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Evaluation of the Lymphocyte Trafficking Drug FTY720 in Vaginal Tissues

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Abstract

Background—FTY720 is an immunomodulatory agent that reduces lymphocytes in peripheral tissues and circulation. Such agents may be effective as vaginal microbicides for HIV prevention. Systemic or vaginal application of FTY720 may reduce lymphocyte concentrations in genital tissues, reducing HIV target cell numbers.

Methods—Five female pigtail macaques received topical vaginal gel FTY720 (n=2), intravenous (IV) FTY720 (n=2), or placebo gel (n=1) in this pilot study. Circulating and mucosal lymphocytes and genital mucosa, cytokines, and tissue histology were analyzed to document topical and IV FTY720 effects.

Results—Topical and IV FTY720 appeared to decrease levels of cervicovaginal IL-8, IL-1ra, and genital inflammatory cells. Small sample size precluded statistical analysis. Topical administration had no overt adverse effects.

Conclusion—This study introduces FTY720 as an immunomodulatory agent for the vaginal mucosa, compares topical effects to those of IV administration, and provides the basis for future studies involving FTY720 for HIV prevention.

Keywords

Genital; nonhuman primates; topical gel; HIV

Introduction

WHO and UNAIDS estimate there were 34.2 million people worldwide living with HIV at the end of 2011 [1]. Slightly more than half of the people living with HIV infections are females. Particularly in sub-Saharan Africa and the Caribbean, females are disproportionately infected with HIV compared to males [2]. In the United States, females account for one in four new HIV diagnoses, with almost 75% of female infections attributed to heterosexual contact [3]. There are female-specific factors that contribute to their susceptibility to HIV, including their anatomy and physiology, traditional gender norms, power inequalities in relationships, economics, and the violence against females [4, 5]. Thus,

HIV prevention efforts targeting females should be tailored to their individual needs. Although condom use is an effective HIV prevention method [6-8], many females are unable to negotiate condom use due to gender-based power imbalance in their relationships [4, 5]. Furthermore, male to female heterosexual HIV transmission is considered more efficient than female to male transmission [5, 6]. There is a rapidly evolving need for development of effective, acceptable, and affordable female-controlled HIV prevention methods [9-11]. Vaginally administered microbicides and discreetly administered drugs are prime candidates for a female-controlled prevention method. Our goal is to enable females to self-administer agents designed to prevent or reduce the risk of mucosal HIV transmission [10, 11], and a number of studies and trials have evaluated various drug candidates [12-15].

HIV is transmitted in the female genital tract by translocating through endocervical columnar epithelium, or ectocervical and/or vaginal squamous epithelia. HIV may directly move across these tissue layers through transcytosis to infect target cells in the tissues such as CD4⁺ T cells, macrophages, or dendritic cells [16]. Early studies in macaque models described intraepithelial CD4⁺ T cells of the genital mucosa as the first target of productive SHIV replication [17, 18]. It is believed the presence of CD4⁺ T cells in the cervicovaginal epithelium and their trafficking pattern to lymph nodes play a significant role in HIV transmission [17, 19]. Collectively, these findings suggest the reduction of HIV target cells in the genital mucosa may aid in preventing or reducing rates of mucosal HIV transmission.

FTY720 is a sphingosine analog and immunomodulatory agent derived from the fungus *Iscaria sinclarii*. It alters the migration pattern of lymphocytes by binding to sphingosine-1-phosphate receptors (S1PR) on lymphocytes, causing the receptors to internalize and degrade [20]. Since S1PRs normally control cell egress from lymphoid tissues into the lymphatic system, internalization of S1PRs by FTY720 results in the sequestration of lymphocytes in secondary lymphoid organs [21]. This leads to a disappearance of lymphocytes from circulation and blood. FTY720 has also been shown to block dendritic cell trafficking [22], and a recent study by Zeng et al. showed that FTY720 down-regulated cytokine production in a mouse model [23]. The drug's diverse immunological effects have been extensively studied and shown to be effective in suppressing autoimmunity in systemic lupus erythematosus and experimental autoimmune myocarditis in mice [24, 25]. It has also been studied for its applications in tumor immunology and extension of allograft survival in allotransplantation models [26]. Moreover, FTY720 is currently FDA approved as an oral therapy (Gilenya®) for relapsing multiple sclerosis [27]. Applied topically, FTY720 has also been shown to impair migration of dendritic cells to the skin in mice [28]. Despite the numerous studies of FTY720 applications, its effect on HIV infection has not been clinically evaluated. We previously studied FTY720 in a rhesus macaque model of HIV infection [29]. Macaques previously infected with SHIV_{SF162P3} were treated with 0.1mg/kg IV FTY720, which resulted in a significant reduction in circulating lymphocytes without lowering plasma SHIV_{SF162P3} levels [29]. In addition, Murooka et al. recently demonstrated the effectiveness of intraperitoneal FTY720 injection (1mg/kg) in limiting viral dissemination in a humanized mouse model [30].

We evaluated FTY720 administered systemically or by a novel topical vaginal gel formulation in female pigtail macaques. We hypothesized that FTY720 would reduce the number of HIV target lymphocytes in the female genital mucosa, and thus could be a feasible candidate for an HIV microbicide.

Materials and Methods

Macaques

We studied adult female pigtail macaques (*Macaca nemestrina*) in this study. All macaques were housed and cared for at the Centers for Disease Control and Prevention (CDC), according to the standards published in the Guide for the Care and Use of Laboratory Animals [31]. The Institutional Animal Care and Use Committee of the CDC approved all procedures used in the study design. Two pigtail macaques were given FTY720 vaginal gel preparation (identification (ID): POh2, PKi2), two were given intravenous (IV) administration of FTY720 (PDt1, PPI2), and one was given placebo, hydroxyl-ethyl cellulose (HEC) gel and was assigned as a control (PVi2). Macaques were anesthetized with ketamine (10 mg/kg) prior to all procedures with the exception of biopsies when Telazol (5 mg/kg) was used.

FTY720 Vaginal Gel Preparation

FTY720 (0.02%; w/v) vaginal HEC gel formulations were prepared by dissolving 20 mg of FTY720 (Cayman Chemical, Ann Arbor, MI, catalog # 10006292) in 100 ml HEC gel (pH 6.5) [32]. FTY720 gel appeared faintly opaque and odorless. Due to reportedly low stability of FTY720, vaginal gels were prepared within 24 hours of each week's application and stored at 4°C until 30 minutes prior to application. The presence of drug in gel was confirmed by drug detection (see below) following storage (data not shown). A gel volume of 3 ml was used for each vaginal application (0.6 mg FTY720/ gel application). Thus, with an average macaque weight of 6 kg, we achieved an approximate topical dose of 0.1 mg/kg. The application schedule is shown in Figure 1.

IV FTY720 Solution Preparation

For systemic FTY720 delivery, we chose intravenous over oral administration due to earlier experience with macaques inconsistently consuming drugs with food. Macaques were administered IV FTY720 at a dose of 0.1 mg/kg, a dose previously found to be effective at reducing peripheral blood CD4⁺ T and B cells in macaques [29]. The IV administration schedule is also shown in Figure 1. Both FTY720 vaginal gel and IV FTY720 were given twice a week (day 0, day 1, day 7, day 8, day 14, day 15, day 21, and day 22) concurrent in part with sample collections at 24 hours, 48 hours, and 72 hours after the first treatments in attempt to determine the effects at various time points and to capture cumulative effects of FTY720 seen in earlier study by Quesniaux et al [33].

FTY720 Drug Concentrations

Modulation of lymphocyte trafficking by FTY720 is detected after FTY720 is converted into FTY720-phosphate and binds to sphingosine-1-phosphate receptors. FTY720 is phosphorylated *ex vivo* in lymphoid tissues of rodents and whole blood of several different species including human [34]. Following oral dosing, FTY720 is readily phosphorylated *in vivo*, especially in the liver [35]. Slow IV FTY720 infusions in humans lead to rapid peak drug concentrations at the end of infusions (within 2 hours) followed by a rapid decline [36]. Both FTY720 and FTY720-phosphate levels in plasma or cervicovaginal lavages (CVL) were measured using high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) as follows. FTY720 and FTY720-phosphate from plasma or CVL samples (200 µL) were extracted with 760 µL HPLC solvent B (10 mM ammonium acetate, 0.08% formic acid in methanol) containing 400 µg of Efavirenz (EFV) as the internal standard. Protein precipitants were removed by centrifugation and 600 µL of the supernatant was transferred to a 96-well polypropylene plate, evaporated to near dryness, and re-suspended in 125 µL of solvent C (10 mM ammonium acetate, 0.08% formic acid in 70% methanol).

Fifty microliters of the extracted material was injected into a Luna C8(2) column (Phenomenex, Torrance CA) connected to Prominence HPLC system (Shimadzu, Columbia, MD) for the separation and Model 3200 QTrap mass spectrometer (ABSciex, Foster City, CA) for the quantification. A linear gradient of 70% B to 98% B (solvent A = 10 mM ammonium acetate, 0.08% formic acid in water) and a flow rate of 0.3 ml/min were used for the separation. Standard curves of known concentrations of FTY720 and FTY720-phosphate (0.5 – 2000 ng/ml) were constructed for each run to quantify analyses. The FTY720 limit of detection (LOD) was 0.5 ng/ml, while the limit of quantification (LOQ) was 5 ng/ml.

Cervicovaginal Mucosa Assessments

Visual assessments of the cervicovaginal compartment were performed using a colposcope and pediatric vaginal speculum at each time point at the baseline, treatment, and follow-up phases. Vaginal and cervical mucosal color, tissue friability, presence of erythema and discharge, discharge consistency and color, and the presence of menstrual bleeding were assessed. During the treatment phase, visual assessment was performed and documented three times per week: prior to FTY720 or placebo treatment, and then 24 and 48 hours post-treatment.

Effect of FTY720 on Circulating Lymphocytes

Blood was collected from the femoral vein at the baseline, treatment, and follow-up phases as outlined in Figure 1. Peripheral blood mononuclear cells (PBMCs) were purified from blood after centrifugation. CD3 cell surface expression was analyzed in PBMC samples by flow cytometry (FACS Calibur, CD3 APC; Becton Dickinson Biosciences, San Jose, CA), and levels of CD3+ cells were determined by FlowJo analysis, as previously described [29].

Collection of vaginal secretions

Vaginal secretions were collected by cervicovaginal lavage (CVL). Samples collections were performed by infusing 5 ml of phosphate-buffered saline (PBS, pH 7.2) into the vagina using 10ml syringes with a rubber tipped catheter. Pooled fluid was then aspirated to evaluate FTY720 drug concentrations and cytokine levels. CVLs were collected at baseline, treatment, and follow-up phases as shown in Figure 1. During the treatment phase, CVLs were collected following colposcopic cervicovaginal mucosa assessments and vaginal swab collections when scheduled, but prior to FTY720 administrations.

Cytokine and Progesterone Measurements

IL-8 and IL-1ra (a=antagonist) were previously found at detectable levels in vaginal secretions of untreated, regularly cycling pigtail macaques (data not shown). IL-8 is a chemo-taxis inducing chemokine, and causes recruitment of inflammatory cells. Various cell types including immune and epithelial cells make the IL-1ra cytokine; it impacts a variety of immune and inflammatory pathways. Both IL-8 and IL-1ra effectively induce lymphocyte mobilization in vitro [37] and a recent European study showed positive association between the level of endocervical CD3+ cells and the concentration of IL-1ra in human CVL [38]. To address whether FTY720 decreases baseline cytokines, we measured IL-8 and IL-1ra in CVL samples using the Milliplex platform (Millipore, Billerica, MA) and analyzed by Luminex system software (Bio-Rad Laboratories, Hercules, CA). Cytokine levels were measured in supernatant fluids of centrifuged CVL and quantitated as picogram per milliliter of CVL fluid. Progesterone level analyses were performed on plasma samples using a progesterone EIA Kit (Cayman Chemical Company, Ann Arbor, MI, item # 582601) to determine the phase of menstrual cycle at the time biopsies were performed. Phases of the menstrual cycle were determined by fluctuation of progesterone levels. Follicular phase was

defined by a decrease of progesterone level and luteal phase was defined by increasing progesterone level.

Histological Evaluation of Cervical and Vaginal Biopsy Samples

Biopsy specimens were collected following vaginal secretion and CVL collections at the baseline and four weeks post-baseline collection, during the treatment phase. Biopsy samples were collected according to this schedule in order to space them at one-month intervals, or approximately one complete menstrual cycle apart, to avoid natural tissue composition fluctuations that might arise due to hormonal changes during the menstrual cycle. Biopsied tissues were fixed in 10% buffered formalin for 24 hours then placed in 4% ethanol. Tissues were then paraffin-embedded, thin-sectioned (4 μ m), stained with hematoxylin and eosin and analyzed by light microscopy (Olympus BX41) by a veterinary pathologist blinded to treatment status.

Statistical methods

This is a pilot study in nature and has served to generate and further define the hypothesis that FTY720 affects and reduces vaginal lymphocytes. Due to the small group sizes, statistical significance was not computed, and the IV and gel groups (each with n=2 macaques) were not compared to each other.

Results

Five female pigtail macaques were enrolled in this pilot study to evaluate the effects of FTY720 administration by both topical vaginal gel formulation and IV administration, in a three-phase study design (baseline, treatment, and follow-up) (Figure 1).

FTY720 Drug Concentrations

FTY720 was measurable in CVL at a level higher than the limit of quantification (5 ng/ml) at 24 and 48 hours after application of vaginal gel (Figure 2A), indicating that the drug was present and not degraded at the indicated time points. The bio-active phosphate form of FTY720 was also detectable in vaginal secretions (Figure 2B), albeit at very low levels, and only at one time point. Mean FTY720-phosphate levels were above the limit of detection one day after FTY720-gel treatment, but remained below this limit on all other time points. This indicates that FTY720 can be taken up through the genital mucosa and can be phosphorylated in local tissues. Gel application did not lead to detection of systemic FTY720 or FTY720-phosphate in blood (data not shown). Intravenous FTY720 administration led to the detection of bio-active FTY720-phosphate in circulation (Figure 2C), as expected due to rapid conversion of FTY720 to FTY720-phosphate following IV administration [36]. Vaginal FTY720 and FTY720-phosphate were undetectable after IV administration of FTY720 (data not shown). The placebo-treated macaque did not have detectable FTY720 or FTY720-phosphate in either compartment (data not shown).

Effect of FTY720 on Circulating and Lymphocyte Populations

We measured CD3+ cells in blood to further analyze systemic effects of FTY720 drug delivery methods. FTY720 administration has been shown to cause a substantial decrease in circulating blood CD3+ cells, because the lymphocytes are retained in lymphoid tissues, and are therefore depleted from circulation and blood [39]. Topical vaginal application had no effect on circulating CD3+ cells, demonstrating that the drug did not have these systemic effects (Figure 3A). In contrast, the percentage of CD3+ cells in PBMCs dropped two and three days after IV FTY720 administrations (Fig. 3B), indicating that the drug was

systemically effective as expected. This reduction was not seen following placebo gel treatment (Figure 3C).

Histological Analysis

Biopsies, including both cervical and vaginal, were collected at baseline and treatment phase time points and were evaluated for lymphocytic infiltration in blinded fashion. Figure 4 shows a representative image of a baseline and treatment biopsy from each macaque, and Table 1 summarizes results from all biopsies. At baseline, two macaques exhibited cellular inflammatory infiltrates (PKi2 and PDt1; Figure 4C, D and Figure 4G, H, respectively). Decreases in cellular inflammatory infiltrate were observed in both of these macaques following FTY720 treatment. Inflammatory cellular infiltrates were not observed in POH2 (Figure 4A, B) nor PKi2 (Figure 4E, F) in both the baseline and treatment phase samples. Notably, there were higher levels of inflammatory cellular infiltrates after treatment when compared to baseline phase samples in PVi2, the macaque which received the placebo gel (Figure 4I, J; Table I). Higher magnification in Figure 4K and 4L (PDt1) illustrate the numerous localized aggregates of inflammatory cells present at baseline and the reduction of the aggregates after IV FTY720 treatment, respectively. Both baseline and treatment biopsies were taken during follicular phase in PDt1, thus the fluctuation of inflammatory cells due to transitioning phases of the menstrual cycle is not reflected.

Cervicovaginal Cytokines

Cervicovaginal IL-8 and IL-1ra, two factors with robust detectable baseline levels, were measured at baseline, treatment, and follow-up time points. In the FTY720 vaginal gel treated group, the mean IL-8 concentration at the baseline was 612 pg/ml and 484 pg/ml during FTY720 treatment phase (Figure 5A). Mean IL-1ra concentrations changed from 1704 pg/ml at the baseline to 808 pg/ml during FTY720 vaginal gel treatment (Figure 5B). In the IV FTY720 treatment group, differences in mean concentration of both IL-8 and IL-1ra (Figure 5C and 5D) were observed between baseline and treatment phase (Figure 5C). Mean IL-8 concentration during IV FTY720 treatment was 1764 pg/ml at the baseline; however, it was only 833 pg/ml during the treatment phase (Figure 5C). Mean IL-1ra concentration during IV FTY720 treatment also decreased from the concentration of 2495 pg/ml at baseline to 1559 pg/ml (Figure 5D). Interestingly, in the placebo macaque, mean IL-8 levels increased from 226 pg/ml at baseline to 655 pg/ml during the treatment phase. Mean IL-1ra levels remained nearly constant, 2777 pg/ml at baseline to 2874 pg/ml during the treatment phase (Figure 5E, F, respectively).

Genital Mucosa and Microenvironment

Visual analysis and characterization of cervicovaginal tissue and discharge were performed by colposcopy and observations were recorded throughout (data not shown). No specific patterns of change in mucosal color, presence of erythema and discharge, or discharge color and consistency were observed with FTY720 vaginal gel, IV FTY720, or placebo gel use, compared to baseline measures (data not shown).

Vaginal Microflora Analysis

Vaginal secretions were collected and analyzed by bacterial culture to assess the impact of FTY720 gel treatment on the genital microenvironment. Figure 6 shows longitudinal data from FTY720 gel treated macaque POH2 as an example. Fluctuations in the number of bacterial species and bacterial concentrations occurred before FTY720 gel treatment (study days -21 to 0 in Figure 6A, B), and continued during (study days 2 – 21), and after FTY720 gel treatment (study days 24, 49). Changes were also observed during HEC gel application (data not shown). Neither treatment resulted in consistent loss or growth of vaginal bacteria

(Figure 6A and data not shown), and no overt adverse effects of FTY720 gel treatment were noted.

Discussion

This study presents novel proof-of-concept data on the effects of FTY720 on female genital tissues in pigtail macaques and introduces the possibility of developing this lymphocyte trafficking drug as a potential HIV microbicide candidate. In this macaque model, we investigated the effects of both a vaginal gel and an IV formulation of FTY720. We observed effects on several inflammatory markers in these pilot analyses which establish the framework of this model for additional studies.

The current study is the first to evaluate the potential use of this drug as an immunosuppressive agent for the female genital tract intended for HIV prevention. CD4⁺ T cell activation and upregulation of the cellular immune response is associated with increased risk of HIV infection [39-41]. Recent data from the CAPRISA 004 study also demonstrated inflammation and immune activation increases HIV infection risk in their cohort, and the team proposed incorporating an immunosuppressant or immunomodulatory component into microbicide gel formulations to increase efficacy [15, 42]. Prior to the CAPRISA data, Li et al. reported the use of topical glycerol monolaurate (GML) to prevent SIV infection in Rhesus macaques via vaginal challenge [12]. GML is an antimicrobial agent that blocks the MIP-3 α signaling pathway, thereby inhibiting innate inflammatory responses and the recruitment of SIV target cells to the vaginal mucosa. Similarly, we anticipated the application of FTY720 to vaginal tissues would result in the absence of (S)HIV target cells from the mucosa [12]. Both topical and IV FTY720 treatment induced a mild decrease in inflammatory cytokines and inflammatory cells in cervical and vaginal epithelial tissues. Neither drug delivery technique induced adverse clinical effects, even after repeated applications. Additionally, we did not observe any significant changes in the state of the genital microenvironment. IV FTY720 reduced numbers of circulating blood lymphocytes; however, vaginal application of the drug did not alter the concentration of circulating lymphocytes. The absence of adverse tissue effects and the reduction of inflammatory mediators in the tissue provide promising results and support the further study of FTY720 as a novel HIV microbicide candidate.

Very little is known about lymphocyte trafficking to the vaginal epithelium. In particular, it is not clear how often lymphocytes travel in and out of the tissue in the absence of a stimulus such as infection. Whether topical or IV FTY720 treatment removes lymphocytes from vaginal tissues or if the observed mean cytokine level decrease will result in effective immunomodulation could not be definitively ascertained in this study. Modest post-treatment lymphocyte reductions in cervicovaginal tissues were seen with both formulations, but not for both macaques in the two treatment groups (Table 1). Variance among individual macaques was observed in lymphocyte infiltration in cervical and vaginal biopsies taken before/after FTY720 treatment. This could be attributed to menstrual cycle, which was not synchronized in this study. But, on a more fundamental note, it would be difficult to show a treatment-induced reduction of tissue lymphocytes if infiltrate was not present at baseline (Table 1: POH2, PPI2). For its licensed use in multiple sclerosis, FTY720 reduces existing inflammation [27, 43]. Nevertheless, a possible interpretation of our histology results is that FTY720 reduced lymphocyte infiltration in both macaques with marked infiltration at study outset, regardless of delivery method.

From this study, it was not clear whether topical FTY720 drug delivery would affect local lymphocyte infiltration, or whether systemic drug delivery to surrounding lymphoid tissues was needed for the drug to be effective. There was a limitation in how much FTY720 could

be delivered by gel due to limited drug solubility, and the small size of the vaginal canal in macaques. However, our data did indicate that topical delivery of FTY720 affects vaginal inflammatory markers by lowering levels of inflammatory cytokines. These data are consistent with the report by Reines et al. on a contact dermatitis mouse model evaluating topical FTY720 (10 μ g/80 μ l)'s effect on dendritic cell migration. In this study, the inflammatory response was also reduced after FTY720 was applied to mouse ears, causing a reduction in dendritic cell migration as well as circulating lymphocyte levels [28].

We acknowledge a limitation of this study is the number of macaques studied (n=5). The macaques were further divided into treatment groups of n=2 macaques, thus precluding statistical analyses of significance for our findings, and definitive comparisons between treatment arms. For example, we refrained from statistical analyses of mean reductions in cytokine levels, because our small animal groups showed variable cytokine distributions at baseline. However, a pilot study with small animal numbers was justified based on limited availability of pigtail macaques in general, their high cost, and the need for safety testing before larger macaque groups are subjected to any treatment. While larger animal numbers, higher dose and/or frequency of FTY720 application may have provided more definitive outcomes and statistical analyses of significance, these pilot data still provide valuable insight into model development and the potential use of FTY720 in a vaginal gel formulation. Additional terminal pharmacokinetic studies are underway to supplement these data and, collectively, will better guide the design of future studies evaluating topical FTY720's ability to prevent (S)HIV transmission.

In conclusion, we demonstrate that vaginal application of FTY720 gel induces a mild decrease in inflammatory cytokines and inflammatory cells without any adverse clinical effects to the cervicovaginal mucosa. In order to strengthen HIV prevention efforts, the development of effective, affordable, acceptable and female-controlled HIV prevention methods are needed. Based on the findings from this study, our previous findings that FTY720 decreases lymphocytes in SHIV infected macaques, and on the promising data from Murooka et al. on limiting HIV-1 dissemination in a mouse model using FTY720, more studies on the immunomodulation effects of FTY720 in the pigtail macaque model should be conducted. This study introduces FTY720 in a vaginal gel formulation, and while further study of FTY720 vaginal gel as an HIV microbicide is necessary, the preliminary data presented here suggests a potential use of this product in biomedical prevention strategies.

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References

1. WHO. [cited 2012] HIV/AIDS - Fact sheet N°360. 2012. Available from:<http://www.who.int/mediacentre/factsheets/fs360/en/index.html>
2. UNAIDS. [cited 2012 April] Global Report - UNAIDS Report on the Global AIDS Epidemic. 2010. Available from: http://www.unaids.org/globalreport/Global_report.htm

3. CDC. HIV Surveillance Report - Diagnoses of HIV infection and AIDS in the United States and Dependent Areas. 2010; 22 Available from:http://www.cdc.gov/hiv/surveillance/resources/reports/2010report/pdf/2010_HIV_Surveillance_Report_vol_22.pdf#Page=1.
4. Gomez CA. Preventing HIV in U.S. women and girls: a call for social action. *Womens Health Issues*. 2011; 21(6 Suppl):S287–94. [PubMed: 22055680]
5. Turmen T. Gender and HIV/AIDS. *Int J Gynaecol Obstet*. 2003; 82(3):411–8. [PubMed: 14499987]
6. Ngugi EN, et al. Prevention of transmission of human immunodeficiency virus in Africa: effectiveness of condom promotion and health education among prostitutes. *Lancet*. 1988; 2(8616):887–90. [PubMed: 2902326]
7. Weller S, Davis K. Condom effectiveness in reducing heterosexual HIV transmission. *Cochrane Database Syst Rev*. 2002; (1):CD003255. [PubMed: 11869658]
8. Hira SK, et al. Condom and nonoxynol-9 use and the incidence of HIV infection in serodiscordant couples in Zambia. *Int J STD AIDS*. 1997; 8(4):243–50. [PubMed: 9147157]
9. McCormack S, et al. Microbicides in HIV prevention. *BMJ*. 2001; 322(7283):410–3. [PubMed: 11179162]
10. Abdool Karim SS, Baxter C. Overview of microbicides for the prevention of human immunodeficiency virus. *Best Pract Res Clin Obstet Gynaecol*. 2012
11. Mayer KH, et al. Safety and tolerability of tenofovir vaginal gel in abstinent and sexually active HIV-infected and uninfected women. *AIDS*. 2006; 20(4):543–51. [PubMed: 16470118]
12. Li Q, et al. Glycerol monolaurate prevents mucosal SIV transmission. *Nature*. 2009; 458(7241):1034–8. [PubMed: 19262509]
13. Rohan LC, et al. In vitro and ex vivo testing of tenofovir shows it is effective as an HIV-1 microbicide. *PLoS One*. 2010; 5(2):e9310. [PubMed: 20174579]
14. Kamali A, et al. A randomised placebo-controlled safety and acceptability trial of PRO 2000 vaginal microbicide gel in sexually active women in Uganda. *Sex Transm Infect*. 2010; 86(3):222–6. [PubMed: 20444744]
15. Abdool Karim Q, et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science*. 2010; 329(5996):1168–74. [PubMed: 20643915]
16. Pope M, Haase AT. Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. *Nat Med*. 2003; 9(7):847–52. [PubMed: 12835704]
17. Miller CJ, et al. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J Virol*. 2005; 79(14):9217–27. [PubMed: 15994816]
18. Zhang Z, et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science*. 1999; 286(5443):1353–7. [PubMed: 10558989]
19. Miller CJ, Shattock RJ. Target cells in vaginal HIV transmission. *Microbes Infect*. 2003; 5(1):59–67. [PubMed: 12593974]
20. Graler MH, Goetzl EJ. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J*. 2004; 18(3):551–3. [PubMed: 14715694]
21. Chiba K. FTY720, a new class of immunomodulator, inhibits lymphocyte egress from secondary lymphoid tissues and thymus by agonistic activity at sphingosine 1-phosphate receptors. *Pharmacol Ther*. 2005; 108(3):308–19. [PubMed: 15951022]
22. Lan YY, et al. The sphingosine-1-phosphate receptor agonist FTY720 modulates dendritic cell trafficking in vivo. *Am J Transplant*. 2005; 5(11):2649–59. [PubMed: 16212624]
23. Zeng X, et al. Topographical and Biological Evidence Revealed FTY720-Mediated Anergy-Polarization of Mouse Bone Marrow-Derived Dendritic Cells In Vitro. *PLoS One*. 2012; 7(5):e34830. [PubMed: 22693544]
24. Okazaki H, et al. Effects of FTY720 in MRL-lpr/lpr mice: therapeutic potential in systemic lupus erythematosus. *J Rheumatol*. 2002; 29(4):707–16. [PubMed: 11950011]
25. Kitabayashi H, et al. FTY720 prevents development of experimental autoimmune myocarditis through reduction of circulating lymphocytes. *J Cardiovasc Pharmacol*. 2000; 35(3):410–6. [PubMed: 10710126]

26. Brinkmann V, et al. FTY720: a novel transplantation drug that modulates lymphocyte traffic rather than activation. *Trends Pharmacol Sci.* 2000; 21(2):49–52. [PubMed: 10664606]
27. Brinkmann V, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov.* 2010; 9(11):883–97. [PubMed: 21031003]
28. Reines I, et al. Topical application of sphingosine-1-phosphate and FTY720 attenuate allergic contact dermatitis reaction through inhibition of dendritic cell migration. *J Invest Dermatol.* 2009; 129(8):1954–62. [PubMed: 19194476]
29. Kersh EN, et al. Evaluation of the lymphocyte trafficking drug FTY720 in SHIVSF162P3-infected rhesus macaques. *J Antimicrob Chemother.* 2009; 63(4):758–62. [PubMed: 19218272]
30. Murooka TT, et al. HIV-infected T cells are migratory vehicles for viral dissemination. *Nature.* 2012
31. Council, NR. Guide for the Care and Use of Laboratory Animals. Eighth Edition. National Academy Press; Washington, D.C.: 2011.
32. Parikh UM, et al. Complete protection from repeated vaginal simian-human immunodeficiency virus exposures in macaques by a topical gel containing tenofovir alone or with emtricitabine. *J Virol.* 2009; 83(20):10358–65. [PubMed: 19656878]
33. Quesniaux V, et al. A novel immunosuppressant, FTY720, induces peripheral lymphodepletion of both T- and B cells and immunosuppression in baboons. *Transpl Immunol.* 1999; 7(3):149–57. [PubMed: 10608298]
34. Billich A, et al. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem.* 2003; 278(48):47408–15. [PubMed: 13129923]
35. Gasperini C, Ruggieri S. Development of oral agent in the treatment of multiple sclerosis: how the first available oral therapy, fingolimod will change therapeutic paradigm approach. *Drug Des Devel Ther.* 2012; 6:175–86.
36. Kovarik JM, et al. Oral-intravenous crossover study of fingolimod pharmacokinetics, lymphocyte responses and cardiac effects. *Biopharm Drug Dispos.* 2007; 28(2):97–104. [PubMed: 17230596]
37. Pleass R, Camp R. Cytokines induce lymphocyte migration in vitro by direct, receptor-specific mechanisms. *Eur J Immunol.* 1994; 24(1):273–6. [PubMed: 8020566]
38. Kyongo JK, et al. Searching for lower female genital tract soluble and cellular biomarkers: defining levels and predictors in a cohort of healthy Caucasian women. *PLoS One.* 2012; 7(8):e43951. [PubMed: 22952818]
39. Begaud E, et al. Reduced CD4 T cell activation and in vitro susceptibility to HIV-1 infection in exposed uninfected Central Africans. *Retrovirology.* 2006; 3:35. [PubMed: 16792805]
40. McLaren PJ, et al. HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. *J Infect Dis.* 2010; 202(Suppl 3):S339–44. [PubMed: 20887221]
41. Jennes W, et al. Suppressed cellular alloimmune responses in HIV-exposed seronegative female sex workers. *Clin Exp Immunol.* 2006; 143(3):435–44. [PubMed: 16487242]
42. Naranbhai V, et al. Innate Immune Activation Enhances HIV Acquisition in Women, Diminishing the Effectiveness of Tenofovir Microbicide Gel. *J Infect Dis.* 2012; 206(7):993–1001. [PubMed: 22829639]
43. Devonshire V, et al. Relapse and disability outcomes in patients with multiple sclerosis treated with fingolimod: subgroup analyses of the double-blind, randomised, placebo-controlled FREEDOMS study. *Lancet Neurol.* 2012; 11(5):420–8. [PubMed: 22494956]

Figure 1. Study Design

The three-phase study course is shown. Two pigtail macaques were given FTY720 vaginal gel preparation, two were given IV administration of FTY720, and one was given placebo, hydroxyl-ethyl cellulose (HEC) gel. During treatment phase, sample collections were performed three times per week: prior to application/administration of FTY720 and at 24, 48 or 72 hours post-treatment.

Figure 2. FTY720 Concentrations in Plasma and Cervicovaginal Lavage Supernatants
 FTY720 drug levels were only quantifiable in CVL samples after vaginal gel application (A), but not in plasma after IV administration (not shown). FTY720-phosphate levels were detectable in vaginal secretions after gel treatment (B), and in plasma after intravenous administration (C), indicating the presence of bio-active drug in these compartments. Limit of quantification (LOQ) and the limit of detection (LOD) are indicated by dashed lines. X-axis labels: bsl = baseline, FU = follow-up; day 0 combines samples prior to weekly treatment administration from days 0, 7, 14, and 21, day 1 combines samples collected 24 hours after the treatment administration from days 1, 8, 15, and 22, day 2 combines samples from days 2 and 9, and day 3 combines samples from days 17 and 23 as identified in the study design, Figure 1.

Figure 3. Circulating Levels of CD3+ Cells

Percentage of CD3+ cells was measured by flow cytometry from PBMC collected at day 0 (prior to weekly treatment administration, and combining data from day 0, 7, 14, and 21 as identified in Figure 1 legend), 24 hours (+1 day), 48 hours (+2 days), 72 hours (+3 days) post-treatment and follow-up (FU). (A) FTY720 vaginal gel treatment group, (B) IV FTY720 treatment group, and (C) placebo macaques are shown. A decrease in CD3+ cell levels was observed 48 hours after IV FTY720 treatment (B), but no differences were observed after application of FTY720 or placebo vaginal gels (A and C).

Figure 4. Histological Evaluation of Cervical and Vaginal Biopsies with Hematoxylin and Eosin Staining

Three biopsies were taken from each macaque with emphasis placed on vaginal tissues. To avoid menstrual cycle-related tissue changes, biopsies were taken one month apart, the first during baseline period (left panels) and the second during treatment period (after FTY720 administration when applicable, right panels). Each row depicts sets of representative images (10x magnification) of baseline/treatment biopsy sections for an individual macaque. Macaques shown in top panels (A, B, C, D) received FTY720 vaginal gel (POh2 and PKi2), macaques in the middle panels (E, F, G, H) received IV FTY720 (PPi2 and PDt1), and the macaque shown in the bottom panels (I, J) received a placebo gel (PVi2). Tissue sections were evaluated and scored on the basis of cellular infiltrate severity by a blinded veterinary pathologist.

Higher magnification (20x) images of vaginal biopsies from PDt1 represents moderate multifocal lymphocytic and neutrophilic infiltrates in lamina propria at baseline (K), and absence of inflammatory infiltrate in lamina propria during IV FTY720 treatment (L).

Figure 5. Cytokine measurement before and after FTY720/placebo treatment

IL-8 (A, C, E) and IL-1ra (B, D, F) levels were measured in cervicovaginal lavage (CVL) samples during baseline, treatment, and follow up. Top panels (A and B) are from FTY720 vaginal gel group, middle panels (C and D) are from IV FTY720 group, and bottom panels (E and F) are from placebo macaque.

Figure 6. Longitudinal analysis of vaginal bacterial microflora population from one FTY720 gel treated macaque, POh2

Data were harvested from vaginal swabs taken at the time points before (study days -21 to 0), during (study days 2 – 21), and after FTY720 gel treatment (study days 24, 49). Neither consistent loss nor growth of vaginal bacteria was noted throughout the study. A: the number of bacteria cultured from the swabs, B: percentage of cultured bacteria composition. Coag = Coagulase, Neg = Negative, Strep = Streptococcus, Ana = Anaerobic, GNR = Gram Negative Rod

Table I
Blinded Histopathology Scoring of Cervical and Vaginal Biopsy Samples

Biopsy results were scored for inflammatory cellular infiltrates by a blinded veterinary pathologist. Infiltrate severity scoring: none (–), mild (+), moderate (++), and severe (+++). Cell types: lymphocytes (LYC), polymorphonuclear cells/neutrophils (PMNs), and plasma cells (PCs). Each row refers to results from one of three separately biopsied sites (e.g., left or right side of vagina, cervix), presented in slide format to the pathologist who was blinded to identification of macaques and treatment group.

	ID	Baseline	Post-treatment
FTY720 vaginal gel	POh2: vaginal 1	–	–
	POh2: vaginal 2	–	–
	POh2: cervix	–	–
	PKi2: vaginal 1	++ (LYC)	+ (PMN, LYC, PC)
	PKi2: vaginal 2	++ (LYC)	++ (PMN, LYC)
	PKi2: cervix	++ (LYC)	+ (PMN, few LYC)
FTY720 IV	PPi2: vaginal 1	–	–
	PPi2: vaginal 2	–	–
	PPi2: cervix	–	–
	PDt1: vaginal 1	++ (LYC, PMN)	–
	PDt1: vaginal 2	++ (LYC, PMN)	+ (LYC)
	PDt1: cervix	++ (LYC, PMN)	–
Placebo	PVi2: vaginal 1	–	–
	PVi2: vaginal 2	–	+ (LYC, PMN, PC)
	PVi2: cervix	–	–