HIV-infected cells. No HIV-infected cells were found in the 3.0% HEC IQP-0528 formulation-treated tissue samples ($n = 3$).

endpoint IHC of the explants; the control and HEC placebo-treated explants harbored HIV-1-infected cells, whereas no infected cells were detected in the HEC IQP-0528-treated explants (Fig. 8d).

DISCUSSION

The field of rectal and vaginal microbicides is a new therapeutic area with several candidates in clinical trials and no registered products. Many of these candidates, with the exception of a 1% tenofovir gel evaluated in the recent CAPRISA 004 trial (1), have failed to demonstrate safety or efficacy. Despite these setbacks, the field is still progressing rapidly due to the pressing need for prophylactic technologies and the advantages of vaginal route of delivery for antiretroviral drugs (9, 16). Typically, drugs delivered through the vaginal route encounter an acidic environment with pH ranging from 4.0 to 5.0 (37, 40). However, soon after intercourse, the vaginal pH increases to a neutral pH due to the introduction of semen. The vaginal environment also hosts several *Lactobacillus* strains that constantly generate lactic acid and hydrogen peroxide, maintaining an acidic pH in the vaginal cavity (37, 40). Therefore, successful development of a vaginal microbicide

product requires an active ingredient that can withstand both neutral and acidic pH in the presence of hydrogen peroxide. For this reason, we chose to evaluate the stability of our drug candidates at pH 4.2, pH 4.2 with 0.1% H_2O_2 , and pH 7.0. The results from our stability study on the nine active PYD congeners suggested that substitutions at the N-1 site play a critical role in determining the stability of the compounds in solution. Cyclopropyl (IQP-0406, -0407, -0408, and -0528) and cyclobutyl (IQP-0530 and -0532) substitutions demonstrated better stability compared to the 1- or 3-cyclopenten-1-yl substitutions (IQP-0558, -0410, and -1187) at the N-1 site. Furthermore, isopropyl substitutions in the heterocyclic ring (IQP-0528 and -1187) resulted in compounds with greater stability compared to drugs with ethyl substitutions (IQP-0408 and -0410). As seen in Fig. 1, the most potent PYD congeners failed to show the desired stability profile and thus were eliminated from consideration for formulation development. The combination of the antiviral activity, the therapeutic index and, most importantly, the stability of the molecules under the physiologically relevant conditions tested (shown in Fig. 1) made IQP-0528 a promising candidate to be explored further down the formulation development pipeline.

In addition to the stability and activity of the antiviral agent,

the effectiveness of a vaginally applied microbicide gel also depends on the ability of the gel to coat the vaginal epithelium and be retained at the target site for the desired duration of action (25). Formulations with high viscosity and yield stress would most likely result in gels that provide poor coating and, consequently, suboptimal release of the active ingredient, since flux is proportional to contact surface area. On the other hand, low-viscosity gels often lead to excessive coating and leakage, resulting in poor user compliance and partial dose delivery. We have previously optimized semisolid vaginal gel compositions based on a detailed exploration of the composition, properties, and performance of vaginal gels (23). Our results indicated the 3.0% HEC gel, followed by the 0.65% Carbopol gel, as the near-ideal microbicide gel candidates at a 3.5-ml volume of administration because of their spreadability and coating properties (23).

To design a sustained-release formulation for vaginal microbicide delivery, a thorough understanding of the vaginal anatomy and the barrier properties of the vaginal epithelium is imperative (18, 29). Transport across the vaginal epithelium is largely driven by diffusion, which is governed by the physicochemical properties of the active ingredient and the barrier through which the drug diffuses. The drug entrapped in the gel network diffuses to the boundary layer and partitions into the lipid and protein domains of the vaginal epithelium. The drug diffuses through the lipid and protein domains to repartition into the microcirculation provided by the local capillary network, which offers an infinite sink. This repeated diffusion-partition process drives the diffusion of the drug into and out of the vaginal tissue (29). The rate at which each of these steps occurs depends on several factors, such as the partition coefficient (Log P), solubility (melting point), polarity (hydrogen bond-forming functional groups), degree of ionization (pK_a/pK_b), and molecular weight of the drug. IQP-0528 has a molecular weight of 340.42 g/mol and is practically insoluble in water, with a calculated Log P of 4.1. Of all of these factors, the one with perhaps the greatest influence on drug transport is the ability of the drug to partition into host tissues (40). Typically, drugs with Log P values in the range of 2 to 3 show optimal permeability across the stratum corneum (SC), as well as moderate partitioning out of the SC. On the contrary, drugs with a Log P of >3 are expected to exhibit high partitioning into the SC but poor partitioning into the systemic circulation (22). It is known that lipophilic compounds have higher tissue residence times and therefore lower systemic exposure (10). The lipophilicity of IQP-0528 could similarly limit its systemic exposure when administered vaginally and therefore could be advantageous for a prophylactic antiretroviral strategy where there is concern over generating resistant viral mutants in infected individuals exposed to the gel (39).

In vitro release studies are an important precursor to *ex vivo* or *in vivo* studies as they permit the determination of release kinetics and serve to evaluate the mechanism of drug release (13). *In vitro* release studies on gels typically use synthetic membranes chosen to provide minimal resistance to drug diffusion (13). Flux in the three sink solutions in our study varied dramatically. We believe that the high flux in 1:1 IPA-PBS solution can be attributed to the observation that IPA causes the polymer in the gel to swell, resulting in rapid depletion of drug from the polymer matrix. Ideally, release studies should

be conducted under sink conditions that do not interfere with or swell the release matrix (13); therefore, liposomes and Solurol offered suitable sink conditions.

Porcine vaginal tissue is a convenient alternative to human vaginal tissue (29, 36) since human vaginal tissue is difficult to obtain in sufficient quantities to conduct drug transport studies with multiple replicates. There is significant similarity between the architecture of human and porcine vaginal tissue and, for studies investigating drug-release kinetics, excised porcine tissues have provided good correlation with human tissue studies. This is because the permeability of the SC has been shown to be unaltered in a nonviable tissue sample after removal from the body (29). IQP-0528 could not be detected in the receptor fluid from the *ex vivo* release studies using HPLC. However, the receptor fluid showed complete inhibition of viral replication and therefore inhibitory concentrations of drug. The concentration of IQP-0528 permeated across the tissue was estimated by comparing the extent of viral inhibition by the receptor fluid with a standard viral inhibition curve for IQP-0528. Using the method described above for the HEC formulation, we estimate that approximately 0.005 and 0.01 μM concentrations of IQP-0528 permeated the ectocervical tissue after 4 and 6 h, respectively. For the Carbopol formulation, we estimate approximately 0.003 μM IQP-0528 permeated the ectocervical tissue after 6 h. After comparison of the drug release profiles of IQP-0528 from the HEC and the Carbopol gels, we observed that the HEC gel demonstrated a shorter lag time (4 h) and greater permeation across the tissue. However, for the IQP-0528 HEC formulation, the lag time, which is the time needed to attain inhibitory concentrations of drug in the basal compartment, was eight times longer than that of the 1% tenofovir gel (lag time < 30 min) (26). This delay can be attributed to the low permeability of IQP-0528.

Thorough investigation of the toxicity and irritation potential of microbicide formulations is critical, since in the past some microbicides that have caused irritation to genital tissue also led to higher infection rates in women compared to placebos (2, 6). VEC-100 tissue treated with an IQP-0528 HEC gel formulation showed no significant induction of cytokines or chemokines (Student *t* test, $P > 0.05$), suggesting the absence of any significant inflammatory response. Triton X-100 (a 1% solution), which was used as the toxic control, induced a significant increase in the release of IL-1 α , IL-6, and IL-8 and a decrease in the levels of TNF- α (Fig. 6). Similar results have been reported previously (2). To further support these *in vitro* results, we evaluated the safety and efficacy of the 3.0% HEC gel on ectocervical tissue explants. The 3.0% HEC gel was chosen over the 0.65% Carbopol gel as the lead formulation based on our previous study that optimized gels based on their coating and retention properties (23) and higher release of IQP-0528 from the HEC gel. Tissues exposed to the IQP-0528 HEC formulation showed no significant loss in viability or change in tissue morphology. A similar study evaluating the safety of a 1% tenofovir gel showed an initial loss in TEER of the tissue, likely due to the hyperosmolarity of the formulation ($\sim 3,000$ mOsm) (26). The formulation developed in our study was designed to have an osmolarity of $< 1,000$ mOsm (~ 850 mOsm). As a result, although the composition of the 3.0% HEC IQP-0528 gel was similar to that of the tenofovir gel, the IQP-0528 formulation showed no signs of loss in tissue viability

or barrier functions after 4 h of exposure. We have observed that IQP-0528, when unformulated, does not show any protection in a polarized ectocervical tissue model. However, when formulated in a semisolid gel formulation as described here, IQP-0528 showed complete protection of the tissue against HIV infection. This emphasizes the role of formulation in enhancing the efficacy of APIs with poor aqueous solubility and low tissue permeability.

The formulation developed in the present study is composed of GRAS polymers HEC and Carbopol for the delivery of IQP-0528. These polymers are acceptable drug delivery vehicles, because of their cost-effectiveness, bioadhesivity, and biocompatibility. The estimated cost of a single dose of this gel is 30¢/dose, similar to the 1% tenofovir gel (26a). Based on our *in vitro* and *ex vivo* evaluations, we conclude that our gels provide diffusion-controlled release of the active ingredient. In addition, this formulation is expected to provide complete protection against infection with no significant toxicity or irritation to vaginal tissue. Our findings indicate that the formulation developed here provides a simple, inexpensive, stable, and efficacious microbicide gel for the prevention of heterosexual transmission of HIV-1.

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