Structure-Based Design of HIV Protease Inhibitors: 4-Hydroxycoumarins and 4-Hydroxy-2-pyrones as Non-peptidic Inhibitors#

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The rapid spread of the acquired immunodeficiency syndrome (AIDS) epidemic has stimulated discovery for therapeutic agents to arrest the replication of the causative virus, human immunodeficiency virus (HIV). One promising possibility to interrupt the viral life cycle is the use of inhibitors of the virally encoded protease responsible for viral maturation.[1,2] Among the most potent inhibitors reported thus far are peptidomimetic compounds containing transition-state inserts in place of the dipeptidic cleavage sites of the substrates.[3–6] The low oral bioavailability and rapid biliary excretion of peptide-derived compounds[7] have limited their utility as potential therapeutic agents. Recent advances have resulted in HIV protease inhibitors with reduced peptidic character and non-peptidic inhibitors that are more orally bioavailable, and an increasing number of HIV protease inhibitors[8–14] are currently undergoing clinical evaluations. We have previously reported the potent peptidomimetic inhibitor U-75875 (Noa-His-Chaψ[CH-(OH)CH(OH)]Val-Ile-Amp)[15,16] which was shown to have inhibitory effect on SIV in Rhesus monkeys only after continuous intravenous infusion.[17] Interest in orally bioavailable HIV protease inhibitors led us to search for inhibitors in non-peptidic templates that might offer superior biopharmaceutic properties.

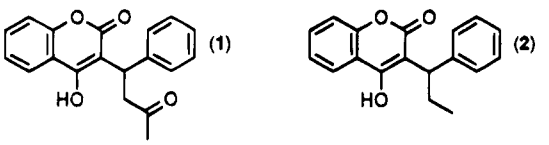


Figure 1. Structures of **1** (warfarin) and **2** (phenprocoumon).

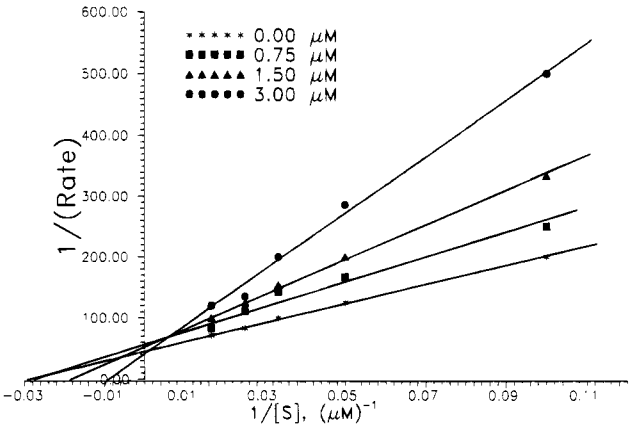
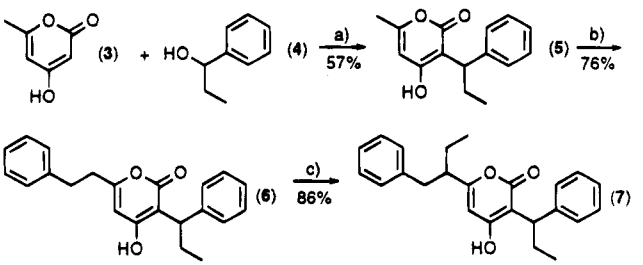


Figure 2. Lineweaver–Burk plot for inhibitor **2**. Initial rates of substrate hydrolysis by HIV-1 protease were determined at various substrate and inhibitor concentrations at 37 °C. The substrate, Lys-Ala-Arg-Val-Nle-ϱNO$_2$Phe-Glu-Ala-Nle, generates an increase in absorbance at 300 nm upon cleavage. The assay buffer consisted of 50 mM NaOAc, 50 mM MES, 100 mM TES, 1 mM EDTA, and 1 M NaCl at pH 5.0. A Lineweaver–Burk plot of the data yielded a K_i value of 421 ± 55 nM.

Scheme 1. Syntheses of **6** and **7** (U-96988)[a]



[a] (a) Cat. TsOH, toluene, reflux; (b) 2 equiv of LiNPr$^i_2$, $C_6H_5CH_2Br$, THF, −30 °C; (c) 2 equiv of LiNPr$^i_2$, CH_3CH_2I, THF, −30 °C.

From a fluorescence-based high-volume broad screening for HIV-1 protease inhibitory activity[18] of a set of 5000 dissimilar compounds from Upjohn compound collection, the 4-hydroxycoumarin **1** (warfarin, see Figure 1) was identified as a weak inhibitor ($IC_{50} \approx 30$ μM). Warfarin has also been reported as having antiviral effect on HIV-1 replication and spread.[19] More recently, independent studies[20,21] have described 4-hydroxybenzopyran-2-ones (warfarin and derivatives) and 4-hydroxypyran-2-ones as competitive inhibitors of HIV protease. On the basis of the 4-hydroxycoumarin structure, additional compounds from a similarity search of the Upjohn compound collection were then tested as

Dedicated to Professor Robert E. Ireland on the occasion of his 65th birthday.

† Medicinal Chemistry Research.
‡ Chemical & Biological Screen.
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∥ Computer-Aided Drug Design.
@ Drug Delivery System Research.
¥ Pharmaceutics.
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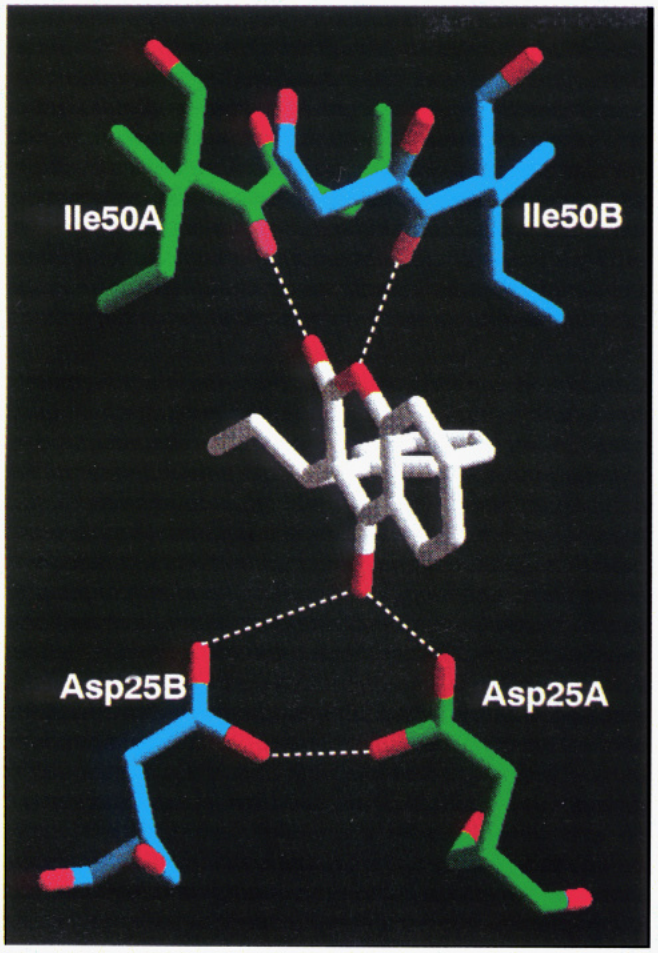

Figure 3. Crystal structure of inhibitor **2**/HIV-1 protease complex. Four residues at the enzyme active site are shown (residues from one monomeric unit are in green and labeled A while residues from the second monomeric unit are in blue and labeled as B): the catalytically essential aspartic acid residues (Asp25's) and the two isoleucine residues on the flap of the enzyme (Ile50's). Hydrogen bonding of inhibitor **2** (in white) to the enzyme active site is shown with dashed lines.

potential inhibitors, and another 4-hydroxycoumarin **2** (phenprocoumon, see Figure 1) was found with significantly improved inhibitory activity (K_i = 1 μM).[22] A Lineweaver–Burk plot illustrating competitive inhibition by compound **2** is shown in Figure 2. This inhibitor **2** also showed antiviral activity (ED_{50} = 100–300 μM), *albeit* weak potency, in HIV-1 infected PBMC. Very importantly, warfarin and phenprocoumon have already been in use as therapeutic agents in humans,[23] with high oral bioavailability and low clearance, and, therefore, are promising lead structures for the discovery of orally bioavailable non-peptidic HIV protease inhibitors.

To facilitate the lead optimization process, a crystal structure of inhibitor **2**/HIV-1 protease complex was determined[24] at 2.5 Å resolution. Due to the C_2 symmetry of the HIV-1 protease, the asymmetric inhibitor **2** could be found in two orientations related by a 180° rotation. Figure 3 shows one orientation of compound **2** in the enzyme active site, in which the C-4 hydroxyl group was located within hydrogen-bonding distance to the two catalytic aspartic acid residues (Asp25A and Asp25B). The two oxygen atoms of the lactone functionality were positioned within hydrogen-bonding distance to the two NH amides of the two isoleucine residues (Ile50A and Ile50B) on the flap of the enzyme. The lactone oxygen atoms in compound **2**, therefore, replaced the ubiquitous water molecule found in complexes of peptide-derivative inhibitors.[25,26] This hydrogen-bonding network of the 4-hydroxycoumarin defines the essential pharmacophore of this new class of inhibitors. Information from the crystal structure of inhibitor **2**/HIV-1 protease complex forms the basis of iterative cycles of structure-based design of more active analogues.

In this crystal structure of compound **2**/HIV-1 protease complex (Figure 4), the α-ethyl group and the α-phenyl ring at the C-3 position lie approximately in the S_1 and S_2 subsites,[27] respectively. The positioning of the benzene ring of the 4-hydroxycoumarin in the S_1' subsite does not readily accommodate the placement of substituents that might extend into the S_2' subsite. It was reasoned that removal of this fused benzene ring, resulting in the 4-hydroxy-2-pyrone ring, might offer the opportunity to place substituents that could be positioned into both the S_1' and S_2' subsites. As shown in Scheme 1, acid-catalyzed condensation of 4-hydroxy-6-methyl-2-pyrone (**3**) and α-ethylbenzyl alcohol (**4**) gave the substituted 2-pyrone **5**,[28] the dianion of which was alkylated at the C-6α position with benzyl bromide to give compound **6**.[28] This 4-hydroxy-2-pyrone **6** (K_i = 0.5 μM) was found to possess similar activity as inhibitor **2** and supported the viability of the 4-hydroxy-2-pyrone as a template for the preparation of HIV protease inhibitors. A crystal structure of inhibitor **6**/HIV-1 protease complex was then determined[24] at 2.3 Å resolution, and the structure in Figure 5 showed direct analogy to the hydrogen-bonding network found with inhibitor **2**. As shown in Figure 6, the phenethyl group at C-6 of compound **6** is located near the S_2' subsite.

It was further reasoned that an added ethyl group at the C-6α position of compound **6** might place this added group into the S_1' subsite and moves the phenyl ring closer into the S_2' subsite. As shown in Scheme 1, the dianion of compound **6** was alkylated at the C-6α position with ethyl iodide to give compound **7**.[28] The sequential alkylation of compound **5** can also be performed in the same reaction vessel with benzyl chloride and then ethyl iodide, without isolation of the intermediate compound **6**. The structure of compound **7** can be viewed as having pseudosymmetric substitutions; the C-3α substituents consist of an ethyl and a phenyl group, while an ethyl and a benzyl group can be found at the C-6α position. These four substituents are designed to extend into the central core of S_2 to S_2' enzyme pockets. Compound **7** was found to be an inhibitor with further significant improvement in binding affinity (K_i = 38 nM). Compound **7** has two chiral centers and, therefore, is a mixture of four stereoisomers. These individual isomers were isolated from a preparative HPLC procedure[29] on a chiral column. These individual diastereomers[30] were all found to be HIV-1 protease inhibitors and showed K_i values of 14, 43, 80, and 109 nM.

Compound **7** was also found to be equally effective against HIV-2 protease (K_i = 32 nM). It is selective for

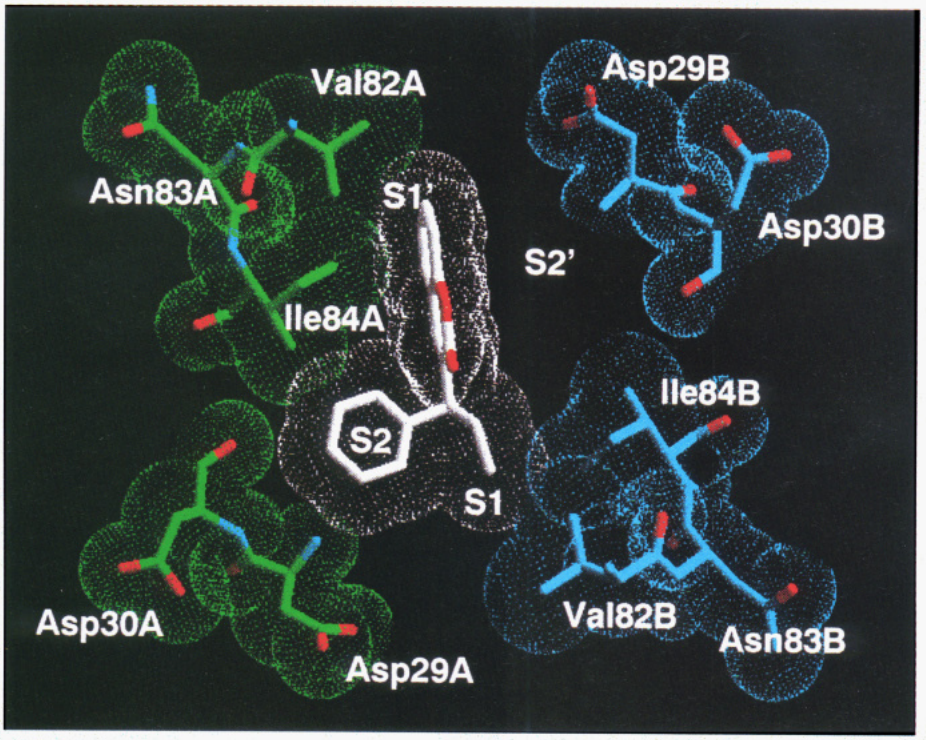

Figure 4. Surface representation of inhibitor **2** in the enzyme active site. The van der Waal surfaces of inhibitor **2** (in white) and 10 residues of the enzyme active site are shown (residues from one monomeric unit are in green and labeled A while residues from the second monomeric unit are in blue and labeled as B). The S_2, S_1, S_1', and S_2' subsites of the enzyme are also indicated.

Table 1. Selected Pharmacokinetic Parameters for Inhibitor **7** Calculated from Time-Course Plasma Concentrations (mean ± sd)[a]

species	dose (mg/kg)	$t_{1/2\beta}$ (hr)	Cl_T (mL/min/kg)	V_{SS} (L/kg)
dog ($n = 3$)	2.5	6.0 ± 1.2	0.7 ± 0.2	0.26 ± 0.03
rat ($n = 6$)	12	4.0 ± 0.76	0.8 ± 0.2	0.10 ± 0.02

[a] The plasma concentration–time data were fitted to mathematical equations using nonlinear regression analysis, and the distribution rate constant (β) and half-life ($t_{1/2\beta}$) were estimated. The total body clearance (Cl_T) and steady-state volume of distribution (V_{SS}) were also calculated.

HIV proteases; at 10 μM, it inhibited the following human aspartyl proteases: renin, gastricsin, pepsin, cathepsin D, and cathepsin E to the extent of 0%, 0%, 53%, 52%, and 72%, respectively.[31] The antiviral activity was assessed against HIV-1_{IIIB}-infected MT4 and H9 cells.[32] The dose–effect curves in these acutely infected cells are shown in Figure 7, and the ED_{50} values were determined to be 3 μM. Compound **7** was also shown to be effective against clinical isolates ($ED_{50} = 4\mu$M), including AZT resistant strains.[33] There was no cytotoxicity noted at 10 μM of compound **7**, and the $TCID_{50}$ was estimated to be 20 μM. Pharmacokinetic properties and absolute oral bioavailability of compound **7** were determined in rats and dogs. Time-course blood levels of compound **7** in dogs after intravenous (2.5 mg/kg) and oral (10 mg/kg) administrations are shown in Figure 8. An oral dose of 10 mg/kg resulted in C_{max} above 50 μM, and blood levels above 10 μM (the *in vitro* ED_{90} value) could be maintained for 6 h. Selected pharmacokinetic parameters for inhibitor **7** in rats and dogs are shown in Table 1. The oral bioavailability of compound **7** in rats and dogs was 76% and 45%, respectively. After additional extensive preclinical studies, compound **7** (U-

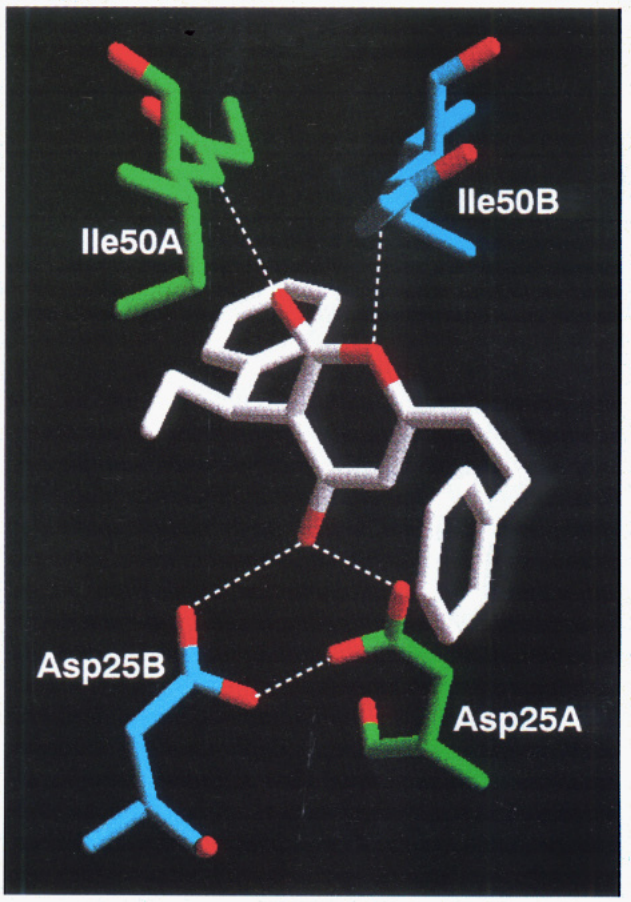

Figure 5. Crystal structure of inhibitor **6**/HIV-1 protease complex. Four residues at the enzyme active site are shown (residues from one monomeric unit are in green and labeled A while residues from the second monomeric unit are in blue and labeled as B): the catalytically essential aspartic acid residues (Asp25's) and the two isoleucine residues on the flap of the enzyme (Ile50's). Hydrogen bonding of inhibitor **6** (in white) to the enzyme active site is shown with dashed lines.

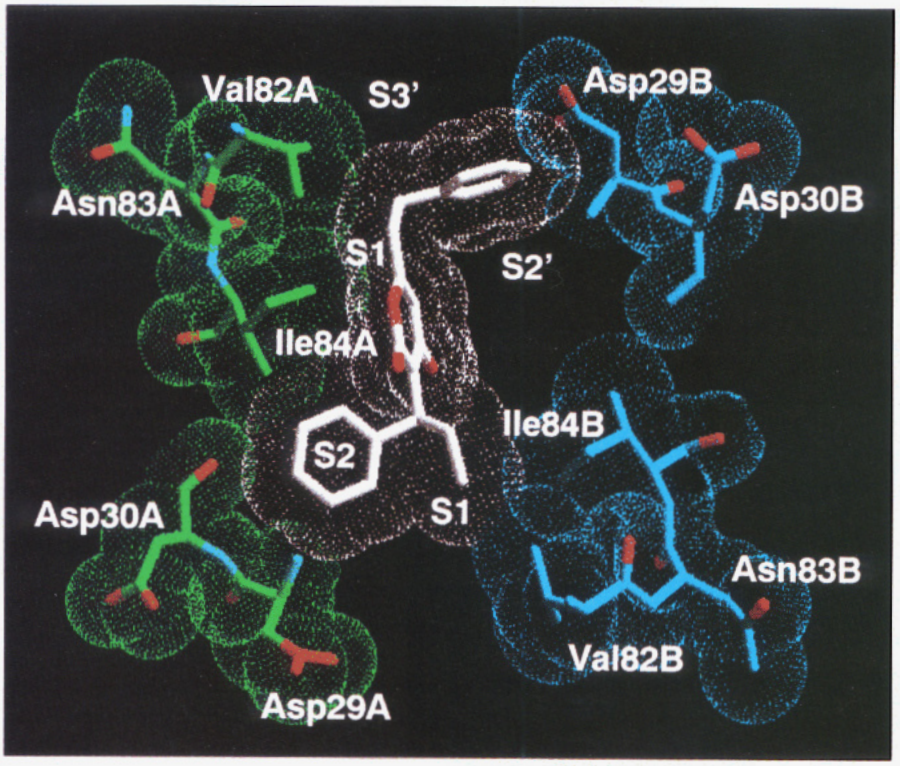

Figure 6. Surface representation of inhibitor **6** in the enzyme active site. The van der Waal surfaces of inhibitor **6** (in white) and 10 residues of the enzyme active site are shown (residues from one monomeric unit are in green and labeled A while residues from the second monomeric unit are in blue and labeled as B). The S_2, S_1, S_1', S_2', and S_3' subsites of the enzyme are also indicated.

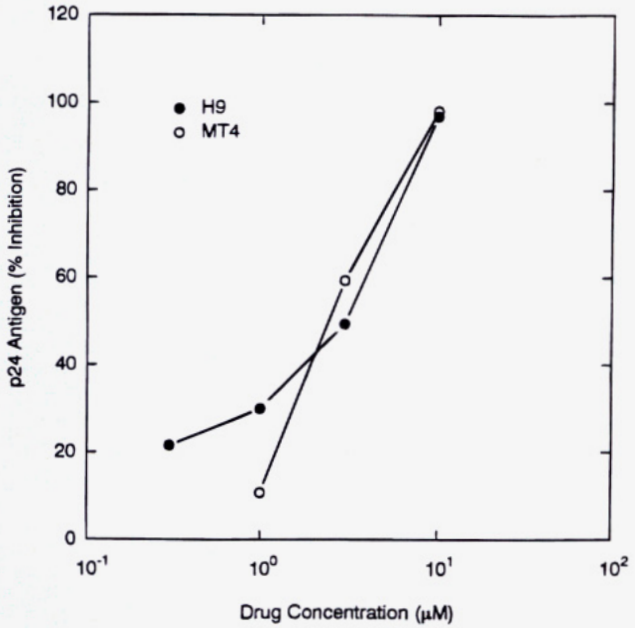

Figure 7. Dose effect of inhibitor **7** on p24 inhibition in HIV-1_{IIIB}-infected MT4 and H9 cells. MT4 or H9 cells (5×10^4 cells) were seeded into 96-well culture dishes and infected by incubating for 90 min with stock virus at 0.003 multiplicity of infection. Stock solution at 10 mM of inhibitor **7** in DMSO were diluted into culture medium. Infected cells in triplicate wells were maintained in the absence (controls) or continued presence of inhibitor **7** for 5 days in a humidified CO_2 incubator. Culture supernatants were harvested and the extent of p24 core antigen production was measured by an ELISA specific for HIV-1 p24 antigen (Coulter).

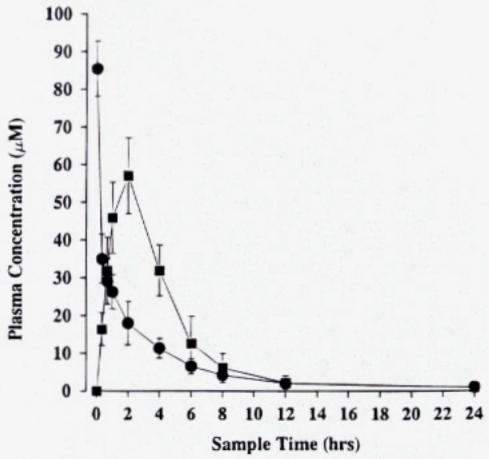

Figure 8. Plasma–time profile of inhibitor **7** (mean ± sd) in beagle dogs. Three fasted male beagle dogs received a 10 mg/kg dose of inhibitor **7** orally in no. 12 hard gelatin capsules formulated in 80:20 propylene glycol/0.1 N aqueous NaOH (8.4 mg/mL). Another three dogs received at 2.5 mg/kg bolus intravenous injection of inhibitor **7** formulated in 25% aqueous (hydroxypropyl)-β-cyclodextrin (9.7 mg/mL). Blood samples were collected from the jugular vein into heparinized tubes. Plasma was harvested by centrifugation at 1200*g* for 15 min, and 200 μL of plasma was mixed with 1 mL of acetonitrile (containing an internal standard) to precipitate plasma proteins and centrifuged. The supernatant was evaporated to dryness under nitrogen and reconstituted in mobile phase consisting of 55:45 acetonitrile/0.5% acetic acid. The prepared samples were chromatographed on a reverse-phase column (Rx-SB-Phenyl, 250 × 44 mm id, 5 μM) and a mobile phase flow rate of 1.0 mL/min. The UV absorbance of the column effluent was monitored at 295 nm. The circle and square symbols represent the plasma–time profile after intravenous and oral administration, respectively.

96988, MW 362), which is synthesized in two chemical steps, entered phase I clinical testing as the first in a series of this promising class of non-peptidic HIV protease inhibitors as potential therapeutic agents for the treatment of HIV infection.

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(18) HIV-1 protease was purified and refolded from *E. coli* inclusion bodies. The substrate used spans the p17–p24 processing site (R-V-S-Q-N-Y-P-I-V-Q-N-K) and was derivatized with biotin and fluorescein isothiocyanate at the amino and carboxy termini, respectively. The reaction was performed in assay buffer at substrate concentrations below K_m concentrations for 60 min at room temperature in the dark. The assay buffer consists of 0.1 M sodium acetate, 1.0 M NaCl, 0.05% NP40. Enzyme, substrate, and inhibitor concentrations are 10 nM, 50 nM, and 100 μM respectively. Inhibitors are dissolved in dimethyl sulfoxide, and the amount in the assay is 2%. After incubation the reaction is stopped by addition of fluoricon avidin beads at 0.5% (w/v). The residual bound fluorescence is obtained by processing on an IDEXX Screen Machine from which percent inhibition values are calculated.

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(22) The same experimental condition as described for the screen is utilized in the determination of K_i values except that a range of inhibitor concentrations are used and the assay time has been expanded to 90 or 180 min. Determination of K_i values with this assay requires analysis under conditions in which substrate concentration resides substantially below K_m and the inhibitor concentrations greatly exceeds the K_i value and the enzyme concentration. One can solve the remaining substrate after prolonged incubation times as a quadratic, exponential function in terms of inhibitor concentrations and time to generate calculated values for the kinetic constants K_m, k_{cat}, and K_i as well as the enzyme concentration.

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(28) Compound **5**: mp 168–170 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (t, 3 H, J = 7.34 Hz), 2.12 (s, 3 H), 2.1–2.4 (m, 2 H), 4.22 (dd, 1 H, J = 9.3 and 6.8 Hz), 6.10 (s, 1 H), 7.1–7.3 (m, 3 H), 7.46 (m, 2 H), 10.7 (s, 1 H). Anal. Calcd for $C_{15}H_{16}O_3$: C, 73.75; H, 6.60. Found: C, 73.48; H, 6.75. Compound **6**: mp 148–150 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.93 (t, 3 H, J = 7.32 Hz), 2.1–2.4 (m, 2 H), 2.63 (m, 2 H), 2.84 (m, 2 H), 4.22 (dd, 1 H, J = 9.1 and 6.9 Hz), 6.13 (s, 1 H), 7.0–7.3 (m, 8 H), 7.45 (m, 2 H), 10.9 (s, 1 H). Anal. Calcd for $C_{22}H_{22}O_3$: C, 79.02; H, 6.63. Found: C, 79.03; H, 6.66. Compound **7**: mp 112–115 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.80 (m, 3 H), 0.91 (m, 3 H), 1.55 (m, 2 H), 2.1–2.4 (m, 2 H), 2.48 (m, 1 H), 2.7–2.9 (m, 2 H), 4.22 (m, 1 H), 6.0 (s, 1 H), 7.0 (m, 2 H), 7.1–7.3 (m, 6 H), 7.43 (m, 2 H), 10.3 (s, 1 H). Anal. Calcd for $C_{24}H_{26}O_3$: C, 79.53; H, 7.23. Found: C, 79.16; H, 7.32.

(29) Chiracel OD semi-prep column; mobile phase of 96% hexane, 3% ethanol, 1% $CHCl_3$ and 0.1% acetic acid; flow rate of 5 mL/min at 380 psi; UV detector at 288 nm.

(30) For synthesis and assignment of stereochemistry of these individual diastereomers, see: Gammill, R. B. Manuscript in preparation.

(31) We thank Dr. Alfredo G. Tomasselli for renin inhibition data and Professor Ben M. Dunn, University of Florida, Gainesville, FL, for the remaining enzyme inhibition data.

(32) Chong, K.-T.; Pagano, P. J.; Hinshaw, R. R. Bisheteroarylpiperazine Reverse Transcriptase Inhibitor in Combination with 3′-Azido-3′-Deoxythymidine or 2′,3′-Dideoxycytidine Synergistically Inhibits Human Immunodeficiency Virus Type 1 Replication In Vitro. *Antimicrob. Agents Chermother.* **1994**, *38*, 288–293.

(33) Experiments (13 clinical isolates) performed at SRA Technologies, Inc., Rockville, MD.