Supporting Information

Preclinical Optimization of gp120 Entry Antagonists as anti-HIV-1 Agents with Improved Cytotoxicity and ADME Properties through Rational Design, Synthesis, and Antiviral Evaluation

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Contents:

EXPERIMENTAL

ADME

Solubility

The equilibrium solubility of one test article was measured in pH 7.4 aqueous buffer. The buffer was prepared by combining 50 mL of 0.2 M KH_2PO_4 with 150 mL of H_2O and then adjusting to pH 7.4 with 10 N NaOH. At least 1 mg of powder test article was combined with 1 mL of buffer to make a ≥ 1 mg/mL mixture. This sample was shaken on a Thermomixer® overnight at room temperature. The samples were then passed through a 0.45 µm PTFE syringe filter. The filtrate was sampled and diluted in duplicate 10-, 100-, 1,000-, and 10,000-fold into a mixture of 1:1 buffer:acetonitrile (ACN) prior to analysis. All samples were assayed by LC-MS/MS using electrospray ionization against standards prepared in a mixture of 1:1 assay buffer:ACN. Standard concentrations ranged from 1.0 µM to 0.1 nM.

CACO-2 Permeability

Caco-2 cells (clone C2BBe1) were obtained from American Type Culture Collection (Manassas, VA). Cell monolayers were grown to confluence on collagen-coated, microporous membranes in 12-well assay plates. Details of the plates and their certification are shown below. The permeability assay buffer was Hanks' balanced salt solution (HBSS) containing 10 mM HEPES and 15 mM glucose at a pH of 7.4. The buffer in the receiver chamber also contained 1% bovine serum albumin. The dosing solution concentration was 5 µM of test article in the assay buffer ±-1 µM valspodar. Cells were first pre-incubated for 30 minutes with HBSS containing ± 1 µM valspodar. Cell monolayers were dosed on the apical side (A-to-B) or basolateral side (B-to-A) and incubated at 37°C with 5% CO_2 in a humidified incubator. Samples were taken from the donor and receiver chambers at 120 minutes. Each determination was performed in duplicate. The flux of lucifer yellow was also measured post-experimentally for each monolayer to ensure no damage was inflicted on the cell monolayers during the flux period. All samples were assayed

by LC-MS/MS using electrospray ionization. Analytical conditions are outlined in Appendix 1. Please note that the cell lines did not have mycoplasma contamination.

The apparent permeability (Papp) and percent recovery were calculated as follows:

$$P_{app} = (dC_r/dt) \times V_r/(A \times CA) \quad (1)$$

$$\text{Percent Recovery} = 100 \times ((V_r \times C_r^{final}) + (V_d \times C_d^{final}))/(V_d \times C_N) \quad (2)$$

Where,

dC_r/dt is the slope of the cumulative concentration in the receiver compartment versus time in μM s^{-1};

V_r is the volume of the receiver compartment in cm^3;

V_d is the volume of the donor compartment in cm^3;

A is the area of the insert (1.13 cm2 for 12-well);

C_A is the average of the nominal dosing concentration and the measured 120-minute donor concentration in μM;

C_N is the nominal concentration of the dosing solution in μM;

C_r^{final} is the cumulative receiver concentration in μM at the end of the incubation period;

C_d^{final} is the concentration of the donor in μM at the end of the incubation period.

Efflux ratio (ER) is defined as P_{app} (B-to-A) / P_{app} (A-to-B).

Stability in liver microsomes

Mixed-gender human liver microsomes (Lot# 1010420) were purchased from XenoTech. The reaction mixture, minus NADPH, was prepared as described below. The test article was added into the reaction mixture at a final concentration of 3 μM. The control compound, testosterone, was run simultaneously with the test article in a separate reaction. An aliquot of the reaction mixture (without cofactor) was equilibrated in a shaking water bath at 37°C for 3 minutes. The reaction was initiated by the addition of the cofactor, and the mixture was incubated in a shaking water bath at 37°C. Aliquots (100 μL) were withdrawn at 0, 15, 30, 60, 90, and 120 minutes. Samples were immediately combined with 400 μL of ice-cold 50/50 acetonitrile (ACN)/H_2O

containing 0.1% formic acid and internal standard to terminate the reaction. The samples were then mixed and centrifuged to precipitate proteins. All samples were assayed by LC-MS/MS using electrospray ionization. Analytical conditions are outlined in Appendix 1. The peak area response ratio (PARR) to internal standard was compared to the PARR at time 0 to determine the percent remaining at each time point. Half-lives and clearance were calculated using GraphPad software, fitting to a single-phase exponential decay equation.

When the calculated half-life is longer than the duration of the experiment, the half-life is expressed as > the longest incubation time. Then, if the calculated half-life is < 2x the duration of the experiment, the calculated half-life is listed in parentheses.

Intrinsic clearance (CL_{int}) was calculated based on $CL_{int} = k/P$, where k is the elimination rate constant, and P is the protein concentration in the incubation.

Plasma protein binding

Studies were carried out in mixed-gender human plasma (Lot# AS1465-88), obtained from BioIVT and collected on sodium heparin. A Pierce Rapid Equilibrium Dialysis (RED) device

was used for all experiments. Stock solutions of the test articles and control compounds were first prepared in DMSO. Aliquots of the DMSO solutions were dosed into 1.0 mL of plasma at a dosing concentration of 5 µM for the test article and 10 µM for the co-dosed control compound, to each corresponding receiver chamber. The device was then placed into an enclosed heated rocker that was pre-warmed to 37°C and allowed to incubate for four hours. After four hours of incubation, both sides were sampled.

Aliquots (50 µL for the donor, 200 µL for receiver) were removed from the chambers and placed into a 96-well plate. Plasma (50 µL) was added to the wells containing the receiver samples, and 200 µL of PBS was added to the wells containing the donor samples. Two volumes of acetonitrile (ACN) were added to each well, and the plate was mixed and then centrifuged at

3,000 rpm for 10 minutes. Aliquots of the supernatant were removed, diluted 1:1 into the water, and analyzed by LC-MS/MS.

Protein binding values were calculated as follows:

% Bound = [(PARR in Donor – PARR in Receiver) / (PARR in Donor)] × 100

PARR = peak area response ratio to the internal standard, including applicable dilution factors.

Cyp450 (CYP) inhibition

The test article, at eight concentrations (0-100 µM), was incubated with pooled HLM (0.25 mg protein/mL) in phosphate buffer (100 mM, pH 7.4) containing $MgCl_2$ (5 mM), NADPH (1 mM), and an individual CYP probe substrate (at approximately Km). The reaction mixture minus NADPH was equilibrated in a shaking water bath at 37ºC for 5 minutes. The reaction was initiated by the addition of NADPH, followed by incubation at 37°C for 10-30 minutes, depending on the individual CYP isoform. The reaction was terminated by the addition of two volumes of ice-cold acetonitrile. Negative (vehicle) controls were conducted without the test article. Positive controls were performed in parallel at a single concentration using known CYP inhibitors. After the removal of protein by centrifugation at 1,640*g* (3,000 rpm) for 10 minutes at 4ºC, the supernatants were transferred to a 96-well plate and diluted with water containing internal standard (stable isotope-labeled CYP probe metabolite). The formation of the CYP probe metabolite was determined by LC-MS/MS.

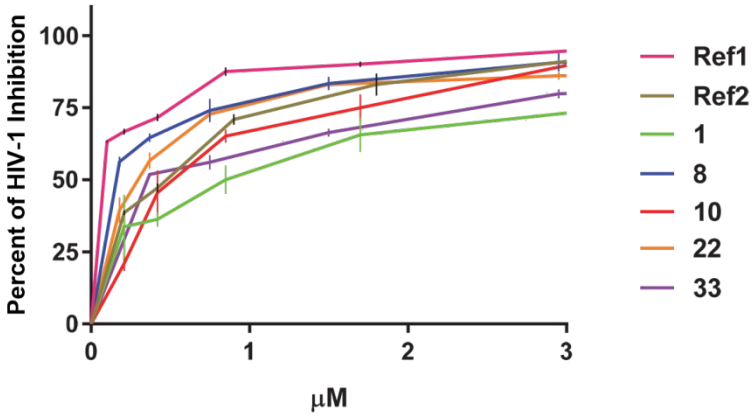

Figure S1. Dose-percent inhibition plots of two Reference compounds and some most active [with high selectivity index(SI)] inhibitors.

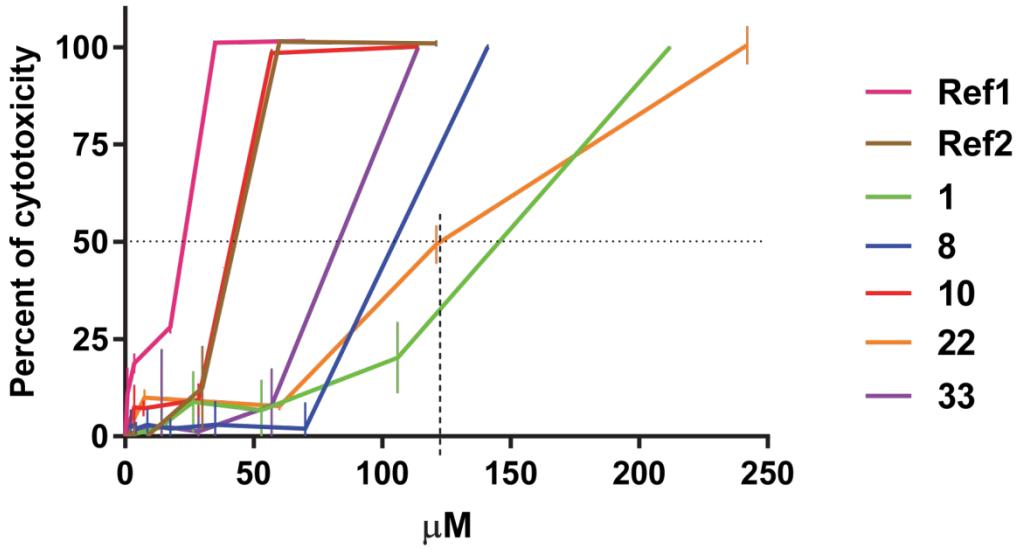

Figure S2. Dose-percent cytotoxicity plots of two Reference compounds and some most active [with high selectivity index(SI)] inhibitors.