Supporting Information

Synthesis and Evaluation of Orally Active Small Molecule HIV-1 Nef Antagonists

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General Chemistry Procedures

^{1}H NMR spectra were recorded on a Varian Oxford 300 MHz instrument in DMSO-d_6. Chemical shifts (δ) are expressed in ppm downfield from tetramethylsilane (TMS). LC/MS data (ESI+) for compounds **3** and **5**-**7** were obtained with a Waters Alliance 2695 HPLC/MS (Waters Symmetry C18, 4.6 × 75 mm, 3.5 µm) with a 2996 diode array detector from 210−400 nm; the solvent system was 5−95% MeCN in water (with 0.1% TFA) over nine minutes using a linear gradient, and retention times are in minutes. Mass spectrometry was performed on a Waters ZQ instrument using electrospray in positive ion mode. MS data (ESI-) for compounds **2** and **4** were recorded on an AccuTOF.

Synthesis of Compound 2

4-[2-(3-Chlorophenyl)-ethyl]-5-hydroxy-3-(4-nitrophenyl)-pyrazole-1-carbothioic acid amide

Step 1. 4-(3-Chlorophenyl)-2-(4-nitrobenzoyl)butyric acid ethyl ester

A mixture of ethyl 4-nitrobenzoylacetate (7.00 g, 29.5 mmol), 3-chlorophenethyl bromide (6.80 g, 31.0 mmol), potassium carbonate (4.49 g, 32.5 mmol) and sodium iodide (4.87 g, 32.5 mmol) in DMF (100 mL) was stirred at 60 °C overnight. The reaction was quenched with water and the product was extracted with EtOAc. The combined organic extracts were washed with brine and the crude material was purified by flash chromatography (120 g silica, 2-10% EtOAc/Hexane gradient) to obtain 3.56 g (32% yield) of a pale yellow oil. ^{1}H NMR (300MHz, $CDCl_3$) δ = 8.33 - 8.07 (m, 2H), 7.52 (d, *J*=7.3 Hz, 2H), 7.26 - 7.19 (m, 3H), 7.19 - 7.01 (m, 1H), 5.79 - 5.61 (m, 1H), 4.40 - 4.10 (m, 4H), 3.20 - 2.93 (m, 2H), 1.38 - 1.28 (m, 3H).

Step 2. 4-[2-(3-Chlorophenyl)-ethyl]-5-hydroxy-3-(4-nitrophenyl)-pyrazole-1-carbothioic acid amide

4-(3-Chlorophenyl)-2-(4-nitrobenzoyl)butyric acid ethyl ester (3.11 g, 8.28 mmol), thiosemicarbazide (1.51 g, 16.6 mmol) and Amberlyst-15 Resin (500 mg) were combined in Ethanol (41 mL) and heated at 60 °C for one week. The reaction was quenched with water and the product was extracted with EtOAc. The combined organic extracts were washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to a yellow solid. The crude product was purified by flash chromatography (80 g silica, 0-5% MeOH/DCM gradient) to obtain 1.17 g (35% yield) of a pale yellow solid. ^{1}H NMR (300MHz, CD_3OD) δ = 8.33 - 8.16 (m, 2H), 8.00 - 7.36 (m, 2H), 7.17 - 7.03 (m, 2H), 7.02 - 6.96 (m, 1H), 6.95 - 6.87 (m, 1H), 2.91 - 2.68 (m, 4H). MS (ESI-) *m*/*z* 401 (44%, M-1), 403 (16%, M-1), 342 (100%), 344 (32%).

Compound 3: 4-[2-(3-Chlorophenyl)ethyl]-5-(4-nitrophenyl)-2*H*-pyrazol-3-ol

^{1}H NMR (300MHz, CD_3OD) δ = 8.33 - 8.11 (m, 2H), 7.55 - 7.33 (m, 2H), 7.16 - 6.78 (m, 4H), 2.94 - 2.65 (m, 4H). LCMS (ESI) *m*/*z* 343.9, 346.0 (M, M+2)$^+$.

Compound 4: 4-(3-Chlorobenzyl)-5-hydroxy-3-(4-nitrophenyl)pyrazole-1-carbothioic acid amide

^{1}H NMR (300MHz, DMSO-d6) δ = 8.36 - 8.14 (m, 2H), 7.80 (d, *J*=8.2 Hz, 2H), 7.47 - 7.30 (m, 1H), 7.24 - 7.02 (m, 3H), 3.83 (s, 2H). MS (ESI-) m/z 387 (93%, M-1) 389 (35%, M-1), 328 (100%) 330 (33%).

Compound 5: 4-(3-Chlorobenzyl)-5-(4-nitrophenyl)-2*H*-pyrazol-3-ol

^{1}H NMR (300MHz, DMSO-d6) δ = 8.30 - 8.08 (m, 2H), 7.60 (d, *J*=8.5 Hz, 2H), 7.47 - 7.32 (m, 1H), 7.25 - 7.09 (m, 2H), 7.03 - 6.82 (m, 1H), 3.88 (s, 2H). LCMS (ESI) *m*/*z* 330.0, 331.9 (M, M+1)$^+$.

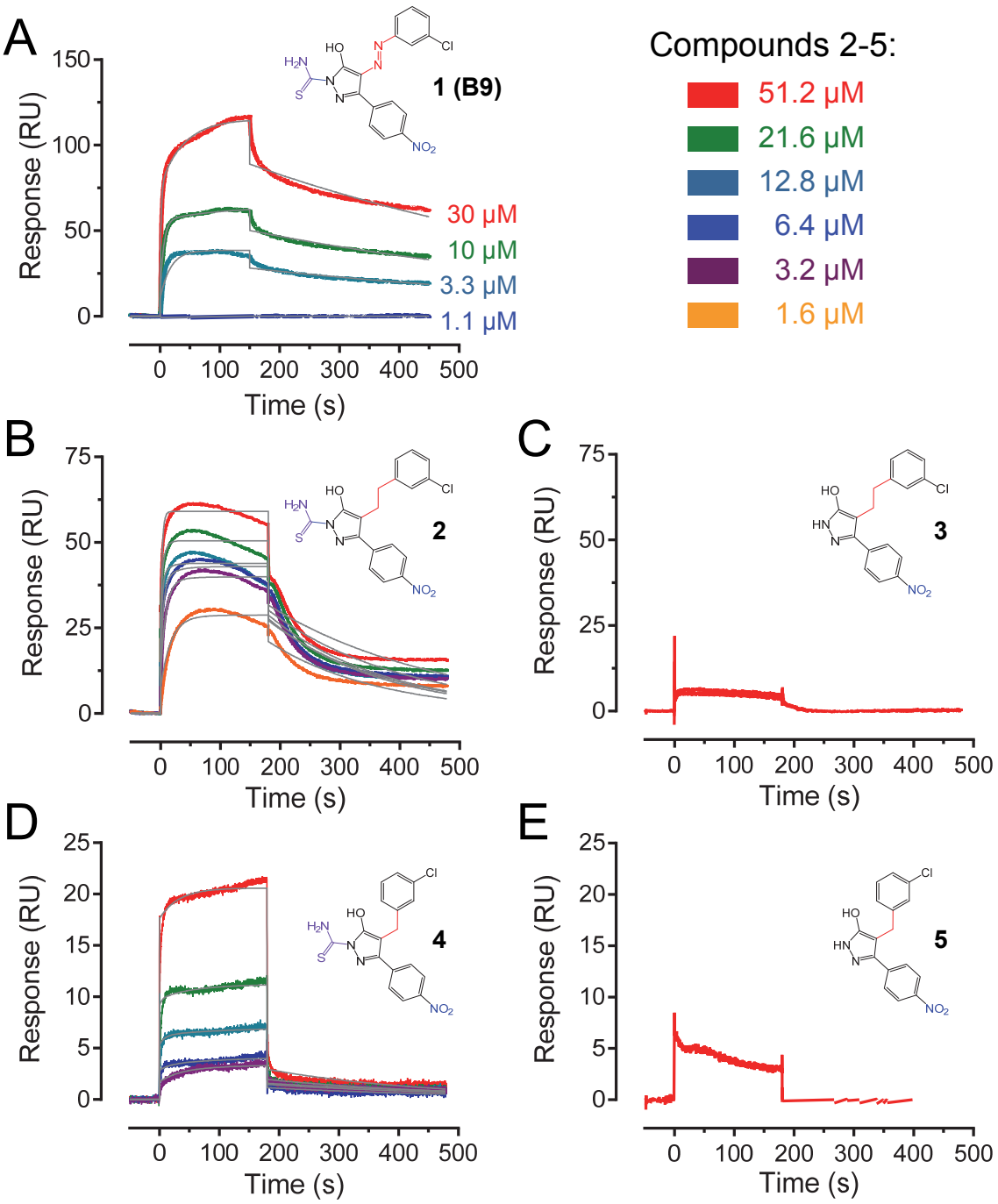
Computational Docking Methods

The X-ray crystal coordinates of the HIV-1 Nef protein dimer were downloaded from the protein data bank (PDB ID: 1EFN). The Protein Preparation Wizard, a module in Schrödinger's Small Molecule Drug Discovery Suite (Release 2015-1), was used to add hydrogens to the structure. The loops missing from the Nef structure are not at the dimer interface and were not modeled. The OPLS2005 force field was used. For refinement, the optimization tool was used and within the binding pocket, Q107 was flipped. In addition, D111, which is within the binding pocket, was predicted by EPIC to be neutral and kept neutral during docking. Other changes were outside the binding pocket. Finally, a restrained minimization was run that converged the heavy atoms to an RMSD of 0.2 Å. Possible conformational states were generated at a target pH of 7.0 ± 2.0 and tautomers were generated. Docking grids were centered on the middle of the Nef dimer interface and box dimensions were 36x36x36 Å, thus sampling the entire dimer interface. Ligands were docked using extra precise docking, and Epik state penalties were added to the docking score. The ligand sampling was flexible, and post-docking minimization was done with the addition of strain correction terms. Induced-fit docking was done with default settings. RRHO and SASA scripts were downloaded from Schrödinger. Hot-spot mapping was done as previously described (Kulp III *et al*. *J. Am. Chem. Soc.* **133**: 10740, 2011).

Table S1. Docking scores, induced-fit docking (IFD) scores, and rigid-rotor-harmonic-oscillator energies (entropy) for compounds **1**, **2**, and **4**.

Compound	Docking Score	IFD score	Entropy (TΔS, kcal/mol)
1	-4.3	-6.6	-14.6
2	-3.5	-6.8	-16.3
4	-3.3	-6.7	-15.6

Figure S1. Representative sensorgrams for the interactions of compounds **1-5** with recombinant Nef.



SPR analyses were performed on a BIAcore T100 instrument (GE Healthcare) using four-channel CM5 biosensor chips with recombinant HIV-1 Nef covalently attached as ligand via amine coupling chemistry. Compounds were injected and flowed past immobilized Nef at a rate of 10 μl/min for 3 min, followed by dissociation and surface regeneration. Binding curves recorded at each compound concentration were assessed in triplicate, corrected for buffer effects, and best-fit to a heterogeneous ligand binding model using the BIAcore T100 evaluation software (version 2.0.4; GE Healthcare). Panel A shows representative sensorgrams obtained with compound **1** (B9 parent) at the indicated concentrations. Panels B-E show sensorgrams for compounds **2**-**5**, at the concentrations indicated at the upper right. For compounds **3** and **5**, the result with highest compound concentration is shown. In all cases a single trace is shown for each compound concentration, and the fitted curves are overlaid as grey lines.

HIV assays

Stocks of HIV-1 were generated by transfection of 293T cells (ATCC) with wild-type and Nef-defective (ΔNef) proviral genomes (NL4-3 strain) followed by amplification in the T-cell line, MT2 (NIH AIDS Research and Reference Reagent Program) as described elsewhere.[1-3] HIV-1 replication was monitored in U87MG astroglioma cells stably transfected with expression vectors for CD4 and CXCR4[1-3] (NIH AIDS Research and Reference Reagent Program). HIV-1 replication is strongly enhanced by Nef in these cells.[1-3] U87MG/CD4/CXCR4 cells (2.0 x 10^4/well in 96-well plates) were allowed to attach overnight prior to infection. Compounds (solubilized in DMSO) were added to the culture medium 1 h prior to infection with HIV-1_{NL4-3} (50 pg p24/ml). Viral replication was assessed 4 d later as p24 Gag protein levels in the culture supernatant using the AlphaLISA method (Perkin Elmer). The AlphaLISA assay offers a high-sensitivity alternative to standard ELISA methods by utilizing p24-mediated streptavidin/biotin bead conjugation to induce singlet oxygen energy transfer, resulting in light emission at 615 nm.[4] HIV-1 infectivity was measured using TZM-bl reporter cells [5,6] (NIH AIDS Research and Reference Reagent Program) in which the HIV-1 LTR is linked to the expression of firefly luciferase. TZM-bl cells (2.5 x 10^4/well in 96-well plates) were allowed to adhere overnight prior to infection. Cells and HIV-1_{NL4-3} (9000 pg p24/ml) were incubated separately with compounds for 3 h and then combined to a final volume of 200 µl in each well. Following incubation for 48 h at 37 $^{\circ}$C, the cells were lysed in 50 µl luciferase cell culture lysis reagent (Promega). Lysates (40 µl) were then transferred to white 96-well plates followed by 50 µl injections of luciferase reagent per well (Promega). Luminescence was then recorded with a delay time of 2 s and an integration period of 10 s. Cytotoxicity of each compound was evaluated in both cell lines using the Cell Titer Blue cell viability assay (Promega).

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Pharmacokinetics and toxicity studies

Pharmacokinetic studies were conducted by SAI Life Sciences Ltd., Hyderabad, India. Pharmacokinetics experiments were performed in male C3H mice following a single intravenous bolus or oral administration. For each experiment, eighteen male mice were divided into two groups of nine; animals in each group were administered compounds by either the intravenous (IV) or oral (PO) routes at 2 mg/kg and 10 mg/kg, respectively. Compound formulations are provided in the legend to Table 2 in the main text. Blood samples (approximately 60 µL) were collected from the retro-orbital plexus pre-dose, 0.08, 0.25, 0.5, 1, 2, 4, 8 and 24 h later for the IV group and pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h later for the PO group. Plasma samples were separated by centrifugation of whole blood and stored below -70 $^{\circ}$C until bioanalysis. Plasma proteins were precipitated using acetonitrile and analyzed via LC/MS/MS with a lower detection limit of 1.00 ng/mL. Pharmacokinetic parameters were calculated using the non-compartmental analysis tool of Phoenix WinNonlin (Version 6.3). For acute toxicity studies, male and female C3H mice were administered compounds either once or twice per day at various doses. Animals were assessed daily for changes in body weight, body condition, and overall behavior for 4 to 12 days (Table S2).

Table S2. Acute toxicity testing of compounds **1**, **2** and **4** in C3H mice.[a]

Compound	Route	Schedule	Dose (mg/kg)	Vehicle	Duration (days)	Sex	Acute toxicity
1 (B9)	IV	SID	2.5	PEG[b]	4	3M/1F	None
	IV	SID	2.5	PEG	4	3M/1F	None
2	IV	SID	5.0	PEG	4	2M/2F	None
	PO	BID	10	Na-CMC[c]	4	1M/3F	None
	PO	BID	10	Na-CMC	4	2M/3F	None
	PO	SID	50	Na-CMC	5	2M	None
4	PO	SID	100	Na-CMC	12	1M/2F	None

[a]Each row shows the results of an independent experiment.
[b]PEG: 40% polyethylene glycol 400 with 0.1% Tween 80 in isotonic saline.
[c]Na-CMC: 0.5% w/v sodium carboxymethyl cellulose with 0.1% v/v Tween 80 in isotonic saline.