Supporting Information

Toward Eradicating HIV Reservoirs in the Brain: Inhibiting P-glycoprotein at the Blood-Brain Barrier with Prodrug Abacavir Dimers

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Materials. Abacavir was obtained from the NIH AIDS Research and Reagent Program (Germantown, MD), [^{125}I]-Iodoarylazidoprazosin was obtained from Perkin Elmer (Waltham, MA), Calcein-AM was purchased from Invitrogen (Carlsbad, CA), 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was obtained from TCI America (Portland, OR), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 4- dimethylaminopyridine (DMAP) were purchased from GenScript Corporation (Piscataway, NJ), α-methyl-γ-butyrolactone, α,α-dimethyl-γ-butyrolactone, 4,4'-dithiobutyric acid and diisopropylamine (DIEA) were purchased from Sigma–Aldrich (St. Louis, MO). All other materials were purchased from Sigma-Aldrich (St. Louis, MO) and used without purification.

Cell Culture. 2D7-MDR cells ($CD4^+$ human T-lymphocytic cell line expressing P-gp)[1-2] were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum (Cambrex bioscience, WalkersVille, Inc.), 5 mM L-glutamine, 50 units/mL penicillin and 50 µg/mL streptomycin (Cellgro Mediatech). Cells were incubated at 37 °C with 5% carbon dioxide. Sf9 cells were cultured at 27 °C in Sf-900 II SFM medium supplemented with 0.5× antibiotic-antimycotic (Invitrogen).

Flow Cytometry Assays. Flow cytometry assays were perfomed as previously described, with a few modifications[4]. 12D7-MDR cells (125 000) were incubated with calcein-AM (0.25 µM) or NBD-Abacavir (0.5 µM) in the presence of increasing concentrations of the compounds of interest and incubated at 37 °C for 30 min, keeping the DMSO concentration constant at 1%. The known P-gp inhibitor GF120918 (1 µM) was used as a positive control. Cells were collected by centrifugation at 300 x*g* and re-suspended in ice cold phosphate buffered saline (PBS, pH 7.4). Followed by analysis for fluorescent substrate accumulation using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser and a 530 nm

band pass filter (FL1). Ten thousand cells were counted for each data point and the mean fluorescence was utilized to determine the IC_{50} values using Sigma plot.

Expression of P-gp in Insect Cells. Sf9 cells in 150-cm^2 flasks (1.86 x 10^7 cells/flask) were infected with BV-*MDR1 th*at expresses human P-gp at a multiplicity of infection of five in 5 mL of culture as described before[3]. After 2 hours of incubation at 27 °C, cells were fed with 15 mL culture medium and incubated at 27 °C for an additional 72 hours.

Preparation of Crude Insect Cell Membranes

BV-*MDR1* infected Sf9 cells were collected and crude membrane extracts prepared as described previously with minor modifications.[3] As our compounds contain disulfide bonds, crude membrane extracts were prepared without the addition of dithiothreitol (DTT). P-gp expression was verified by immunoblot analysis with C219 primary antibody (1:8000) and HRP-conjugated antimouse secondary antibody (1:8000). Protein bands were visualized using enhanced chemiluminescence (ECL) (Pierce).

IAAP Photoaffinity Labeling. Crude Sf9 membranes expressing P-gp (25 µg) were incubated for 10 min at room temperature in the dark in assay buffer (50 mM Tris-HCl, pH 7.5, 1% (v:v) aprotinin and 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) with either DMSO, GF120918 (10 µM), or increasing concentrations of the compounds of interest and [^{125}I]-IAAP (1 µL, specific activity of ~2200 Ci/mmol). The DMSO concentration was kept constant at 2.5%. The protein samples were illuminated with UV light (365 nm) for 20 min on ice and separated by 7.5% SDS-PAGE. Following autoradiography of the fixed and dried gel, the band corresponding to P-gp was analyzed using ImageJ (NIH). Values are represented as a percent of the DMSO control sample, and the IC_{50} values were determined by GraphPad Prism 4.

Human Plasma Stability. Plasma stability studies were performed as described previously, with some modifications[5-6]. Abacavir dimers (60 μM) were incubated at 37 °C in 55% human plasma, 45% PBS, pH 7.4 and the DMSO concentration was kept constant at 0.3%. At various time points, ice cold acetonitrile containing internal standard (bromobenzene, 1.2 mM) was added to deactivate proteins. The solution was vortexed for 20 sec, then centrifuged at 6000 x*g* for 10 min. The supernatant was stored at -80 °C until analysis. For analysis, the samples were analyzed by HPLC with a C5 analytical column (Phenomenex), with an eluent consisting of solvent A (acetonitrile and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 30 min gradient of 5-70% solvent A, a flow rate of 1.20 mL/min and UV detection at 254 nm. The peak area corresponding to abacavir dimer and released abacavir monomer was quantified. All assays were performed in duplicate, and half-lives were obtained by fitting the time-dependent data using Graph Pad Prism 4.

Dithiothreitol (DTT) Stability. Abacavir dimers (70 μM) were incubated at 37 °C with 10 mM DTT in previously degassed phosphate buffer (pH 7.4) containing 1 mM bromobenzyl alcohol (internal standard). At different time points, aliquots from the reaction mixture were removed and immediately analyzed by HPLC using a C5 analytical column (Phenomenex), with an eluent consisting of solvent A (acetonitrile and 0.1% TFA)) and solvent B (water and 0.1% TFA) with a 30 min gradient of 5–70% solvent A, a flow rate of 1.20 mL/min and UV detection at 254 nm. The peak area corresponding to monomeric abacavir released was calculated. All assays were performed in duplicate, and half-lives were obtained by fitting the time-dependent data using Graph Pad Prism 4.

Rat Brain Capillary Isolation. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences and conform to the

guidelines and standards of the National Institutes of Health. Male Sprague Dawley rats (retired breeders) (Taconic, Hudson, NY) were sacrificed by CO_2, decapitated, and the brains removed for capillary isolation as described previously[7,8]. Brains from 5-10 rats were placed in ice cold PBS complete (2.7 mM KCl, 1.46 mM KH2PO4, 139.9 mM NaCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$), pH 7.4, supplemented with 5 mM glucose and 1 mM sodium pyruvate. After removal of the cerebellum, choroid plexuses, meninges, and white matter, the gray matter was homogenized in a four-fold volume of PBS complete using ~30 strokes of a Teflon-tipped drill mounted homogenizer (200 μm clearance) followed by ~5 strokes of a dounce homogenizer (150 μm clearance). Ficoll solution (30% w/v) was added to the homogenate with a ~1.2:1 ratio of ficoll to homogenate, and the samples were centrifuged for 20 min at 5800 x*g*, 4 °C. The pellet containing capillaries was resuspended in PBS complete containing 1% BSA (w/v) and applied to a column containing 40 mL of glass beads (Sartorius, Goettingen, Germany) pre-equilibrated with PBS complete containing 1% BSA. Following several washes with PBS complete with 1% BSA, capillaries were collected from the beads by gentle agitation and with several washes with ice cold PBS complete. The capillaries were then collected by centrifugation at 500 x*g* for 5 min at 4 °C and washed three times with PBS complete. The final capillary containing pellet was resuspended in 300-500 μL ice cold PBS complete and stored on ice until ready for experimentation.

Rat Brain Capillary P-gp Transport Assay. Capillaries were incubated at room temperature in PBS complete for 5 min before the addition of PBS complete (470 μL) containing various concentrations of the compounds of interest. Following a 45 min incubation at room temperature, PBS complete (500 μL) containing NBD-abacavir (2 μM) was added and the capillaries incubated another 30 min at room temperature. The capillaries were then analyzed for fluorescence using a Zeiss 510 NLO confocal scanning microscope using an Argon laser and 40× water-immersion objective. The pinhole diameter, photomultiplier gain, and laser

power were the same for all samples analyzed. Luminal fluorescence was quantified using ImageJ and the data represent the quantification of at least seven capillaries per data point.

HIV-1 cell inhibition assay

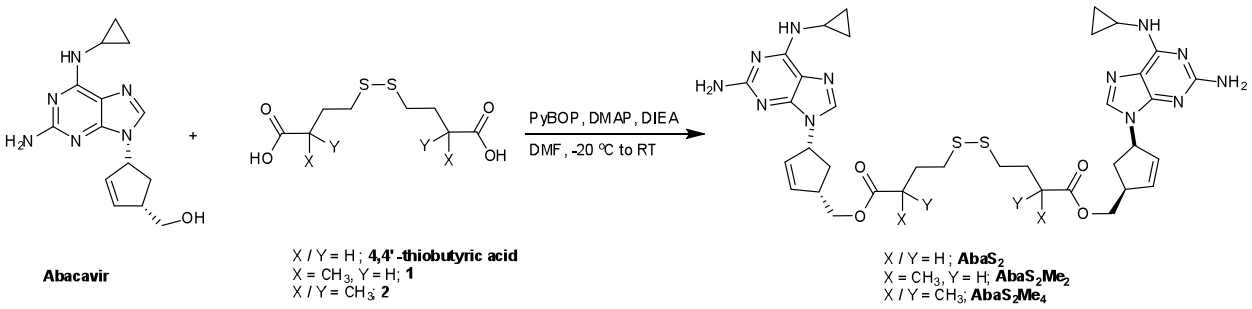
12D7 T-cells were maintained in RPMI 1640 medium supplemented with antibiotics/glutamine (100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 292 µg ml^{-1} glutamine, all from Invitrogen Corp., Carlsbad, CA) and 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT) at 37ºC. Cells in the log phase were pelleted, washed once with medium and resuspended at 1 million cells ml^{-1} and infected with 500 $TCID_{50}$ HIV-1 (Advanced Biotechnology Inc, Columbia, MD). After 4 hours, cells were washed three times with cell culture media and plated at a density of 75,000 cells ml^{-1}. Triplicate wells were then treated with increasing concentrations of abacavir, $AbaS_2Me_4$ or vehicle control (DMSO), keeping the DMSO concentration constant at 0.05%. Every four days half the media was exchanged and drug added to maintain treatment concentration. On day 12-14, an aliquot of the media was removed and the level of virus capsid protein in the media analyzed by ELISA as described previously.[9] Values represent the average p24 +/- the standard deviation.

HIV-1 Induced cell cytotoxicity assay

To assess the effect of compounds on acute HIV-1 infection, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to determine the ability of compounds to protect MT-2 cells from the cytopathic effect of $HIV\text{-}1_{LAI}$.[10] MT-2 cells (4×10^3 cells in 200 µl complete medium per well) grown in RPMI-based culture medium with10% fetal calf serum (HyClone, Logan, UT) with 50 units per ml penicillin and 50 mg per ml streptomycin were incubated with 100 $TCID_{50}$ of $HIV\text{-}1_{LAI}$ for 2 hrs and then added to 96 well plates containing the test compounds dissolved in DMSO. The final DMSO concentration of 0.5% was kept constant throughout the course of the experiment. Seven days later, 100 µl of medium was

removed and MTT solution (10 µl, 7.5 mg per ml) was added to each well. The plates were incubated at 37º C for 2 h and then each well treated with 100 µl of acidified isopropyl alcohol containing 4% (v/v) Triton X-100 to dissolve the formazan crystals. The absorbance at 570 nm was then determined in a microplate reader (Spectramax). Each assay was performed in duplicate. In this assay, the cytopathic effect of HIV-1 requires several rounds of infection and therefore is susceptible to inhibitors that act on the late stages of viral replication. The concentration of each compound required to inhibit HIV-1 induced cytotoxicity by 50% was calculated from the plot of drug concentration verses the percent viable cells compared to the untreated HIV-1 infected control.

Synthesis of Abacavir Prodrug dimers (**$AbaS_2$** , **$AbaS_2Me_2$** and **$AbaS_2Me_4$**)



Abacavir

X / Y = H ; **4,4' -thiobutyric acid**
X = CH_3, Y = H; **1**
X / Y = CH_3; **2**

X / Y = H ; **$AbaS_2$**
X = CH_3, Y = H; **$AbaS_2Me_2$**
X / Y = CH_3; **$AbaS_2Me_4$**

Supporting Scheme 1. A synthetic route to obtain Abacavir dimers, **$AbaS_2$, $AbaS_2Me_2$** and **$AbaS_2Me_4$**.

General Synthesis of Abacavir Prodrug Dimers

To a solution of bis-carboxylic acid (0.06 mmol) in dry dimethylformamide (DMF) (2 mL) at -20°C was added PyBOP (0.18 mmol), DMAP (0.03 mmol), and DIEA (0.6 mmol). After 20 min at -20°C, abacavir (0.18 mmol) was added. The mixture was stirred for 8 hours at -20°C, and then allowed to warm up to room temperature and stirred for 8 hours. The solvent was removed *in vacuo* and the resulting material was dissolved in DMSO and purified by reverse phase HPLC using a C5 column (Phenomenex, USA) with an eluent consisting of solvent A (methanol and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 60 min gradient of

20–95% solvent A, a flow rate of 20.0 mL/min and UV detection at 214 nm and 285 nm to give the title compound $AbaS_2$ (65%) / $AbaS_2Me_2$ (48%) or $AbaS_2Me_4$ (32%)

$AbaS_2$

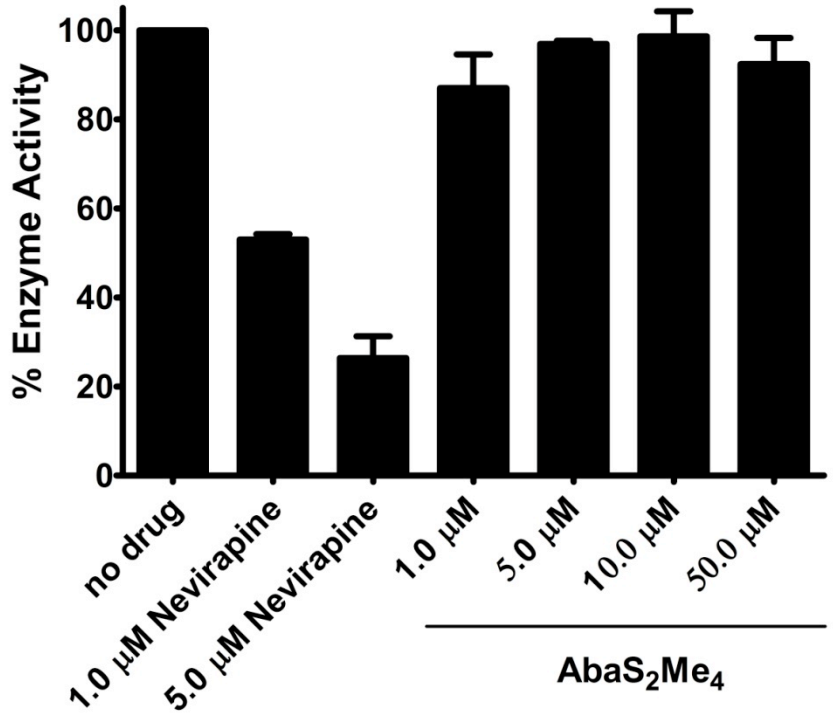
^{1}H NMR (300 MHz, DMSO-*d6*) δ 9.80 (broad singlet, 2H), 7.96 (s, 2H), 7.60 (broad singlet, 4H), 6.14 – 6.12 (m, 2H), 5.98 – 5.96 (m, 2H), 5.42 (m, 2H), 4.08 (d, *J* = 5.7 Hz, 4H), 3.09 (m, 4H), 2.80 (m, 2H), 2.72 – 2.62 (m, 4H), 2.39 – 2.35 (m, 4H), 1.84 – 1.79 (m, 4H), 1.70 (m, 2H), 0.90 – 0.88 (m, 4H), 0.76 (m, 4H). ^{13}C NMR (300 MHz, DMSO-d_6) δ172.4, 152.5, 139.0, 138.5, 130.0, 110.0, 66.2, 59.2, 45.8, 44.0, 40.3, 36.5, 34.2, 31.8, 25.9, 25.8, 23.8, 6.9 MS (MALDI-TOF) *m/z* 776 (M+2H) $^{2+}$. HRMS (ESI) calculated for $C_{36}H_{46}N_{12}O_4\ S_2$ 775.3285, found 775.3281 $(M+H)^+$ Purity (~99%) was determined by analytical HPLC, retention time 23.72 min using a C5 analytical column Phenomenex, USA) with an eluent consisting of solvent A (methanol and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 30 min gradient of 20–95% solvent A, a flow rate of 1.20 mL/min.

$AbaS_2Me_2$

^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.80 (broad singlet, 2H), 7.92 (s, 2H), 7.59 (broad singlet, 4H), 6.13 – 6.12 (m, 2H), 5.99 – 5.98 (m, 2H), 5.43 (m, 2H), 4.10 – 4.08 (d, *J* = 5.8 Hz, 4H), 3.10 (m, 4H), 2.90 (m, 2H), 2.73 – 2.69 (m, 4H), 2.63 – 2.53 (m, 2H), 1.85 (m, 2H), 1.67 – 1.60 (m, 4H), 1.06 (d, *J* = 6.8, 6H), 0.87 – 0.86 (m, 4H), 0.74 (m, 4H). ^{13}C NMR (300 MHz, DMSO-d_6) δ175.1, 158.0, 152.0, 137.2, 130.1, 111.9, 66.3, 59.2, 44.0, 37.5, 35.0, 34.3, 32.5, 23.2, 16.6, 6.9. MS (MALDI-TOF) *m/z* 804 $(M+2H)^{2+}$. HRMS (ESI) calculated for $C_{38}H_{50}N_{12}O_4\ S_2$ 803.3598 found 803.3593 $(M+H)^+$. Purity (~92%) was determined by analytical HPLC, retention time 15.54 min using a C5 analytical column (Phenomenex, USA) with an eluent consisting of solvent A (acetonitrile and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 30 min gradient of 5–95% solvent A, a flow rate of 1.20 mL/min.

Aba$S_2$$Me_4$

^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.70 (broad singlet, 2H), 7.97 (s, 2H), 7.50 (broad singlet, 4H), 6.15 – 6.14 (m, 2H), 6.02 – 6.00 (m, 2H), 5.47 (m, 2H), 4.11 – 4.09 (d, *J* = 6.3Hz, 4H), 3.10 (m, 2H), 3.0 (m, 2H), 2.70 (m, 2H), 2.59 – 2.52 (m, 4H), 1.82 – 1.78 (m, 4H), 1.70 (m, 2H), 1.15 (s, 12H), 0.91 – 0.90 (m, 4H), 0.77 (m, 4H). ^{13}C NMR (300 MHz, DMSO-d_6) δ178.6, 154.0, 152.0, 140.5, 138.9, 131.0, 113.5, 68.0, 61.6, 46.0, 43.6, 41.6, 36.1, 35.3, 25.7, 25.6, 24.0, 8.0. (MALDI-TOF) *m/z* 832 $(M+2H)^{2+}$. HRMS (ESI) calculated for $C_{40}H_{54}N_{12}O_4S_2$ 831.3911 found 831.3901 $(M+H)^+$. Purity (~99%) was determined by analytical HPLC, retention time 25.85 min using a C5 analytical column (Phenomenex, USA) with an eluent consisting of solvent A (acetonitrile and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 30 min gradient of 2-50% solvent A, a flow rate of 1.20 mL/min.



Supporting Figure 1. Assessment of Inhibitory activity of $AbaS_2Me_4$ against HIV-1 Reverse Transcriptase *in vitro* was determined using a reverse transcriptase (RT) assay kit, colometric (Roche, USA). The non nucleoside reverse transcriptase inhibitor of HIV-1 RT, Nevirapine, was used as a positive control. The abacavir monomer was not tested as it is not active in this assay – needs to be phosphorylated.

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