Supporting Information

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SI Text

Synthesis of a Bifunctional Protease Inhibitor. N-[(2R,3S)-3-amino-2-hydroxy-4-phenylbutyl]-N-isobutyl-4-methoxybenzenesulfonamide (**3**). (2S, 3S)-1,2-Epoxy-3-(Boc-amino)-4-phenylbutane (Sigma, 50 mg, 0.1898 mmol) and isobutylamine (Sigma; 38.4 μl, 0.3797 mmol, 2 equivalents) were stirred in 2.0 ml of methanol overnight at room temperature. The reaction was then concentrated in vacuo and brought up in 3.0 ml of dry dichloromethane (DCM). Et_3N (29.7 μl, 0.2128 mmol, 1.1 equivalents) was added dropwise, followed by reaction with 4-methoxybenzenesulfonyl chloride (43.9 mg. 0.2128 mmol, 1.1 equivalents) and the mixture was stirred overnight at room temperature to generate compound **2**. This crude compound was carried forward by addition of 3.0 ml of trifluoroacetic acid and stirring for another 3 h. The mixture was then brought up in 8.0 ml of DCM and the organic phase was washed once with 50 ml of H_2O, once with 50 ml of saturated $NaHCO_3$, once with 50 ml of H_2O, once with brine and dried over Na_2SO_4. The crude product was filtered and evaporated to dryness. After drying, **3** (58.6 mg, 0.1441 mmol, 76%; 3 steps) was obtained as a white solid. *m/z* 407.05 [M + H, calculated 406.54].

Tetrahydrofuran-3-yl (2S,3R)-3-hydroxy-4-(N-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-ylcarbamate (4; 4-methoxy amprenavir). 2,5-dioxopyrrolidin-1-yl tetrahydrofuran-3-yl carbonate (22.5 mg, 0.0984 mmol, 2 equivalents) and EtN_3 (27.4 μl, 0.1968 mmol, 4 equivalents) were added to a stirred solution of **3** (20.0 mg, 0.0492 mmol) in 2.0 ml of DCM and allowed to proceed 3 h at room temperature. The reaction was brought up in 6.0 ml of DCM, washed 3 times with 5% $NaHCO_3$, once with brine, and dried over Na_2SO_4. The crude product was then filtered and evaporated to dryness. After drying, **4** (25.1 mg, 0.0482 mmol, 98%) was obtained as an oily residue. *m/z* 521.20 [M + H, calculated 520.64].

4-[(2S,3R)-3-hydroxy-4-(N-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-ylamino]-4-oxobutanoic acid (5). Succinic anhydride (49.9 mg, 0.4986 mmol, 5 equivalents) was added to **3** (40.6 mg, 0.0998 mmol) in 3.0 ml of DCM and allowed to stir at room temperature. After 2 h, the reaction was placed in ice bath to precipitate unreacted succinic anhydride, followed by vacuum filtration. The organic layer washed three times with 5% citric acid, once with brine, and dried over Na_2SO_4. The crude product was then filtered and evaporated to dryness. After drying, **5** (48.6 mg, 0.0961 mmol, 96%) was obtained as a residue. *m/z* 507.10 [M + H, calculated 506.61].

(R)-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(4-((2S,3R)-3-hydroxy-4-(N-isobutyl-4-nitrophenylsulfonamido)-1-phenylbutan-2-ylamino)-4-oxobutanamido)phenyl)propyl) 1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (6; SLFavir). Diisopropylcarbodiimide (115 μl, 0.7324 mmol, 10 equivalents) was added to **5** (38.2 mg, 0.0732 mmol) in 3.0 ml of dimethylformamide (DMF), and stirred for 20 min at room temperature. SLF (38.4 mg, 0.0732 mmol, 1.0 equivalent) was then added with dimethylaminopyridine (9.0 mg, 0.0732 mmol, 1.0 equivalent) and the reaction was stirred overnight at room temperature. The reaction mixture was then brought up in 8.0 ml of DCM, washed 3 times with H_2O, once with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The product was further purified by preparative chromatography on a Waters Spherisorb S10 ODS2 column (10 × 250 mm) to yield **6** (48.1 mg, 0.04384 mmol, 64%) as a pale yellow solid. *m/z* 1028.50 [M + H, calculated 1028.22].

HIV Protease Inhibition Assay. To determine the inhibitory potency of PIs, we used a commercially available, FRET-based assay (Bachem). Briefly, 5.0 μL of a 120 nM HIV-1 protease solution in Buffer P (20% glycerol, 0.1% CHAPS, 20 mM K2HPO4, 1.0 mM EDTA, 1.0 mM DTT at pH 5.5) was preincubated with 2.0 μL of inhibitor and 1.0 μL of a 2% PEG-400 solution for 60 min at 37 °C. Then, 12.0 μL of HIV-1 protease substrate 1 (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(dabcyl)-Arg) (Molecular Probes) diluted in Buffer P was added to the wells to a final concentration of 30 nM. The change in fluorescence was monitored at 490 nm on a SpectraMax M5 plate reader (Molecular Devices) for 60 min at 37 °C.

Determination of Inhibitor Concentrations in Plasma and Blood. Both blood and plasma inhibitor levels were determined by using a modified version of an established and validated LC-MS method (Annesley TM, Clayton L (2004) Simple extraction protocol for analysis of immunosuppressant drugs in whole blood. *Clin Chem* 50:1845–1848.) Briefly, 100 μL of blood or plasma was added to a 1.5 μL polypropylene microcentrifuge tube, followed by protein precipitation with 100 μL of a 0.1 M aqueous zinc sulfate and immediate vortexing for 30 s. Analytical grade acetonitrile (ACN) was then added (800 μL) and the sample was vortexed again for 30 s. After high-speed centrifugation at 13,200 rpm for 10 minutes, the supernatant was transferred to a glass vial and subjected to LC-MS analysis. The LC-MS system used for these studies was a Shimadzu series 2010EV instrument equipped with an APCI probe to minimize ion suppression. Quantification was performed using LCMSolution Version 2.05 and a set of external standards.

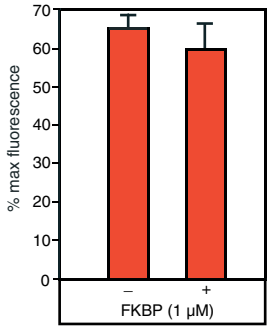
Fluorescent Probe: Bodipy-SLF. SLF (1.50 mg, 0.00286 mmol) was dissolved in 2.0 ml of DMF and then added to Bodipy FL C_5 SE (Molecular Probes; 1.31 mg, 0.00314 mmol, 1.1 equivalents) with stirring. EtN_3 (10.0 μl, 0.07175 mmol, 25 equivalents) was then added dropwise and the reaction was allowed to proceed at room temperature for 12 h. The reaction was quenched by adding Tris-amine Polystyrene HL Resin (Biotage; 1.0 mg, 1.0 equivalents) and stirring for 60 min, followed by vacuum filtration. The filtrate was brought up in 8.0 ml of DCM, washed three times with H_2O, once with brine, and dried over Na_2SO_4. The crude product was then evaporated under reduced pressure to yield Bodipy-SLF as a purple solid.

Cell Culture. Mouse embryonic fibroblast (MEF) cell lines were derived from wild-type (WT) mice as well as mice in which both alleles of the FKBP12 gene were disrupted; these mutant MEFs fail to express FKBP12 (35). MEF cells were cultured in Dulbecco's modified essential medium (DMEM) with high glucose supplemented with 10% FBS, 25 μg/ml Pen-Strep/Fungizone, and 55 μM β-mercaptoethanol. Cells were grown at 37°C in 5% CO_2 and 90% humidity.

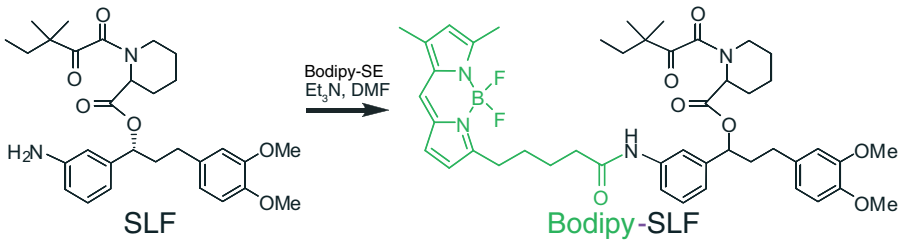
Confocal Microscopy. MEFs were seeded in 35-mm poly-D-lysine coated, glass-bottomed culture dishes at 100,000 cells per plate. Before microscopy, the media was replaced with Opti-MEM I reduced serum medium. The cells were then treated with Hoescht stain and either the mock-conjugated control probe, which lacks the SLF group, or Bodipy-SLF at a final concentra-

tion of 100 μM for 30 min and then washed with media. Confocal microscopy was performed on an Olympus laser-scanning confocal microscopy (FV500) by using single line excitation (488 nm). In the competition experiments, FK506 (LC Labs; 100 μM) was preincubated with the MEFs for 30 min.

(A) FKBP does not block the activity of SLFavir



(B) Synthesis of a fluorescent FKBP probe



(C) Model for selective protection of FKBP-binding protease inhibitors

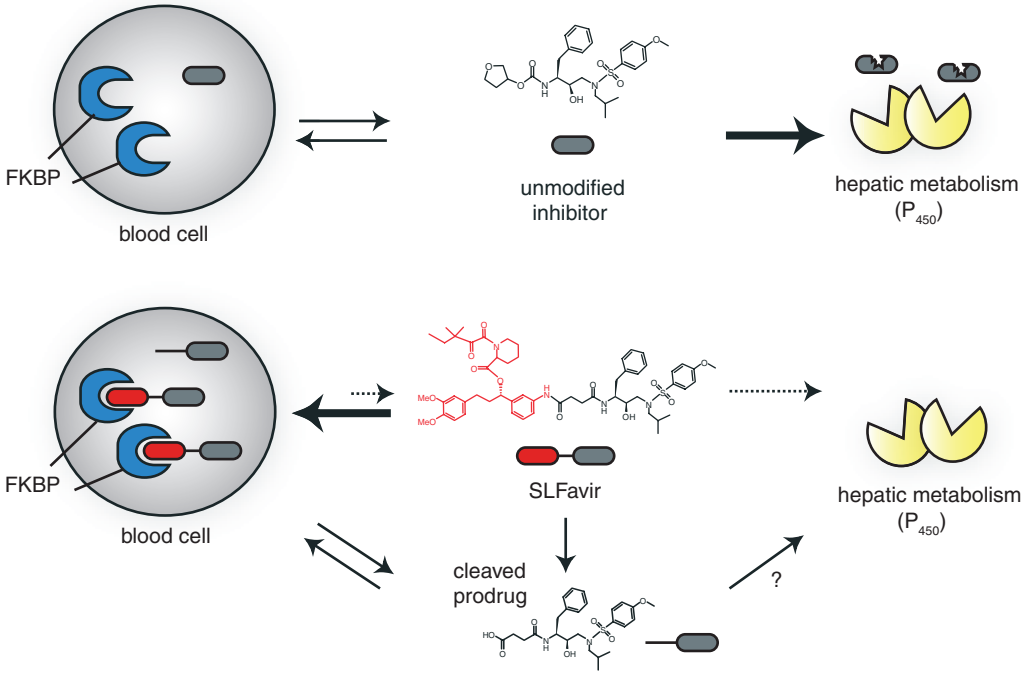


Fig. S1. (*A*) Addition of recombinant, FKBP (1 μM) did not reduce the anti-protease activity of SLFavir, which suggests that binding is not disrupted by recruitment of FKBP. (*B*) Synthesis of a fluorophore-labeled SLF. Purified yield was ≈75% (*C*) Model for selective protection of SLFavir, in which the cleaved prodrug and the bifunctional compound contribute to anti-viral activity.