Supplementary Materials for

Long-Acting Integrase Inhibitor Protects Macaques from Intrarectal Simian/Human Immunodeficiency Virus Challenges

Authors: Chasity D. Andrews[1], William R. Spreen[2], Hiroshi Mohri[1], Lee Moss[2], Susan Ford[2], Agegnehu Gettie[1], Kasi Russell-Lodrigue[3], Rudolf P. Bohm[3], Cecilia Cheng-Mayer[1], Zhi Hong[2], Martin Markowitz[1], David D. Ho[1]*

correspondence to: dho@adarc.org

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Materials and Methods

Rhesus Macaques and the Challenge Virus

All studies were approved by the Institutional Animal Care and Use Committee of the Tulane National Primate Research Center, which is a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC # 000594). The facility's OLAW animal welfare assurance number is A4499-01, and its USDA registration number is 72-R-0002. Macaques were anesthetized with tiletamine/zolazepam (Telazol; 8 mg/kg) or ketamine-HCl (10 mg/kg). All challenges were performed on the same day with the same virus stock and inoculation method. Animals were considered infected and virus challenges were stopped following two positive plasma viral RNA results. The SHIV162P3 *(30, 31)* challenge stock was expanded and titrated in rhesus macaque PBMCs prior to this study. The virus infectious titer of the challenge stock was calculated as 5180 $TCID_{50}$/mL by the method of Reed and Muench *(32)*.

GSK744 Levels in Plasma and Tissues

Whole blood was collected using K2EDTA tubes, centrifuged to obtain plasma and stored at -80°C until analysis. Tissue samples were rinsed in saline, blotted dry, weighed, snap frozen and stored at -80°C until analysis. Prior to analysis, tissue biopsy samples were transferred into vials containing metal beads. Water containing EDTA and formic acid was added to each tube and samples were homogenized using a FastPrep™ homogenizer (MP Biomedicals, Solon, OH) by processing for two cycles (60 sec/cycle; 6.5 m/sec), followed by two additional cycles (30 sec/cycle; 6.5 m/sec). Additional 30 sec cycles were applied as necessary to provide complete homogenization. Homogenates were then diluted with water containing EDTA and formic acid for a final dilution of 20:1, equivalent to ~ 50 mg tissue mass per mL of homogenate. The necropsy tissues were homogenized by using a Polytron™ probe homogenizer (Kinematica Inc., Bohemia, NY) in water containing EDTA and formic acid. Plasma and tissue homogenates were analyzed for GSK744 using an investigative liquid chromatography and tandem mass spectrometry (LC-MS/MS) method. Samples were extracted by protein precipitation with acetonitrile containing [$^{13}C^{15}N^{2}H_2$] GSK744 as the internal standard. The extract was injected onto an Acquity™ HPLC system (Waters Associates, Milford, MA), and a mobile phase of 37% acetonitrile in aqueous 0.1% formic acid was used to elute components from a 2.1 x 50 mm 3.5-micron XBridge™ C18 column (Waters Associates). The eluate was detected by using a Sciex API-4000 (AB Sciex, Framingham, MA) equipped with a TurboIonspray™ ionization source using positive ion mode and multiple reaction monitoring (GSK744 m/z 406>263; internal standard m/z 410>263). Data acquisition and processing were performed with Analyst 1.4.1 software (AB Sciex). The calibration range for GSK744 was 10 to 10,000 ng/mL for plasma and 2.5 to 1000 ng/mL for tissues.

PK Methods

Individual plasma concentration-time data were analyzed with model 200 for extravascular administration of the WinNonlin Professional software (version 5.2; Pharsight Corp, Mountain View, CA). Actual recorded sampling times for each

individual profile were used to determine plasma GSK744 parameters, which included the area under the curve (AUC) from time zero until the end of the dosage interval (AUC[0-t]) using the linear-up/log-down approach to the trapezoidal rule, the observed maximum plasma concentration (C_{max}), the time to observed maximum plasma concentration (t_{max}), and the plasma concentration at the end of the dosage interval ($C\tau$).

Plasma SHIV RNA Virus Load Assay

Plasma SHIV RNA was quantified using a real-time RT-PCR assay. Plasma (1.5 mL) was centrifuged ≥18,000 × g for at least one hour at 4°C to pellet virus. RNA was extracted from virus pellets using the QIAamp Viral RNA Mini kit (Qiagen) as per the manufacturer's instructions. Viral RNA was reversely transcribed in 30 μL reactions containing 1X TaqMan Buffer A (containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 μM EDTA, 60 nM passive reference ROX) with 4.2 mM $MgCl_2$, 333 μM of each dATP, dCTP, dGTP and dTTP, 1.67 μM random hexamer, 20 U RNAsin (Promega) and 20 U Superscript II reverse transcriptase (Life Technologies). Cycling conditions were 10 min at 25°C, 50 min at 42°C and 10 min at 85°C. Real-time PCR was performed by adding 20 μL of master mix to cDNA for a final volume of 50 μL containing a final concentration of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 μM EDTA, 60 nM passive reference ROX, 2.5 mM $MgCl_2$, 200 μM of each dATP, dCTP, dGTP and dTTP, 400 nM forward primer (gag-3.2: 5'- TGGAGAACAAAGAAGGATGTCAAA-3'), 400 nM reverse primer (gag-5.2: 5'- CACCAGATGACGCAGACAGTATTAT-3'), 100 nM probe (Gag-Btaq.2: 6FAM-TTGGCACTAATGGAGCTAAGACCGAAAGTATT-BHQ1) and 1.25 U AmpliTaq Gold (Life Technologies). Real time PCR was run using Mx3000P (Stratagene) with the conditions of 95°C for 10 minutes, followed by 55 cycles of 95°C for 15 sec and 60°C for 50 sec. Duplicate samples were analyzed and the limit of detection was 40 SHIV RNA copies/mL plasma.

PCR Amplification of Proviral DNA

DNA was isolated from PBMCs using the QIAamp DNA Blood Mini Kit (Qiagen). Samples were analyzed in duplicate with 200 ng of DNA to amplify SHIV proviral DNA with the same primers and probes as for the viral load assay above. Real-time PCR was run using Mx3000P (Stratagene) with the conditions of 95°C for 10 minutes, followed by 55 cycles of 95°C for 30 sec and 60°C for 1 min. Following necropsy, mucosal mononuclear cells were enriched from rectal and colon tissue using collagenase extraction for 55 minutes and Ficoll density gradient separation as previously described *(33)*. The remaining tissues were snap frozen and stored at -80°C until DNA extraction. DNA was extracted from mucosal mononuclear cells using QIAamp DNA Blood Mini Kit or from tissues using the DNeasy Blood and Tissue Kit (Qiagen). Ten replicates per sample were analyzed as described above.

Integrase sequence analysis for resistance mutations

cDNA was generated as described above from cell-free viral RNA. Integrase was amplified using Phusion Hot Start Flex DNA Polymerase (NEB) as per the manufacturer's instructions using a final concentration of 400 nM each primer, macIN.F1 (5'- AGCATGGGTACCAGCACACAAAGG-3') and macIN.R1 (5'- CCGCTGTAAAGCAAGGGAAATA-3'). The reaction was incubated at 98°C for 30 sec

followed by 30 cycles at 98°C for 8 sec, 68°C for 30 sec, 72 °C for 40 sec and a final extension at 72°C for 5 minutes. The product was used for nested PCR with primers, macIN.F3 (5'- GGTATAGGAGGAAACCAAGAAATAGA-3') and macIN.R3 (5'-AGTGGGAAGATTACTCTGCTGC-3') incubating the mixture at 98°C for 30 sec followed by 30 cycles at 98°C for 8 sec, 65°C for 30 sec, 72 °C for 30 sec and a final extension at 72°C for 5 minutes. PCR products were sequenced by Genewiz Inc. using primers macIN.Fseq (5'- GCTTCGCAAGAAGTAAAGATG-3') and macIN.Rseq (5'-ATTAATCTTTCTGCTGGAGTCATA-3'), and sequence analysis was performed using Geneious software (version 6.1.7).

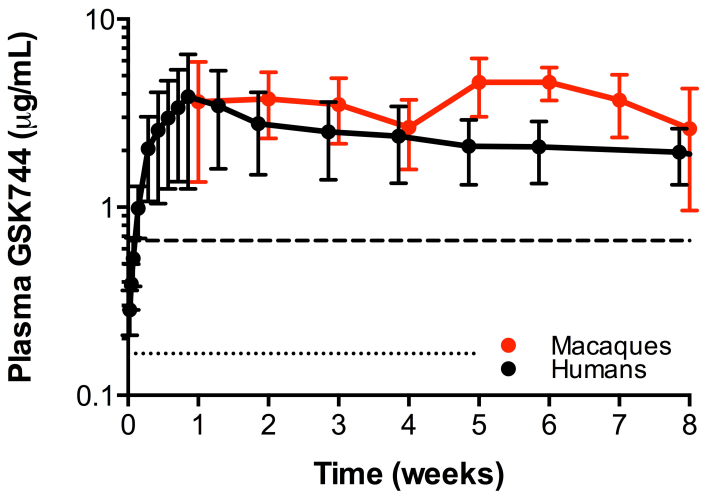
Serology

Virus-specific antibody responses (IgG and IgM) were measured using a synthetic-peptide enzyme immunoassay as per the manufacturer's instructions (Genetic Systems HIV-1/HIV-2 Plus O; Bio-Rad).

Statistical Methods

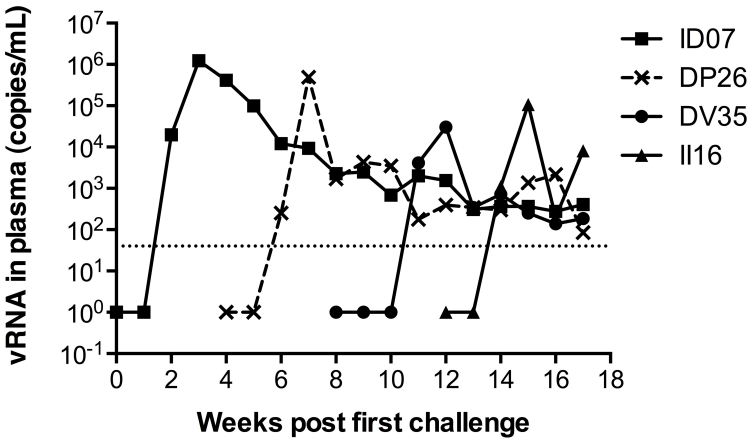
The log-rank test was used to discern statistical differences between GSK744 LA-treated macaques and untreated control macaques. Hazard ratio was estimated by the Mantel-Haenszel model. A two-sided Fisher's exact t-test was used for categorical analysis of number of infections per total exposures in each group relative to other groups. All statistical analyses were performed using GraphPad Prism software (version 6.0).

Fig. S1.



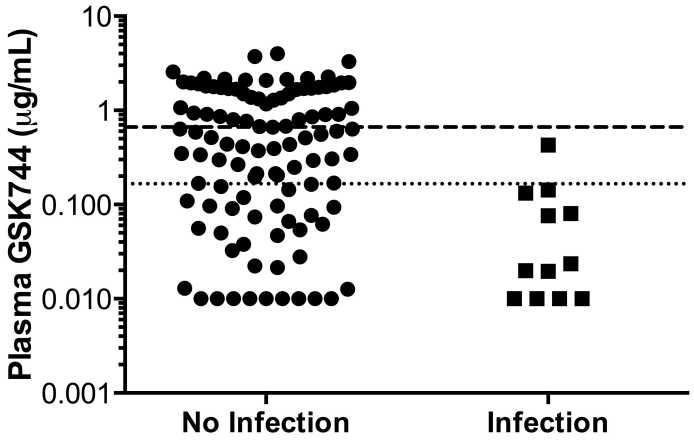
Overlay of GSK744 LA PK profiles from rhesus macaques (n=8) treated with 50 mg/kg of GSK744 LA on week 0 and week 4 and human volunteers (n=6) treated with 800 mg of GSK744 LA on week 0. Mean ± SD are shown. Dotted and dashed horizontal lines represent 1X and 4X $PAIC_{90}$, respectively. LOQ >0.01 μg/mL.

Fig. S2



Viral loads of individual untreated control macaques shown relative to weeks post first challenge of GSK744 LA-treated macaques. Dotted line represents the LOQ, >40 SHIV RNA copies per milliliter of plasma.

Fig. S3



Collective GSK744 plasma concentrations from twelve macaques treated with a single dose of GSK744 LA. Each data point represents the GSK744 plasma concentration from an individual macaque at the time of challenge, and the data are grouped based on the outcome of the inoculation. GSK744 plasma concentrations are reported at the time of infection assuming a two-week viral eclipse phase. Dotted and dashed horizontal lines represent 1X and 4X $PAIC_{90}$, respectively. LOQ >0.01 μg/mL. Samples below LOQ are reported as 0.01 μg/mL.

Table S1.

	T:P	
Tissue	**Mean**	**SD**
Rectum	0.26	0.03
Colon	0.28	0.08
Ileum	0.31	0.06
Jejunum	0.21	0.04
Duodenum	0.25	0.08
Cervical LN[a]	0.23	0.06
Inguinal LN	0.15	0.03
Mesenteric LN	0.15	0.04
Axillary LN	0.21	0.07
Tonsil	0.30	0.11
Spleen	0.16	0.03
Liver	0.41	0.11
Muscle[b]	0.08	0.01

a: n=3
b: injection site

Tissue distribution of drug was evaluated following IM injection into the quadriceps of 30 mg/kg of GSK744 LA (n=4, [2 × 15 mg/kg]) as a split injection. GSK744 LA was administered on week 0 and 4, and tissues were collected at necropsy on week 7. Plasma and tissue concentrations were assessed by HPLC-MS/MS with a LOQ >0.01 μg/mL and >0.05 μg/g, respectively. Ratios of tissue:plasma were calculated for individual animals. Mean ± SD for the group are shown.

Table S2.

	Macaque ID							
	EL11	CM10	FC98	EJ49	FF78	DP48	CM98	EI35
Serology[1]								
	-	-	-	-	-	-	-	-
Real Time-PCR								
Viral RNA in:								
Plasma[1]	-	-	-	-	-	-	-	-
Proviral DNA in:								
PBMCs[1]	-	-	-	-	-	-	-	-
Rectum tissue[2]	-	-	-	-	-	-	-	-
Rectum MMCs[2]	-[3]	-	-	-	-	-	-	-
Colon tissues[2]	-	-	-	-	-	-	-	-
Colon MMCs[2]	-	-	-	-	-	-	-	-
Mesenteric LN[2]	-	-	-	-	-	-	-	-
Inguinal LN[2]	-	-	-	-	-	-	-	-
External iliac LN[2]	-	-	-	-	-	-	-	-
Internal iliac LN[2]	-	NA	-	NA	NA	NA	NA	NA
Colonic LN[2]	NA	NA	-	NA	-	NA	NA	-

[1] Assessed weekly throughout study
[2] Assessed at necropsy
[3] 1 copy/2 μg DNA
NA: lymph nodes not identified during necropsy

Summary of serologic and virologic analyses for evidence of virus infection in GSK744 LA-treated macaques. SHIV antibodies and RNA in plasma, as well as SHIV proviral DNA in PBMCs, were monitored weekly throughout the challenge and follow-up periods. Animals were necropsied at week 19, and multiple tissues were analyzed for the presence of proviral DNA by means of quantitative real-time PCR assays. Mucosal mononuclear cells (MMCs) were enriched from colon and rectum for increased assay sensitivity.

Table S3.

GSK744 LA-treated macaque ID	Integrase sequence changes
EP55	-
FH38	-
HI04	D256E
HN88	-
HV38	-
IG71	-
IH77	-
II33	-
II64	A122T
IK79	-
IK98	-
IP06	G27R, E173K

-: No change in integrase sequence compared with viral stock.

Integrase amino acid sequences from plasma of GSK744 LA-treated macaques were compared with SHIV162P3 viral stock integrase consensus sequence. Cell-free plasma was analyzed within one week of vRNA detection. No primary integrase resistance conferring mutations were observed.