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Charles Dobard *et al.*

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Editor's Summary

## An After-Sex Gel to Protect Against HIV

Vaginal gels containing antiretroviral (ARV) drugs are important for HIV prevention but are all designed to be applied by women before sex, which can interfere with sex practices and limit their use. A gel that can be applied after sex would be more desirable because it will have more user control and less need for sex anticipation and partner acceptance. However, all ARV-based gels in development contain ARV drugs that block the early steps in virus infection and thus require application before sex. Dobard *et al*. in their new study reasoned that HIV integrase inhibitors, which belong to a different ARV class that blocks later steps in virus infection, may be more suitable for application after HIV exposure during sex. They first confirmed that HIV integration into cellular DNA begins about 6 hours after virus infection, providing a wide window for dosing with integrase inhibitors after sex. Using a macaque model to assess efficacy, the authors further showed that a vaginal gel containing an integrase inhibitor protected macaques when applied 30 min before or 3 hours after vaginal challenge with simian HIV. The study supports evaluation of topical integrase inhibitors for HIV prevention and the assessment of after-sex use for improved acceptability by women.



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HIV

# Postexposure Protection of Macaques from Vaginal SHIV Infection by Topical Integrase Inhibitors

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**Coitally delivered microbicide gels containing antiretroviral drugs are important for HIV prevention. However, to date, microbicides have contained entry or reverse transcriptase inhibitors that block early steps in virus infection and thus need to be given as a preexposure dose that interferes with sexual practices and may limit compliance. Integrase inhibitors block late steps after virus infection and therefore are more suitable for post-coital dosing. We first determined the kinetics of strand transfer in vitro and confirmed that integration begins about 6 hours after infection. We then used a repeat-challenge macaque model to assess efficacy of vaginal gels containing integrase strand transfer inhibitors when applied before or after simian/human immunodeficiency virus (SHIV) challenge. We showed that gel containing the strand transfer inhibitor L-870812 protected two of three macaques when applied 30 min before SHIV challenge. We next evaluated the efficacy of 1% raltegravir gel and demonstrated its ability to protect macaques when applied 3 hours after SHIV exposure (five of six protected; $P < 0.05$, Fisher's exact test). Breakthrough infections showed no evidence of drug resistance in plasma or vaginal secretions despite continued gel dosing after infection. We documented rapid vaginal absorption reflecting a short pharmacological lag time and noted that vaginal, but not plasma, virus load was substantially reduced in the breakthrough infection after raltegravir gel treatment. We provide a proof of concept that topically applied integrase inhibitors protect against vaginal SHIV infection when administered shortly before or 3 hours after virus exposure.**

## INTRODUCTION

HIV-1 continues to spread globally, highlighting the need to develop efficacious biomedical interventions to limit its transmission. In addition to vaccine development, there are several antiretroviral (ARV) drug–based intervention strategies currently being advanced, including oral preexposure prophylaxis (PrEP) treatment as prevention as well as topical PrEP with gels or other ARV delivery methods such as intravaginal rings and tablets (*1*–*3*). ARV gels have several advantages for HIV prevention. When applied to the mucosal site of virus exposure, gels can rapidly dose tissues with drug concentrations that are much higher than those achieved by oral dosing and with minimal systemic drug exposures and drug toxicities (*3*–*9*). Topical gels are user-controlled and can be optimally formulated with a single drug or in combination for both vaginal and rectal application (*4*–*6*, *10*).

The CAPRISA 004 trial evaluated the safety and effectiveness of a vaginal gel containing 1% tenofovir (TFV) and provided the first evidence of efficacy by this intervention strategy. The study demonstrated a 39% reduction of HIV acquisition in women who applied the gel pericoitally (*1*). Effectiveness was dependent on adherence and was found to increase to 54% in women who reported frequent gel use (>80% adherence) compared to only 28% in women who were <50% adherent. A subsequent study (VOICE) evaluating the same gel formulation in a noncoital daily dosing modality was stopped because of the absence of protection (*2*). TFV testing in plasma revealed poor adherence among participants, likely explaining the lack of efficacy (*3*). These data and similar findings from oral PrEP studies highlight the challenges of identifying intervention strategies that have both high biological efficacy and enhanced user compliance (*4*).

Coitally used topical gels are important HIV prevention methods. However, to date, all such products rely on a preexposure dose that can interfere with sexual practices, and may be limited by partner's acceptance and the need to anticipate sex. We reasoned that a topical product that can be applied by women after sex will have less impact on sexual practices, have better user control, and circumvent the need for sex anticipation and partner acceptance. These advantages can all potentially enhance compliance and effectiveness. However, all topical ARV-based gels in development use entry or reverse transcriptase (RT) inhibitors that block early steps in the virus infection cycle, which raises questions about their suitability for postexposure prophylaxis (PEP) (*1*, *5*–*7*). Macaque models of rectal or vaginal transmission of the simian immunodeficiency virus (SIV) or the chimeric simian/human immunodeficiency virus (SHIV) have provided important data confirming the efficacy of topical gels with RT inhibitors such as TFV and MIV150 when applied before virus challenge, but demonstrated loss or substantial reduction in efficacy when applied 1 to 2 hours after virus challenge (*8*–*10*). These data likely reflect the need to adequately dose mucosal tissues with RT inhibitors before virus exposure and suggest that drugs from different classes that target later steps in virus infection may be more efficacious for PEP.

HIV or SIV integration into the host DNA is the last step before an irreversible infection of a cell takes place. Integration is a multistep process catalyzed by the viral integrase that follows reverse transcription and starts in the cytoplasm with 3′-end processing of viral DNA, continues with translocation of the processed DNA to the nucleus, and is completed by integrating the viral DNA into the host chromosome by a strand transfer mechanism (*11*, *12*). The integration process can

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be inhibited at various stages by three main classes of integrase inhibitors: 3′-processing inhibitors (*13*), strand transfer inhibitors (*14*), and macromolecular complex LEDGF/p75-IN inhibitors (*15*). Strand transfer inhibitors are the most developed and have been validated as potent inhibitors of HIV replication in vitro, ex vivo, in animal studies, and in clinical trials (*16*, *17*). The mechanism of action of strand transfer inhibitors is characterized by their ability to chelate the $Mg^{2+}$ ions in the catalytic core domain of integrase, thereby disrupting the interaction between the integrase/viral complementary DNA preintegration complex and the target DNA (*18*). The only clinically approved integrase inhibitors licensed for the treatment of HIV-infected persons are raltegravir (RAL), elvitegravir, and dolutegravir, all of which are strand transfer inhibitors (*14*, *19*). The compound L-870812 (L-812) is a potent investigational strand transfer inhibitor and has been shown to be effective in reducing SIV replication in macaques (*16*). For PEP, blocking the last step in virus integration by strand transfer inhibitors can be advantageous because it can extend the post-coital dosing window (*14*, *20*). RAL and L-812 are equally potent on both HIV and SIV integrase, with $IC_{50}$ (50% inhibitory concentration) in the nanomolar range (1 to 4 nM), which provides an opportunity to test in macaque models the concept of topical prophylaxis by this class of drugs for both pre- and postexposure protection (*16*, *17*).

Here, we used a repeat low-dose vaginal challenge macaque model to evaluate the efficacy of gels containing L-812 or RAL administered before or after virus challenge, respectively. This well-established macaque model has several advantages, including the use of pigtailed macaques, which have a menstrual cycle and vaginal anatomy similar to women, an inoculum dose with viral RNA levels in the range similar to those detected in seminal fluid, twice-weekly virus challenges to mimic high-risk human exposure, and an $SHIV_{SF162p3}$ inoculum that uses an R5-tropic envelope similar to that of most transmitted HIV (*10*, *21*, *22*). The repeated challenges in this model have the added advantage of increasing statistical power without requiring large groups of animals because they allow measurement of protection over more than one transmission event per animal. We also performed time-of-drug addition studies in vitro to define the window for strand transfer inhibition and to better inform in vivo dosing.

## RESULTS

### Gel formulation and impact of vaginal fluids on antiviral activity

L-812 was successfully formulated in a hydroxyethyl cellulose (HEC) gel at a maximum solubility of 2.3 mg/ml (0.23%). Because of higher water solubility, RAL was formulated in a HEC gel at a concentration of 10 mg/ml (1%). Both gels were clear, viscous, and free of particulates and remained highly active for up to 1 year at room temperature. To investigate whether biological factors present in the vagina, including mucus, enzymes, and microflora, may compromise the stability and potency of RAL gel, we assessed the antiviral activity of RAL gel incubated in cervicovaginal fluids (CVFs). Figure 1 shows that CVF had no impact on RAL activity because no difference in $IC_{50}$ was observed between RAL incubated in CVF ($IC_{50}$ = 2.6 ± 1.1 nM) or saline (1.7 ± 1.2 nM).

### Time-of-drug addition reveals a wide window for inhibition by integrase inhibitors

To better define the timing of reverse transcription and integration and to inform the dosing modality in vivo, we performed time-of-drug addition experiments using single-cycle infections of TZM-bl cells with vesicular stomatitis virus (VSV)–pseudotyped HIV-1. The kinetics of postexposure inhibition by the RT inhibitor TFV was compared with that of RAL. Figure 2 shows that TFV maintained high (>95%) protection up to 2 hours after infection, but was only ~50% protective when added at 5 hours after infection. In contrast, RAL provided high protection (>90%) at 6 hours and remained above 50% protection for up to 10 hours after infection. These data suggest that strand transfer is delayed by at least 4 hours after reverse transcription, providing a longer window to block infection with integrase inhibitors than inhibitors of reverse transcription.

### Preexposure protection by a vaginal integrase inhibitor gel

We first evaluated whether topically applied integrase inhibitors administered before virus challenge protected macaques from SHIV infection. Three pigtailed macaques received vaginal gel containing 0.23% L-812 30 min before vaginal $SHIV_{SF162p3}$ exposure. Infection outcome

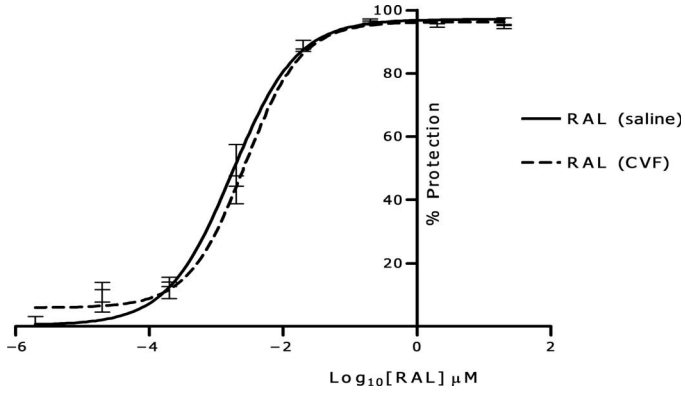


**Fig. 1. Antiviral activity of RAL gel is maintained in the presence of vaginal fluids.** A TZM-bl reporter cell line was used to evaluate antiviral activity ($IC_{50}$) of RAL gel preincubated (24 hours) in saline buffer or CVF. Data are means ± SE of three independent experiments.

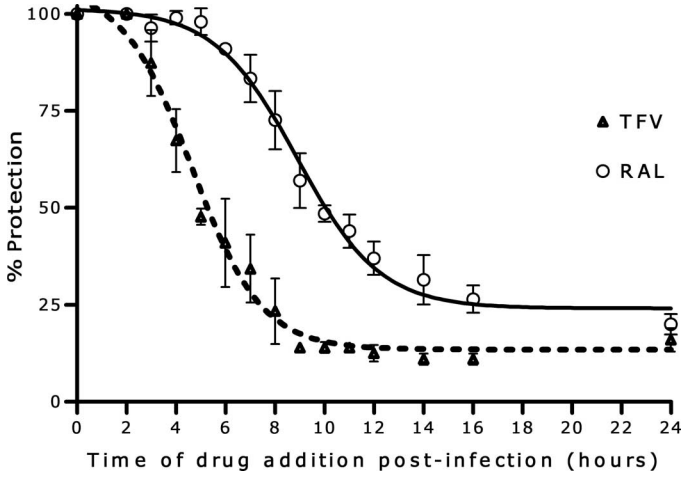


**Fig. 2. Kinetics of the window of inhibition by RT and integrase inhibitors.** Protection of TZM-bl cells from infection with VSV-HIV was evaluated after addition of the RT inhibitor TFV or the integrase inhibitor RAL at the indicated times after infection. Data are means ± SE of three independent experiments.

after 14 twice-weekly vaginal challenges was evaluated against 10 untreated controls receiving placebo gel (1 real-time and 9 historic controls). All controls received the same placebo gel and were challenged with the same virus stock under identical experimental conditions. In this model, pigtailed macaques are more susceptible to infection during the late luteal phase of the menstrual cycle (*10*, *23*). As previously described, the risk for infection was calculated per month or menstrual cycle (*10*). Figure 3A shows the infection rates in animals treated with L-812 and placebo gel. Nine of 10 controls (90%) became infected after 14 challenges over a 2-month period; seven, including the real-time control, were infected after eight challenges (1 month), and two were infected during the second month. In contrast, two of the three macaques treated with L-812 gel remained uninfected after 14 challenges (estimated efficacy = 76%; $P = 0.057$, Fisher's exact test); the breakthrough infection occurred after nine challenges (week 5), whereas both protected animals remained seronegative and had undetectable SHIV sequences in plasma or PBMCs throughout the 10-week follow-up period. L-812 gel efficacy was estimated at 76% on the basis of 9 of 13 infections per months (or cycles) at risk among controls and 1 of 6 for treated animals.

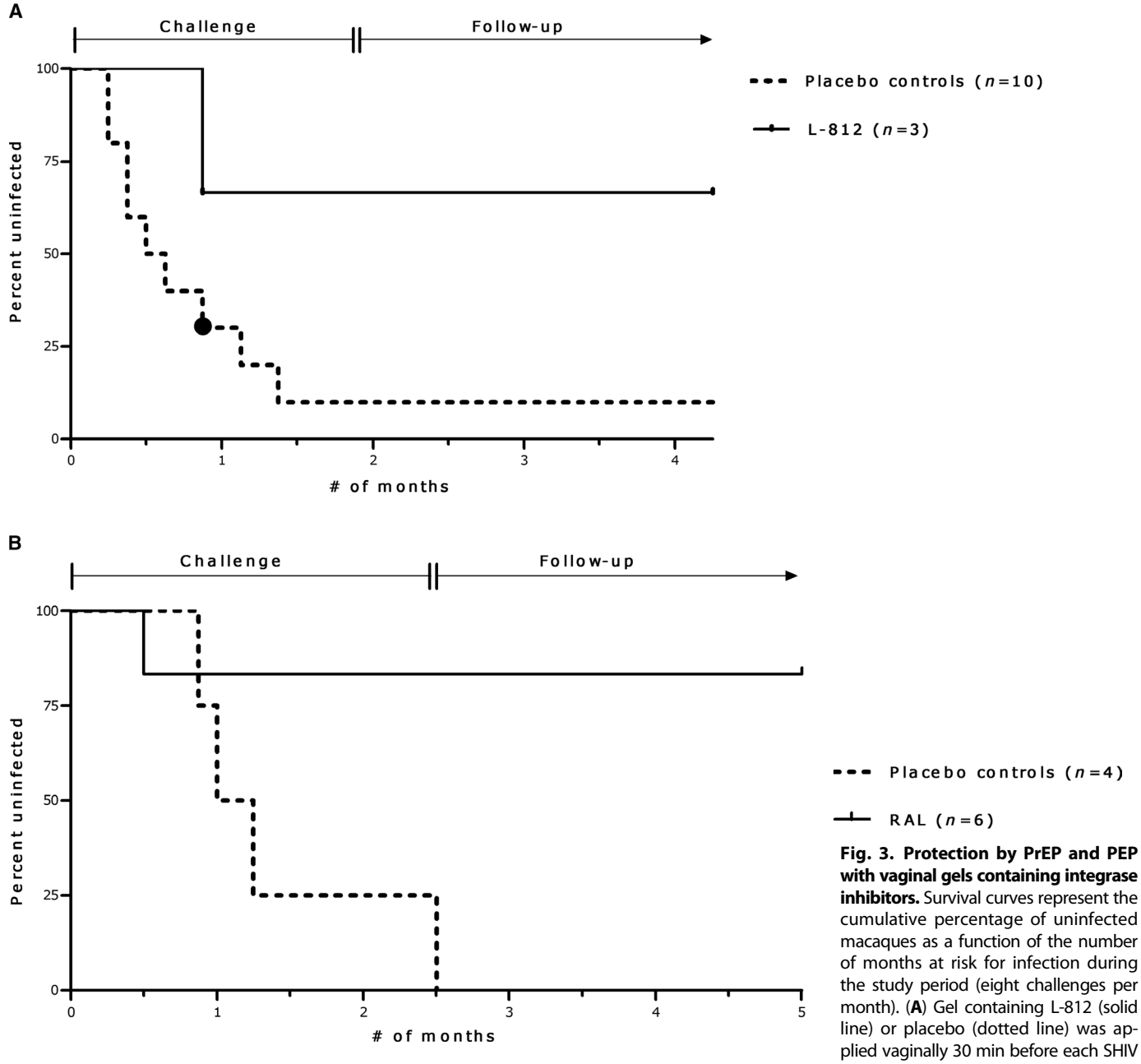


**Fig. 3. Protection by PrEP and PEP with vaginal gels containing integrase inhibitors.** Survival curves represent the cumulative percentage of uninfected macaques as a function of the number of months at risk for infection during the study period (eight challenges per month). (**A**) Gel containing L-812 (solid line) or placebo (dotted line) was applied vaginally 30 min before each SHIV exposure (up to 14 SHIV exposures). Controls include one real-time (solid circle) and nine historic controls. (**B**) Gel containing RAL (solid line) or placebo (dotted line) was applied vaginally 3 hours after SHIV exposure (up to 20 SHIV exposures).

### Postexposure protection by RAL gel

We next explored the efficacy of 1% RAL gel applied 3 hours after vaginal SHIV exposure. The 3-hour dosing interval falls within the dosing window for maximal in vitro inhibition of strand transfer and is behaviorally feasible for post-coital application. Figure 3B shows infection outcomes in macaques that were challenged twice weekly for 2.5 months (about two menstrual cycles) with $SHIV_{SF162p3}$ and administered placebo ($n = 4$) or 1% RAL ($n = 6$) gel 3 hours after each virus challenge. All four macaques that received placebo gel became infected by 10 weeks (20 challenges). In contrast, five of six macaques treated with RAL gel 3 hours after SHIV exposure remained uninfected after 20 SHIV exposures and the 10-week follow-up period. All infected macaques, including the breakthrough infection, exhibited typical SHIV RNA viremia and detectable proviral DNA in PBMCs and seroconverted 1 to 3 weeks after first detectable plasma SHIV RNA was observed. Risk for infection differed by study group ($P < 0.05$, Fisher's exact test), with RAL gel efficacy estimated at 84% on the basis of 4 of 7 infections per months (or cycles) at risk among controls and 1 of 11 for treated animals. Together, these data suggest that topically applied integrase inhibitors can prevent vaginal infection when administered shortly before or up to 3 hours after SHIV exposure.

### Systemic drug concentrations after vaginal gel dosing

Measuring drug exposure in blood is critical to understanding kinetics of drug release and systemic absorption after vaginal gel dosing. Systemic drug exposures are also important for determining impact on acute viremia and emergence of drug resistance in animals that fail gel prophylaxis and continue receiving gel treatment. We measured L-812 and RAL concentrations in plasma 30 min after each gel administration to determine systemic drug exposure at time of challenge and also assessed the impact of the menstrual cycle on vaginal drug absorption. We have previously noted with gels containing TFV or emtricitabine (FTC) that systemic drug exposure peaks within 1 hour after gel dosing and is highly influenced by the menstrual cycle with higher drug concentrations in plasma observed during the progesterone-dominated luteal phase when the vaginal epithelium is thin (*24*, *25*). We analyzed longitudinally both progesterone and drug concentrations in plasma in all macaques that were treated with L-812 or RAL gel (Fig. 4, A and B). With the exception of one (PMl), all macaques experienced at least two menstrual cycles during the study period. Plasma drug concentrations 30 min after vaginal dosing showed similar trends in all animals with substantially higher concentrations consistently detected after peak progesterone. The mean concentration of L-812 and RAL 1 week after peak progesterone (late luteal phase) was 115.3 ± 20.8 ng/ml (range, 74 to 140) and 111 ± 31.3 ng/ml (range, 0 to 260) compared to only 1.5 ± 0.5 ng/ml (range, 0 to 6) and 5.8 ± 4.9 ng/ml (range, 0 to 39.8) 1 week before peak progesterone (late follicular phase). The data confirm that both drugs were rapidly released from the HEC-based gel formulation. The longitudinal analysis of plasma drug concentrations in the two breakthrough infections (PBg-2 and PSz-1) revealed similar peak drug levels and absorption trends to animals that were protected. PSz-1 had detectable RAL in plasma at the estimated time of infection, 7 days (226 ng/ml) to 10 days (259 ng/ml) before the first detectable SHIV RNA. However, PBg-2 had undetectable L-812 at day 7 and 17 ng/ml at day 10. Although limited to only two animals, these results do not show a clear association between drug concentrations in plasma and protection.

### Virus replication in breakthrough infections

We next explored the impact of continued vaginal gel dosing on plasma and vaginal virus shedding in macaques PBg-2 and PSz-1, which became infected despite receiving L-812 or RAL gel, respectively. Infected macaques continued to receive twice-weekly placebo ($n = 5$), L-812 ($n = 1$), or RAL ($n = 1$) gel treatment for up to 8 weeks (16 gel applications) after the first SHIV RNA was detected in plasma. Plasma SHIV RNA detected in both breakthrough infections was evaluated against the five real-time controls. Virus shedding was assessed only in the RAL failure and controls receiving placebo gel. Figure 5A shows the virus load kinetics in both breakthrough infections compared to controls. The peak viremia in PBg-2 (6.08 $\log_{10}$ RNA copies/ml) and PSz-1 (6.7 $\log_{10}$ RNA copies/ml) was similar to the median peak viremia of the five untreated controls (7.01 ± 0.98 $\log_{10}$ RNA copies/ml), showing no difference in acute viremia ($P = 0.29$, Wilcoxon rank sum test). In contrast, Fig. 5B reveals that the peak vaginal SHIV RNA (3.5 $\log_{10}$ copies/ml) and the frequency (4 of 15) of SHIV RNA detected in vaginal fluids were both significantly lower in PSz-1 than in placebo-treated animals that had a median SHIV RNA peak of 5.2 $\log_{10}$ ± 0.42 copies/ml ($P = 0.007$) and frequency of 36 of 60 ($P < 0.0001$), respectively. The reduction in vaginal SHIV shedding could reflect the antiviral activity by RAL gel.

### No evidence of emerging drug resistance after continued gel dosing in breakthrough infections

Because L-812 and RAL were detected in plasma in the breakthrough infections, it was important to determine whether these systemic drug exposures led to drug resistance. Sequence analysis of the integrase region spanning the N-terminal and core domain (amino acids 1 to 234) in virus first detected in plasma showed that both breakthrough infections were initiated with wild-type virus (fig. S1). Plasma specimens collected at peak viremia and up to 8 weeks after infection all revealed wild-type genotypes (Fig. 5A). To examine drug resistance emergence at the site of gel application, we also genotyped SHIV RNA isolated from vaginal secretions in the RAL breakthrough infection (fig. S1). Vaginal fluids with detectable SHIV ($n = 4$) were genotyped, and all found to be wild type (Fig. 5B). Thus, despite twice-weekly vaginal dosing for 8 weeks after infection, no evidence of drug resistance in plasma or genital secretions was observed (fig. S1).

## DISCUSSION

This study provides a proof of concept that topically applied strand transfer integrase inhibitors protect macaques against repeated vaginal SHIV challenges. Protection was observed when the vaginal gel was applied shortly before and, more importantly, 3 hours after virus challenge. The protection by postexposure dosing reflects the advantages of the late-acting strand transfer inhibitors that are needed several hours after virus entry, thus providing an optimal window for post-coital dosing that was previously not feasible with entry or RT inhibitors (*8*, *9*). Because applying gel after sex may interfere less with sexual practices, our data support exploring new post-coital modalities with integrase inhibitors for enhanced acceptance and compliance in women.

We used single-round infections to determine the kinetics of reverse transcription and integration and show that in the HeLa-derived TZM-bl cells, strand transfer starts more than 6 hours after infection, consistent with previous findings in different cell infection systems

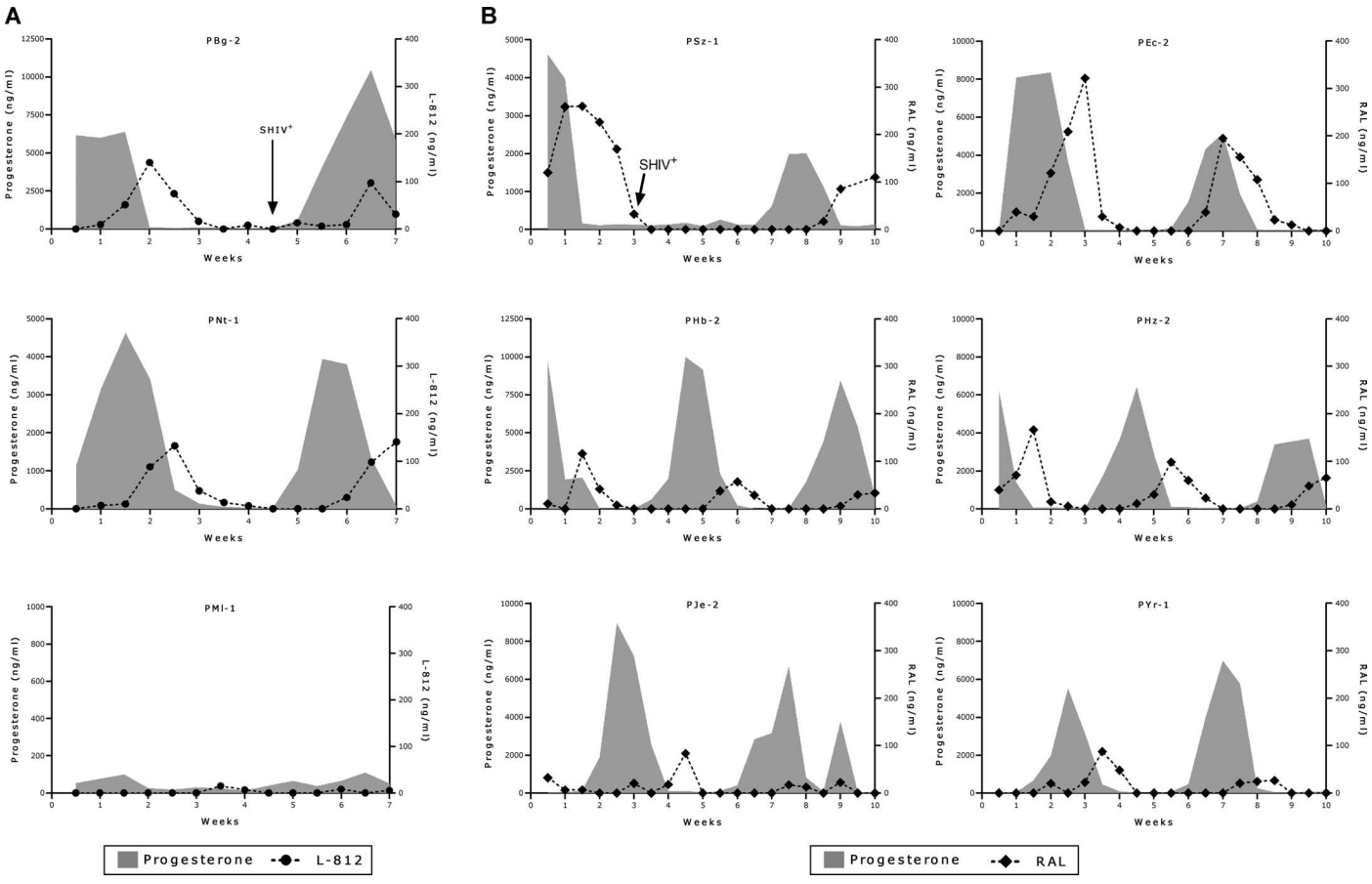


**Fig. 4. Longitudinal analysis of plasma drug and progesterone concentrations in pigtailed macaques dosed with vaginal gels containing integrase inhibitors.** (**A**) Three macaques were dosed twice weekly with L-812 gel (dotted line); shaded areas show progesterone concentrations. (**B**) Six macaques dosed twice weekly with RAL gel (dotted line); shaded areas show progesterone concentrations. All drug concentrations were analyzed from plasma collected 30 min after each vaginal gel application. $SHIV^+$ denotes first detectable SHIV RNA in plasma.

(*21*, *22*, *26*, *27*). The integration step may take longer in less activated primary cells that are commonly present in vivo in which reverse transcription and integration proceed more slowly than in transformed cell lines (*26*, *28*). Thus, it is likely that the dosing window in vivo may be longer than the 3 hours evaluated in this study. Integrase inhibitors, including RAL, are known to bind tightly to preintegration complexes with dissociation half-lives of >7 hours, which ensures longer antiviral activity in infected cells even if tissue drug concentrations subsequently decay to suboptimal levels (*19*). We also note the detection of drug in plasma within 30 min of vaginal gel dosing, which reflects rapid absorption through vaginal tissues. The rapid absorption of RAL and the lack of intracellular activation suggest that the pharmacological lag time for in vivo activity may be minimal. Additional macaque studies of longer postexposure gel applications are needed to further define the post-coital window of protection by strand transfer inhibitors.

We also document in pigtailed macaques changes in plasma drug levels during the menstrual cycle and observe higher concentrations during the progesterone-dominated luteal phase. These results reflect a higher vaginal permeability peaking during the luteal phase likely due to thinner epithelium, increased porosity, changes in mucus composition, and other factors (*23*, *24*, *29*). The influence of the menstrual cycle on vaginal drug absorption also explains the cyclical changes in plasma concentrations of TFV and FTC we have previously observed in pigtailed macaques dosed with gels containing TFV and FTC (*6*). We also examined the relationship between plasma drug concentrations and vaginal efficacy because Cranage *et al.* previously found a positive association between TFV concentration in plasma and the degree of rectal protection by rectal TFV gel (*9*). With only two breakthrough infections, we were unable to discern any trend because one animal had high drug concentrations around the estimated time of infection and one did not. These results likely highlight the added complexity in identifying pharmacological markers of vaginal protection relative to rectal protection and the confounding role of the menstrual cycle modulating both susceptibility to infection and drug permeability (*23*). Nevertheless, the data show the usefulness of the pigtailed macaque model that has a menstrual cycle similar to that of women, and support similar evaluations in women to better define the impact of menstrual cycle and hormonal contraceptives on vaginal pharmacokinetic parameters and efficacy.

Both L-812 and RAL are potent drugs with $IC_{50}$ in nanomoles; however, we found that the concentrations in gels that conferred high, yet incomplete, in vivo protection were in the micromolar range, or about 1 million–fold higher than those concentrations needed for

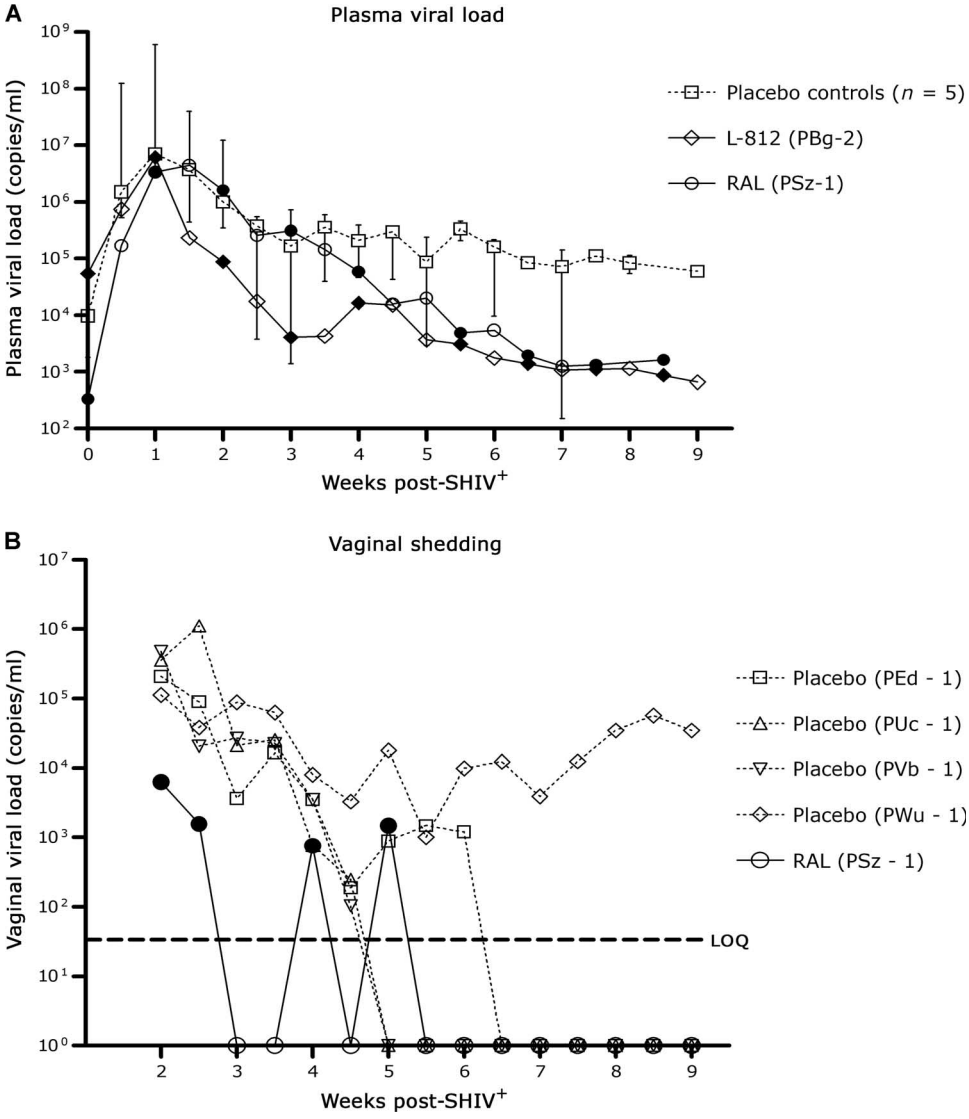


**Fig. 5. Plasma and vaginal virus loads in macaques with breakthrough infection and controls.** (**A**) Individual virus load kinetics in breakthrough infections under continued twice-weekly dosing with gel containing L-812 (diamonds) or RAL (circles) compared to median virus loads of controls ($n$ = 5, dotted black line). Time 0 indicates first detection of SHIV in plasma. (**B**) Individual virus load kinetics in vaginal secretions collected twice weekly before dosing with gel containing RAL (solid line; large circles) or placebo ($n$ = 4, black dotted lines). Filled symbols indicate samples sequenced for drug resistance in plasma and vaginal fluids. The broken line denotes the limit of quantitation of the virus load assay (50 copies/ml).

in vitro inhibition. The higher drug concentrations required for in vivo efficacy have been consistently observed with other gels containing entry or RT inhibitors and likely reflect the distribution kinetics of the drug through the mucosa to achieve concentrations needed to protect the large surface area of the vagina for a significant period of time (*6*, *7*, *10*). Previous work with TFV gels in macaques has shown that the pharmacologically active TFV-diphosphate (TFV-DP) concentrations in vaginal lymphocytes predict in vivo efficacy, and topical dosing by gel or intravaginal ring that achieved TFV-DP concentrations exceeding the in vitro $IC_{95}$ values in these lymphocytes was associated with complete protection (*10*, *30*). Thus, it is tempting to surmise that a similar pharmacological correlate will also apply to RAL. However, confirming this correlate will require measuring intracellular RAL concentrations in vaginal lymphocytes, which could be challenging because unlike intracellularly bound TFV-DP, RAL may diffuse out during the cell purification process as extracellular RAL concentrations decrease. Thus, alternative tissue testing methods are needed.

The potential for drug resistance to develop when prophylaxis fails raises concerns, especially with drugs that are also used for treatment. Prolonged systemic treatment of SHIV-infected macaques with L-812 has been found to select drug-resistant variants with the integrase mutation (N155H) (*20*). We show no evidence of drug resistance emergence in plasma from either the L-812 or RAL breakthrough infection despite continued twice-weekly gel dosing for up to 8 weeks after infection. Although larger numbers of animals are needed to define incidence of resistance in animals failing prophylaxis, the finding of wild-type SHIV suggests that both gel failures are not due to a selection of a low-frequency drug-resistant variant in the challenge virus, and likely reflects insufficient selective pressure by the systemically absorbed drug from the twice-weekly gels. These systemic drug exposures are much lower than those observed from oral dosing and likely contribute to lower risks of drug resistance (*20*). Indeed, we found no reduction in the acute viremia in both breakthrough infections compared to controls, further supporting the minimal systemic antiviral activity. These findings are similar to TFV gel breakthrough infections but differ from those in macaques that fail oral PrEP in which reductions in virus loads and selection of drug-resistant variants have been observed (*7*, *9*). Given that the antiviral activity from gels remains largely concentrated in the vaginal compartment, we also evaluated the viral population in the genital secretions from the RAL breakthrough infection. We detected only wild-type SHIV, but noted that, in contrast to plasma virus load, vaginal virus shedding was substantially reduced in this macaque. The suppression of virus shedding may reflect the antiviral activity from gel dosing, which may also have reduced drug resistance selection. These data are only from one animal and require confirmation, but they do point to broader implications because reductions in vaginal virus shedding during acute infection may lower transmissibility.

Our study has several limitations. The virus challenges did not include cell-associated virus and were done in the absence of semen or semen-derived factors shown to enhance HIV infection in vitro (*18*). However, recent data suggest little impact of semen on vaginal SIV

transmission in rhesus macaques (*31*). Also, SHIV exposures were made on intact vaginal mucosa without trauma associated with coitus, concurrent genital ulcers, or bacterial vaginosis, all of which can increase the risk of HIV acquisition (*26*, *27*). As previously noted, only two breakthrough infections were available to study vaginal SHIV shedding and drug resistance, and thus, these observations require confirmation in larger numbers of animals.

In conclusion, we show preclinical in vivo data that support the use of topical integrase inhibitors for HIV prevention. We highlight the advantage of using this class of drugs in new post-coital modalities that were previously not possible with entry or RT inhibitors. This study supports evaluation of this class of drug for HIV prevention, including small molecules that do not overlap with clinically approved integrase inhibitors in optimized gels or other dosage forms such as vaginal rings or tablets (*28*, *32*, *33*). The study also supports further evaluation of post-coital modalities in women for enhanced acceptability and compliance.

## MATERIALS AND METHODS

### Gel formulations

L-812 and RAL were provided by Merck and the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, respectively. L-812 was formulated at 0.23% (w/w) in a 2% HEC gel containing 20% propylene glycol, 0.02% propylparaben, 0.18% methylparaben, and 0.02% EDTA. RAL was formulated at 1% (w/w) in a 2% HEC gel containing 0.017 M phosphate-buffered saline (PBS) (pH 7.4), 0.02% propylparaben, 0.18% methylparaben, and 0.02% EDTA. A 2% HEC gel formulated without drug was used as a placebo control. Gels were stored at room temperature, and antiviral activity was confirmed throughout the course of the study with a TZM-bl reporter assay to measure drug susceptibility.

### Virus stock

$SHIV_{SF162P3}$ (SIVmac239 backbone with an HIV-1 subtype B, CCR5-tropic envelope) was obtained from the NIH AIDS Research and Reference Reagent Program and was propagated in peripheral blood mononuclear cells (PBMCs) from pigtailed macaques as previously described (*34*, *35*). The virus stock titer was calculated in pigtailed macaque PBMCs and diluted to the challenge dose of 10 $TCID_{50}$ (median tissue culture infectious dose) ($1.5 \times 10^6$ RNA copies per exposure).

### Antiviral activity and stability of RAL gel

TZM-bl cells (NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH) were used to determine the $IC_{50}$ of gels containing integrase inhibitors, and data were analyzed by GraphPad Prism (v5.02) software. For stability testing, 1% RAL gel (20 mM) was diluted 10-fold in PBS or macaque CVF and incubated overnight at 37°C. RAL-saline and RAL-CVF were then serially diluted (20-0 μM) in complete medium, and 100 μl was added to wells of a 96-well plate in triplicate. Complete medium (50 μl) containing TZM-bl cells ($3 \times 10^6$/ml) was plated in each well. After 15 min of incubation, 50 μl of $SHIV_{SF162p3}$ stock, diluted to a final concentration resulting in a signal of about $10^5$ relative light units, was added to each well. After 48 hours of incubation at 37°C, 100 μl of medium was removed and replaced with 100 μl of Bright-Glo (Promega Corp.), and the luminescence was measured with PerkinElmer Victor X2 Multilabel Plate Reader. Inhibition was determined on the basis of reduction from the virus-only control and presented as the percentage of cells protected (mean ± SEM).

### Kinetics of postexposure inhibition

Time-of-drug addition experiments were carried out with single-cycle infections to determine the inhibition window for RT and integrase inhibitors. TFV or RAL was added to infected TZM-bl cells at concentrations exceeding $IC_{99}$ (200 μM TFV or 20 μM RAL) at different times after infection (0 to 24 hours). Briefly, TZM-bl cells were preincubated with VSV-pseudotyped HIV-1 NL4-3-ΔE-EGFP (VSV-HIV) for 1 hour to establish infection. A VSV-pseudotyped virus was used to ensure that all reported infectivity was derived from a single round of infection. Cells were washed twice with PBS and treated with 0.25% trypsin (Cellgro) to remove all cell-bound and cell-free viruses. Infected cells were resuspended in complete medium and plated ($3 \times 10^4$ per well) in triplicates in a 96-well plate. Drug was added at 1-hour time increments up to 24 hours. The percentage of cells protected at each time point was determined and plotted as % cells protected ± SEM relative to time when drug was added after infection.

### Efficacy studies

The efficacy of integrase inhibitors against vaginal transmission was evaluated in female pigtailed macaques under conditions similar to those described in other studies (*6*, *10*). Macaques were administered gel (3 ml) vaginally and challenged with a low-dose $SHIV_{SF162P3}$ inoculum (10 $TCID_{50}$) twice weekly (every 3 to 4 days) for up to 10 weeks (about two menstrual cycles). All animals were anesthetized and remained recumbent for 30 min after each gel application and 15 min after virus inoculation to minimize leakage. For preexposure efficacy measurements, macaques received placebo ($n = 1$) or 0.23% L-812 ($n = 3$) gel vaginally 30 min before each vaginal SHIV exposure (up to 14 challenges). For postexposure efficacy, anesthetized animals were challenged with SHIV (up to 20 challenges) and remained incumbent for 15 min after receiving the virus to minimize leakage and then returned to their cages. After 3 hours, macaques were anesthetized and administered 3 ml of placebo ($n = 4$) or 1% RAL ($n = 6$) gel. For each study, blood was collected 30 min after gel dosing to monitor for drug levels and SHIV infection. Virus challenges were stopped when a macaque became SHIV RNA-positive ($SHIV^+$) in plasma. All infected macaques continued to receive study gel (placebo, L-812, or RAL) twice weekly for up to 8 weeks after infection. All experiments were done under highly controlled conditions by the same personnel with the same virus stock, inoculum dose, and procedures as described in previous studies (*6*). These studies adhered to the *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, 1996); all procedures were approved by the Institutional Animal Care and Use Committees of both the Centers for Disease Control and Prevention (CDC) and the Yerkes National Primate Research Center, Emory University.

### Monitoring systemic infection, virus shedding, and drug resistance

Systemic infection was monitored twice weekly by screening for SHIV RNA in plasma with a quantitative real-time reverse transcription polymerase chain reaction (PCR) assay with a sensitivity of 50 RNA copies/ml, as previously described (*6*, *34*, *36*). The estimated time of infection was defined as 7 to 10 days before the first SHIV-positive specimen to account for the eclipse period between infection and detection of SHIV

RNA in plasma. Infected macaques were monitored for emergence of drug resistance in both plasma and CVF by standard sequence analysis of SIVmac239 integrase reference sequence base pairs 3624 to 4332 (amino acids 1 to 234). CVF specimens were collected by instilling and recollecting about 5 ml of sterile PBS in the vaginal cavity. CVFs were centrifuged for 15 min at 400*g* to pellet cells and debris. Virion-associated RNA was extracted from CVF supernatant (1 ml) with a Qiagen viral RNA kit. PCR amplification of PBMC proviral DNA was done with primers and probes specific for SIVmac239 pol (*6*, *34*). Serologic testing was performed with a synthetic peptide enzyme immunoassay (Genetic Systems HIV-1/HIV-2 Plus O; Bio-Rad). Animals were considered protected if they tested negative for SHIV RNA in plasma and SHIV DNA in PBMCs and remained seronegative during the challenge period of the study and the 10-week follow-up in the absence of challenge and gel application.

### Measurement of drug concentrations in plasma

L-812 and RAL levels in plasma were measured in macaques 30 min after vaginal administration of 0.2% L-812 and 1% RAL gel, respectively. Briefly, drug was extracted from 100 μl of plasma by protein precipitation with 350 μl of methanol containing 200 ng of the unstudied analyte as the internal standard (RAL was used as an internal standard for L-812 and vice versa). Supernatant containing the drug from precipitation was evaporated to near dryness under vacuum and then resuspended in high-performance liquid chromatography (HPLC) buffer A (9.9 mM acetic acid, 5.9 mM ammonium hydroxide, and 9.4 mM formic acid in $H_2O$). Drug concentrations were analyzed by HPLC–tandem mass spectrometry method with a Shimadzu Prominence HPLC (Shimadzu Scientific) system equipped with a 150 × 2.0–mm Ascentis Phenyl column (Sigma-Aldrich) and an AB Sciex 3200 QTRAP mass spectrometer. Chromatography was done with a linear gradient of 20 to 90% buffer B (acetonitrile containing 9.9 mM acetic acid, 5.9 mM ammonium hydroxide, and 9.4 mM formic acid). Mass spectrometer was run in positive MRM (multiple reaction monitoring) mode, and the following transitions were monitored: for RAL, mass/charge ratio (*m/z*) 445.2/109.0 and 445.2/361.5, and for L-812, *m/z* 354.1/109.0 and 354.1/203.0. The assay had a lower limit of quantification of 5 ng/ml for both analytes and standard curve $R^2$ values greater than 0.99.

### Menstrual cycle and drug absorption monitoring

A longitudinal assessment of drug absorption in relation to menstrual cycle was done by measuring plasma drug and progesterone levels 30 min after vaginal gel application throughout the challenge period for each animal and each cycle. Plasma samples collected twice per week (every 3 to 4 days) were analyzed for drug levels as described above, and progesterone levels were measured at the University of Wisconsin National Primate Research Center with an enzyme immunoassay (*37*).

### Statistical methods

Fisher's exact test was used to compare the number of infections per number of months at risk by study group. Risk for infection per month, or approximately per menstrual cycle, with biweekly challenge doses throughout each month, considers that cycling pigtailed macaques are more susceptible to infection during the luteal phase of the menstrual cycle as previously demonstrated in this model (*23*). Intervention efficacy was calculated as $1 - (p_1/p_0)$, where $p_1$ and $p_0$ denote the proportion of months with incident infection for intervention and control animals, respectively (*38*). SAS version 9.2.1 or GraphPad Prism 5 for Windows (GraphPad Software, http://www.graphpad.com) was used for all statistical analyses.

## SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/6/227/227ra35/DC1
Fig. S1. Analysis of SHIV integrase amino acid sequences in breakthrough infections.

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