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In-Press Preview

Long-acting antiretroviral agents for pre-exposure prophylaxis (PrEP) represent a promising new alternative to daily oral regimens for HIV prevention. Lenacapavir (LEN) is a first-in-class long-acting capsid inhibitor approved for the treatment of HIV-1 infection. Here, we assessed the efficacy of LEN for PrEP using a single high-dose simian-human immunodeficiency virus (SHIV) rectal challenge macaque model. In vitro, LEN showed potent antiviral activity against SHIV, similar to HIV-1. In macaques, a single subcutaneous administration of LEN demonstrated dose proportional increases in and durability of drug plasma levels. A high-dose SHIV inoculum for the PrEP efficacy evaluation was identified via virus titration in untreated macaques. LEN-treated macaques were challenged with high-dose SHIV 7 weeks post drug administration and the majority remained protected from infection as confirmed by plasma PCR, cell-associated proviral DNA, and serology testing. Complete protection and superiority to the untreated group was observed among animals whose LEN plasma exposure exceeded its model-adjusted clinical efficacy target at the time of challenge. All infected animals had subprotective LEN concentrations and showed no emergent resistance. These data demonstrate effective SHIV prophylaxis in a stringent macaque model at clinically relevant LEN exposures and support the clinical evaluation of LEN for HIV PrEP in humans.

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Long-acting lenacapavir acts as an effective pre-exposure prophylaxis in a rectal SHIV challenge macaque model

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Abstract

Long-acting antiretroviral agents for pre-exposure prophylaxis (PrEP) represent a promising new alternative to daily oral regimens for HIV prevention. Lenacapavir (LEN) is a first-in-class long-acting capsid inhibitor approved for the treatment of HIV-1 infection. Here, we assessed the efficacy of LEN for PrEP using a single high-dose simian-human immunodeficiency virus (SHIV) rectal challenge macaque model. In vitro, LEN showed potent antiviral activity against SHIV, similar to HIV-1. In macaques, a single subcutaneous administration of LEN demonstrated dose proportional increases in and durability of drug plasma levels. A high-dose SHIV inoculum for the PrEP efficacy evaluation was identified via virus titration in untreated macaques. LEN-treated macaques were challenged with high-dose SHIV 7 weeks post drug administration and the majority remained protected from infection as confirmed by plasma PCR, cell-associated proviral DNA, and serology testing. Complete protection and superiority to the untreated group was observed among animals whose LEN plasma exposure exceeded its model-adjusted clinical efficacy target at the time of challenge. All infected animals had subprotective LEN concentrations and showed no emergent resistance. These data demonstrate effective SHIV prophylaxis in a stringent macaque model at clinically relevant LEN exposures and support the clinical evaluation of LEN for HIV PrEP in humans.
Introduction

HIV/AIDS continues to pose a major public health threat globally, particularly in low- and middle-income countries. In the absence of a curative therapy or an effective prophylactic vaccine other modes of HIV infection prevention are critical to combating the HIV/AIDS epidemic. Daily oral pre-exposure prophylaxis (PrEP) has proven highly effective at preventing new infections when taken as instructed. However, out of 1.2 million people with PrEP indications in the US, less than 25% use PrEP as of 2020 and coverage remains uneven across racial and ethnic groups, age groups, and for transgender persons (1). Low uptake is driven, in part, by the challenges associated with medication adherence crucial for the prophylactic effect of the daily oral PrEP, social stigma and health system demands. Less frequent dosing alternatives can help address the unmet medical need among those not benefiting from currently available PrEP options (2). Long-acting PrEP agents may increase convenience, lower stigma associated with daily pill taking, reduce clinical visits, and hence, increase adherence and outcomes.

HIV capsid inhibitors represent a novel class of highly potent antivirals with biophysical and biochemical properties which make them amenable to long-acting formulation (3). Lenacapvir (LEN) is a potent and selective multi-stage inhibitor of HIV-1 capsid function with demonstrated safety and antiviral efficacy following twice-yearly subcutaneous dosing in people living with HIV (4). Based on these data, LEN has been approved for the treatment of multidrug-resistant HIV-1 infection in combination with other antiretroviral(s) in multiple countries.

Macaque models for PrEP have been used to evaluate the efficacy of antiretroviral agents against various modes of virus transmission for over 2 decades (5-8). These models have been used to establish drug correlates of protection, defined prophylactic windows of protection post-exposure and informed the design of clinical PrEP trials. Clinical trial data have in turn validated the high predictive value of the macaque models for PrEP (9). We previously reported that GS-CA1, a structural analog of LEN exhibiting the same capsid-dependent multistage mechanism of action, provided significant protection against new SHIV infection in a repeat rectal challenge macaque model (10). The use of GS-CA1, whose plasma half-life in macaques is shorter as compared to LEN, allowed us to efficiently examine protection
against repeat challenges across a wide range of drug exposures. However, a limitation of the repeat SHIV challenge model is its reliance on an assumption regarding the infection-to-detection lag time, generally 1-2 weeks, to estimate protective drug levels. A long-acting antiretroviral drug can suppress viral replication resulting in a diagnostic delay of a new infection, and hence inability to establish drug exposure at the time of seeding of an infection. To overcome this limitation in the current study, we set out to characterize LEN’s pharmacokinetic profile in rhesus macaques and evaluate its prophylactic efficacy at clinically relevant drug exposures using a high-dose single rectal challenge model. We confirmed that similarly to GS-CA1, LEN offered significant protection against SHIV challenge in macaques. After mathematically adjusting for the lower potency of LEN against SHIV in macaques compared to HIV-1 in humans, we observed complete protection against infection at drug exposures at or above the minimum clinical target achievable with twice-yearly subcutaneous injectable LEN.

Results

In vivo SHIV titration to select high challenge dose

While a relatively low virus inoculum paired with multiple transmission events provides a physiologically relevant preclinical model to evaluate PrEP efficacy, the determination of actual drug exposure at the time of infection is confounded by the inability to precisely establish the timing of infection using existing analytical tools. To circumvent this challenge while evaluating the PrEP efficacy of LEN, we opted to perform a single “high-dose” challenge study. To this end, a SHIV.SF162.P3 stock expanded in activated rhesus PBMCs (50% Tissue Culture Infectious Dose, TCID₅₀ = 1.024 x 10⁴/mL) was titrated in vivo over 5 rounds of increasing rectal mucosal exposure. Eight rhesus macaques per round were challenged with titers ranging from 0.625 to 100 TCID₅₀ in order to select a “high” dose yielding at least 50% infection rate per challenge (Table 1). Plasma viral loads were quantified weekly to monitor the infection status and calculate infectivity at each inoculum level (Figure 1). Peak viremia was reached by week 2 or 3 post challenge and began to decline by week 4, at which point all animals confirmed SHIV-positive were placed on daily antiretroviral therapy (ART) to prevent disease progression.
An initial inoculum of 0.625 TCID$_{50}$ yielded no infections, however, all subsequent higher doses resulted in increasing fraction of animals becoming viremic (Table 1, Figure 1). After achieving 50% animal infection rate (4 out of 8 infected) on the first round of challenge with 100 TCID$_{50}$, this inoculum was re-tested in another group of 8 animals to increase the confidence in the virus dose selection for the efficacy study. The latter resulted in 6 out of 8 animals becoming infected (i.e. 75% infection rate). At the conclusion of the titration, 14 new infections were recorded out of a total of 40 challenges and displayed similar kinetics of replication with peak viremia ranging between $4 \times 10^5$ and $5 \times 10^7$ SHIV copies/mL across study groups (Figure 1). The logistic regression model was then applied to compute the half-maximal animal infectious dose (AID$_{50}$), equal to 77 TCID$_{50}$, as well as the animal infection rate corresponding to the 100 TCID$_{50}$ inoculum, 65.3% (40.3, 84.0 CI) (Supplemental Figure 1, Table 1).

Since the infection rate with 100 TCID$_{50}$ inoculum exceeded the 50% infection target, it was selected for the single high-dose challenge efficacy study. The 16 animals inoculated with 100 TCID$_{50}$ during the titration served as controls for estimating LEN PrEP efficacy.

**Antiviral activity and pharmacokinetic profile of LEN in macaques**

The in vitro antiviral potency of LEN against HIV-1 has already been assessed in MT-4 cells and previously reported to have a half-maximal effective concentration (EC$_{50}$) of 0.1 nM (11, 12). Because SHIV does not replicate in MT-4 cells, to measure anti-SHIV activity of LEN we performed an antiviral assay in primary rhesus PBMCs (Figure 2A). The resulting mean EC$_{50}$ and EC$_{95}$ values for LEN were 0.39 and 0.91 nM, respectively, against SHIV in rhesus PBMCs as compared to 0.05 and 0.12 nM, respectively, against a panel of 23 HIV-1 isolates of differing subtype as previously reported for primary human PBMCs (11). After correcting for rhesus and human plasma protein binding, which informs the free drug concentration in plasma, LEN was predicted to be approximately 4.4-fold less potent against SHIV vs HIV *in vivo* (8.80 nM vs 2.01 nM protein-adjusted EC$_{95}$ or paEC$_{95}$) (Table 2). The estimated LEN potency difference between SHIV in rhesus vs HIV in human cells allowed us to compute the
macaque model-adjusted clinical minimum target exposure as 70 nM (i.e. 4.4 x 16 nM human target C\text{\textsubscript{trough}} exposure with 6-month dosing regimen).

Human exposure of LEN with twice-yearly injection at 927 mg in a clinical formulation following 2 oral lead-in doses (on days 1 and 2) at 600 mg was previously described (13). In short, LEN concentrations reached an efficacious target of 15.5 ng/mL (or 16 nM) within 2 hours post-injection and were maintained above this level through the dosing interval. Next, we characterized the pharmacokinetic profile of LEN preclinical formulation in macaques following a single subcutaneous administration of five dose levels, ranging from 5 to 75 mg per kilogram, in 4 animals per group. Animals dosed with 5-20 mg/kg of LEN were monitored weekly for a minimum of 14 weeks, after which their exposures dipped below the paEC\textsubscript{95} (8.8 nM) mark, while the 2 higher dose groups were followed through study week 25. Lenacapavir had a slow, sustained release and dose-proportional increase in exposure from 5 to 20 mg/kg and more than dose proportional increase from 50 to 75 mg/kg in macaque plasma (Figure 2B and Supplemental Table 2). The slow release of LEN was demonstrated by a long half-life in the range of 17-53 days following a single subcutaneous dosing. Lenacapavir plasma drug levels peaked 4-17 days post administration on average for the 5, 10, 20, 50 and 75 mg/kg dosing groups.

Safety and tolerability profile of subcutaneous LEN dosing in macaques

Safety and tolerability of LEN was monitored via daily animal cage-side observations, injection site scoring according to the Draize Grading scale daily for 2 weeks then weekly through week 14 post-dose, hematology and clinical chemistry testing performed every other week for the first 4 weeks followed by every 4 weeks through week 16. Injections at all dose levels were generally well tolerated without notable clinical observations. Grade 2-4 edema and erythema were noted at a single time point on week 1 post-dose among 2 animals in the 75 mg/kg group and 1 animal in the 50 mg/kg group. Grade 1 edema and/or erythema was observed across multiple time points and animals from all 5 dose groups, and fully resolved by week 11 post-dose. Hematology analyses yielded no abnormal values throughout the study. Blood chemistry parameters remained within the normal range across all analytes, except for a transient minimal
elevation in aspartate aminotransferase in 2 animals in the 75 and 5 mg/kg groups on weeks 2 and 4 post-dose, respectively. Overall, the subcutaneous injection of long-acting LEN was safe and well tolerated in rhesus macaques at all dose levels tested.

**SHIV infectivity in LEN-treated macaques**

To assess prophylactic efficacy of LEN a subset of 20 animals from the PK study was challenged rectally with SHIV on week 7 post LEN dosing. Based on “real-time” measurements through study week 5, LEN plasma exposures among 9 out of 20 animals fell below 17.6nM or 2x paEC95 for LEN inhibition of SHIV, an index estimated to be protective with GS-CA1 in a prior repeat challenge study (10). Hence, 9 animals with insufficient LEN exposure were excluded from the efficacy assessment, while the remaining 11 animals from the groups receiving 20-75 mg/kg LEN doses received a single SHIV challenge (Figure 3A). The SHIV infection status in each challenged animal was initially established via weekly monitoring of plasma viral loads (Figure 3B). Of 11 animals, 3 became viremic 2 weeks post challenge (V229, BM55, and V435). Similar to the early SHIV replication kinetics observed among untreated animals in the virus titration study, viral loads peaked on week 3 and declined thereafter. Peak viremia was highly variable between animals and no significant differences were observed between the untreated and LEN-dosed infected animals challenged with the same viral inoculum (Supplementary figure 2). Between weeks 8 and 10 post-infection, the 3 viremic animals were placed on ART to comply with a pre-specified study criteria to prevent disease progression. Animals V229 and BM55 spontaneously controlled SHIV viremia ahead of ART initiation, likely owing to their expression of the major histocompatibility complex class I allele Mamu-B*17 implicated in the control of chronic-phase SIV replication (Supplemental Table 1) (14).

As a secondary measure of infection, we evaluated the seroconversion status of 11 SHIV-exposed animals using an anti-capsid (p27) antibody ELISA. All 3 animals positive for SHIV by qPCR produced measurable antibodies by week 4 post challenge, while the other 8 animals remained undetectable through the end of the monitoring period on week 10 post challenge (Figure 3C). Thirdly, we employed an intact
proviral DNA assay (IPDA) to confirm the infection status and establish the timing of detectable reservoir establishment. We assayed PBMCs on weeks 1, 2, and 3 post challenge from the 3 animals with confirmed viremia and measured first detectable intact SHIV DNA coincidental with detectable viremia at week 2 post-challenge (Figure 3D). Eight animals SHIV negative by plasma PCR and serology were assayed by IPDA on weeks 2 and 10 to capture the early and late time points post-challenge. No signal was detected at either time point consistent with other assays, thus confirming uninfected status of 8 out of 11 LEN-dosed animals challenged with SHIV.

We went on to characterize the viral capsid sequence from plasma-derived virus isolated from infected animals to look for any resistance emergence to LEN. Samples yielding sufficient SHIV RNA quantity and sequence data revealed no emerging mutations in capsid among the infected animals throughout the monitoring period (Figure 3C).

LEN prophylactic efficacy relative to exposure

To establish the precise LEN plasma levels at the time of challenge and compute LEN prophylactic efficacy, we plotted week 7 post-dose drug exposure values for the infected and non-infected groups (Figure 4A). LEN exposures among 11 challenged animals ranged from 18 to 177 nM and were significantly lower in the infected group (p = 0.005, unpaired t-test with Welch's correction). To determine whether LEN offered significant protection against infection, we compared the rates of infection between total LEN-treated animals and untreated animals challenged with the same SHIV inoculum in the titration study. The percent infection per exposure decreased from 62.5 % among untreated animals (10 infections / 16 challenges) to 27.2% (3 infections / 11 challenges) among animals treated with LEN, though this infection rate reduction did not meet an alpha of 0.05 cutoff for significance (p = 0.08, one-tailed Fisher’s exact test) (Figure 4B). However, considering that the LEN-treated group encompassed animals with exposures below the protective target, we performed a subgroup analysis based on the actual LEN exposure at the time of challenge. Hence, we evaluated protection above and below the model-adjusted clinical minimum target exposure of 70 nM calculated above. Above this
target exposure cutoff, LEN provided complete protection against infection, with 0/6 animals becoming infected. When comparing the rate of infection in the untreated group to the LEN-treated group with exposures above the minimum target, LEN demonstrated significant protection against SHIV infection in this stringent nonhuman primate challenge model (p = 0.012, one-tailed Fisher’s exact test).

Discussion

Despite the high efficacy of currently available PrEP, new options are still needed as only a minority of the people vulnerable to HIV infection are taking PrEP. Long-acting agents could address some of the reasons people have not been persistent in adherence to oral PrEP. Based on surveys, long-acting agents represent an acceptable and, in certain cases, the preferred option for people who may benefit from PrEP (15). Lenacapavir, a first-in-class HIV capsid inhibitor suitable for twice-yearly subcutaneous administration, was recently approved for the treatment of multi-drug-resistant HIV infection in combination with other antiretrovirals. Here we examined the potential utility of LEN for HIV prophylaxis using a preclinical macaque SHIV challenge model.

We confirmed potent antiviral activity of LEN against SHIV in PBMCs, albeit with a slight increase in EC_{50} compared to HIV-1 as was the case for GS-CA1 (10). Amino acid variations within the capsid-encoding region between HIV-1 and SHIV, most notably at HIV-1 positions K70 and E180 (relative to the HXB2 reference strain) located within the LEN binging site, may account for lower potency against SHIV compared to HIV-1 (Supplementary Figure 3). Substitutions at both positions have been previously implicated in loss of susceptibility of HIV-1 to LEN in vitro and are also present in HIV-2, which is less susceptible to LEN (11, 16, 17).

We characterized the pharmacokinetic profile of a long-acting preclinical LEN formulation in rhesus macaques dosed with 5 to 75 mg/kg of LEN establishing a plasma drug half-life of 2-8 weeks in this species. Although we observed variability in key pharmacokinetic parameters such as maximum concentration (C_{max}) or area under the plasma concentration–time curve from time 0 to infinity (AUC_{inf}) across animals, in particular in the 75 mg/kg dosing group due to animal V435 displaying a lower
exposure profile, the overall group variance was consistent with that reported in the human clinical studies (13). Eleven animals belonging to the 3 highest LEN dosing groups maintained plasma exposures above the paEC$_{95}$ or 8.8 nM for over 10 weeks and were chosen for the SHIV challenge study to establish LEN prophylactic efficacy. While a low virus dose repeat challenge model is useful in evaluating protection against multiple transmission events with a more physiologic virus inoculum, it can complicate accurate estimation of the time of infection and hence determination of the drug exposure that is expected to be protective. With only a single high-dose SHIV challenge we interrogated protection with the precise knowledge of plasma drug exposure at the time of challenge. Compared to the untreated group, LEN administration reduced the rate of infection per exposure by more than 2-fold and was fully protective against SHIV acquisition at plasma drug exposures above the rhesus-adjusted C$_{\text{trough}}$ concentrations targeted with twice-yearly formulations of LEN in the clinic. Unlike in the prior study with GS-CA1 peak viremia among LEN-dosed animals was not significantly inhibited relative to the control, which is likely a consequence of a smaller sample size in the current study (10).

A long-acting formulation of the integrase strand transfer inhibitor (INSTI) cabotegravir (CAB-LA) administered intramuscularly at 8-week intervals by a healthcare provider remains the only long-acting PrEP option approved to date. CAB-LA demonstrated superiority to the oral standard of care attributed primarily to inadequate adherence to the daily tablet regimen (18). Lenacapavir, if demonstrated to be efficacious for PrEP, would have the potential to further transform the PrEP landscape with just twice-yearly dosing and simple subcutaneous administration.

In addition to LEN, various reverse transcriptase inhibitors, protease inhibitors, INSTIs and entry inhibitors with long-acting potential are being evaluated as oral, injectable, and/or implantable agents for PrEP (19). Given that HIV prevention medicines are prescribed to an otherwise healthy population that may benefit from PrEP, a particularly high safety bar must be met to achieve a favorable risk-benefit ratio. Among the long-acting agents already in clinical development are broadly neutralizing antibodies (bNAbS) such as VRC01 to inhibit HIV entry and the nucleoside reverse transcriptase translocation inhibitor islatravir to suppress virus replication. Although bNAbS are generally well tolerated, these
biologics are limited in the breadth of viral strain coverage due to high diversity of HIV envelope sequence and are vulnerable to viral escape. VRC01 monotherapy did not prevent the overall HIV-1 acquisition but demonstrated reduced incidence of infection with a subset of sensitive strains (20). These data indicated that a combination of two or more bNAbs with broader strain coverage is likely needed to achieve clinical validation in future trials. Islatravir’s long half-life and high potency against HIV combined with its proven efficacy at preventing infection in a macaque model supported its evaluation for PrEP (21). However, unanticipated lymphopenia observed during clinical development resulted in a halt of ongoing PrEP studies with islatravir (22). Consistent with previously approved PrEP medicines, there have been no serious adverse events or laboratory abnormalities related to LEN reported through week 52 of treatment across clinical studies (4).

An additional important consideration for any PrEP medicine is the potential for selection of resistance in the case of breakthrough infections on PrEP, during the pharmacokinetic tail (i.e. long-term persistence of subprotective drug levels after the last dose), or when the initiation of PrEP coincides with an undetected HIV infection (23). Resistance to the first-line ART is of particular concern as it would limit treatment options post-infection (24). In study HPTN-083 evaluating CAB-LA for PrEP in cisgender men and transgender women who have sex with men, new infections and selection of INSTI resistant variants were reported while on PrEP (25). Retrospective testing revealed that viral replication was suppressed for long periods by CAB-LA and that seroconversion, and hence HIV diagnosis, was significantly delayed (26). Results of phenotypic studies of emergent virus in these individuals confirmed resistance to CAB and, in some cases, other INSTI class drugs. Although follow up studies have suggested that earlier infection detection via an HIV RNA detection assay may help reduce the risk of resistance, the feasibility and merit of this approach remains to be addressed (27).

No resistance was selected among the 3 infections that occurred at suboptimal LEN exposures in the current study consistent with our previous macaque PrEP study conducted with the capsid inhibitor GS-CA1 (10). In addition, unlike with CAB-LA, none of the capsid mutations associated with resistance to LEN display cross-resistance to existing ART classes in vitro (11, 28). Resistance to LEN in HIV
treatment clinical studies has been characterized and will be important to continue monitoring in ongoing trials for the treatment and PrEP as well as in routine clinical practice (29, 30).

Together with our previous preclinical PrEP efficacy studies using the LEN analog GS-CA1, we established an important proof of concept that LEN can offer effective long-lasting HIV prophylaxis as a monotherapy. The measured half-life of LEN in macaques, though longer than that of GS-CA1, is still shorter than the 7-11 week range measured with the twice-yearly LEN formulation in human clinical studies (13, 31). Thus, human dosing is likely to provide longer-lasting protection against HIV infection in people than achievable in this macaque model. Two Phase 3 clinical studies, PURPOSE 1 (NCT04994509) and PURPOSE 2 (NCT04925752), are currently underway to determine the safety and efficacy of subcutaneous long-acting LEN for PrEP among cisgender adolescent girls and young women in South Africa and Uganda, and in cisgender men, transgender women, transgender men, and gender non-binary individuals who have sex with men in North and South America, Africa, and Asia, respectively (32). Data presented in this manuscript further support the merit of evaluating LEN for PrEP in the clinic.

**Methods**

**Drug and formulation**

Lenacapavir and the liquid chromatography-mass spectrometry (LC-MS) internal standard GS-224337 were synthesized at Gilead Sciences, Inc. (Foster City, CA) and subjected to a standard quality control analysis. For antiviral assays, LEN was dissolved in dimethyl sulfoxide (DMSO) to produce a 10 mM stock concentration and stored frozen at -20°C. For animal dosing studies, LEN was dissolved in vehicle (58.03% polyethylene glycol 300, 27.1% water, 6.78% ethanol, 6.61% poloxamer 188, 1.48% sodium hydroxide) at 300 mg/mL and stored at ambient temperature, protected from light, until dosing. The formulation contained additional excipients absent from the clinical formulation in order to tailor the PK profile in macaques.
The SHIV-162P3 virus was derived from SIV<sub>mac239</sub> and encodes the HIV genes env, tat, rev, and vpu. The molecular clone was generated through the intravenous infusion and infection of rhesus macaques followed by three consecutive serial blood/bone marrow transfusions. The parent stock was obtained from the laboratory of Dr. Dan Barouch at Beth Israel Deaconess Medical Center, Harvard Medical School (Boston, MA) and in vitro expanded in concanavalin A-activated PBMCs from Specific Pathogen Free (SPF) rhesus macaques. In vitro titration was performed in PHA-stimulated rhesus PBMCs infected with a serial dilutional series of viral harvest. The cultures were tested for the presence of SIV capsid (p27) in the supernatant using an antigen capture assay (ABL, Inc). Wells that showed an assay OD value greater than the virus alone control wells were considered positive. The number of SIV positive versus negative wells at each serial dilution were enumerated and the half-maximal tissue culture infectious dose (TCID<sub>50</sub>) was calculated.

**Primary cells**

Freshly isolated peripheral blood mononuclear cells (PBMCs) from 3 male Indian rhesus macaque (Macaca mulatta) donors were obtained from Human Cells Biosciences (San Jose, CA). Rhesus PBMCs were cultured in RPMI-1640 cell culture media (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 2 mM glutamine, and 100 units/mL penicillin plus 100 μg/mL streptomycin (complete RPMI). Prior to their use in antiviral assays, PBMCs from three independent donors were pooled and activated at a density of 3 × 10<sup>6</sup> cells per mL for 72 hours at 37°C by addition of 1 μg/mL phytohemagglutinin (PHA, Sigma-Aldrich) and 50 units/mL recombinant human interleukin-2 (IL-2) (Roche Diagnostics).

**Antiviral assay in PBMCs**

Antiviral assays for LEN in rhesus PBMCs acutely infected with SHIV.SF162P3 were performed as previously described for HIV-1 in human PBMCs, with minor modifications (11). Briefly, PHA/IL-2
stimulated rhesus PBMCs were infected in bulk culture with SHIV.SF162P3 at a concentration of 130 pg p27 equivalent per million PBMCs. Cells were maintained in suspension by gently rocking the cultures mixed with virus inoculum for 3 hours at 37°C. Cells were then pelleted by centrifugation at 500 x g for 5 minutes, washed twice with complete RPMI to remove any un-adsorbed virus and seeded into 96-well plates at a cell density of $2 \times 10^5$ cells per well in 100 μl. Eight-point 3-fold serial dilutions of LEN were made in complete RPMI containing 50 units/mL IL-2 and added in triplicate to wells containing cells (100 μl per well). Cultures were incubated in a 5% CO₂ incubator at 37°C for 7 days. The cell-free supernatants derived from the PBMC cultures were harvested 7 days post-infection and the amount of SHIV present was quantified using a SIV p27 antigen capture ELISA assay (Item #5436, Advanced Bioscience Laboratories, Inc., Rockville, MD) performed according to the manufacturer’s protocol. The mean EC₅₀ value for LEN, determined from a total of 7 assays across two independent experiments, was calculated from the dose-response curves using XLfit software (IDBS, Boston, MA). The Hill coefficient ($n$) for LEN was measured from the slope of antiviral dose response curves ($n = 3.51 \pm 0.31$) and used to calculate its EC₉₅ value using the equation: $EC_{95} = EC_{50} \times (95/5)^{1/n}$.

**Equilibrium dialysis shift assay**

Rhesus plasma protein binding to LEN was determined by competitive equilibrium dialysis. Pooled rhesus plasma (10%) from 12 animals was spiked with LEN (2 μM) and blank RPMI cell culture medium containing 10% FBS (CCM) were placed into opposite sides of assembled dialysis cells, and incubations were performed in triplicate. After a 24-hour equilibration period at 37°C, samples were collected and mixed with opposite side of blank matrix. Samples were deproteinated with four volume equivalents of acetonitrile containing internal standard and the resulting supernatant was analyzed by LC-MS/MS. Lenacapavir and the internal standard were detected using a source which was configured with Turbo Ion Spray® ionization in positive mode and using multiple-reaction monitoring. The fold change value in 100% rhesus plasma was then calculated using the plasma/CCM ratio after correcting for the sample dilution factor and the percent free fraction in the matrix.
**Animal studies**

All animals were housed at Bioqual, Inc. (Rockville, MD) and all procedures were conducted in compliance with the relevant local, state, and federal regulations and were approved by the Bioqual, Inc. IACUC. For the in vivo SHIV stock titration study, 8 untreated outbred Indian-origin male rhesus macaques aged 3-5 years were challenged intrarectally per round for a total of 5 challenge rounds using increasing virus doses ranging from 0.625 to 100 TCID$_{50}$, with the 100 TCID$_{50}$ round performed twice for increased resolution (Supplemental Table 1). Plasma viral load was measured to confirm the infection status. For LEN pharmacokinetics and PrEP efficacy determination, 20 outbred Indian-origin male rhesus macaques aged 3-5 years were assigned to 5 study groups with an even weight distribution (Supplemental Table 1). On study week 0, 4 animals per group were administered LEN at 5, 10, 20, 50 or 75 mg/kg in the scapular region by subcutaneous injection. LEN was prepared as a 300 mg/mL stock solution and no more than 2 mL of solution was injected into a single subcutaneous site. Injection sites were monitored daily by veterinary staff for 2 weeks, then weekly through the end of study. On week 7, 11 animals were challenged by the intrarectal route with 1 mL of RPMI containing 100 TCID$_{50}$ SHIV-SF162P3. Whole blood was collected and processed into plasma and PBMCs as necessary for the assessment of routine hematology and clinical chemistry, viral load analysis, serology, and the bioanalysis of drug levels. Animals were considered protected if they remained SHIV-negative by a plasma PCR assay and seronegative by enzyme immunoassay through week 10 post-challenge.

Animals confirmed as SHIV-positive in both the virus titration and the PrEP efficacy studies were placed on a daily subcutaneous ART regimen between weeks 4 and 10 post-infection to prevent AIDS disease progression. The formulated ART cocktail (Gilead Sciences, Inc.) contained tenofovir disoproxil fumarate (TDF, 5.1 mg/mL), emtricitabine (FTC, 40 mg/mL), and dolutegravir (DTG, 2.5 mg/mL) and was administered subcutaneously once daily at 1 mL/kg.
Bioanalysis of LEN in macaque plasma

Rhesus plasma samples were stored frozen at -80°C, thawed, and a 50 µL aliquot of each was treated with 200 µL acetonitrile containing an internal standard. After precipitation of the protein component, a 100 µL aliquot of the supernatant was transferred to a clean 96-well plate and mixed with 200 µL water. A 10 µl aliquot of the above solution was then injected into a Q-Exactive high resolution mass spectrometer from Thermo Electron (San Jose, CA) with electrospray ionization in positive mode. Quantification was performed using an accurate mass ([M+H]+) of 968.1508 for LEN and 756.3289 for the internal standard. The lower and upper limits of quantitation for LEN were 1 nM and 10,000 nM, respectively.

Plasma viral load assay

A QIAsymphony SP (Qiagen, Hilden, Germany) automated sample preparation platform along with a Virus/Pathogen DSP midi kit and the cellfree500 protocol were used to extract viral RNA from 500 µL of plasma. A reverse primer specific to the gag gene of SIVmac251 (5’- CAC TAG GTG TCT CTG CAC TAT CTG TTT TG -3’) was annealed to the extracted RNA and then reverse transcribed into cDNA using SuperScriptTM III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) along with RNAse Out (Thermo Fisher Scientific). The resulting cDNA was treated with RNase H (Thermo Fisher Scientific) and then added (2 replicates) to a custom 4x TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific) containing primers and a fluorescently labeled hydrolysis probe specific for the gag gene of SIVmac251 (forward primer 5’- GTC TGC GTC ATC TGG TGC ATT C -3’, reverse primer 5’- CAC TAG GTG TCT CTG CAC TAT CTG TTT TG -3’, probe 5’- /56-FAM/CTT CCT CAG TGT GTT TCA CTT TCT CTG CG/3BHQ_1/-3’). The qPCR was then carried out on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). Mean SIV gag RNA copies per reaction were interpolated using quantification cycle data and a serial dilution of a highly characterized custom RNA transcript containing a 730 bp sequence of the SIV gag gene. The assay limit of quantification (LOQ) is approximately 62 RNA copies per milliliter of sample.
Enzyme-linked immunosorbent assay (ELISA)

Rhesus serum samples from viremic study animals were tested for the presence of antibodies to HIV-1 by ELISA using the GS HIV-1/HIV-2 PLUS O EIA assay kit from Bio-Rad (catalog # 32588). Individual macaque sera (150 μl) mixed with 50 μl specimen diluent supplied in the kit were added to assay plates precoated with recombinant purified HIV-1 capsid (p24) protein and transmembrane glycoprotein (gp160) and incubated for 1 hour at room temperature. The plates were then washed 3 times with a sodium chloride and Tween 20-containing wash buffer from the kit and incubated for 1 hour with a horseradish peroxidase (HRP)-conjugated antigen solution containing peptides mimicking various immunodominant epitopes of HIV-1 gp160 and p24 proteins. Wells with antibody to HIV-1 bound to the antigen coating the wells and to the peroxidase-conjugated antigens in the conjugate solution to form immobilized stable antigen-antibody-antigen complexes. The plates were washed 3 more times with the above wash buffer, developed with a working solution of tetramethylbenzidine (TMB), stopped by the addition of 1N sulfuric acid, and analyzed at 450 nm using a Versamax microplate reader (Molecular Devices) using the Softmax Pro 6.5.1 software. Samples with an OD450 nm absorbance value >0.2 were considered positive.

Intact proviral DNA assay

SHIV-adapted version of IPDA (SHIV-IPDA) was used to determine the number of intact SHIV proviruses. Total genomic DNA was extracted from unfractionated PBMCs using a QIAamp DNA Mini kit (Qiagen). DNA quality and quantity were evaluated by spectrophotometry and fluorometry, respectively, and SHIV-IPDA was then performed on the isolated DNA. In brief, SHIV-IPDA consists of a three-component multiplex droplet digital PCR (ddPCR) reaction. The first is a SHIV proviral discrimination reaction targeting two conserved, frequently deleted regions of the SHIV genome to determine the intact provirus count; the second is a two-long terminal repeat (2-LTR) DNA circle reaction to determine 2-LTR circle counts; and the third is a copy reference/DNA shearing reaction targeting ribonuclease P/MRP subunit P30 (RPP30) to determine assay input cell equivalents and the DNA.
shearing index (DSI). All ddPCR reactions were performed using a Bio-Rad QX200 AutoDG ddPCR system with Bio-Rad ddPCR supermix for probes with no dUTP. After DSI correction and subtraction of intact 2-LTR circles, the intact proviral frequencies were reported per million input cells. The endpoint ddPCR data were collected using Bio-Rad QuantaSoft version 1.7.4.0917.

**Plasma virus genotypic analysis**

Total RNA was extracted from 50 µL plasma aliquots obtained from each viremic monkey using the MagMAX-96 Viral RNA Isolation Kit (Life Technologies) in conjunction with the Thermo Scientific KingFisher Flex automated extraction platform and eluted in 60 µL of AVE buffer. The capsid coding area of gag in each sample was then individually amplified by RT-PCR using the SuperScript IV One-Step RT-PCR System (Life Technologies) Qiagen OneStep RT-PCR Kit performed according to the manufacturer’s recommended protocol. Amplification of the SHIV capsid coding region in each sample was performed using primers SIV-CA-F [5’-CCAAAAACAAATAGACCAACAG-3’] and SIV-CA-R [5’-TGCAAAAGGGATTGGCAC-3’] and the products subjected to population-level bulk sequencing at Elim Biopharmaceuticals, Inc. (Hayward, CA) using the same primer set. To identify codon changes, capsid encoding sequences for each sample were aligned using DNA Sequencher Software (Gene Codes Corporation) with that of the parent challenge virus stock. A sequence alignment for major consensus HIV-1 subtype, HIV-2 and SHIV-SF162P3 capsid amino acid sequences was performed using BioEdit Sequence Alignment Editor version 7.2.6.

**Statistics**

Protection against acquisition of infection was analyzed using Fisher’s exact test. Comparisons were considered statistically significant at a two-sided alpha level of 0.05 (p < 0.05). Animal infectious dose modeling was done based on logistic regression represented by the following formula:

\[
\log \left( \frac{p(x_i)}{1 - p(x_i)} \right) = b_0 + b_1 x_i
\]
Where \( p(x) \) represents the infection rate at dose \( x_i \), \( i=1,2,3 \). The response is binary with responder represented as 1 and non-responder represented as 0. The parameter estimates were obtained from the dose-response curve package in R.

**Study approval**

All animal procedures were conducted in compliance with the relevant local, state, and federal regulations and were approved by the Bioqual, Inc. IACUC (Rockville, MD). Human PBMCs were collected from healthy volunteers under informed consent and their use approved by an institutional review board at AllCells (Alameda, CA).

**Data availability**

Raw experimental data can be accessed via the “Supporting data values” XLS file.

**Author contributions**

EB designed and oversaw the animal virus titration study. EB, SY and CC designed the pharmacokinetic and efficacy animal studies. EB and LV oversaw the PK and efficacy study execution. DH performed and analyzed the antiviral and serology assays and viral sequencing. KW performed and analyzed the equilibrium dialysis shift assays. WR supported drug formulation. BL and SRY analyzed the plasma LEN concentrations. EB interpreted the data and wrote the manuscript with input from all authors.

**Acknowledgements**

The authors would like to thank members of Bioqual Inc (Rockville, MD) for performing the in-life portion of the animal studies, Duke Human Vaccine Institute (Durham, NC) for SHIV plasma viral load analysis and AccelevirDx (Baltimore, MD) for IPDA measurements. All studies were funded by Gilead Sciences, Inc.
References


Figure Legends

**Figure 1. Early infection viral load kinetics among untreated animals during virus titration study.**

Plasma SHIV viral loads measured by gag RT-qPCR among untreated rhesus macaques that became infected following a single rectal challenge with the specified inoculum of SHIV.SF162P3. The number of infections observed per total challenges performed with a given inoculum is noted in parentheses. Plasma viral RNA monitoring was performed through week 4 post challenge, at which time viremic animals were placed on combination antiretroviral therapy (ART). Each symbol represents an average of 3 technical assay replicates. Assay limit of detection is 62 copies per mL.

**Figure 2. LEN antiviral activity in vitro and long-acting pharmacokinetics profile in rhesus macaques.** (A) Representative antiviral dose–response curve for LEN in rhesus peripheral blood mononuclear cells (rhPBMCs) acutely infected with SHIV-SF162P3. Data are shown as mean ± s.d. values from one of seven assays ($n = 3$ biological replicates each). (B) Plasma LEN levels measured by mass spectrometry following a single subcutaneous administration at specified dose levels ($n = 4$ /group). Data represent mean ± s.d. values. The bottom dashed line represents the assay limit of detection (LOD, 1 nM). The top dotted line represents the rhesus PBMC paEC$_{95}$ for LEN (8.8 nM).
Figure 3. Study design, SHIV infectivity, seroconversion status and CA resistance profile post single challenge of LEN-treated macaques. (A) Study design. Rhesus macaques of Indian origin were treated with a single subcutaneous administration of LEN at the specified dose level on week 0 and challenged with a high-dose of SHIV on week 7. (B) Plasma SHIV viral loads measured by gag RT-qPCR among LEN-treated rhesus macaques a single challenge with 100 TCID₅₀ of SHIV (plotted on the left y-axis) versus plasma LEN exposure (plotted on the right y-axis). Animals dosed with 20, 50 and 75 mg/kg LEN are represented by the top, middle, and bottom rows, respectively. Each symbol represents an average of 3 assay replicates. Dotted lines represent the assay limit of detection (LOD, 62 copies per mL). Asterisks indicate the timing of ART initiation among the 3 viremic animals. (C) The timing of p27 antibody detection (i.e. seroconversion) measured via serum ELISA and depicted by the grey shaded regions on top of corresponding plasma viral load curves (only animals with detectable signal shown out of 11 assayed). Capsid-encoding gene sequencing results are noted above the viral load curves as applicable. WT, wild
type sequence; SF, sequence failure. (D) SHIV intact proviral DNA counts from peripheral blood mononuclear cells (PBMCs) determined by intact proviral DNA assay (IPDA) among 11 SHIV-challenged rhesus macaques at the indicated timepoints. Each symbol represents an average of 12 technical replicates in a single experiment. Red symbols represent animals that were viremic by qPCR and seropositive by p27 serum ELISA. Dotted line represents the assay limit of detection (LOD, 2 copies per million PBMCs).

Figure 4. Animal infection status relative to LEN exposure at the time of challenge. (A) Plasma levels of LEN measured by mass spectrometry among SHIV-challenged animals at the time of challenge vs. final infection status. Symbols represent plasma LEN concentration derived from an average of 3 biological assay replicates for individual study animals and are color-coded by the corresponding LEN-dosing groups. The horizontal bars represent group average. The P value comparing exposures among infected vs uninfected animals computed via an unpaired t-test with Welch's correction is shown. The bottom dotted line represents the rhesus paEC95 for LEN (8.8 nM), while the top dotted line represents the rhesus adjusted clinical target C_{\text{trough}} concentration for LEN (70 nM). (B) Infection rates among the different study animal subgroups. Bars represent the number of infected (red) or uninfected (grey) study animals that were untreated, total LEN-treated, or LEN-treated and split into subsets with exposures below or above the adjusted LEN target concentration. P values computed via Fisher’s exact test are shown.
Tables

Table 1. Infection frequencies in rhesus macaques following a single rectal challenge with increasing titers of SHIV-SF162P3.

<table>
<thead>
<tr>
<th>SHIV Inoculum (x TCID&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proportion of Challenged Animals Infected (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AID&lt;sub&gt;50&lt;/sub&gt; c</th>
<th>% AID at 100 TCID&lt;sub&gt;50&lt;/sub&gt; (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.625</td>
<td>0/8 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1/8 (12.5%)</td>
<td>77 TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>65.3% (40.3, 84.0)</td>
</tr>
<tr>
<td>30</td>
<td>3/8 (37.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4/8 (50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6/8 (75%)</td>
<td></td>
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</tr>
</tbody>
</table>

a. Indian-origin male rhesus macaques were challenged with increasing doses of SHIV-SF162P3. TCID<sub>50</sub>, half-maximal tissue culture infectious dose
b. Rhesus macaques (n=8/group) were rectally challenged once with SHIV. Fraction and percentage of animals infected post challenge as confirmed by plasma viral load assay
c. AID<sub>50</sub>, half-maximal animal infectious dose computed via logistic regression analysis
d. Percentage of animals predicted by logistic regression analysis to become infected per challenge using a 100 TCID<sub>50</sub> SHIV inoculum; CI, 95% confidence interval

Table 2. Comparison of predicted LEN antiviral activity in rhesus and human in vitro and in vivo.

<table>
<thead>
<tr>
<th>LEN Antiviral Potency</th>
<th>Rhesus PBMC (SHIV-infected)</th>
<th>Human PBMC (HIV-1-infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (nM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.39 ± 0.16</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>EC&lt;sub&gt;95&lt;/sub&gt; (nM)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91 ± 0.38</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>Plasma binding shift&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.7 (rhesus)</td>
<td>17.4 (human)</td>
</tr>
<tr>
<td>paEC&lt;sub&gt;95&lt;/sub&gt; (nM)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.80 ± 3.69&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.01 ± 1.22</td>
</tr>
</tbody>
</table>

a. EC<sub>50</sub> against SHIV-SF162P3 in rhesus and HIV-1 in human mitogen-activated PMBCs using a 7-day replication assay.
Rhesus PBMC/SHIV data are mean ± s.d. values from 7 assays (n = 3 biological replicates each) across 2 independent experiments. Human PBMC/HIV-1 data are mean ± s.d. values from 23 HIV-1 Group M (subtypes A-G, circulating recombinant forms), N and O clinical isolates (n = 3 biological replicates for each isolate), as previously reported by Link et al (11).
b. EC<sub>95</sub> values (mean ± s.d.) were calculated using the formula: EC<sub>95</sub> = EC<sub>50</sub> x (95/5)<sup>n</sup>, where n represents the Hill coefficient for LEN measured against HIV-1<sub>1HIV</sub> in MT-4 cells (n=3.51 ± 0.31).
c. Mean competitive equilibrium dialysis (EQD) plasma shift assays for LEN in rhesus and human plasma, each determined from 3 independent experiments performed in singlet.
d. Protein-adjusted EC<sub>50</sub> (paEC<sub>50</sub>) values derived from the EC<sub>50</sub> multiplied by the corresponding plasma shift value.
e. LEN displays 4.4-fold lower protein-adjusted potency against SHIV vs. HIV-1 (8.8 vs. 2.0 nM), resulting in a rhesus-adjusted in vivo target trough concentration of 16 nM (i.e., LEN human target C<sub>trough</sub>) x 4.4 = 70 nM.