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Switching between Allosteric and Dimerization Inhibition of HIV-1 Protease

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Summary

Refining the functional groups on a phenethylamine moiety within an inhibitor of HIV-1 protease led to a switch in the mechanism of inhibition from competitive and allosteric to dimerization inhibition. Phenylether extensions to the phenethylamine group led to agents that target the dimerization interface of HIV-1 protease with high potency.

Introduction

HIV-1 protease (HIV-1 PR) exists as a homodimeric enzyme [1, 2] in which half of the active site and the substrate binding site is found within each monomer [3, 4]. The primary function of HIV PR is site-specific cleavage of the polypeptide products of the viral *gag* and *gag-pol* genes [5]; without proper processing the virion remains in an immature form [6–9]. As such, virions arising from transfection with a provirus mutated in the protease coding region contained unprocessed viral proteins and were noninfectious [8, 10, 11]. FDA-approved agents that combat HIV infection through inhibition of the active site of HIV PR have been shown to be potent in vivo [12, 13]. However, the effect is often short lived as strains containing mutations around the active site of HIV PR become more prevalent [14–16].

A potential alternative mode of inhibition would be to target conserved elements of the virus, and an attractive target for such a strategy would be the dimerization interface of HIV PR. A major portion of the stabilizing interactions for dimerization is generated by a four-stranded β sheet structure [17] that is formed by interdigitation of the C- and N-terminal residues of HIV PR. The dimerization interface has been identified to be highly conserved among HIV-1 isolates [18, 19]; presumably a mutation in any position (except Leu97) would require a complementary mutation on the opposite terminus of the enzyme to confer dimer stability.

In order to mimic the native structure of the conserved β sheet region of HIV PR, a non-peptide-based linker was used to connect the two N termini of the interfacial peptides, leading to a dimerization inhibitor (**1**) with good potency against HIV PR (Figure 1) [20]. Truncation of the interdigitating peptides and modification of a tryptophan residue produced a lower molecular weight inhibitor (**2**) that also functioned by a dissociative mechanism [21]. The desire to reduce the number of amide linkages present in inhibitor **2** led to the removal of the terminal carboxamide within the northern tripeptide (compound **3**, Figure 1), as our model suggests that the carboxamide is not involved in hydrogen bonding. The removal of the carboxamide would lead to simplified diversity elements in this position based on the phenethylamine group. Compound **3** was found to be a modest inhibitor of HIV-1 PR with an IC_{50} value of 5.8 μM.

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Results and Discussion

Although compound **3** was found to inhibit HIV-1 PR, this agent did not act as a dimerization inhibitor using the assay of Zhang and Poorman [22], despite being equipotent to the dimerization inhibitor **2** (IC_{50} = 5.5 μM, K_i = 3.0 μM), as nonparallel lines between the protease in an inhibited and noninhibited form were obtained (Figure 2A). In an effort to determine the mode of inhibition for compound **3**, further assays were performed. A double reciprocal Lineweaver-Burk plot for **3** with HIV-1 PR showed the intersection of the uninhibited and inhibited lines on the *y* axis, which is characteristic of a purely competitive mechanism (K_c) of inhibition (Figure 2B). It seemed unlikely that **3** was acting by a traditional mode of competitive, active site inhibition because it lacks the heptapeptide structure associated with recognition and active site binding [23] or the equivalent tetrapeptide mimic required for active site inhibition [24].

Competitive inhibition can manifest itself in several forms and herein two variations are described. Classical competitive inhibition involves the inhibitor binding to the active site of the enzyme thereby excluding the substrate from the binding site. Alternatively, binding to an allosteric site that alters the conformation of the enzyme, leading to reduced affinity for the substrate, can appear to be competitive inhibition. It is plausible that the competitive inhibitor described is binding to the β sheet target, but lacks the ability to either disrupt the dimer or prevent dimer formation. Rather this agent may cause a change in the substrate binding pocket leading to reduced affinity for the substrate. In order to distinguish between the two scenarios, we carried out the crosscompetition assay as described by Yontani and Theorell [25] to test whether **3** was binding to the active site or to an allosteric site.

The crosscompetitive assay requires two inhibitors that act by a purely competitive mechanism, whereby the binding site of one of the inhibitors has been established. Acetyl-pepstatin was chosen as one of the inhibitors for this assay since it has been shown to be a competitive inhibitor of HIV-1 PR with K_c values of 20 nM and 35 nM at pH 4.7 and pH 5.0, respectively [26]. Additionally, the crystal structure of acetyl-pepstatin with HIV-1 PR has been solved, demonstrating that the location of binding is in fact the active site [27]. Due to

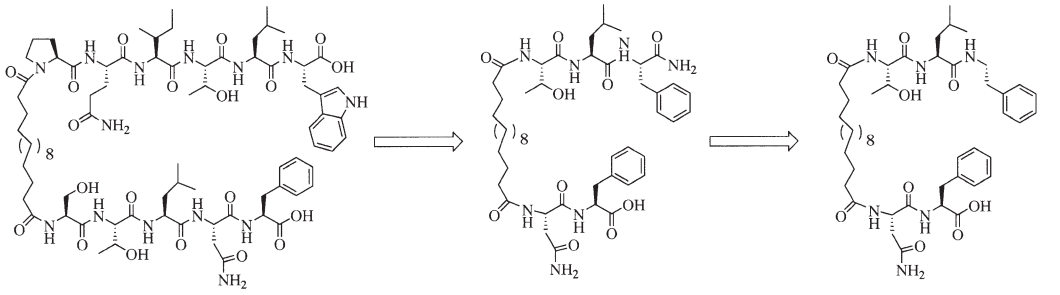


Figure 1. Structure of Compounds 1–3
See text for details.

pH and ionic strength differences in the assay conditions of Richards et al. and the conditions employed by Chmielewski et al., it was necessary to assay acetyl-pepstatin to determine the inhibitory constant for ensuing calculations. Acetyl-pepstatin was found to be a competitive inhibitor of HIV-1 PR under our conditions with a K_c value of 77 nM. After establishing acetyl-pepstatin as a viable competitive inhibitor of HIV-1 PR, the crosscompetitive assay was performed using acetyl-pepstatin and competitive inhibitor **3**.

Nonexclusive binding can be shown in a Dixon plot of 1/*v* versus [**3**] for multiple constant concentrations of acetyl-pepstatin or 1/*v* versus [acetyl-pepstatin] for multiple constant concentrations of **3**. The slope of this plot will depend on the concentration of the varying inhibitor as described by Yonetani and Theorell [25]. Consequently, the data obtained at different fixed concentrations of one inhibitor and varied second inhibitor concentrations will not be parallel if synergistic binding is occurring. A crosscompetitive experiment was performed to evaluate the nature of the binding of competitive inhibitor **3** to HIV-1 PR in the presence of acetyl-pepstatin (Figure 3A), as well as acetyl-pepstatin in the presence of **3** (Figure 3B). In each case, nonparallel lines were obtained indicating simultaneous and synergistic binding. These data provide evidence that inhibitor **3** is not binding to the active site of the enzyme as with acetyl-pepstatin, but is acting by binding to an allosteric site.

One may also obtain information on the effect of binding of one inhibitor or the simultaneous binding of the second inhibitor by using the method of Yontani and Theorell to obtain α—the constant defining the interaction between the two inhibitors. The value of α will equal 1 if binding of the two inhibitors occurs independently, <1 if the binding of one inhibitor facilitates the binding of the second inhibitor, and >1 if the binding of one inhibitor makes the binding of the second more difficult. An infinite α value indicates that the two inhibitors bind in a mutually exclusive manner and compete for the same binding site. Calculating the α value from the Dixon plots, the α value for acetyl-pepstatin was determined to be 0.05 and the α value for **3** was 140. These data indicate that the effect of **3** binding to

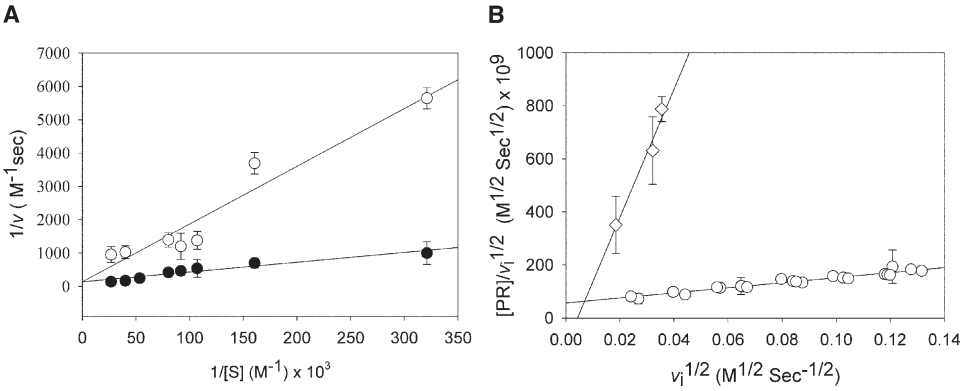


Figure 2. Inhibition Analysis of Compound **3**

(A) Lineweaver-Burk double reciprocal plot for inhibitor **3** with 25 nM HIV-1 PR. ●, uninhibited HIV PR; ○, 6 μM **3**.
(B) Zhang-Poorman analysis for **3** (5–25 nM HIV-1 PR, 25 μM substrate). ○, uninhibited HIV PR; ◇, 6 μM **3**.
Error bars were obtained from an average of two trials.

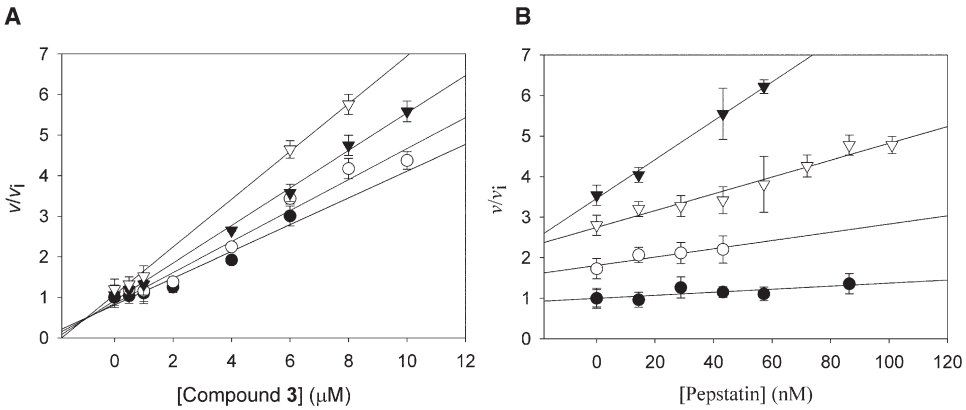


Figure 3. Yonetani-Theorell Inhibition Analysis of Compound 3

(A) Yonetani-Theorell plot of v/v_i versus concentration of compound **3** in systems containing constant acetyl-pepstatin concentrations. ●, 0 nM acetyl-pepstatin; ○, 72 nM acetyl-pepstatin; ▼, 109 nM acetyl-pepstatin; ▽, 145.5 nM acetyl-pepstatin. (25 nM HIV-1 PR, 12.5 μM substrate).

(B) Yonetani-Theorell plot of v/v_i versus concentration of acetyl-pepstatin in systems containing constant concentrations of compound **3**. ●, 0 μM compound **3**; ○, 4 μM compound **3**; ▽, 6 μM compound **3**; ▼, 8 μM compound **3** (25 nM HIV-1 PR, 12.5 μM substrate).

Error bars were obtained from an average of two trials.

HIV-1 PR has a positive impact on the binding of acetyl-pepstatin, perhaps due to a change in the substrate recognition site of HIV-1 PR, as the $K_{m(app)}$ of HIV-1 PR·**3** is increased by definition as a competitive inhibitor. On the other hand acetyl-pepstatin binding has the opposite effect on the binding of **3**. This may be due to the cooperative stabilization of residues at the dimeric interface caused by the binding of acetyl-pepstatin to the active site, thereby reducing the ability of **3** to potentially bind in this region [28].

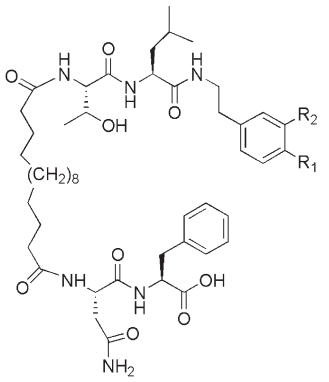
Many phenethylamine derivatives are known, and this provided the possibility to prepare a small library of agents based on the core structure of **3**, but with modifications to the terminal phenyl ring (Figure 4A). A solution-based strategy was used for the synthesis of seven analogs, utilizing a Cbz-protected dipeptide (Z-Thr (Ot-Bu)-Leu-OH) for reaction with the phenethylamine derivatives. Various phenethylamine derivatives were reacted with this invariant dipeptide to generate the desired amide, followed by reaction with Asn-Phe(Ot-Bu) and the di-N-hydroxysuccinimide ester of hexadecanodioic acid to provide the desired target molecules after treatment with TFA. Several exceptions to the general synthesis were required due to variable functionality of ring. In most cases, the removal of the Cbz protecting group was affected by hydrogenation to provide the desired northern fragments. The synthesis of the desired tripeptide containing an –OH in the R_2 position was completed by the use of BBr_3 [29] to convert the methoxy group of the tripeptide to a hydroxyl group while simultaneously removing the protecting groups.

The potency of compounds **4–10** against HIV-1 PR was initially determined using the fluorogenic assay developed by Toth and Marshall (Figure 4C) [30]. Substitutions to the phenyl ring in the meta-position (R2) of a hydroxyl group (**4**) or a methoxy group (**5**) had little effect on the potency of these compounds, and kinetic analyses (Zhang Poorman and Lineweaver Burk) confirmed a competitive inhibition mechanism. Interestingly, substitition at the para-position (R1) with a hydroxyl group (**6**) provided no increase in potency, but neither purely dimerization nor competitive inhibition was observed. Instead, this compound was found to be a mixed type inhibitor by Lineweaver-Burk analysis. The methoxy compound (**7**), however, was approximately 2-fold more potent than **6**, and was found to inhibit HIV-1 PR as a dimerization inhibitor by Zhang Poorman analysis (Figure 4B). Similarly, an amino group was well accommodated in the dimerization inhibitor **10**. Combining the two hydroxyl groups by using the dopamine analog **8** did not increase potency over compound **7**, although the dissociative mechanism of inhibition was maintained, whereas the bis-methoxy derivative **9** lost potency and a mixed type of inhibition was observed.

These data confirmed that increased potency could be obtained by substitution at the para-position of inhibitor **3**, and that the allosteric mechanism of inhibition may be switched to a dissociative mechanism by small modifications. The successful addition of the methyl group in inhibitor **7**, and the increased potency observed with phenyl ether substitution in northern peptide of compound **2** [31] led to the synthesis of two additional analogs (**11**, **12**) with phenyl ether functionality at position R1 (Figure 5A). The synthesis of the desired biphenyl ethers was accomplished as described above. However, the phenol functionality of tyramine was reacted with a copper activated aryl boronic acid [32, 33], to produce Cbz-(4-(4-methoxyphenoxy)-tyramine, which was simultaneously deprotected and the methoxy group was converted to a hydroxyl group using BBr_3 [29].

Compounds **11** and **12** were potent inhibitors of HIV-1 PR with IC_{50} values in the high nM range (Figure 5C). A Zhang-Poorman analysis provided evidence for dimerization inhibition (Figure 5B) and provided K_i values that are close to some of the most potent dimerization inhibitors reported to date [31, 34]. Compound **12** (K_i = 96 nM) is nearly twice as potent as **11** (K_i = 175 nM),

A



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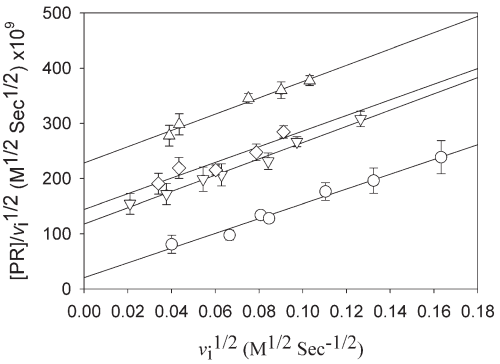


C

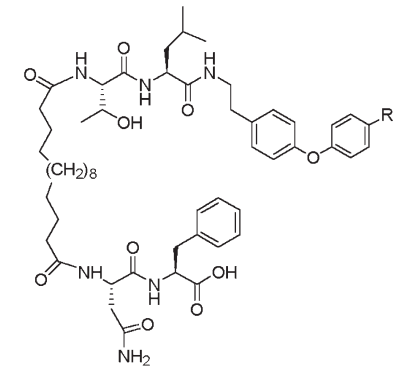
Compound	R_1	R_2	IC_{50} (μM)	Type of Inhibition	K_i (nM)
4	H	OH	4.4 ± 0.1	Competitive	1130 ± 20
5	H	OCH_3	4.9 ± 0.5	Competitive	1290 ± 10
6	OH	H	5.8 ± 0.2	Mixed	N/A
7	OCH_3	H	2.7 ± 0.2	Dimerization	720 ± 135
8	OH	OH	2.9 ± 0.3	Dimerization	600 ± 100
9	OCH_3	OCH_3	4.3 ± 0.1	Mixed	N/A
10	NH_2	H	2.5 ± 0.2	Dimerization	650 ± 70

Figure 4. Inhibition Analysis of Compounds **4–10**

(A) Structural data for compounds **4–10**. (B) Zhang-Poorman assay of compounds **7, 8,** and **10**. ○, uninhibited HIV-1 PR; ◇, 1.6 μM compound **7**; △, 2.9 μM compound **8**; ▽, 1.4 μM compound **10** (3–36 nM HIV-1 PR, 25 μM substrate). (C) Kinetic data for compounds **4–10**. Error bars were obtained from an average of two trials.

whereas these agents are approximately 15- and 30-fold more potent, respectively, than **2**. This increased potency may be due to the presence of the biaryl ether moiety binding on the surface of HIV-1 PR in a hydrophobic pocket in close proximity to polar, uncharged residues. In this binding orientation the extended aro-

A



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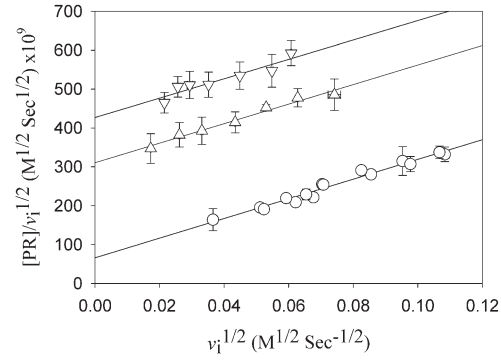

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Compound	R	IC_{50} (μM)	Type of Inhibition	K_i (nM)
11	H	0.73 ± 0.03	Dimerization	175 ± 20
12	OH	0.36 ± 0.03	Dimerization	96 ± 10

Figure 5. Inhibition Analysis of Compounds **11** and **12**

(A) Structural data for compounds **11** and **12**. (B) Zhang-Poorman assay of compounds **11** and **12**. ○, uninhibited HIV-1 PR; △, 660 nM compound **11**; ▽ 500 nM compound **12** (3–36 nM HIV-1 PR, 25 μM substrate). (C) Kinetic data for compounds **11** and **12**. Error bars were obtained from an average of two trials.

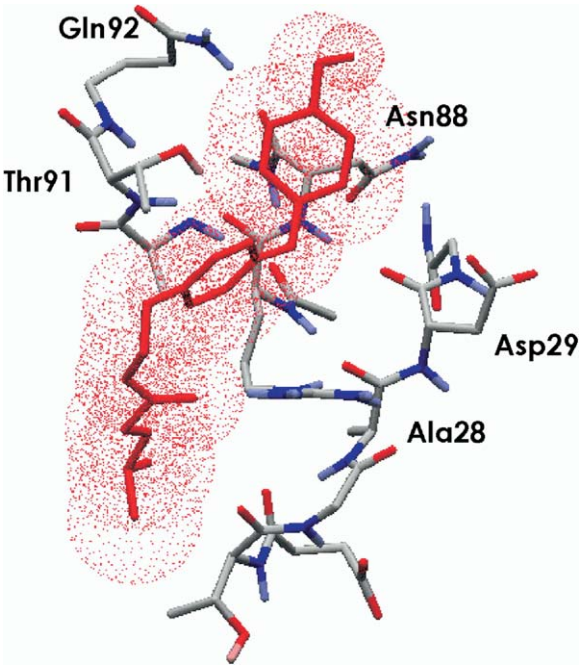

Figure 6. A model of the biphenylether portion of compound **12** bound to the protease monomer.
See text for details.

matic ring also brings the hydroxyl group of **12** in close proximity to Asp29, Gln92, and Asn88 of HIV-1 PR according to our model (Figure 6).

Significance

Herein we describe an inhibitor (3) of HIV-1 protease (HIV-1 PR) that functions by allosteric binding, a mode of inhibition that may be more effective than dimerization inhibition in cases of high substrate concentration. Small modifications to the phenethylamine portion of compound 3 led to significant increases in potency, and, interestingly, a switch in the mechanism of inhibition against HIV-1 PR from allosteric to dimerization inhibition. This class of inhibitors provides a more easily modified system for the synthesis of extended aromatic groups than previously reported compounds with a terminal amino acid, and may lead to a new source for the generation of potent inhibitors.

Experimental Procedures

Fluorescence-Based Kinetic Assay

All solutions were transferred using siliconized low retention pipette tips (Fischer). 1.2 ml polypropylene tubes were used for incubation of the inhibitor and HIV-1 PR prior to addition to substrate. The substrate was added to a polypropylene fluorescence 96-well plate (Greigner) prior to addition of the inhibited and uninhibited HIV-1 PR samples. The change in fluorescence was measured at 430 nm (λ_{ex} = 355 nm) for 20 min at 30°C, using a Tecan SPECTRAFluoro Plus 96-well plate reader. Raw kinetic data was imported into Microsoft Excel, converted to the appropriate format, and plotted. Slope, y-intercept, and R^2 values were determined by linear regression. Resultant data was plotted in SigmaPlot to display error bars.

Determination of IC_{50} Values Using Fluorogenic Assay

A solution of 180 μl of 50 nM HIV-1 protease in buffer (20 mM phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.1% CHAPS at pH 5.52; final concentration 25 nM protease) was incubated with 36 μl of the appropriate concentration of inhibitor solution in DMSO for 1 hr at room temperature. 60 μl of this solution was added to 40 μl of a 150 μM substrate solution (final concentration 60 μM), and the fluorescence was measured. The change in fluorescence values was compared to sample containing no inhibitor.

Determination of K_d and K_i Using the Zhang-Poorman Assay

Solutions of 180 μl of various concentrations of HIV-1 protease in buffer (20 mM phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.1% CHAPS at pH 5.52; final concentration 3-36 nM protease) were incubated with 36 μl of each inhibitor solution (concentration = IC_{50} value and 0.75 × IC_{50} value) in DMSO for 1 hr at room temperature. 60 μl of this solution was added to 40 μl of a 62.5 μM substrate solution (final concentration 25 μM), and the fluorescence was measured. Values obtained from the kinetic cleavage of the protease substrate were converted to μmol/sec cleaved using a response curve (full cleavage of various substrate concentrations versus relative fluorescence units). The initial velocity of the protease was used (<5% substrate consumed). The concentration of HIV-1 protease divided by the square root of the converted slopes was plotted versus the square root of the converted slopes.

Determination of K_m and K_c Using Double-Reciprocal Plots

Solutions of 180 μl of HIV-1 protease in buffer (20 mM phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.1% CHAPS at pH 5.52; final concentration 25 nM protease) were incubated with 36 μl of each inhibitor solution (concentration = IC_{50} value) in DMSO for 1 hr at room temperature. 60 μl of this solution was added to 40 μl of a 2.5–30 μM substrate solution and the fluorescence was measured. Values obtained from the kinetic cleavage of the protease substrate were converted to μmol/sec cleaved using a response curve (full cleavage of various substrate concentrations versus relative fluorescence units). The initial velocity of the protease was used (<5% substrate consumed). The inverse of the initial velocity was plotted versus the inverse of the substrate concentration.

Determination of Crosscompetitive Inhibition

Solutions of 180 μl of HIV-1 protease in buffer (20 mM phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.1% CHAPS at pH 5.52; final concentration 25 nM protease) were incubated with 36 μl of each inhibitor solutions varying in the concentration of a known active site competitive inhibitor (pepstatin-(0–100.8 nM) with constant concentrations of compound **2** (0, 4, 6, 8 μM) in DMSO for 1 hr at room temperature. Alternatively, solutions of 180 μl of HIV-1 protease in buffer (20 mM phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, and 0.1% CHAPS at pH 5.52; final concentration 25 nM protease) were incubated with 36 μl of each inhibitor solutions a constant concentration of a known active site competitive inhibitor (pepstatin, 72, 109, 145 nM) with varying concentrations of compound **2** (0–10 μM) in DMSO for 1 hr at room temperature. 60 μl of this solution was added to 40 μl of a 30 μM substrate solution and the fluorescence was measured. Values obtained from the kinetic cleavage of the protease substrate were converted to μmol/sec cleaved using a response curve (full cleavage of various substrate concentrations versus relative fluorescence units). The inverse of the initial velocity was plotted versus the concentration of the varying competitive inhibitor.

Supplemental Data

A list of the compound characterizations is available online at http://www.structure.org/cgi/content/full/12/4/439/DC1/.

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