

HIV

A long-acting integrase inhibitor protects female macaques from repeated high-dose intravaginal SHIV challenge

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Long-acting GSK1265744 (GSK744 LA) is a strand transfer inhibitor of the HIV/SIV (simian immunodeficiency virus) integrase and was shown to be an effective preexposure prophylaxis (PrEP) agent in a low-dose intrarectal SHIV (simian-human immunodeficiency virus) rhesus macaque challenge model. We examined the pharmacokinetics and efficacy of GSK744 LA as PrEP against repeat high-dose intravaginal SHIV challenge in female rhesus macaques treated with Depo-Provera (depot medroxyprogesterone acetate), which promotes viral transmission vaginally. When Depo-Provera-treated female rhesus macaques were dosed with GSK744 LA (50 mg/kg) monthly, systemic and tissue drug concentrations were lower than previously observed in male rhesus macaques. GSK744 concentrations were fivefold lower on average in cervical tissues than in rectal tissues. Eight female rhesus macaques were treated with GSK744 LA at week 0, and four female rhesus macaques served as controls. All animals received a high-dose challenge of SHIV162P3 at week 1. No infection was detected in GSK744 LA-treated rhesus macaques, whereas viremia was detected 1 to 2 weeks after SHIV challenge in all control animals. The GSK744 LA-treated rhesus macaques were given a second administration of drug at week 4 and further challenged at weeks 5 and 7. GSK744 LA treatment protected six of eight female rhesus macaques against three high-dose SHIV challenges, whereas all control animals became infected after the first challenge ($P = 0.0003$, log-rank test). These results support further clinical development of GSK744 LA for PrEP.

INTRODUCTION

The use of antiretroviral (ARV) drugs as preexposure prophylaxis (PrEP) has been shown to be an effective strategy for preventing HIV-1 acquisition. Daily oral emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) as PrEP prevented HIV-1 acquisition in men who have sex with men in the iPrEx trial (1). In addition, FTC/TDF and TDF alone prevented infection in heterosexual men and women in the Partners PrEP and the TDF2 trials (2, 3). Finally, TDF alone reduced HIV infection by 49% in injection drug users in the Bangkok Tenofovir Study (4). Studies performed exclusively in women have demonstrated mixed results. CAPRISA 004 using 1% tenofovir gel before and after sex demonstrated protection (5), whereas no protection was observed with daily regimens in the FEM-PrEP (oral FTC/TDF) or the VOICE trials (oral FTC/TDF or TDF, or tenofovir gel) because of low adherence to study drug (6, 7). Together, these studies have demonstrated that the efficacy of a given PrEP regimen is proportional to the degree of adherence to the intervention. We hypothesized that long-acting (LA) ARV formulations requiring less frequent dosing may improve both adherence and PrEP efficacy across groups at high risk for HIV-1 infection.

GSK1265744 (GSK744), an analogue of dolutegravir, is a potent integrase strand transfer inhibitor with physicochemical properties that permit nanomilling of the crystalline free acid to a median particle size of 200 nm in the presence of surfactant, polymer, mannitol, and water for injection (8). The resulting nanoparticles are essentially 100% ac-

tive drug and formulated as a GSK744 LA (200 mg/ml) injectable suspension. The same formulation is under evaluation in multiple clinical studies (8). In healthy volunteers, single GSK744 LA injections were well tolerated; the most common adverse event reported was pain at the injection site (9). The apparent terminal half-life ($t_{1/2}$) of GSK744 LA ranged from 21 to 50 days, compared to about 40 hours for oral GSK744 (8, 10). The long $t_{1/2}$ of GSK744 LA makes it suitable for administration every 3 months in the clinic.

We previously demonstrated that GSK744 LA is an effective PrEP agent against repeated low-dose intrarectal simian-human immunodeficiency virus (SHIV) challenge (11) in a model that was developed to more closely mimic human exposure and infection by HIV-1 via unprotected receptive anal intercourse (12–15). Our results showed that monthly administration of GSK744 LA beginning 1 week before intrarectal inoculation provided complete protection against eight weekly challenges (11). In a follow-up experiment, we defined the correlate of protection against intrarectal inoculation in this model as GSK744 plasma concentrations $>3\times$ protein-adjusted IC_{90} (PAIC₉₀) resulting in 100% protection; concentrations $\geq 1\times$ PAIC₉₀ yielded 97% efficacy (11). These plasma concentrations are readily achievable in humans with quarterly 800-mg intramuscular injections (11). These data support the preclinical evaluation of GSK744 LA as PrEP in other transmission models. Because about half of new HIV-1 transmissions occur in heterosexual women (16), and rectal prevention efficacy cannot predict efficacy against vaginal transmission, we evaluated the protective efficacy of GSK744 LA using an intravaginal challenge model in rhesus macaques.

Unlike the rectum, which is composed of a single layer of columnar epithelium, the vagina is a multilayered stratified squamous epithelium with a thickness that varies during the menstrual cycle (17).

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The multilayered epithelium provides a more substantial physical barrier to infection during repeated exposure to foreign materials as part of its normal function. Intravaginal HIV-1 transmission is typically studied in the rhesus macaque (*Macaca mulatta*) or pigtail macaque (*Macaca nemestrina*). Rhesus macaques are typically seasonal breeders, and the physical barrier of the macaque genital tract requires up to 10,000-fold more cell-free simian immunodeficiency virus (SIV) to infect 100% of macaques via the vagina compared to an intravenous route (18). To increase the susceptibility of macaques to vaginal infection, pretreatment with progesterone has been used to thin the vaginal and ectocervical epithelium (19, 20). The Depo-Provera (depot medroxyprogesterone acetate) macaque model has been used to assess the efficacy of vaccines, neutralizing antibodies, ARVs, and vaginal microbicides against challenge with both SIV and SHIV (including SHIV162P3) (21–26). SHIV162P3 challenge has been most recently used in studies of topically applied ARVs, including maraviroc (27–31). Protection in these studies was time-dependent, with protection waning as time increased between microbicide application and viral challenge (31). The Depo-Provera rhesus macaque model results in infection of >90% of control animals after one challenge. Conversely, pigtail macaques have a menstrual cycle similar to humans and can be infected in a repeat low-dose vaginal challenge model that has been established to resemble vaginal HIV-1 transmission without Depo-Provera pretreatment (32, 33). The variables relevant to vaginal SIV/SHIV susceptibility in the rhesus macaque and pigtail macaque models have been discussed (34). The use of pigtail macaques is limited by the low number of breeding facilities in the United States. Because our primary data were generated in male rhesus macaques, we evaluated the efficacy of GSK744 LA at preventing vaginal SHIV infection in a stringent high-dose female rhesus macaque model pretreated with Depo-Provera to ensure efficient infection of control macaques. These data are complementary to the results provided by Radzio *et al.* (35) demonstrating complete protection by GSK744 LA against SHIV162P3 low-dose intravaginal challenge in pigtail macaques. Together, these data support further testing of GSK744 LA as PrEP in high-risk women.

RESULTS

Pharmacokinetic evaluation of GSK744 LA in Depo-Provera-treated rhesus macaques

We previously evaluated the pharmacokinetic (PK) profile of GSK744 LA in male rhesus macaques and established that monthly dosing with 50 mg/kg maintained plasma concentrations similar to those achieved in healthy human volunteers dosed intramuscularly with 800 mg of

GSK744 LA quarterly (11). Here, we first explored the impact of Depo-Provera pretreatment on the GSK744 PK profile. Female rhesus macaques ($n = 8$) were treated with Depo-Provera on weeks –3 and 2 and with GSK744 LA (50 mg/kg) on weeks 0 and 4. Mean plasma GSK744 concentrations in Depo-Provera-treated female rhesus macaques were lower than the mean plasma GSK744 concentrations observed in male rhesus macaques that protected against low-dose intrarectal challenge (Fig. 1A). When compared to male rhesus macaques, Depo-Provera-treated female rhesus macaques had comparable drug exposures after the first GSK744 LA dose ($AUC_{Dose1} = 2047 \pm 772 \mu\text{g} \times \text{hour/ml}$ and $1698 \pm 455 \mu\text{g} \times \text{hour/ml}$, respectively; $P = 0.27$, Mann-Whitney two-tailed t test) (Table 1); however, the mean plasma trough concentrations (C_t) were lower in the female macaques after the first dose ($2.66 \pm 1.07 \mu\text{g/ml}$ and $1.20 \pm 0.74 \mu\text{g/ml}$, male and female, respectively; $P = 0.007$, Mann-Whitney two-tailed t test). After the second dose of GSK744 LA, a 38% lower AUC was observed in the female macaques compared to that in the male macaques ($AUC_{Dose2} = 1616 \pm 591 \mu\text{g} \times \text{hour/ml}$ and $2593 \pm 648 \mu\text{g} \times \text{hour/ml}$, respectively; $P = 0.02$, Mann-Whitney two-tailed t test), whereas a lower but not statistically different C_t was observed ($1.29 \pm 0.59 \mu\text{g/ml}$ and $2.62 \pm 1.66 \mu\text{g/ml}$, female and male, respectively; $P = 0.08$, Mann-Whitney two-tailed t test). PK studies in male rhesus macaques established that

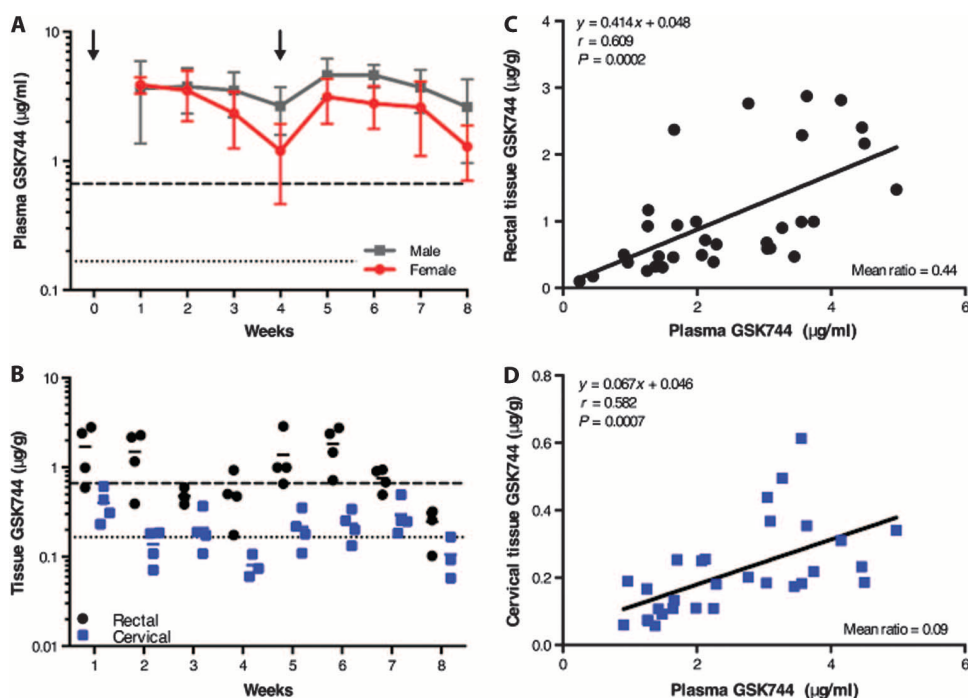


Fig. 1. GSK744 LA plasma PK profile and tissue distribution in Depo-Provera-treated female rhesus macaques. Eight female rhesus macaques were injected intramuscularly with 30 mg of Depo-Provera on weeks –3 and 2 and with GSK744 LA (50 mg/kg) on weeks 0 and 4. (A) GSK744 plasma concentrations from Depo-Provera-treated female rhesus macaques (red) were compared with those from male rhesus macaques (gray) dosed with GSK744 LA (50 mg/kg) on weeks 0 and 4. Means \pm SD are shown. Dotted and dashed horizontal lines represent 1 \times and 4 \times PAIC₉₀, respectively. (B) Rectal and cervical tissue distribution of GSK744 was assessed from pinch biopsies each week in a subset ($n = 4$) of Depo-Provera-treated rhesus macaques. Each symbol represents tissue concentrations from an individual macaque. Solid lines represent the mean for the group. Dotted and dashed horizontal lines represent 1 \times and 4 \times PAIC₉₀, respectively. (C and D) Correlation of (C) rectal tissue GSK744 or (D) cervical tissue GSK744 and plasma GSK744 concentrations. Each symbol represents the simultaneous plasma and tissue concentrations of an individual macaque.

Table 1. PK of GSK744 in rhesus macaques dosed with GSK744 LA (50 mg/kg). ND, not determined.

GSK744 LA dose	AUC (0–τ) (μg × hour/ml)		C _τ (μg/ml)		C _{max} (μg/ml)		T _{max} (day)*		t _{1/2} (day) [†]
	1	2	1	2	1	2	1	2	2
50 mg/kg (male)	2047 (772) [‡]	2593 (648) ^{‡§}	2.66 (1.07) ^{‡§}	2.62 (1.66)	4.53 (2.12)	5.17 (1.20) ^{‡§}	14 (7–28)	42 (35–56)	8.6 (3.7)
50 mg/kg (female), Depo-Provera–treated (PK study)	1698 (455) [¶]	1616 (591)	1.20 (0.74)	1.29 (0.59)	4.21 (0.84) [¶]	3.47 (1.45)	10.5 (7–21)	38.5 (35–49)	ND
50 mg/kg (female), Depo-Provera–treated (challenge study)	1247 (236)	1587 (415)	0.85 (0.51)	1.39 (0.75)	3.03 (0.71)	3.45 (0.98)	14 (7–21)	42 (35–49)	9.6 (7.8)

*T_{max} reported in median (range) and not subjected to statistical analyses. †t_{1/2} estimated for the second dose only because terminal phases were long enough to estimate parameter. ‡P < 0.05 when compared with female challenge study. §P < 0.05 when compared with female PK study. ¶P < 0.05 when compared with female challenge study. ||t_{1/2} was not estimated for PK study because sufficient time points were not collected to estimate parameter.

dosing with GSK744 LA (50 mg/kg) would maintain plasma concentrations $\geq 3 \times$ PAIC₉₀ throughout dosing, a value that correlated with 100% protection in that model. However, in two Depo-Provera–treated female macaques, three C_τ measurements were $< 3 \times$ PAIC₉₀ (fig. S1).

Tissue penetration of GSK744

ARVs exhibit differential mucosal tissue penetration (36–39), a characteristic we believe to be highly relevant to the PK profile of a potential PrEP agent. We determined the GSK744 distribution in rectal and cervical tissues. In Depo-Provera–treated macaques, GSK744 penetrated rectal tissue more efficiently than cervical tissue (Fig. 1B). As observed in male macaques (11), higher plasma concentrations correlated with higher rectal tissue concentrations (Fig. 1C). In Depo-Provera–treated female macaques, the mean rectal tissue/plasma (T/P) ratio was 0.44 (range, 0.14 to 1.43). To make certain that rinsing rectal tissues in saline before GSK744 concentration analysis had no effect on drug concentration determinations, pinch biopsies ($n = 16$ to 20) from each animal at each time point were processed in parallel with half of the biopsies subjected to a saline rinse. A linear correlation (slope = 0.88) was observed between processing methods (fig. S2), indicating similar GSK744 values in rectal tissues irrespective of the processing method. The mean T/P ratio in unwashed rectal tissues was 0.37 (range, 0.15 to 1.13). These values were not statistically different ($P = 0.6$, Mann-Whitney two-tailed t test). GSK744 concentrations were analyzed from two cervical biopsies from each individual animal at each time point (fig. S3), and the mean concentrations were calculated (Fig. 1, B and D). As with rectal tissue, cervical tissue GSK744 concentrations increased with plasma GSK744 concentrations (Fig. 1D). The mean cervical T/P ratio was 0.09 (range, below detection to 0.20). In some animals, GSK744 concentrations were not detectable in cervical tissues collected at weeks 4 and 8, thus not allowing for the determination of the T/P ratio.

PrEP efficacy of GSK744 LA

Although systemic GSK744 concentrations were lower than observed in the male rhesus macaques and GSK744 penetrated the cervical tissue with less efficiency than rectal tissue in Depo-Provera–treated female rhesus macaques, we nevertheless performed a challenge experiment to determine the efficacy of protection against SHIV infection at the observed GSK744 tissue concentrations. Twelve rhesus macaques were injected with Depo-Provera on weeks –3 and 2, and eight of the rhesus macaques were subsequently administered GSK744 LA (50 mg/kg) on

week 0, with the remaining four rhesus macaques serving as controls (Fig. 2A). Both groups of rhesus macaques were challenged by non-traumatic inoculation with high-dose 300 TCID₅₀ (median tissue culture infectious dose) SHIV162P3 on week 1. Control rhesus macaques became infected during the first challenge, with viral RNA (vRNA) detected 1 week ($n = 3$) to 2 weeks ($n = 1$) after inoculation (Fig. 2, B and C). GSK744 LA–treated rhesus macaques remained aviremic after the first challenge and were subsequently dosed with GSK744 LA on week 4 and similarly challenged on weeks 5 and 7 (Fig. 2A). All GSK744 LA–treated rhesus macaques remained aviremic throughout the challenge phase of the experiment. Plasma vRNA was detected in two GSK744 LA–treated macaques (FG95 and FM26), 10 and 14 weeks after the first GSK744 LA dose and 3 and 7 weeks after the last viral challenge, respectively (Fig. 2C). The GSK744 LA–treated rhesus macaques had an 8.8-fold (hazard ratio; 95% confidence interval, 7.7 to 1019.0) lower risk of infection compared with control rhesus macaques ($P = 0.0003$, log-rank test). At the time of each challenge, plasma GSK744 concentrations were 3.06, 2.93, and 2.22 μg/ml (13.3× to 18.4× PAIC₉₀) and 2.47, 3.02, and 2.07 μg/ml (12.4× to 18.1× PAIC₉₀) for FG95 and FM26, respectively (Fig. 2D). Plasma GSK744 concentrations at the time of first vRNA detection were 0.15 μg/ml ($< 1 \times$ PAIC₉₀) and 0.37 μg/ml ($\approx 2.2 \times$ PAIC₉₀) for FG95 and FM26, respectively (Fig. 2D), with cervical tissue concentrations expected to be ~10% of plasma concentrations (Fig. 1D). In the six aviremic rhesus macaques, GSK744 plasma concentrations fell below the $1 \times$ PAIC₉₀ between weeks 9 and 16. Plasma vRNA, proviral DNA, and anti-SHIV antibodies were not detected in the six aviremic rhesus macaques through week 24.

In the GSK744 LA–treated macaques that became infected, the peak viral loads (2.81×10^5 and 1.16×10^7 vRNA copies/ml plasma, FG95 and FM26, respectively) were comparable to those measured in the control rhesus macaques (mean, 9.39×10^5 vRNA copies/ml plasma). Anti-SHIV antibodies were detected in the two infected GSK744 LA–treated rhesus macaques 2 weeks after vRNA detection compared with 2 to 3 weeks in the control rhesus macaques. In all infected rhesus macaques, treated and controls, proviral DNA and vRNA became detectable simultaneously (table S1).

The t_{1/2} of GSK744 LA in Depo-Provera–treated female rhesus macaques in the challenge experiment was 9.6 ± 7.8 days, which was similar to 8.6 ± 3.7 days ($P = 0.70$, Mann-Whitney two-tailed t test) previously observed in male rhesus macaques treated with GSK744 LA (50 mg/kg) (Table 1). In the challenge experiment, lower AUC_{Dose1}

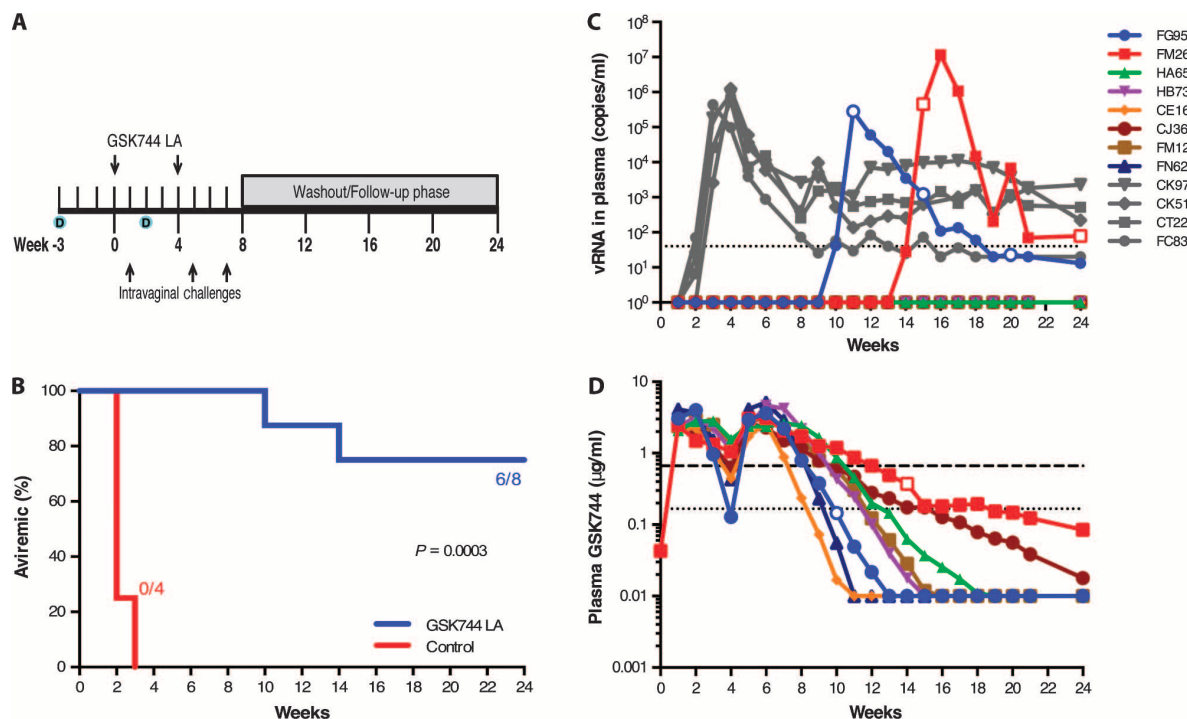


Fig. 2. Monthly injections of GSK744 LA protect rhesus macaques against three intravaginal SHIV challenges. (A) Study design. Twelve female rhesus macaques were injected intramuscularly with 30 mg of Depo-Provera on weeks -3 and 2 . Eight rhesus macaques were injected intramuscularly in the quadriceps with GSK744 LA (50 mg/kg) at two time points, weeks 0 and 4 . Four Depo-Provera-treated rhesus macaques served as controls. All animals were challenged intravaginally on week 1 with 300 TCID₅₀ of SHIV162P3. GSK744 LA-treated rhesus macaques were further challenged on weeks 5 and 7 . All rhesus macaques were followed for 24 weeks.

and $C_{\max(\text{Dose}1)}$ were observed compared to the PK experiment in female rhesus macaques pretreated with Depo-Provera (Table 1). The same rhesus macaques were used in both experiments with a 5-month washout phase between studies. Overall, these PK data demonstrate that there was some degree of inter- and intra-animal variability. With the exception of the GSK744 plasma C_{τ} at week 4 , the PK profiles obtained in the Depo-Provera-treated rhesus macaques corresponded well with plasma concentrations observed in healthy human volunteers dosed intramuscularly with 800 mg of GSK744 LA (fig. S4).

Integrase resistance mutants not identified in breakthrough infections

Hypothetically, when ARVs used as PrEP fail to prevent transmission, drug-resistant viruses may either establish infection or emerge because of viral replication in the presence of subinhibitory drug concentrations. We performed consensus sequencing of the integrase-coding region 1 week after the first detectable viremia (weeks 11 and 15 for FG95 and FM26, respectively) and at subsequent time points as indicated by open symbols in Fig. 2C. Consensus sequencing at the time of detectable viremia revealed infection by SHIV162P3 virus lacking known resistance-conferring amino acid substitutions to integrase inhibitors (fig. S5). One mutation, E198G, was identified in FG95 at week 20 . This E198G mutation did not decrease viral susceptibility to GSK744 (table S2).

(B) Kaplan-Meier plot of GSK744 LA-treated and control rhesus macaques remaining aviremic after three intravaginal SHIV challenges. (C) Viral loads of control rhesus macaques (in gray) and GSK744 LA-treated rhesus macaques (in color). Open symbols represent samples sequenced for the integrase-coding region. Dotted line represents the limit of quantitation (LOQ), >40 SHIV RNA copies/ml plasma. (D) Plasma PK of individual GSK744 LA-treated rhesus macaques throughout the study. Open symbols represent drug concentrations at the time of first vRNA detection. Dotted and dashed horizontal lines represent $1\times$ and $4\times$ PAIC₉₀, respectively. LOQ >0.01 $\mu\text{g/ml}$.

We further analyzed the integrase-coding region 1 week after the first detectable viremia using single-genome analysis. About 30 single genomes from each infected GSK744 LA-treated rhesus macaque were analyzed, providing 95% confidence that integrase variants comprising $\geq 10\%$ of the total virus population were assessed (40). Thirty-one and 29 single genomes were obtained from FG95 (week 11) and FM26 (week 15), respectively. Two nonsynonymous mutations were identified in the viral population from FG95 but none in the viral population from FM26. The two mutations identified in plasma from FG95 (P142S and I210V) were found on different genomes, and both were only identified once. These mutations did not decrease the susceptibility of FG95 to GSK744 in vitro (table S2).

Transmitted/founder virus analyses

To investigate the diversity of the viral populations that infected the rhesus macaques, we compared the number of transmitted/founder (T/F) viruses that established infection in the drug-treated and control rhesus macaques. The diversity of the SHIV162P3 challenge stock was characterized by generating 25 *env* sequences (3102 nucleotides), using single-genome analysis as previously described (26). The SHIV162P3 challenge stock used in this study had a mean diversity of 0.24% (range, 0 to 0.52%), which is within the range of other challenge stocks used in such studies (24, 26, 41). From six infected rhesus macaques, 159 (median, 26 sequences per rhesus macaque; range, 24 to 30) full-length

env sequences were amplified from plasma collected within 1 week of first detectable viremia (table S3). Analyzing at least 20 plasma vRNA sequences provided 95% confidence that variants representing $\geq 15\%$ of the viral population were sampled (40). The minimum number of T/F variants was reported, but it is possible that some variants were not detected by single-genome analysis. A maximum likelihood phylogenetic tree was generated illustrating the relationships among SHIV162P3 challenge stock sequences and variants from each infected rhesus macaque (Fig. 3). The two GSK744 LA-treated rhesus macaques were each infected with a minimum of 1 T/F variant resulting in a median of 0 T/F variants in the GSK744 LA-treated group ($n = 8$), compared with a minimum of 1 and up to 8 T/F variants in the control rhesus macaques ($n = 4$; median T/F, 2; $P = 0.006$, Mann-Whitney two-tailed t test) (fig. S6). The viral eclipse phase length may correlate with the number of T/F variants (42). The control rhesus macaque that became infected with 1 T/F variant had an eclipse phase of 2 weeks, compared with an eclipse phase of 1 week in the rhesus macaques that were infected with >1 T/F variant (table S3). The number of animals infected in this study was small; however, the number of T/F variants correlated well with what was previously observed for this model (24, 26). The transmission in GSK744 LA-treated animals limited the variants to one founder virus, suggesting a pharmacodynamic bottleneck to virus infection and subsequent transmission.

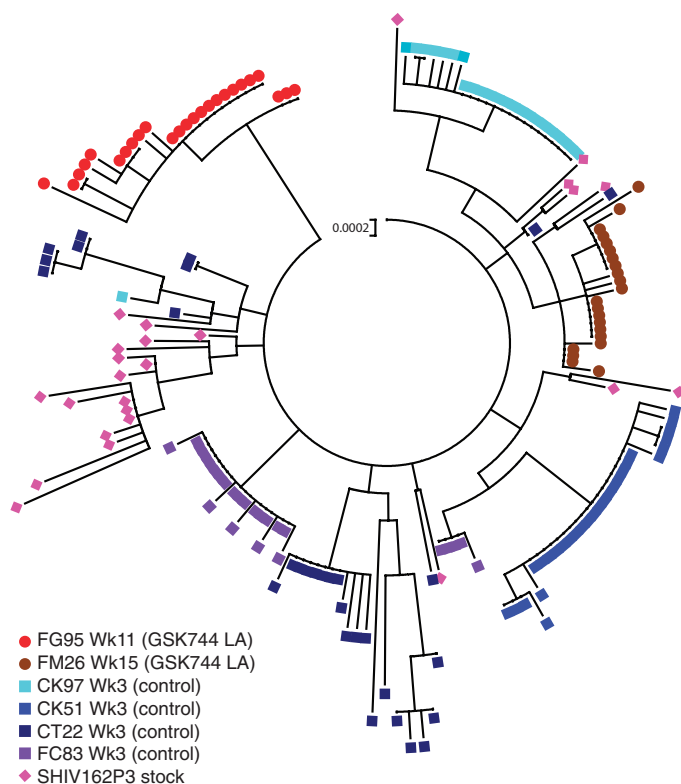


Fig. 3. Phylogram of SHIV162P3 sequences. Maximum likelihood phylogenetic tree of SHIV162P3 sequences from viral stock and from each of the six infected rhesus macaques. Hypermutated sequences were excluded. Red and brown circles represent sequences from GSK744 LA-treated rhesus macaques, squares represent sequences from control rhesus macaques, and pink diamonds represent SHIV162P3 viral stock. The vertical scale bar represents 0.0002 nucleotide substitutions per site.

DISCUSSION

Here, we investigated the PK and protective efficacy of GSK744 LA as a PrEP agent in a high-dose vaginal challenge model. Female rhesus macaques were dosed with GSK744 LA (50 mg/kg), a dose identified in male rhesus macaques that provided plasma concentrations similar to those observed in humans (11). Overall, lower systemic exposures were observed in the Depo-Provera-treated female rhesus macaques, which may be due to a drug interaction between Depo-Provera and GSK744, or perhaps a potential gender difference in the absence of Depo-Provera. PK parameters appeared to be more influenced by the second dose of Depo-Provera, including AUC_{Dose2} , $C_{t(Dose1)}$, and $C_{max(Dose2)}$ (Table 1). GSK744 is metabolized via the hepatic glucuronidation pathway, primarily by UGT1A1, with some involvement from UGT1A9 (43). Studies have shown that UGT1A1 expression is up-regulated by high progesterone concentrations via a nuclear receptor, pregnane X receptor (44). Thus, the lower systemic concentration of GSK744 may be due to increased metabolism induced by UGT1A1. That said, we do not believe that this will be a significant factor in clinical trials of GSK744 LA in women because progesterone exposures in macaque models are an order of magnitude higher than those seen in women treated with progesterone-containing birth control agents (45). However, detailed drug-drug interaction studies between GSK744 and progesterone-containing birth control agents will be a component of the drug development path.

In addition to lower systemic concentrations observed in the Depo-Provera-treated female rhesus macaques, GSK744 penetrated the cervical tissue less efficiently than it penetrated rectal tissue. However, although data are limited, tissue drug concentrations in human studies suggest that GSK744 penetrates cervical tissue slightly more efficiently than rectal tissue (46). The discrepancy in the relative GSK744 mucosal penetration may be due to species differences or possibly the influence of Depo-Provera pretreatment, highlighting perhaps one limitation of this animal model. Previous studies with CCR5 inhibitors (CMPD167 and maraviroc) have shown lower drug concentrations in vaginal tissue and vaginal fluid after drug administration via vaginal rings in Depo-Provera-treated rhesus macaques compared with rhesus macaques that were not pretreated with Depo-Provera (47).

The efficacy of GSK744 LA as PrEP was demonstrated in a stringent high-dose intravaginal SHIV challenge model. Protection was observed in six of the eight GSK744 LA-treated rhesus macaques against three high-dose challenges, whereas all control rhesus macaques became infected after one challenge. We did not identify known drug resistance-associated mutations in the integrase-coding region of the viruses establishing infection in the GSK744 LA-treated animals, and we believe that drug-sensitive virus established infection. The failure of GSK744 LA as PrEP in this model can be explained by a combination of factors including the low cervical tissue penetration of drug (about 10% of plasma concentration on average), the high viral inoculum used in the presence of Depo-Provera, and the mechanism of action of the integrase inhibitor. Because of the high inocula used in this model, it is likely that virus enters a number of susceptible cells locally resulting in the generation of more reverse-transcribed SHIV DNA as compared to the low-dose challenge models (11, 35). Coupled with the low tissue penetration of drug, proviral DNA may have either integrated immediately or perhaps persisted preintegration until drug decreased to concentrations permitting integration. Subsequent reductions in drug concentrations then could have allowed infection to first spread locally

and then disseminate, by which time the viremia would be readily detectable. The drug effect is consistent with a genetic bottleneck in the T/F virus analysis and also accounts for the delay observed between the time of virus challenge and the subsequent appearance of viremia.

Questions remain regarding the validity of the high-dose challenge model in the rhesus macaque (33). It could be argued that the challenge doses used in this model simulating vaginal infection with HIV-1 are higher than the inoculum that women are exposed to during intercourse and could therefore underestimate the potential efficacy of the candidate being tested. The result that GSK744 LA was at least 90% protective in this model may indeed underestimate its potential, at least quantitatively, and the animal models using lower inocula would suggest that this is the case (35). It is worth noting that nearly one-half of HIV-1 transmission events could be ascribed to a sex partner with acute HIV-1 infection (48), when the blood and genital tract HIV-1 burden is the highest (49). The high-dose challenge model may also reflect HIV-1 acquisition in several high-progesterone situations including pregnancy and during injectable contraceptive use (50). This model also may mimic an important stage of the menstrual cycle when the risk of HIV/SHIV infection increases (51, 52). The relevance of this model is further supported by recent data showing that only one virus, or perhaps a few viruses, is transmitted (24), a biological feature mirroring what happens in HIV-1-infected women (40) and rhesus macaques not pretreated with Depo-Provera (53). It is notable, however, that unlike the low-dose intrarectal challenge model in which we were able to determine a systemic threshold concentration of drug at which protection is maintained, this model does not allow for such an experimental design.

In summary, our findings, along with the complementary results provided by Radzio *et al.* (35) demonstrating complete protection by GSK744 LA against SHIV162P3 low-dose intravaginal challenge in pigtail macaques, suggest that GSK744 LA is suitable for clinical testing as a next-generation PrEP agent. Its activity ranged from highly to completely protective in animal models simulating HIV-1 transmission. In addition, early-phase clinical trials suggest that the drug is well tolerated, and its PK in man suggests that it is amenable to episodic use. Although LA formulations have improved adherence to drug regimens in a variety of settings including contraception (54), schizophrenia (55), and male hypogonadism (56), clinical trials must be performed to determine whether LA formulations will improve adherence to ARV agents used as PrEP while still demonstrating safety and efficacy. Phase 2 clinical trials to assess the safety and acceptability of GSK744 LA are under way, and should these studies prove successful, then large phase 3 efficacy studies will soon follow.

MATERIALS AND METHODS

Study design

The PK study was designed as a single-arm study in eight female Indian rhesus macaques dosed with 50 mg/kg split into four 12.5 mg/kg injections. The primary endpoints were drug concentrations in plasma, cervical tissue, and rectal tissue at time points as described below. The study was descriptive and therefore not powered. A total of eight animals were treated. To permit tissue healing, an animal was biopsied every 2 weeks. Staggering the biopsies yielded tissue drug concentration results from four animals at each time point.

The intravaginal SHIV162P3 challenge study was a two-arm study that included 12 female Indian rhesus macaques treated with Depo-Provera; 8 were treated with GSK744 LA as described below and 4 were controls. Animal handlers and laboratory staff performing assays were aware of each animal's assigned study arm throughout the course of the study. Assuming that 100% of challenges would result in infection in the control animals, the study had 100% power to detect a 90% effective PrEP agent, using Fisher's exact *t* test with a *P* value of 0.05. The primary endpoint of the challenge study was the presence or absence of SHIV162P3 infection in all SHIV162P3-exposed animals on the basis of the presence or absence of plasma viremia as determined by real-time reverse transcription polymerase chain reaction (RT-PCR) as described below. Secondary endpoints included qualitative determinations of proviral DNA in peripheral blood mononuclear cells (PBMCs), presence of anti-SHIV antibodies in plasma, characterization of viral populations in GSK744 LA-treated animals that became infected for evidence of resistance to GSK744, and phylogenetic characterization of the SHIV162P3 stock and T/F viruses in all infected animals.

PK evaluation of GSK744 LA in rhesus macaques

Eight Indian rhesus macaques (*M. mulatta*) were treated intramuscularly with 30 mg of Depo-Provera on weeks -3 and 2 and GSK744 LA on weeks 0 and 4. GSK744 LA is a 200 mg/ml nanosuspension that was administered on the basis of body weights measured at the time of dosing (5.3 to 8.2 kg) with the 50 mg/kg dose split into four 12.5 mg/kg injections, two per quadriceps. Blood was collected weekly from all rhesus macaques (*n* = 8), and rectal and cervical tissues were biopsied from a subset of rhesus macaques (*n* = 4) each week permitting a 2-week period between biopsies. Rectal biopsies (*n* = 16 to 20 pieces of tissue per time point) were collected using 1.5 mm × 3 mm forceps, and cervical biopsies were collected from the ectocervix (*n* = 2 pieces of tissue per time point) using 2 mm × 4 mm forceps.

Efficacy of GSK744 LA in preventing intravaginal SHIV transmission

The efficacy of GSK744 LA against vaginal SHIV transmission was evaluated in 12 rhesus macaques treated with 30 mg of Depo-Provera intramuscularly on weeks -3 and 2 and GSK744 LA (50 mg/kg) on weeks 0 and 4. Rhesus macaques were challenged by applying 300 TCID₅₀ of SHIV162P3 in 1 ml to the vaginal vault, using a gastric feeding tube. Rhesus macaques remained recumbent for at least 15 min after inoculation. Challenges were performed on the same day with the same viral stock and inoculation method. The R5 tropic SHIV162P3 (57, 58) challenge stock was expanded and titrated in rhesus macaque PBMCs before this study. The SHIV162P3 challenge stock had a titer of 5180 TCID₅₀/ml by the method of Reed and Muench (59). Systemic infection was monitored weekly for 24 weeks by detection of SHIV RNA in plasma, using real-time RT-PCR assay with a sensitivity of 40 SHIV RNA copies/ml plasma as previously described (11). PBMC proviral DNA amplification was performed as previously described (11). Five replicates of each sample (0.6 to 1 µg per replicate) were analyzed. Virus-specific antibody responses were measured using a synthetic peptide enzyme immunoassay as per manufacturer's instructions (Genetic Systems HIV-1/HIV-2 Plus O, Bio-Rad). The Institutional Animal Care and Use Committee (IACUC) of Tulane National Primate Research Center (TNPRC) approved all studies. TNPRC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care

(AAALAC no. 000594). The TNPRC Office of Laboratory Animal Welfare (OLAW) assurance number is A4499-01 and U.S. Department of Agriculture registration number is 72-R-0002.

Analysis of GSK744 concentrations in plasma and tissues

Whole blood was collected using K2EDTA tubes, centrifuged to obtain plasma, and stored at -80°C until analysis. Tissues were rinsed in saline (unless indicated), blotted dry, weighed, snap-frozen, and stored at -80°C until analysis. GSK744 concentrations were monitored by high-performance liquid chromatography–tandem mass spectrometry after protein precipitation with acetonitrile containing [$^{13}\text{C}^{15}\text{N}_2\text{H}_2$]GSK744 as an internal standard as previously described (11). The calibration range for GSK744 was 10 to 10,000 ng/ml for plasma or 2.5 to 1000 ng/ml for tissues. PK analyses were performed using WinNonlin Professional software (version 5.2, Pharsight Corp.) as previously described (11).

Integrase sequence analysis

Consensus integrase sequences were generated as previously described (11). Briefly, integrase-coding sequences were amplified from cell-free virus using Phusion Hot Start Flex DNA Polymerase (NEB) as per manufacturer's instructions with primers macIN.F1 and macIN.R1. The product was used for nested PCR with primers macIN.F3 and macIN.R3. Second-round PCR products were sequenced by Genewiz Inc. using primers macIN.Fseq and macIN.Rseq, and sequence analysis was performed using Geneious software (version 7.1.4). The same procedure was followed for single genome analysis using complementary DNA (cDNA) diluted to provide $\leq 30\%$ positive reactions representing sequences derived from a single cDNA molecule. Second-round PCR products were sequenced by Genewiz Inc. using second-round primers and sequencing primers.

Construction of single-cycle SIV recombinant viruses and determination of susceptibility to GSK744

To determine the susceptibility of selected viral variants to GSK744, a novel assay was developed to, first, create single-cycle recombinant viruses with the SIV backbone and, then, to measure drug susceptibility in vitro. Inactivation of *env* expression and deletion of the integrase-coding region in full-length SIVmac239 provirus (239-FL SpX, provided by R. Desrosiers) were achieved by ligating its two PCR products, a short fragment [1610 base pairs (bp)] and a long fragment (10,196 bp). The short fragment was produced by PCR with primers F.dIN [5'-CTAGTTAGTCAAGGGCCCCATTTAAGGTCG-GATG-3' (4499–4513 fused with 5466–5485 in SIVmac239)] and 239.1R [5'-ACAAGAACATGCTAGTCTCATTGACCAT-3' (7056–7034)]. The long fragment was produced by PCR with primers 239.2F [5'-TGAGACTAGCATGTTCTTGTATAGCCCAGG-3' (7041–7066)] and R.dIN [5'-CCCTTGACTAACTAGGTGGTC-TATTTCTT-3' (4513–4485)], with the addition of CATG at the Spe I site within *env*, as underlined, introducing a stop codon resulting in a truncated Env protein. The two fragments were ligated using Gibson Assembly Master Mix (NEB) and transformed into STBL2 cells (Life Technologies). A clone, Gi-3, was obtained and used to generate single-cycle recombinant viral variants with an SIV backbone.

Pseudotyped virus with the desired integrase-coding region was obtained by first linearizing Gi-3 by digestion with PspOM I. The second-round PCR product from a limiting-dilution nested PCR assay generating cDNA of the integrase-coding region as detailed above was either reamplified with the same primer pair of macIN.F3 and macIN.R3

(product size of 1053 bp) or amplified with a primer pair of macIN.F4 [5'-CTAGTTAGTCAAGGGATTAGACAAGTTCTCTTC-3' (4499–4531)] and macIN.R4 [5'-GACCTTAAATGGGGCACA-TAGCAAAC-3' (5480–5454)] (product size of 982 bp), which overlap with linearized Gi-3 by 45 or 54 bp and 15 or 13 bp at the 5' or 3' end, respectively. Gel-purified PCR products were ligated with linear Gi-3, using Gibson Assembly Master Mix, and the resultant recombinants and pCI-VSV-G were cotransfected onto human embryonic kidney 293T cells, using jetPRIME (Polyplus transfection, VWR). Culture supernatants were collected 48 hours after transfection and filtered, and aliquots were stored at -150°C for subsequent use. p27 concentrations of viral stocks were measured using RETROtek, SIV p27 Antigen ELISA (ZeptoMetrix).

Sensitivity to GSK744 was assessed using TZM-bl cells (1×10^4 per well) plated overnight in a 96-well plate. Cells were incubated with 20 μg of polybrene (Sigma-Aldrich) in 100 μl of Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 30 min at 37°C and then incubated with either no drug or serially diluted GSK744 for 30 min at 37°C followed by the addition of pseudotyped virus (5 ng of p27 per well). After 48 hours, β -galactosidase expression was measured using Galacto-Star as per manufacturer's instructions (Applied Biosystems). The light signal was measured using MLX Microtiter Luminometer (Dynex Technologies). All assays were performed in triplicate, and the results were analyzed using GraFit 5 (Erithacus Software) with four-parameter fitting. The GSK744 concentration inhibiting 90% luminescence (IC_{90}) was calculated as the concentration of GSK744 that inhibited 50% luminescence (IC_{50}) $\times 9^{1/s}$, where s is the slope of the fitted curve.

Single-genome analysis for T/F analysis

Viral RNA was extracted as described above. cDNA was generated as previously described using primer 11-SIVmac239-R1 (5'-CTGTAA-TAAATCCCTTCCAGTCC-3') (26). cDNA was serially diluted and plated for nested PCR reactions amplifying the 3.1-kb *env* as described using primers 7-F1-SIVsm/macTatF1 (5'-CCTCCCCCTCCAGG-ACTAGC-3') and 11-SIVmac239-R1 for the first round and primers 5-R2-BKSIVsm/macEnvR261 (5'-ATGAGACATRTCTATTGCAATTTGTA-3') and 6-F2-*env*B5in (5'-TTAGGCATCTCC-TATGGCAGGAAGAAG-3') for the second round. cDNA dilutions were identified yielding $\leq 30\%$ positive reactions (26). PCR products were sequenced by Genewiz Inc. using primers Rev14 (5'-ACCATGTTATTTT-TCCACATGTAAA-3'), For13SHIV (5'-CAGAAAGAGCAGAAGAC-AGTGG-3'), Rev1200 (5'-GTGCTTGCTTATATCTCTATTATTTCIC-3'), For15 (5'-CAGCACAGTACAATGTACACATGGAA), For16SHIV (5'-TTTAATTGTTGGAGGGGAATTTTCTA-3'), For17 (5'-AGCAGCAG-GAAGCACTATGGGGCG-3'), For18Bal (5'-CATAACAAAATGGCTGT-GGTATAT-3'), and For19Bal (5'-GGAGCCITGTTCCITTCAGCTACC-3'). Single sequences from each sample were aligned using Geneious software. MEGA (version 5.0) predicted the best model of nucleotide substitution for *env* sequences from each animal was Hasegawa, Kishino, and Yano. Phylogenetic trees were inferred by maximum likelihood analyses with 500 bootstrap replicates and rooted with SHIV162P3 *env* consensus sequence. Viral diversity was calculated by maximum composite likelihood methods.

Statistical methods

PK parameters and tissue processing methods were compared using Mann-Whitney two-tailed t test. The log-rank test was used to discern

statistical differences between GSK744 LA-treated and control macaques. The hazard ratio was estimated by the log-rank model. All statistical analyses were performed using GraphPad Prism software (version 6.0).

SUPPLEMENTARY MATERIALS

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Fig. S1. Plasma PK of individual GSK744 LA-treated rhesus macaques throughout the PK study.

Fig. S2. Correlation of rectal tissue GSK744 concentrations by processing method before freezing.

Fig. S3. Correlation of cervical tissue GSK744 concentrations from individual samples.

Fig. S4. GSK744 plasma concentrations from Depo-Provera-treated rhesus macaques compared with humans.

Fig. S5. Consensus sequence analysis of SHIV integrase-coding regions from plasma of infected GSK744 LA-treated macaques.

Fig. S6. Minimum number of T/F variants was estimated from plasma collected within 1 week of detection of viremia.

Table S1. Summary of time of detection for plasma vRNA, proviral DNA, and anti-SHIV antibodies (Ab).

Table S2. Susceptibility of single-cycle recombinant viruses with the integrase-coding regions of SHIV162P3 viral stock, FM26, FG95, and mutants to GSK744.

Table S3. Env single-genome analysis summary.

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A long-acting integrase inhibitor protects female macaques from repeated high-dose intravaginal SHIV challenge

Chasity D. Andrews, Yun Lan Yueh, William R. Spreen, Leslie St. Bernard, Mar Boente-Carrera, Kristina Rodriguez, Agegnehu Gettie, Kasi Russell-Lodrigue, James Blanchard, Susan Ford, Hiroshi Mohri, Cecilia Cheng-Mayer, Zhi Hong, David D. Ho and Martin Markowitz

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Taking a Shot at HIV

HIV-1 transmission during vaginal intercourse remains a major public health issue. Antiretrovirals can prevent HIV-1 transmission when used as preexposure prophylaxis, but results are highly dependent on patient adherence to prescribed therapy. One possible solution to this limitation is the use of long-acting injectable agents. Andrews *et al.* used an animal model that simulates the heterosexual transmission of HIV-1 to women and found that GSK744 LA, a long-acting integrase inhibitor amenable to dosing every 3 months in humans, is highly protective against viral transmission. These results support further clinical trials in women at risk for HIV-1 infection.

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