Safety and pharmacokinetics of quick dissolving polymeric vaginal films delivering the antiretroviral IQP-0528 for pre-exposure prophylaxis

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Running title: Quick dissolving vaginal IQP-0528 films in macaques

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For HIV prevention, microbicides or drugs delivered as quick-dissolving films may be more acceptable to women than gels because of their compact size, minimal waste, lack of an applicator, and easier storage and transport. This has the potential to improve adherence to promising products for pre-exposure prophylaxis. Vaginal films containing IQP-0528, a non-nucleoside reverse transcriptase inhibitor, were evaluated for their pharmacokinetics in pigtailed macaques. Polymeric films (22x44x0.1mm-75% of a human dose) containing IQP-0528 (1.5% w/w) with and without poly(lactic-co-glycolic acid) nanoparticle encapsulation were inserted vaginally into pigtailed macaques in a crossover study design (n=6). With unencapsulated drug, the median (range) vaginal fluid concentrations of IQP-0528 were, 160.97 (2.73-2,104), 181.79 (1.86-15,800), and 484.50 (8.26-4,045) µg/mL at 1, 4 and 24 hours post film applications, respectively. Median vaginal tissue IQP-0528 concentrations at 24 hours were 3.10 (0.03-222.58) µg/g. Values were similar proximal, medial and distal to the cervix. The IQP-0528 nanoparticle formulated films delivered similar IQP-0528 levels in vaginal tissue and secretions as the unencapsulated formulation. A single application of either formulation did not disturb the vaginal microflora or the pH (7.24±0.84). The high mucosal IQP-0528 levels delivered by both vaginal film formulations were between 1 and 5 logs higher than the in vitro IC$_{90}$ of 0.146 µg/mL. The excellent coverage and high mucosal levels of IQP-0528, well above the IC$_{90}$, suggest that the films may be protective, and warrant further evaluation in a vaginal repeat low-dose SHIV transmission study in macaques and clinically in women.
Introduction

In the absence of an effective vaccine, pre-exposure prophylaxis (PrEP) with antiretroviral (ARV) drugs is currently a very promising biomedical intervention to control the HIV epidemic (1, 2). PrEP involves the use of ARV by high risk HIV-negative individuals to prevent HIV acquisition. Many ARVs that have traditionally been used for HIV treatment have advanced as PrEP agents owing to their safety and potency profiles (1, 3-5). Of the available ARVs, tenofovir disoproxil fumarate (TDF), a nucleoside reverse transcriptase inhibitor used widely in HIV treatment, has been studied extensively in animals and humans as a PrEP agent (3, 5). The success with oral TDF and Truvada®, TDF in combination with emtricitabine (FTC), in inhibiting HIV infection in nonhuman primates led to the clinical trials in humans with TDF and Truvada® (1, 6-11). In stark contrast to the success of TDF and Truvada® in clinical trials in men who have sex with men and heterosexual discordant couples, two large clinical trials in women with oral Truvada®, VOICE and FEM-PrEP, have demonstrated no protection owing to a lack of adherence (12, 13).

Providing optimal dosage forms of potent HIV PrEP agents for women has the potential to improve product adherence (14, 15). Topical dosage forms can be effective in reducing HIV infection, as demonstrated by the CAPRISA 004 vaginal 1% tenofovir (TFV) gel trial where per-protocol use of the gel before and after sex with no more than 2 doses in 24 hours (BAT-24) reduced HIV acquisition by 39% (2). As observed in the oral PrEP trials, a greater efficacy was reported among the high adherers in CAPRISA 004 (54% vs. 28% in the low adherers) (2). Subsequent trials with 1% TFV gel such as FACTS 001 employing the BAT-24 regimen used in CAPRISA-004, and the VOICE trial with a daily application of 1% TFV gel, were not effective in protecting women against HIV acquisition (12, 16). It was estimated based on the returned used applicators that only 13% of the FACTS 001 trial participants were able to use...
the gel in ≥ 80% of sex acts per month (16). Neither the non-coital daily application of the TFV gel with VOICE nor the pericoital vaginal dosing regimen with FACTS 001 demonstrated effectiveness in comparison to the placebo group against HIV acquisition (12, 16). The above studies indicate the need for highly efficacious ARVs and delivery platforms that would improve and enhance user compliance, particularly in women.

Women-initiated HIV prevention strategies are limited and negotiation of safe-sex practices by women is not readily acceptable in many high HIV-incidence settings (17). As with hormonal contraception, regional differences in what is acceptable can vary widely, and women may prefer to choose between multiple dosage forms based on their socioeconomic status and individual preferences (18, 19). Multiple topical PrEP delivery platforms such as gels, intravaginal rings (IVRs), films, vaginal inserts, and soft-gel capsules are currently being developed (20-24). A product that is destined for topical use should be safe, widely acceptable to promote and enhance adherence, cost effective, and deliver high mucosal concentrations of the drug to prevent HIV infection. Microbicide delivery through solid dosage forms such as quick-dissolving vaginal films may be more acceptable to women than gels because of their small size, lack of a need for an applicator, easier storage and transport, and increased product stability.

Vaginal films are polymeric thin solid dosage forms which when applied mucosally undergo hydrolysis to release the active pharmaceutical ingredient (API) incorporated in its matrix. A consumer product preference study conducted among 526 sexually active women in Burkina Faso, Tanzania, and Zambia reported that vaginal films and soft-gels were preferred over vaginal tablets owing to the ease of insertion and faster dissolving time (15). Other promising attributes such as no leakage, lack of feeling the product inside, likelihood to use the product in the future, and acceptability among their male partners were also described for the
films (15). A vaginal contraceptive film (VCF) containing the spermicide nonoxynol-9 was the first commercially available film (25). Other feminine hygiene products including a VCF lubricating film and a vaginal cleansing film are also marketed. Vaginal films to treat bacterial vaginosis and vaginal candidiasis have also been developed (26, 27). A number of promising ARVs and/or microbicides such as dapivirine, TFV, dapivirine in combination with TFV and maraviroc, retrocyclin-101, and cellulose acetate phthalate, have been formulated into films (28-34). A few studies have evaluated the safety, pharmacokinetics (PK) and pharmacodynamics of films in macaques and humans (29, 35).

Films are limited by the amount of API that can be incorporated into them and the API generally does not exceed 50% of the of the product’s dry weight (36, 37). Therefore, the API in films needs to be very potent to overcome this limitation. Pyrimidinediones, an important class of non-nucleoside reverse transcriptase inhibitor (NNRTI), are potent topical PrEP candidates (38, 39). Of the available analogs, IQP-0528 [1-(3-Cyclopropyl)methyl-6-(3,5-dimethylbenzoyl)-5-isopropyl-2,4(1H,3H)-pyrimidinedione], has been formulated for topical PrEP owing to its desirable characteristics such as subnanomolar NNRTI activity, high therapeutic index, chemical stability, and ability to synthesize from readily available precursors thus lowering production costs (40-44). IQP-0528 acts by binding to a hydrophobic pocket of the reverse transcriptase enzyme and a high mucosal concentration is required to prevent local HIV-1 replication. IVRs delivered IQP-0528 to macaque mucosal fluid and tissue at concentrations that were several logs greater than the in vitro IC₅₀ (40). The half maximal effective dose concentration (ED₅₀) of IQP-0528 at 3 nM is 700-fold lower than that of TFV (2000 nM) (42). Unlike TDF, explant tissue pretreated with IQP-0528 provided significant protection to co-cultured T cells against HIV, indicating the ability of IQP-0528 and not TDF to protect newly recruited immune cells (45). The above features of high potency and the ability...
of IQP-0528 to move in and out of cells favor its formulation into polymeric films. In efforts to
broaden the microbicide pipeline and to provide women with as many HIV preventative options
as possible, IQP-0528 has been developed into a vaginal film. Initial formulations
demonstrated its \textit{in vitro} ability to effectively and rapidly deliver IQP-0528 into vaginal tissue
and target cells. The physicochemical properties, safety evaluation in organotypic \textit{in vitro}
tissue models and anti-HIV activity of vaginal films containing IQP-0528 have demonstrated its
potential as an effective microbicide (46). Additionally, by integrating IQP-0528 into
dissolvable nanoparticles, the films offer the potential to confer long-term drug delivery (47).

Nonhuman primates, in particular pigtailed macaques, have been pivotal in evaluating
the PK, safety and efficacy of a number of gel and IVR based microbicide candidates and
informing clinical trial designs due to their great similarity to women in vaginal architecture,
menstrual cycling, and microfloral composition (48-57). Here we further evaluate IQP-0528
released from a quick-dissolving film with and without nanoparticle formulation and report \textit{in vivo}
safety and bioavailability in pigtailed macaques in a multi-day PK with repeated
applications of the film.

\textbf{Materials and Methods}

We previously reported the development and optimization of a polymeric quick-dissolving,
stable, non-toxic film as a solid dosage form for the vaginal delivery of IQP-0528 with high
efficacy \textit{in vitro} against HIV (46). These films contained 0.1\% (w/w) of IQP-0528 and dissolved
at pH 7.

\textbf{IQP-0528 Nanoparticle formulation: Controlled release nanoparticle drug delivery}

Nanoparticles containing the NNRTI IQP-0528 were encapsulated into a combination PLGA:
Eudragit S-100 formulation via a double emulsion process (58). Nanoparticles with Eudragit S-
100 were incorporated 1:1 to MW 5000-20000 Da mPEG-PLGA formulation. The nanoparticles were 434 ± 46 nm in diameter with a zeta potential of -3.26 mV (47).

**Quick-dissolving vaginal films containing IQP-0528**: Vaginal films containing the IQP-0528 nanoparticles (IQP-0528NP) and vaginal films containing unencapsulated IQP-0528 (IQP-0528) were manufactured through solvent casting and evaporation method as previously described (34, 46). Briefly, the polymeric film excipients were mixed in a water solution followed by the addition of 1.5% (film w/w) of IQP-0528 or the equivalent drug concentration of IQP-0528 nanoparticles. The polymeric solution was cast into thin sheets on an Elcometer 4340 Automatic Film Applicator (Elcometer, Rochester Hills, MI) to evaporate the solvents, resulting in the final quick-dissolving polymeric film. The physicochemical properties of the films were determined as previously described with the following characteristics: Area = 1200 mm²; mass = 235 ± 4 mg; opaque white appearance that is smooth and pliable; thickness = 0.22 ± 0.01 mm; visual disintegration time = 8.6 ± 1.5 minutes; water content = 4.635 ± 0.049%; tensile strength = 9.675 ± 0.330 N; puncture strength = 6.710 ± 0.454 N; dissolves at pH 7 (34). The dissolution of the films are pH independent and controlled by the vaginal fluid volumes. The IQP-0528 content of the films was determined by HPLC stability indicating analytical methods previously developed (41). Both the nanoparticle (IQP-0528NP) and the unencapsulated (IQP-0528) films contained a measured IQP-0528 drug content of 1.50 ± 0.08% (w/w). The increase in drug loading from previous published products did not result in any negative effects in stability or performance (*data not shown*) (46). For the non-human primate studies, the human-sized vaginal films were scaled down to proportionally to the size of the primate vagina: a reduction of 25% of the film surface area (22x44x0.1mm, surface area 75% of a human dose).
**In vitro release of IQP-0528 from vaginal films:** The *in vitro* release of IQP-0528 (1.5% w/w) from the quick-dissolving films and the nanoparticle encapsulated (1.5% w/w drug equivalent) quick-dissolving films were evaluated in a class USP apparatus (SOTAX CP7) as previously detailed (34, 41). The flow rate was set to 15 mL/min at a temperature of 37°C. A 10% acetonitrile in Dulbecco’s phosphate-buffered saline solution (pH =7.0) was the dissolution medium. IQP-0528 content was determined by HPLC (41). Release of IQP-0528 from the film was measured to be 90.25 ± 2.32% by 30 minutes with complete drug recovery by 1 hour. Conversely, the *in vitro* release of the IQP-0528 from the nanoparticles resulted in a burst release of 37.72 ± 8.45% available drug at 10 hours and 51.65 ± 7.22% available drug at 24 hours.

**Nonhuman primate studies:** All macaques were housed at the Centers for Disease Control and Prevention according to the *Guide for the Care and Use of Laboratory Animals* (59). All the study procedures were approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee. The sample collection and the timelines are listed below and employed published protocols (60, 61).

**24 hour PKs:** Six sexually mature female pigtailed macaques (*Macaca nemestrina*) were enrolled in a two arm cross-over PK (IQP-0528NP and IQP-0528 film formulations) with a two week washout in between the cross-overs. The cross-over PK was repeated two months later to obtain a greater sample number for the study. Three pigtailed macaques received the IQP-0528NP film (1.5% w/w); the other three pigtailed macaques received the IQP-0528 film (1.5% w/w). The vaginal films were inserted at time 0 in the upper half of the posterior vagina (half way between the cervix and introitus). The vaginal film disintegration was visually inspected at one hour after film application. Visual examination for abrasions or inflammation was also performed. Vaginal secretions were obtained proximal (ectocervix) and distal (introitus) to the...
cervix at 0, 1 (Weck-Cel spears, Beaver Visitec), 4, and 24 hours (eight Ultracell surgical sponges, 3.5 x 4 mm). Three vaginal pinch (proximal, medial and distal to the cervix, 3 mm) and two rectal biopsies were collected at 24 hours with Miltex Townsend #30-1445 biopsy forceps. Variations of the vaginal pH were monitored from vaginal secretions obtained at 0, 1, 4, and 24 hours by rolling a Dacron swab with the secretion onto a pH colorimetric indicator strip (Millipore Billerica, MA, USA). The vaginal pH of animals that were undergoing menstruation were not readable owing to the bleeding and exudation. A second swab sample was collected to evaluate changes in vaginal microflora at baseline (time 0) and 24 hours post film application (one cross-over PK only). These were placed individually in Port-a-Cul (Becton Dickinson, Franklin Lakes, NJ) tubes and transported to Magee Women’s Research Institute (Pittsburgh, PA) within 24 hours of collection for microbial analysis. The swabs were characterized for the presence of aerobic and anaerobic bacteria by semi-quantitative culture as described previously (40, 60). Hydrogen peroxide production by lactobacilli and viridans streptococci were tested qualitatively on tetramethylbenzidene agar plates (62). Based on the absence of significant differences in the vaginal microflora of pigtailed macaques following the presence of IQP-0528 vaginal rings for 28 days, it was demonstrated that IQP-0528 was not detrimental to the native vaginal microflora even when present for a long time (40). Hence in this study acute changes in vaginal microbial milieu were only monitored for the 24 hour PKs.

**Repeated- Dose PK:** The six macaques utilized for the 24 hour PKs were enrolled in a multi-day PK with repeated applications of the film in a 23 day period following a two month washout in between the 24 hour and the repeated-dose PK. As above, three pigtailed macaques received the IQP-0528NP film; the other three animals received the IQP-0528 film on day 0. The sample collection was identical to the 24 hour PKs. Repeated application of the film was carried out on days 7, 10, 14, 17, and 20 to determine the duration of IQP-0528 delivery.
Samples were obtained on days 7, 14, and 23 to quantitate the bioavailability of IQP-0528 seven, four, and three days following the last film application respectively. The sample collection schedule is listed in Table 1. All samples on days 7 and 14 were collected prior to new film insertion. We monitored for mucosal inflammation by the measurement of cytokines in vaginal secretions obtained with Weck-Cel spears (0, 1 hour) and Ultracell surgical sponges (4 hours, days 7, 14, and 23) using a Milliplex™ MAP (Millipore, Billerica, MA, USA) fluorescent multiplexed bead-based assay as previously described (40, 61).

The 24 hour data from the two cross-over PKs (n=12 for each arm, IQP-0528NP film and IQP-0528 film) was combined with the data obtained in the first 24 hours from the repeated-dose PK (n=3 for each arm, IQP-0528NP film and IQP-0528 film) to obtain an n of 15.

Quantitation of pyrimidinedione in plasma, tissue, vaginal lavages and secretions

IQP-0528 was quantitated in plasma, vaginal fluid collected at 0, 1, 4, 24 hours, days 7, 14, and 23, and vaginal and rectal biopsies by LC-MS/MS as described elsewhere (40, 63). Briefly each sample was extracted with 0.2% acetonitrile and analyzed using a Phenomenex phenyl hexyl column with a 0.2% formic acid/acetonitrile mobile phase. The linear gradient of this assay was 20%-98%. IQP-0528 was determined to be stable in freeze thawed plasma and vaginal fluid samples. The lower limit of quantification (LLOQ) was determined to be 5 ng/sample of matrix for tissue and vaginal fluid obtained with spears. The LLOQ for vaginal fluid obtained with the Ultracell surgical sponges was 25 ng/sample of matrix after extraction and values are corrected for the volume collected on the sponge. The LLOQ was 5 ng/mL for plasma. The vaginal fluid and tissue densities were assumed to be 1.0 g/mL to convert weight/weight concentrations of IQP-0528 (nanogram IQP-0528 per gram of vaginal fluid or tissue) to molarity (µM).
Data Analysis

The changes in cytokines and chemokines of the IQP-0528NP and the IQP-0528 film PK macaques (repeated-dose PK) were monitored by Friedman tests of the log-transformed values. These were followed by Wilcoxon signed-rank test with false discovery rate (FDR) adjusted p-values for post-hoc pairwise comparisons between each of 5 time points (1, 4 hours, days 7, 14 and 23) and 0 hours (57). Wilcoxon matched-pairs signed-rank test was used to compare the proximal and distal drug measurements between the IQP-0528NP and the IQP-0528 film groups and within groups as well.

Results

Safety

We analyzed the in vivo safety of the IQP-0528NP and IQP-0528 film formulation through visual examination, vaginal microflora, pH, and cytokine and chemokine measurements. Upon routine visual examinations, while obtaining mucosal samples, no vaginal abnormalities such as tissue abrasion or inflammation were noted with either film application in the 24 hour PKs or following repeated-dosing and at all time points throughout the study including day 23. To evaluate if the IQP-0528 film formulations triggered detrimental changes to the normal vaginal microflora, vaginal fluid at baseline (time 0) and 24 hours post film application was characterized for changes in the occurrence of aerobic and anaerobic microorganisms. The prevalence of normal aerobic microorganisms such as H$_2$O$_2$-producing, and non- H$_2$O$_2$-producing lactobacilli and Viridans streptococci, diphtheroids, and anaerobic species such as anaerobic gram negative rods (black and non-pigmented), was found to be generally stable with sporadic variations 24 hours post-application of either film (Fig 1). A
similar stable distribution was seen among 16 other vaginal microbial species (data not shown). Transient and sporadic variations were noted for some species such as \( \text{H}_2\text{O}_2^- \) producing *Viridans streptococci* and the same has been observed in pigtailed macaques previously (40, 64). The prevalence of the \( \text{H}_2\text{O}_2^- \)-producing, and non- \( \text{H}_2\text{O}_2^- \)-producing lactobacilli and *Viridans streptococci*, diptheriods, anaerobic gram negative rods (black and non-pigmented) species was found to be similar while comparing the IQP-0528NP and the IQP-0528 film groups at 24 hours (data not shown).

The vaginal pH was monitored at baseline, 1, 4 and 24 hours after film application. Neither film produced a significant change in the pH at any of the time points tested (Fig 2). All macaques maintained their pH within the range that we have typically seen in pigtailed macaques of 4.5 - 8 (61). The vaginal pH was monitored at baseline, 1, 4 and 24 hours after film application. The mean pH and standard deviation at baseline for all of the animals was 7.36 ± 0.91 (IQP-0528NP film) and 7.20 ± 0.70 (IQP-0528 film). Friedman’s test found no significant change in the pH between post product application (1, 4, 24 hours) and 0 hours for the IQP-0528NP film and the IQP-0528 film (p=0.0923 and p=0.4753 respectively, Fig 2).

Though 20% of the animals showed a 1-2 log change in pH from baseline to 24 hours post application, it was not statistically significant (Wilcoxon matched-pairs signed rank test, p=0.0585). The pH range noted in these macaques over the course of the study is normally seen in pigtailed macaques (48, 61).

The induction of mucosal inflammation, if any, due to the presence of the IQP-0528NP or IQP-0528 film was monitored by analysis of 18 cytokine and chemokine measurements from vaginal fluid samples obtained from the macaques at 0, 1, and 4 hours, and at days 7, 14...
and 23. Friedman tests found differences over time in the IQP-0528NP film group in levels of MIP-1β, and IL-18 and in the IQP-0528 film macaques in G-CSF, IL-15, IL-1Ra, and IL-13 (supplementary tables S1 and S2). However, when the analysis included Wilcoxon signed-rank test confirmed with false discovery rate (FDR) adjusted p-values for post-hoc pairwise comparisons between each of 5 time points (1, 4 hours, days 7, 14 and 23) and 0 hours, these changes were found to arise due to differences between time points (1, 4 hours, days 7, 14, and 23) suggesting these changes were not due to delivery device use as shown previously (57). No statistically significant differences were noted in any of the other cytokines or chemokines measured in this study including IL-1β which showed increased levels on days 7 and 14 in the IQP-0528NP film macaques.

Pharmacokinetic drug measurements

The film was found to be completely dissolved upon visual examination at 1 hour post film application indicating a disintegration time of less than 60 minutes for both the IQP-0528NP and IQP-0528 films. Tables 2 and 3 summarize the PK parameters of IQP-0528 among both groups across many mucosal compartments at various time points. Drug measurements were below the LLOQ (5 ng/mL) in all plasma samples tested. Drug was detected in all vaginal fluid samples obtained within the first 24 hours (Table 2, Figs 3a and 3b). Median vaginal fluid drug levels were similar among samples obtained proximal and distal to the cervix with the exception of the proximal samples obtained at 4 hours (IQP-0528 film group) and 24 hours (IQP-0528NP film group) showing a statistically significant increase (p≤0.05 and 0.001, respectively) in comparison to the distal samples. Table 3 shows that the maximum concentration (C_{max}) obtained proximal to the cervix was similar among the IQP-0528NP and the IQP-0528 film group macaques (1.06 x 10^4 and 1.09x 10^4 µg/mL,
respectively). The C\text{max} concentration in vaginal fluid samples obtained distally was higher in the IQP-0528 film than the IQP-0528NP group (1.58 \times 10^4 and 3.72 \times 10^3 \mu g/mL, respectively). A two-fold proximal and three-fold distal higher area under the concentration-time curve (AUC\text{0-24}) was noted for the IQP-0528 film when compared to the IQP-0528NP film. Vaginal fluid samples obtained on days 7, 14 and 23 days, to quantitate the bioavailability of IQP-0528 7, 4, and 3 days following the last film application, were highly variable with regards to drug detection (range 0.13 to 238.92 \mu g/mL) with only 25% above LLOQ with the IQP-0528NP films and 31% above LLOQ with IQP-0528 films (data not shown).

Median vaginal tissue concentrations of 1.08, 1.32 and 0.64 \mu g/g were detected in tissue obtained proximal, medial, and distal to the cervix, respectively, in the IQP-0528NP film group (Fig. 3C, Table 2). Proximal, medial, and distal IQP-0528 concentration in the IQP-0528 film macaques were 1.87, 3.82, and 3.09 \mu g/g, respectively. 14 vaginal tissue samples were found to be below LLOQ in the IQP-0528NP film group and 10 in the IQP-0528 film group. A statistically significant increase was only noted in the amount of IQP-0528 detected in vaginal tissue obtained proximal to the cervix in the IQP-0528 film group compared to the IQP-0528NP group (p=0.0494, Wilcoxon matched-pair signed rank test) though a trend towards an increased concentration of IQP-0528 was noted for samples obtained medial and distal to the cervix in the IQP-0528 film group. All vaginal tissue specimens obtained on day 23 (3 days after last film application) were below the LLOQ for IQP-0528 (data not shown). Drug levels were below LLOQ in all rectal tissue samples tested.

Discussion

This study demonstrates that quick dissolving IQP-0528 vaginal film formulations exhibit promising safety and pharmacokinetics. Using the well-defined pigtailed macaque model these
Films were found to exhibit preliminary safety and deliver high mucosal levels of IQP-0528, between 1 and 5 logs greater than the \textit{in vitro} IC$_{90}$ of 0.146 µg/mL as early as one hour post film application and even 24 hours later suggesting that these polymeric quick dissolving vaginal films may deliver IQP-0528 at concentrations sufficient to prevent HIV infection.

Films have traditionally been found to be safe and used in humans for oral and vaginal use. They are used to orally deliver pain medications, vitamins, and supplements (65-67). Microbicide drugs such as dapivirine, retrocyclin-101, cellulose acetate phthalate, and dapivirine in combination with TFV have been formulated into films for vaginal use, and VCF containing nonoxynol-9, a VCF lubricating film, and a vaginal cleansing film (Apothecus Pharmaceuticals) are all available commercially (25, 28-32). It is important to evaluate the safety of topical PrEP products as any breach to the integral mucosal barrier and associated inflammation promotes the recruitment of local CD4 T cells and facilitates HIV infection (68).

The PrEP product should not alter the innate vaginal microbial milieu as altered microbial states may increase the risk of HIV acquisition (69-71). Vaginal facultative and anaerobic bacteria remained stable throughout the study with both the IQP-0528NP and IQP-0528 film formulations (Fig 1). A lactobacilli dominant vaginal microbiome is indicative of a healthy vaginal microenvironment in women and protects against pathogenic microorganisms (72). Pigtailed macaques have a similar frequency of lactobacilli colonization in their vaginal tract (48). A single application of either film formulation did not disturb the vaginal lactobacilli or the pH (Fig 2). The presence of an IQP-0528 IVR for 28 days in pigtailed macaques did not cause a significant change in the vaginal microfloral composition (40). It was demonstrated that IQP-0528 was not detrimental to the native vaginal microflora even when present for a long time.

Therefore the vaginal microbial milieu in this study was only monitored at the time of insertion and 24 hours later. Mucosal cytokine and chemokine concentrations, in particular the pro-
inflammatory cytokines, remained stable throughout the study (Tables S1 and S2). Changes in a few cytokines were noticed; however, these were found to arise due to variability between time points by statistical analysis with FDR adjusted p-values. This observation is typical and has been reported previously in pigtailed macaques (57). Overall, the findings suggest that both film formulations of IQP-0528 are safe and do not perturb the vaginal environment.

The recently completed phase I trial in women with dapivirine film and gels demonstrated a reduction in the innate anti-HIV activity of cervicovaginal lavages (CVL) obtained from the gel and not the film users (35). It was reported that dilution, due to the greater volume used with gels than films, could likely account for the reduction in the innate anti-HIV activity. The reduction in innate anti-HIV activity was also observed three weeks after last gel application. Therefore, use of IQP-0528 films would likely not alter the innate anti-HIV activity in CVL of women.

IQP-0528, was chosen as the lead pyrimidinedione candidate for topical PrEP and has been formulated as a single entity or as a combination microbicide into IVRs, and gels (40-44). IQP-0528 formulated in films showed sub-nanomolar efficacy in CEM-SS and human PBMCs against HIV-1IIIb and clinical strains of HIV-1, respectively (46). These films may offer additional advantages over other dosage forms such as lower production costs owing to its small size. The solid dosage form may increase the drug stability by preventing its precipitation and degradation by hydrolysis or oxidation. Low residence time has been reported with traditional vaginal dosage forms such as suppositories, and liquid formulations, owing to the self-cleaning action of the vaginal tract (73, 74). The bioadhesive properties of films may increase the local retention time and improve clinical performance (65-67). Median vaginal
fluid concentrations of IQP-0528 obtained 60 minutes post film application were 3 logs greater than the *in vitro* IC$_{90}$ of 0.146 µg/mL (45) with a T$_{max}$ of 15,795.08 µg/mL at 4 hours. The median vaginal fluid level obtained 24 hours post application was 3.3 logs higher than the *in vitro* IC$_{90}$ (Fig 3A and 3B). The dynamic range in vaginal fluid concentrations of IQP-0528 might be a result of IQP-0528’s characteristic to move in and out of cells and the *in vivo* variability in the volume of vaginal secretions in macaques as the films, when applied to the mucosa, undergo hydrolysis to release the API. Though no studies to date have quantitated the cervicovaginal fluid volume in macaques, the median volume was estimated to be 0.51 mL in women with an interquartile range of 0.33 to 0.69 mL (75). The median vaginal tissue concentration obtained at 24 hours post film application was 21 times greater than the *in vitro* IC$_{90}$ of 0.146 µg/mL [Fig 3c]. High concentrations of IQP-0528 were detected in vaginal fluid and tissue samples obtained proximal, medial and distal to the cervix. This observation suggests that a uniform distribution of IQP-0528 is obtained in the vaginal cavity with these polymeric films. IQP-0528’s activity is not affected by the presence of semen (6.25% v/v) suggesting that the NNRTI would remain effective during or after coitus (42). Along with coitus, the uniform distribution could potentially minimize areas in the vaginal tract that are more vulnerable to HIV infection owing to a concentration gradient (68). The low clearance (CL) which signifies higher steady state concentrations of IQP-0528, and the low volume of distribution, Vd, the ratio between the dose given and the concentration in the vaginal fluid or tissue, indicates that a large amount of active drug is available at the site of action. We observed that plasma concentrations of IQP-0528 were below the analytical LLOQ, a unique advantage observed with topical PrEP as it could limit the emergence of drug resistance and systemic side effects. The rapid disintegration and the high mucosal fluid and tissue...
concentrations suggest that these films may provide a quick viable option against HIV-1 transmission.

Although vaginal films deliver inherently lower drug dosing levels compared to other delivery methods, the measurable drug concentrations are similar to that observed with gels and intravaginal rings. IQP-0528 delivered through IVR, gels and films have been evaluated in this model. The 14 w/w% IVRs provided a sustained release of 0.5 mg/day IQP-0528 over 28 days (40), whereas gels delivered 15 mg of IQP-0528 in a single application (76). In contrast, the quick dissolving vaginal films described here delivered only 450 µg IQP-0528 upon application. However, the median vaginal fluid IQP-0528 concentration obtained with the films at 4 hours (1.82 x 10^5 ng/mL) was similar to that obtained with IVRs (6.08 x 10^5 ng/mL at day 3) and gels (4.56 x 10^5 ng/mL at 4 hours). Likewise, the median vaginal tissue concentration was comparable among the films (3.10 µg/g), IVRs (2.97 µg/g) and gels (18.8 µg/g). The measurable drug concentrations obtained with the films illustrates that it is likely that the IQP-0528 film could deliver drug at concentrations similar to other delivery modalities such as IVRs and gels up to 24 hours post application.

Nanoparticles are an attractive and efficient mucosal drug delivery system that provide sustained and controlled release of a variety of biologically active agents like small molecules and proteins, with desirable features such as increased solubility, targeted delivery, enhanced cellular uptake, protection of the active ingredient from degradation, deep tissue penetration, and lowered risk of systemic toxicity by reducing the dose of drug needed for therapeutic purposes (77-82). PLGA-based encapsulation of nanoparticles is widely used for drug delivery because of its biodegradability, allowing its reabsorption by the body and hence lowering toxicity (83). PLGA nanoparticles have been demonstrated to intravaginally deliver small interfering RNA (siRNA), and Rantes (58, 81). Therefore, the incorporation of nanoparticle
encapsulation into a microbicide formulation has the potential to confer long-term protection from a single dose.

In vaginal tissue and secretions the IQP-0528NP film formulation was similar to the IQP-0528 film formulation. In \textit{in vitro} drug release studies, the release profile of the IQP-0528NP films was orders of magnitude longer than the IQP-0528 films. The IQP-0528 films released all the drug within an hour. The IQP-0528NP films produced a small burst release of IQP-0528 from the nanoparticles in a similar time frame resulting in only 37.72 ± 8.45% IQP-0528 of the loaded drug available after 10 hours dissolution. Following that burst release, IQP-0528 was constantly and slowly released from the circulating nanoparticles for over 10 days. These data supported that the IQP-0528NP could provide long-term drug delivery. However, the nanoparticle encapsulation did not provide any greater advantage for IQP-0528 distribution \textit{in vivo}. Limited drug detection in vaginal fluid and no detection in vaginal tissues 24 hours post film application even upon repeated film application suggests that the nanoparticles do not have an extended residence time in the vaginal tissue. The IQP-0528 nanoparticles appear to have similar clearance rates in the vaginal tissue as the IQP-0528 molecule itself. The IQP-0528NP may not be able to provide controlled long-term release of IQP-0528 observed during \textit{in vitro} testing where clearance is not an issue. Therefore, there may be no \textit{in vivo} long-term pharmacokinetic benefits of delivering IQP-0528 in the current formulation.

A limitation of the current study is that no pre-clinical or clinical data exist that defines the pharmacologically relevant concentrations of IQP-0528 that are needed for protection against HIV-1. IQP-0528 has been evaluated against a panel of NRTI- and NNRTI-resistant viruses. The compound retains full activity against some NNRTI-resistant viruses but does lose some potency against others. Against the strains where some resistance is observed the compound exhibits EC\textsubscript{50} values that range between 100 and 900 nM. In addition one macaque
in the IQP-0528NP film group menstruated during the analysis. A higher median proximal vaginal fluid IQP-0528 concentration was obtained at 1, 4, 24 hours (126.6, 534.59, and 84.72 µg/mL respectively) while excluding the menstruating animal in comparison to that obtained while including the menstruating animal (Table 2). The distal vaginal fluid concentrations (179.57, 50.23 and 36.06 µg/mL at 1, 4, 24 hours respectively) were similar among the two groups. A greater number of animals would be required to determine the influence of menstruation on drug absorption. Despite these limitations, this study demonstrates that quick-dissolving IQP-0528 vaginal film formulations exhibit a promising safety and pharmacokinetic profile. The IQP-0528 films demonstrated that a relatively low drug dosing levels are able to reach above IC₉₀ values. This formulation compares well with other film formulation developments where a dapivirine film showed high PK values in vaginal tissue. Concentrations were detected at levels to protect against ex vivo HIV infection (35).

The next step in developing this formulation is the performance of additional PK studies in macaques followed by SHIV challenge studies to determine the pharmacodynamics. Subsequent to these studies, PK studies in human tissue will further inform the development of IQP-0528 vaginal films. The excellent coverage and mucosal levels of IQP-0528 well above the IC₉₀ suggest that these films hold potential to serve as a quick, viable women-initiated prevention option in high HIV-incidence settings.

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The findings and conclusions in this paper are those of the authors and do not necessarily
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Department of Health and Human Services.

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Figure legends

Fig 1: Vaginal microbiological composition in macaques from one cross-over 24 hour PK that received the IQP-0528NP (n=6) and IQP-0528 films (n=6): Prevalence (number of macaques that are positive) of a representative number of the 23 microorganisms in macaques with a) IQP-0528NP film, b) IQP-0528 film at baseline (0 hours) and 24 hours post film application. Lacto- Lactobacilli, Viridans- Viridans streptococci, GNR- Gram negative rods, S. aureus- Staphylococcus aureus
Fig 2: Vaginal pH in macaques that received the IQP-0528NP and IQP-0528 films: a). Vaginal pH was measured prior to film application (0 minutes), and 1, 4, and 24 hours post film application. Each symbol represents the mean of 15 measurements and the standard deviation are indicated by bars. b). Difference in vaginal pH between the baseline (0 minutes) and 1, 4, 24 hours post IQP-0528 film application. The symbol represents the mean pH difference from the baseline and the bars indicate the standard deviation.

Fig 3: IQP-0528 concentration in macaques that received the IQP-0528NP and IQP-0528 film: IQP-0528 concentration was measured in vaginal fluid at 1, 4, and 24 hours post film application obtained proximal (A) and distal to the cervix (B) and in vaginal tissue obtained proximal, medial and distal to the cervix at 24 hours (C). The horizontal line represents the median concentration. A statistical significance of $p \leq 0.0001$ and $p \leq 0.05$ is indicated by *** and * respectively. Samples below LLOQ were assigned one-half of the LLOQ (i.e. 25 ng/mL).
Table 1: Repeated-dose PK and safety sample collection schedule [IQP-0528NP film (n=3) and IQP-0528 film (n=3)]

<table>
<thead>
<tr>
<th>Sample</th>
<th>0 min(^1)</th>
<th>60 min</th>
<th>4 hr</th>
<th>24 hr</th>
<th>Day 7(^1)</th>
<th>Day 10</th>
<th>Day 14(^1)</th>
<th>Day 17</th>
<th>Day 20</th>
<th>Day 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert film</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Blood(^2)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal fluid (Weck-Cel spears)(^3)</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal fluid (Ultracell sponges)(^4)</td>
<td>---</td>
<td>---</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Pinch biopsies (vaginal and rectal)</td>
<td>---</td>
<td>---</td>
<td>X</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>X</td>
</tr>
</tbody>
</table>

\(^1\) Samples collected immediately prior to film application

\(^2\) Collected in CPT tubes. Plasma used for drug analysis

\(^3\) Vaginal spears- Proximal and distal to the cervix

\(^4\) Vaginal Ultracell Surgical sponges (8 plugs, 4 proximal and 4 distal to the cervix)

\(^5\) Vaginal pinch biopsies proximal, medial and distal to the cervix; two rectal pinch biopsies
Table 2: IQP-0528 drug concentrations across mucosal sites in macaques that received IQP-0528NP film (n=15) and IQP-0528 film (n=15)

<table>
<thead>
<tr>
<th>Site</th>
<th>IQP-0528NP film</th>
<th>IQP-0528 film</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td>Vaginal fluid -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>150 (13.13-1,213)</td>
<td>300 (8.68-10,560)</td>
</tr>
<tr>
<td>Distal</td>
<td>150 (0.54-726.79)</td>
<td>300 (4.15-3,722)</td>
</tr>
<tr>
<td>Vaginal tissue -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Distal</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Medial</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

1. n - number of samples
2. Lower limit of quantification
3. Not done
Table 3: Summary of vital PK parameters

<table>
<thead>
<tr>
<th>Vaginal fluid</th>
<th>Cmax (µg/mL)</th>
<th>Tmax (hours)</th>
<th>AUC&lt;sub&gt;0-24h&lt;/sub&gt; (hr*µg/mL)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Vd (L)</th>
<th>CL (ml/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQP-0528NP-Proximal</td>
<td>10.561</td>
<td>4</td>
<td>6.481</td>
<td>0.0032</td>
<td></td>
</tr>
<tr>
<td>IQP-0528NP-Distal</td>
<td>3.722</td>
<td>4</td>
<td>1.273</td>
<td>0.0072</td>
<td>0.3534</td>
</tr>
<tr>
<td>IQP-0528 film-Proximal</td>
<td>10.939</td>
<td>4</td>
<td>13.346</td>
<td>0.0013</td>
<td>0.0337</td>
</tr>
<tr>
<td>IQP-0528 film-Distal</td>
<td>15.795</td>
<td>4</td>
<td>4.326</td>
<td>0.0026</td>
<td>0.1040</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vaginal tissue&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cmax (µg/mL)</th>
<th>Tmax (hours)</th>
<th>AUC&lt;sub&gt;0-24h&lt;/sub&gt; (hr*µg/mL)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Vd (L)</th>
<th>CL (ml/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQP-0528NP-Proximal</td>
<td>78.87</td>
<td>N/A</td>
<td>12.99</td>
<td>0.42</td>
<td>34.65</td>
</tr>
<tr>
<td>IQP-0528NP-Medial</td>
<td>38.75</td>
<td>N/A</td>
<td>15.82</td>
<td>0.34</td>
<td>28.44</td>
</tr>
<tr>
<td>IQP-0528NP-Distal</td>
<td>30.25</td>
<td>N/A</td>
<td>7.63</td>
<td>0.71</td>
<td>58.95</td>
</tr>
<tr>
<td>IQP-0528 film-Proximal</td>
<td>222.58</td>
<td>N/A</td>
<td>3.44</td>
<td>0.24</td>
<td>130.96</td>
</tr>
<tr>
<td>IQP-0528 film-Medial</td>
<td>174.60</td>
<td>N/A</td>
<td>45.79</td>
<td>0.12</td>
<td>9.83</td>
</tr>
<tr>
<td>IQP-0528 film-Distal</td>
<td>55.55</td>
<td>N/A</td>
<td>37.06</td>
<td>0.15</td>
<td>12.14</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data from the two arm cross-over 24 hour PKs (n=12) were combined with the data from the first 24 hours of the repeated-dose PK for each arm to generate the PK parameters (n=15). C<sub>max</sub>, maximum concentration, T<sub>max</sub>, time to C<sub>max</sub>, AUC, area under the concentration-time curve; AUC<sub>0-24h</sub> from the time of film placement to the 24 hour sample collection, Vd, volume of distribution, CL, clearance.