

Articles

A Copper(I)-Catalyzed 1,2,3-Triazole Azide–Alkyne Click Compound Is a Potent Inhibitor of a Multidrug-Resistant HIV-1 Protease Variant

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Treatment with HIV-1 protease inhibitors, a component of highly active antiretroviral therapy (HAART), often results in viral resistance. Structural and biochemical characterization of a 6X protease mutant arising from in vitro selection with compound **1**, a C₂-symmetric diol protease inhibitor, has been previously described. We now show that compound **2**, a copper(I)-catalyzed 1,2,3-triazole derived compound previously shown to be potently effective against wild-type protease (IC₅₀ = 6.0 nM), has low nM activity (IC₅₀ = 15.7 nM) against the multidrug-resistant 6X protease mutant. Compound **2** displays similar efficacy against wild-type and 6X HIV-1 in viral replication assays. While structural studies of compound **1** bound to wild type and mutant proteases revealed a progressive change in binding mode in the mutants, the 1.3 Å resolution 6X protease–compound **2** crystal structure reveals nearly identical interactions for **2** as in the wild-type protease complex with very little change in compound **2** or protease conformation.

Introduction

Cumulative deaths from AIDS since its identification rank the disease as one of the deadliest in human history and is one of the leading causes of infectious disease deaths worldwide.¹ Highly active antiretroviral therapy (HAART^a) has vastly improved the life span of HIV-infected individuals.² However, poor patient compliance and high viral mutation rates often lead to protease inhibitor (PI)-resistant HIV strains. The emergence of specific PI-resistant viruses, as well as PI multidrug-resistant viruses, also termed cross-resistance, reduces the efficacy of treatment. The loss of PI efficacy necessitates changes in patient treatments and the need for new and more effective PIs in an ongoing battle against the viral evolution of resistance.^{3,4} While PI resistant forms of protease have been extensively catalogued in HIV⁺ patient populations, the contribution of PI resistance mutations on protease structure and inhibitor interactions is less well understood. Many resistance mutations occur distal to the active site and do not involve amino acids contributing directly to interactions with either substrate or PIs yet have substantial effects on inhibitor binding.^{5–8} Moreover, some mutations are

associated with promoting cross-resistance to numerous PIs, while other mutations are associated with resistance to only one PI.⁹

Compound **1**, a C₂-symmetric diol, is a broad-based protease inhibitor that has nanomolar activity against HIV-1, SIV, FIV, and viral isolates from patients that failed treatment with indinavir, saquinavir, nelfinavir, and ritonavir.^{10,11} To understand the biochemical and structural contributions of mutations in PI-resistance, we have previously generated a temporal series of PI-resistant HIV-1 mutants, 1X (V82A), 3X (V82A, M46I, F53L), and 6X (V82A, M46I, F53L, V77I, L24I, L63P), from wild-type virus under the selective pressure of compound **1**.¹⁰ As reported, the PR mutants, 1X (PR_{1X}), 3X (PR_{3X}), and 6X (PR_{6X}), exhibited 4-, 11-, and 30-fold increases in IC₅₀ to **1**, respectively. Moreover, *K_m* increased for selective substrates for all mutants and *k_{cat}* decreased for PR_{1X} but increased for PR_{3X} and PR_{6X}. The net result was an increased catalytic efficiency for the PR_{3X} and PR_{6X} and improved viral fitness. The recovered enzymatic efficiency, *k_{cat}/K_m*, of the PR_{6X} was 60% of the wild-type protease. Moreover, the PR_{6X} variant was found to be multidrug resistant, with IC₅₀s increasing approximately 9-, 8-, and 14-fold for saquinavir, nelfinavir, and ritonavir, respectively.

We have previously reported on the X-ray crystallographic analyses of PR_{1X-6X} series and showed that the dimeric PR_{6X}–compound **1** complex assumed an asymmetric conformation relative to the wild-type dimer.¹² The PI-induced mutations affected protein core packing and hydrogen bonding networks within the protease, promoting the movement of the monomer B flap region toward monomer A, resulting in a break in symmetry typically observed in dimeric HIV-1 protease structures. This asymmetric arrangement resulted in the disruption of seven hydrogen bonds and a number of the van der Waals interactions with compound **1**. The conformational change in

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^a Abbreviations: HIV-1, human immunodeficiency virus type 1; PI, protease inhibitor; HAART, highly active antiretroviral therapy.

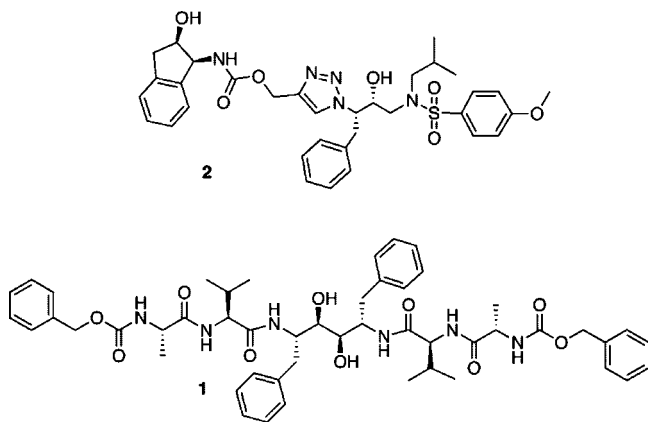


Figure 1. Two dimensional structural representations of **2** and **1**. The central triazole of **2** arises from click chemistry, while **1** is a mechanism-based inhibitor with C_2 symmetry.

the PR_{6X} provides a molecular mechanism of resistance whereby PI binding affinity was considerably decreased, while efficiency of Gag-Pol substrate cleavage remained similar to the wild type enzyme.¹² Therefore, the multidrug resistant PR_{6X} variant-containing virus is useful for evaluating protease inhibitor efficacy and the cloned and expressed PR_{6X} for determining structural mechanisms that afford drug resistance.

As a strategy to develop PIs with potential efficacy against multidrug-resistant viruses, we reported on the feasibility of rapid synthesis and selection of PIs from diverse libraries of small compounds derived from Cu(I)-catalyzed azide–alkyne [3 + 2] cycloaddition chemistry (click chemistry).¹³ The use of click chemistry can aid in synthesizing potent inhibitors by optimizing the diversity of resulting compounds with structures in the P2/P2' positions, regions that have been shown to be critical for inhibitor binding in the protease active site.^{14,15} Moreover, the rapid synthesis of potential PIs allows their use as chemical probes on our panel of PI-resistant viruses and purified PRs, thereby affording biochemical and structural comparisons with reported PIs. We have previously reported that one amprevir-like compound obtained from the synthesis, compound **2** (Figure 1), was found to have an IC₅₀ of 6.0 nM on wild-type PR and was found to be efficacious on the protease inhibitor active single-site mutants, V82F, V82A, and G48V.¹³

We report herein on our studies of the efficacy of compound **2** against the multidrug resistant PR_{6X} containing virus and purified PR_{6X} in biochemical assays. Compound **1** was effective against the PR_{6X} virus in tissue culture and was found to retain low nanomolar potency against PR_{6X} when evaluated on a fluorogenic substrate. X-ray crystallographic analysis of the PR_{6X}–compound **2** complex revealed that the complex does not show the conformational rearrangements seen previously in the PR_{6X}–compound **1** complex and **2** retains essentially all of the interactions with PR_{6X} that were previously observed in the PR_{wt}–compound **2** complex.¹⁶ Our findings provide structural insights by which compound **2**, but not compound **1**, maintains tight binding resulting in potent antiviral activity on a multidrug-resistant protease variant.

Results

Compound 2 Inhibits Viral Replication of the PI-cross-resistant 6X Virus. We have reported that the compound **1**-evolved PR_{1X}, PR_{3X}, and PR_{6X} viruses show increasing degrees of resistance to **1** with increasing resistance mutations, with PR_{6X} virus 17-fold more resistant to **1** than PR_{wt} virus when evaluated in tissue culture replication assays.¹⁰ In addition, PR_{6X}

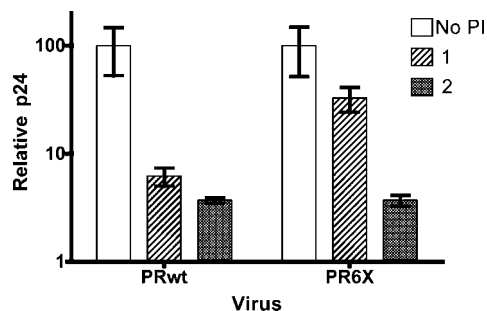


Figure 2. Inhibition of PR_{wt} and PR_{6X} viral replication by **2**. SupT1 cells were infected PR_{wt} and PR_{6X} viruses and then cultured in the presence or absence of 100 ng/mL **1** or **2**. Levels of p24 were quantified 6 days postinfection. Levels of p24 were detected by ELISA and are shown as a percentage of that measured from cells not treated with drug and presented as relative p24 levels.

Table 1. Quantitation of Viral Susceptibility, EC₅₀, to **1** and **2**^a

	HIV-1 _{wt}		HIV-1 _{6X}	
	2 (nM)	1 (nM)	2 (nM)	1 (mM)
p24	30	46	55	> 1 ^b
TZM-bl	83	92	260	> 1 ^b

^a Supernatants from infected SupT1 cells were measured for HIV-1 production by p24 ELISA or HIV-1 LTR-driven luciferase activity using TZM-bl cells. Nonlinear regression results, reported in nM units, are shown. ^b EC₅₀s for **1** against HIV-1_{6X} were not accurately determined, as no significant inhibition of the virus was observed, even at the highest concentration of compound tested (4 μM).

is cross-resistant to saquinavir, ritonavir, and nelfinavir when evaluated in cleavage assays in vitro.¹⁰ Therefore, the compound **1**-resistant viral and purified PR series is useful for evaluating antiviral activity of compound **2**.

To assess the potency of compound **2** to control viral replication as compared to compound **1**, SupT1 cells were infected with PR_{wt} and PR_{6X} and cultured in the absence or presence of **1** or **2** at concentrations of 1–4000 nM. Replication of wild-type virus, as quantified by p24 accumulation over time and presented as relative p24 levels, was greatly reduced in the presence of **1** and **2** (Figure 2) and decreased to background levels at 250 nM (data not shown). Furthermore, viral replication of the PR_{6X} virus was inhibited by **2**, with PR_{6X} viral replication, shown in Figure 2, reduced almost 20-fold at 250 nM compound **2** relative to the infected cells in the absence of inhibitor. In contrast to inhibition of viral replication by **2**, compound **1** had little effect on the compound **1**-resistant PR_{6X} virus (Figure 2).

Quantification of infectious virus production from the cell-free culture supernatants using TZM-bl cells shows EC₅₀ values of approximately 86 nM and 93 nM for wild type virus in the presence of **2** and **1**, respectively, and 260 nM for the PR_{6X}-containing virus cultured in the presence of **2**. The resulting EC₅₀ for compound **1** against the PR_{6X}-containing virus was greater than 1 mM. Thus, compound **2**, in contrast to compound **1**, is a potent inhibitor of the PR_{6X} multidrug resistant virus. EC₅₀ determinations based on p24 quantitation showed a similar trend but deviated slightly in overall value (Table 1). These changes may reflect differences in the sensitivity of each assay.

Compound 2 Inhibits PR_{wt} and PR_{6X} in Vitro. To quantify compound **2** inhibitor efficacy with respect to the PR_{wt} and PR_{6X}, biochemical and kinetic analyses were performed. In a fluorogenic substrate cleavage assay, **2** showed half-maximal inhibition of substrate cleavage at an inhibitor concentration of 6.0 nM for PR_{wt} and 15.7 nM for the PI-cross-resistant PR_{6X} (Figure 3), with K_i values of 2.7 and 7.1 nM, respectively. This finding shows that **2** has similar inhibition potency against PR_{wt} and

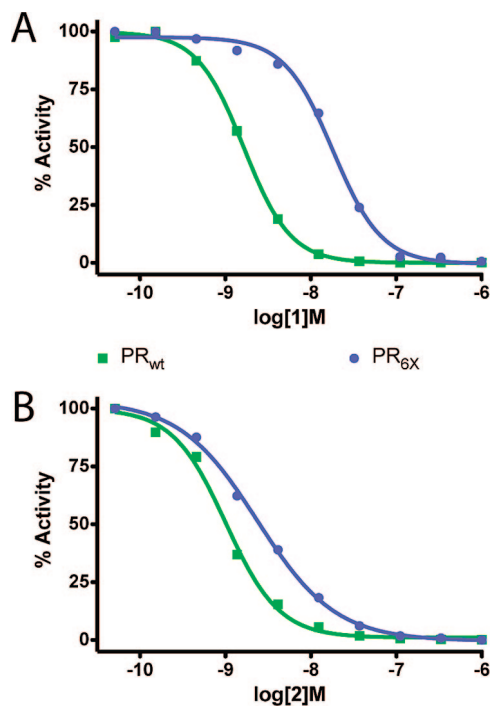


Figure 3. IC₅₀ curves for **1** (A) and **2** (B) based on in vitro assay of enzyme activity. IC₅₀ determinations are shown for each protease inhibitor against PR_{wt} (green squares) and PR_{6X} (blue circles) proteases. Curve fitting analysis results in an IC₅₀ of 6.0 nM for PR_{wt} and 15.6 nM for the PR_{6X}. Nonlinear regression analysis in Prism (Graphpad Software) was used for curve fitting of the substrate cleavage reaction.

the PR_{6X} proteases with IC₅₀s differing by 2.6-fold (Figure 3). This is in contrast to our reported inhibition by compound **1**, saquinavir, ritonavir, and nelfinavir, where the IC₅₀s increased 30-fold, 9-fold, 14-fold, and 8-fold, respectively, for PI activity on PR_{6X} relative to PR_{wt}.¹⁰ These results demonstrate that **2** has a low nanomolar inhibitory efficacy against both PR_{wt} and PR_{6X}, and the constellation of PI-cross-resistant mutations present in PR_{6X} affords less than a 2.6-fold increase in the IC₅₀.

Crystal Structure of the PR_{6X}–Compound 2 Complex. As we reported previously, the PR_{6X}–**1** crystal structure demonstrated conformational asymmetry, present in both inhibitor and protein, consistent with the decreased effectiveness of compound **1** inhibitor binding and function.¹² To provide a structural basis for the differences between PI inhibitor efficacy of compound **2** relative to **1**, we determined the 1.3 Å resolution crystal structure of **2** in complex with PR_{6X}. Refinement of the structure showed that the crystals were in the *P*₄₃ space group, with a dimer in the asymmetric unit (Table 2). Nevertheless, compound **2** in the PR_{6X}–**2** complex was present in two orientations within the active site related by noncrystallographic 2-fold symmetry coincident of the 2-fold axis of the PR_{6X} dimer. Well-defined electron density was observed for both orientations of **2**, which were refined with equal 50% occupancy (Figure 4A). The two orientations of **2** exhibit virtually identical conformations, the only significant differences being the torsional preferences of an isobutyl side chain and a methoxy group (Figure 4B). While the PR_{6X} dimer was refined as monomers A and B in the asymmetric unit, the two subunits are very similar, with an rms difference of 0.23 Å for all C α atoms. Structural superposition shows that the conformations adopted by each monomer near the inhibitor are equivalent, with the protease maintaining equivalent interactions with each orientation of **2** in the active site (Figure 4B).

Table 2. Data Collection and Refinement Statistics

6X HIV PR–2	
crystal form	
space group	<i>P</i> ₄ ₃
unit cell parameters	
<i>a</i> (Å)	86.61
<i>b</i> (Å)	86.61
<i>c</i> (Å)	33.66
$\alpha = \beta = \gamma$ (deg)	90.0
solvent content (%) / <i>V</i> _m (Å ³ /Da)	50.0 (2.46)
no. molecules per asymm. unit	2
data collection	
resolution (Å)	61.2–1.3
no. of unique reflections	57347
no. of observations	95706
<i>R</i> _{merge} ^a (%)	3.9 (43.8)
<i>I</i> / σ _{<i>I</i>}	10.1 (1.6)
data completeness (%)	92.7 (74.1)
mean multiplicity	1.67 (1.30)
refinement	
no. of reflections used in refinement	57347
<i>R</i> _{cryst} ^b (%)	15.6
<i>R</i> _{free} ^b (%)	18.9
% reflections used in <i>R</i> _{free} (<i>I</i> / σ > 0)	5.0
rms deviations, bond lengths (Å)	0.011
rms deviations, bond angles (deg)	1.24
Ramachandran plot	
% most favored	96.8
% additional allowed residues	3.2
% disallowed residues	0.0
estimated standard uncertainty ² (Å)	0.070
coordinate error (Luzatti plot) (Å)	0.237
model	
no. of atoms	
protein (including alternate conformations)	1518
water molecules	286
average <i>B</i> -factors (Å ²)	
protein main chain atoms	16.9
protein side chain atoms	21.5
ligand	15.2
water molecules	29.5

^a $R_{\text{sym}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - I(hkl)|}{\sum_{hkl} \sum_i I_i(hkl)}$ where $I_i(hkl)$ is the intensity of an individual measurement, and $I(hkl)$ is the mean intensity of this reflection. ^b *R* factor = $\frac{\sum_{hkl} |F_{\text{obs}}| - |F_{\text{calc}}|}{\sum_{hkl} |F_{\text{obs}}|}$, where $|F_{\text{obs}}|$ and $|F_{\text{calc}}|$ are observed and calculated structure factor amplitudes, respectively. The values in parentheses for completeness, *R*_{sym} and *I*/ σ correspond to the highest resolution shell. ^c Estimated standard uncertainty, diffraction precision index (DPI), based on *R*_{free}.⁴⁶

Protease–Compound 2 Interactions. Interactions between the **2** compound and protease involve many of the same elements as those seen in protease–substrate complexes, although subtle differences exist. As previously observed in the PR_{wt}–**2** complex, the triazole linker serves to mimic the substrate peptide bond and provides a crucial interaction with the protease flaps via a bridging H₂O molecule.¹³ In particular, the triazole N5 atom and a sulfonamide oxygen accept hydrogen bonds from the “flap water”, while this water accepts hydrogen bonds from the amides of the Ile50_A and Ile50_B in the protease flaps (Figure 5). This tetrahedral coordination by the flap water bridges the flaps and mimics the hydrogen bonding network seen in substrate complexes and other inhibitor complexes.^{17–21} The carbamate carbonyl oxygen in the P2 pocket accepts a hydrogen bond from the amide of Gly48, the hydroxyl nearest the dimer 2-fold axis donates a hydrogen bond to Asp25, and the methoxy-benzyl oxygen accepts a hydrogen bond from the amide of Asp30 (Figure 4B, Figure 5). The latter hydrogen bond is important for high affinity binding in other substrate and inhibitor complexes.^{18–20} The hydrogen bonding interactions,

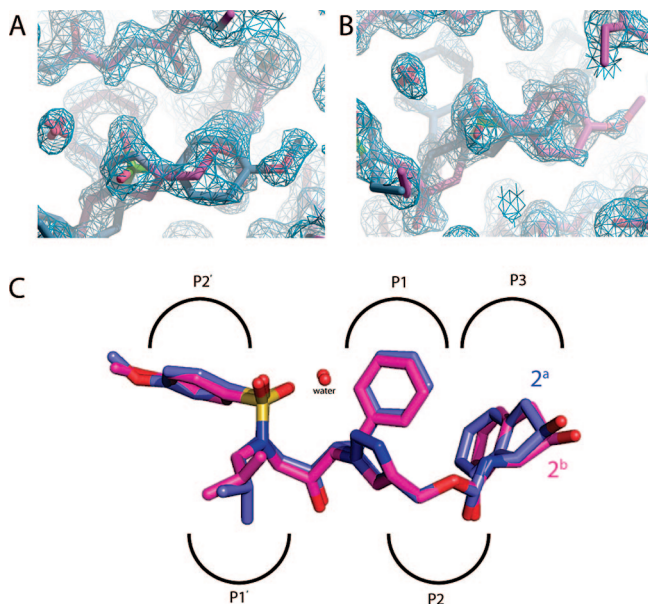


Figure 4. Electron density of **2**. $2F_o - F_c$ electron density map at 1.3 Å resolution contoured at 1.0σ of **2** in the active site of the PR_{6X}-**2** complex. (A) The benzyl-methoxy group of **2^a** and the indene region of **2^b**. (B) The indene region of **2^a** and the benzyl-methoxy region of **2^b**. Some atoms of the PR_{6X} dimer and bound H₂O molecules are also shown. (C) The superposition of **2^a** and **2^b** in the PR_{6X}-**2** complex. The two orientations of **2** exhibit very similar conformations, differing only in the torsion angles of the isobutyl group in the P1' pocket, and the methoxy group in the P2' pocket.

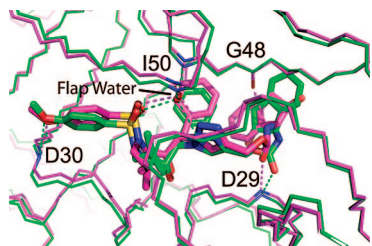


Figure 5. Superposition of structures of PR_{wt} and PR_{6X} in a complex with **2**. Protease is represented by main chain atoms, green for PR_{wt} and magenta for PR_{6X}. The conformation of **2** is represented by stick bonds with carbon atoms in green when bound to PR_{wt} or magenta when bound to PR_{6X}. Hydrogen bonds are represented by dashed lines, with interacting main chain atoms colored by atom type and labeled by residue. The “flap water” is shown forming four hydrogen bonds bridging the amides of Ile50 residues with the sulfonamide and triazole of **2**.

present in both orientations of **2** in the active site, stabilize the closed form of the 2-fold symmetric protease dimer.

Interactions of **2** with PR_{6X} involve several nonpolar contacts. The **2^a** phenyl and isobutyl side chains occupy the P1 and P1' pockets of the PR dimer, with the phenyl ring contacting Gly49_A, Pro81_B, Ala82_B, and Ile84_B and the isobutyl group contacting Leu23_A, Gly27_B, Ala82_A, and Ile84_A (Figures 4B, 5, 6A). The methoxy-benzyl group of **2^a** occupies the P2' hydrophobic pocket formed by the side chains of residues Ala28_B, Val32_B, Ile47_B, Ile50_A, and Ile84_B. At the same time, the **2^a** 2-hydroxy-2,3 dihydro-indene group occupies the P3 specificity pocket, where it is sandwiched by π -stacking interactions with the **2^a** phenyl ring and the guanidinium group of Arg8_B, which is salt-bridged with Asp 29_A. This interaction stabilizes the dimer interface in the PR_{6X}-**2** complex but is absent in the PR_{wt}-**2**

structure, where the indene group is flipped $\sim 180^\circ$ due to rotation about the linking carbamate.

Discussion

Compound 2 Exhibits Low Nanomolar Potency Against PR_{wt} and the Multidrug-Resistant PR_{6X} virus and PR. We have previously reported on the biochemical and structural evolution of HIV-1 protease enzymatic function and resistance to **1**,^{10,12} with potency decreasing with increasing resistance-induced mutations in PR, with the PR_{6X} virus showing only slight inhibition compared to the PR_{wt} virus (Figure 2 and ref 10). The difference is most evident when comparing viral replication of the multidrug-resistant PR_{6X} virus in the presence of **1** or **2** (Figure 2). SupT1 cells infected with PR_{6X} virus cultured in the presence of 100 nM **1**, showed approximately a 2-fold decrease in relative p24 levels when compared to the untreated control, whereas cells treated with **2** had a nearly 20-fold decrease in relative p24 levels at similar compound concentrations (Figure 2). Biochemical and kinetic analyses further underscored the potency of **2** against the PR_{6X} with an IC₅₀ of 15.7 nM, a modest increase relative to the 6.0 nM IC₅₀ observed for PR_{wt} (Figure 3). Other protease inhibitors, including **1**, saquinavir, nelfinavir, and ritonavir, all show greatly increased IC₅₀s (>log) against PR_{6X}, consistent with the PI-cross-resistant nature of PR_{6X} and the PR_{6X} virus.¹⁰ Thus, **2** is effective in inhibiting a multidrug-resistant HIV-1 variant.

Compound 2 Complexes with PR_{wt} and PR_{6X} are Isomorphous. To gain molecular insights into the compound **2**-binding site interactions responsible for its potent antiviral activity against the multidrug-resistant PR_{6X}, we solved the crystal structure of **2** in complex with PR_{6X} at 1.3 Å resolution (Figure 4). Compound **2** binds to the active site of the 2-fold symmetric PR_{6X} dimer in two orientations related by a 2-fold symmetry. Clear electron density is observed for both orientations, which are 50% occupancy in the crystal (Figure 4A). Hence, **2^a** and **2^b** bind to PR_{6X} with equivalent interactions, and superposition reveals that they align very closely (Figure 4B).

Obtaining the PR_{6X}-**2** structure allows comparison to our previously reported PR_{wt}-**2** structure.¹⁶ In the PR_{wt}-**2** complex, which crystallized in a different space group (P6₁22 vs P4₃), the protease dimer is superposed on a crystallographic 2-fold axis; in the PR_{6X}-**2** structure, the dimer represents the crystallographic asymmetric unit (Table 2).¹⁶ Despite the different symmetry restraints and packing environments, superposition of the PR_{wt} and PR_{6X}-**2** structures reveals a high degree of similarity (Figure 5). Main chain atoms show negligible deviations and side chain atoms align well, with both proteins adopting similar side chain rotamer conformations (rms deviations 0.57 Å for 99 monomer C α atoms, 0.60 Å for main chain and C β atoms). This point is further illustrated in a difference distance matrix plot showing the extent to which local 2-fold symmetry, i.e., similarity of monomer chains A and B, is retained in the PR_{6X}-**2** complex relative to the PR_{wt}-**2** complex (Figure S1, Supporting Information). The conformational differences that do occur are small and confined to regions of the fold that exhibit deviations in other PR crystal structures, including the flaps, the loop involving Gly68, and the turn near Arg87.²²

The protein-inhibitor interactions observed in the PR_{wt}-**2** and PR_{6X}-**2** complexes are similar, and the conformation of **2** is similar, with the only significant differences being the torsion angles affecting the indene and isobutyl moieties (Figure 5). Variability in the latter group is also seen for **2^a** vs **2^b** in the PR_{6X} complex (Figure 4B). With respect to the indene group

attached to the carbamate, analysis of the electron density and temperature factors for **2** in the PR_{wt} structure (refined at 2.02 Å resolution; 1ZP8) indicates greater mobility in this structure. In general, the congruence of the PR_{6X}–**2** and PR_{wt}–**2** structures is in accord with the similarity of IC₅₀ values for **2** against PR_{6X}, IC₅₀ = 15.7 nM, and PR_{wt}, IC₅₀ = 6.0 (Figure 3).

Clearly, the PR_{wt}–**2** and PR_{6X}–**2** structures differ at the sites of the six mutations. As noted, the main chain conformations of the PR dimers in each complex are very similar, so that the mutations affect only the side chains, thus limiting loss of binding affinity for compound **2** (Figure 5). Moreover, four of the substitutions, L24I, M46I, L63P, and V77I, are distal to the active site and do not affect compound **2** interactions or conformation. However, the remaining two mutations, F53L in the flap region and V82A, do appear to have an effect. The mutation F53L removes a packing contact with the compound **2** indene ring, which may be correlated with the flipping of the carbamate in the PR_{6X}–**2** structure, shifting the indene ring into an aromatic stack with the **2** phenyl group and Arg8 (Figures 4B, 5). Similarly, mutation of Val82 to Ala in PR_{6X} active site removes a packing contact with the **2**^a isobutyl group in the P1' site, allowing it to adopt another torsion angle and be in contact with Leu23_A, Gly27_B, Ala82_A, and Ile84_A. Leu23_A shifts ~1 Å, and Ile84_A changes its χ₁ torsion angle to favor interaction with the isobutyl group. With respect to the P1 site, the V82A mutation has a lesser effect on interactions of the **2**^a phenyl group with Gly49_A, Pro81_B, Ala82_B, and Ile84_B. The net effect of the V82A and F53L mutations is the loss of four contacts (isobutyl-Val82_A, phenyl-Val82_B, indene-Phe53, and associated carbamate-Asp30 amide hydrogen bond), however, there is a gain of three contacts (isobutyl-Gly27_B, isobutyl-Leu23_A, and indene stacking with Arg8). Overall, these three new contacts in PR_{6X} stabilize compound **2** interactions and are in accordance with the modest 2.6-fold increase in the IC₅₀ for the PR_{6X} as compared to PR_{wt} (Figure 3).

Different Structural Responses of PR_{6X} to **2** and **1**.

Previously, we reported on the crystal structures of the PR_{wt}–**1** and PR_{6X}–**1** complexes and how the constellation of compound **1** resistance-induced mutations contributed to PR_{6X} resistance.¹² While the PR_{wt}–**1** structure is symmetric, such that a crystallographic 2-fold superposes on both the PR dimer and **1**, the PR_{6X}–**1** structure is distinctly asymmetric, with the PR dimer in the asymmetric unit and non-2-fold related changes in conformation in both the protein and **1**. As discussed above, this is not the case for the PR_{6X}–**2** structure. In regards to the PR_{6X}–**1** structure, in particular, side chain packing arising from the six mutations in PR_{6X} alters the dimer interface and disrupts interactions with **1** in one side of the active site, resulting in significant changes to the conformation of one flap and one-half of **1**. In general terms, PR_{6X} binds the C₂-symmetric **1** inhibitor in an asymmetric fashion but it retains the 2-fold symmetry of the PR_{wt} complexes upon binding the distinctly asymmetric inhibitor, **2**.

The structural analysis of PR_{wt}–**1** and PR_{6X}–**1** complexes, when compared to the PR_{6X}–**2** complex, reveals further insights into how PR mutations may reduce affinity for the inhibitor while maintaining enzymatic function.¹² As we have reported, the compound **1** IC₅₀ for the PR_{6X} mutant increases 30-fold as compared to PR_{wt}.¹⁰ In contrast to the PR_{wt}–**1** and PR_{6X}–**1** complexes, the PR_{wt}–**2** and PR_{6X}–**2** structures are very similar and **2** retains high affinity for PR_{6X}, with the IC₅₀ increasing 2.6-fold as compared to PR_{wt}. Differences in the shape and volume of the intermolecular contact surfaces between the PR_{6X} and **2** (Figure 6A) vs PR_{6X} and **1** (Figure 6B) further supports

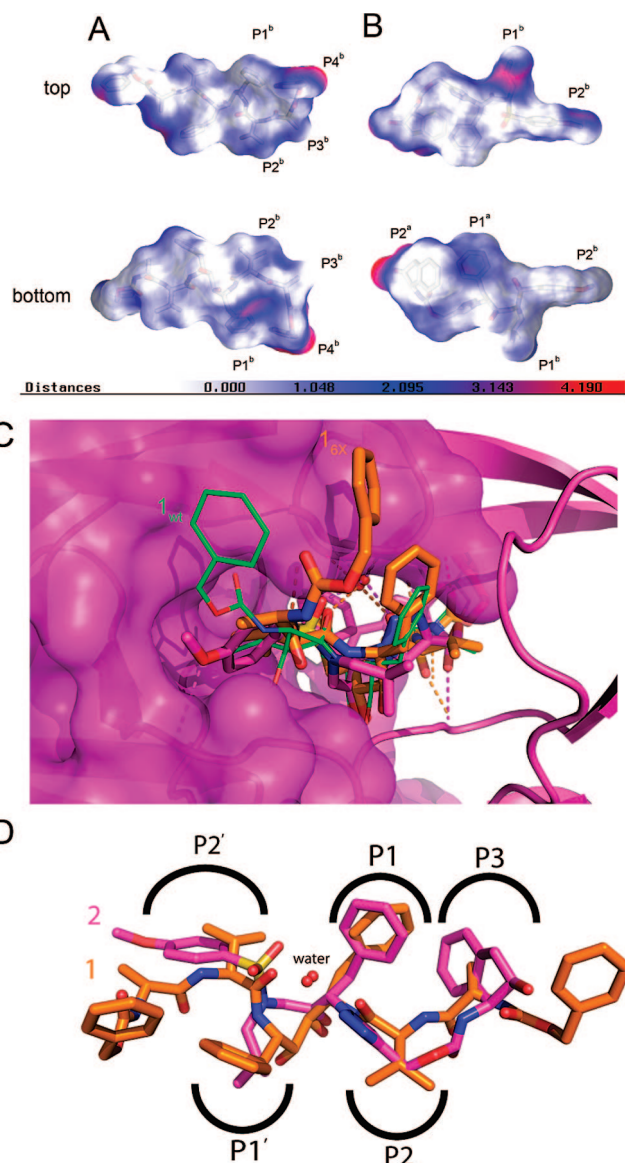


Figure 6. Intermolecular contact surfaces between PR_{6X} and the two PR inhibitors, **2** (A) and **1** (B). Surfaces are colored according to intersurface distances calculated using GRASP, with white indicating an intersurface distance of 0 and red indicating 4 Å. In these views the right half of each inhibitor interacts with the “B” monomer in the X-ray crystal structures, with the “top” imaging showing the flap-proximal surface and the “bottom” showing the D25-proximal surface. (C) Stick representations of **2** (magenta) and **1** (orange) in the active site of PR_{6X}. Hydrogen bonds to the “B” PR monomer, represented as a ribbon with transparent molecular surface, are indicated by dashed lines. The conformation of **1** bound to PR_{wt} is shown as green sticks. (D) Superposition of the PR_{6X}–**2** and **1** structures (rmsd 0.70 Å for C α atoms), as viewed from the protease flaps.

the notion that the active site of the compound **1** complex is expanded due to changes in one flap conformation, thereby accommodating the “flipped” half-of **1**.¹² This configuration of **1** lacks contacts with the core of the enzyme, thereby reducing PI efficacy, whereas with **2**, the methoxy-benzyl with Asp30 and indene-Arg8 interactions (Figure 5) retains contacts in the **2** complex. The net result is maintenance of **2** binding in PR_{6X} and potent antiviral activity.

It should be noted that unliganded PR_{6X} is a symmetrical dimer, therefore the PR_{6X} mutations do not predispose the dimer to be an asymmetric conformation.²³ In particular, comparison of the apo forms of PR_{wt} and PR_{6X} show that the proteins adopt

similar conformations with very similar hydrogen bonding networks in their core regions.²³ Small differences do occur in the flaps, where the PR_{6X} multidrug-resistant mutant exhibits more disorder, consistent with increased flexibility in this region as observed in other PI-resistant PRs.^{6,24,25} Therefore, the PR_{6X} residues occupy the same structural context in both unliganded and **2** bound PR_{6X}, implying that the asymmetric configuration when bound to **1** is a function of interactions with **1** and not an intrinsic conformational preference of the PI cross-resistant PR_{6X} mutant.¹²

Structural Mechanisms of PI Resistance. It has been difficult to precisely define the structural mechanisms by which mutations in protease promote multidrug-resistance and loss of PI efficacy.^{5,22,26,27} Like compound **2**, saquinavir, nelfinavir, and ritonavir lack C₂ symmetry yet lose 7–14-fold affinity for PR_{6X} relative to PR_{wt}, whereas **2** loses only 2.6-fold affinity.¹⁰ The role of conformational flexibility in the inhibitor has been noted in explaining improved binding of the inhibitor darunavir to a multidrug-resistant PR mutant.²⁸ In a similar fashion, **2** exhibits flexibility in both the isobutyl side chain (**2**^a vs **2**^b, Figure 4B) and the carbamate-linked indene (Figure 5). These various degrees of freedom contribute to the configurational entropy of the complex, which should enhance the binding energy of the inhibitor to multidrug-resistant PRs.

Like amprenavir, darunavir, and other recently developed inhibitors,^{28,29} **2** is nonpeptidomimetic, underscoring an advantage for this inhibitor class against multidrug-resistant PRs. Compound **2**, amprenavir, and darunavir each contain a benzyl ring with a hydrogen bonding functional group attached to the isobutyl-sulfonamide, a phenyl group in the P1/P1' pocket, a hydroxyl group to interact with Asp25/Asp25', and a heterocyclic ring system linked to a carbamate at the opposite end of the inhibitor (Figure 1). Compound **2** differs by having a triazole in the inhibitor core and an inverted carbamate.¹⁶ These chemical differences appear to contribute to the higher affinity of **2** for PR_{6X}: the triazole participates in a precise hydrogen bonding network with the "flap water" and the carbamate accepts a hydrogen bond from the amide of Gly48. Both of these interactions feature a conserved element of protease structure. Such interactions are less likely to be influenced by mutations, while limiting conformational change with respect to PR_{wt}. In other words, **2** exploits structural features common to both wild-type and multidrug-resistant proteases to retain high affinity binding.

Conclusion

We have reported that **2** is effective against the single point protease inhibitor mutants, V82F, V82A, and G48V. The biochemical and structure findings reported herein demonstrate that compound **2** retains high affinity for both wild-type and the PR_{6X} multidrug-resistant protease variant as the result of interactions with selected residues and maintenance of hydrogen bonding to main chain atoms. These structural features of **2**–protease interactions resulted in potent antiviral activity to our series of increasing PI-resistant viruses. The importance of maintaining PI interactions with the protein backbone of the active site has been found in PIs that are effective on multidrug-resistant proteases.^{15,30} Furthermore, it is proposed that PIs should be developed to maintain extensive hydrogen bonding with the active site backbone to promote continued binding in the face of PI-resistance mutations.^{14,15,30} On the basis of our structural findings, modifications may be made to **2** to increase hydrogen bonding to backbone atoms. The use of azide–alkyne click chemistry synthesis will allow rapid and diverse chemical

modification of **2**. This will also allow the evaluation of whether such changes improve antiviral performance against newly appearing multidrug-resistant viruses while maintaining efficacy to wild-type and previous versions of multidrug-resistant viruses. Lastly, insights into the contributions of multiple PI-resistance mutations and their resulting effect on protease interactions with inhibitors and substrates will continue to provide the basis for new inhibitors to target wild-type and multidrug resistant viruses.

Experimental Section

Compounds. Compound **1**, phenylmethyl-*N*-[(2*S*)-1-[[[(2*S*)-1-[[[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[[[(2*S*)-3-methyl-2-[[[(2*S*)-2-(phenylmethoxycarbonylamino)propanoyl]amino]butanoyl]amino]-1,6-di(phenyl)hexan-2-yl]amino]-3-methyl-1-oxobutan-2-yl]amino]-1-oxopropan-2-yl]carbamate, synthesis was originally described by Lee et al as compound TL-3.¹¹ Compound **2**, (1-(3-hydroxy-4-(*N*-isobutyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl)-1*H*-1,2,3-triazol-4-yl)methyl-2-hydroxy-2,3-dihydro-1*H*-inden-1-ylcarbamate, was originally described by Brik et al. as compound AB2.^{13,16}

Cloning, Expression, and Purification of PRs. Construct design and synthesis of plasmids for the expression of all PRs as well as the detailed expression and purification protocols have been reported previously.^{10–12}

Protease Activity Assays. HIV-1 protease enzymatic activity was assayed as described previously,^{10,11} using a fluorogenic substrate.³¹ Briefly, purified HIV-1 protease was mixed with inhibitor compounds in a reaction buffer containing 25 mM MES, pH 5.6, 200 mM NaCl, 5% glycerol, and 1 mM DTT in a prewarmed 96-well plate. The reaction was started by the addition of fluorescently labeled anthranilyl protease substrate H-2992 (Bachem, CA), and the reaction progress was measured by fluorescence intensity using an FLx-800 fluorescence plate reader (BioTek, VT). Final reaction concentrations were 25 nM protease, 30 μM substrate, and 0.001–600 μM inhibitor. Kinetics constants were determined by nonlinear regression of initial reaction velocities as a function of inhibitor concentration using Prism v4.0c (Graphpad Software, San Diego, CA). Values for K_i were calculated from the IC₅₀ values using the Cheng–Prusoff equation:³²

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \quad (1)$$

where *S* is the concentration of fluorescent substrate in the reaction.

Cell Culture Activity and Viral Expression Assays. SupT1 cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin. HIV-1 stocks were obtained from the culture supernatant of infected SupT1 cells. Cells were infected by addition of 10–100 ng/mL p24 and incubated at 37 °C. Unadsorbed virus was removed by washing the cells with fresh media and resuspended in media ± protease inhibitors. Culture supernatant samples were removed for p24 quantification every second day, and then media and selected PIs were added back to the culture. Viral replication was quantified by p24 ELISA assay (Perkin-Elmer).

Inhibition of infectious virion production was quantified using the TZM-bl indicator cell line, which contain a luciferase reported driven by the HIV-1 LTR.³³ SupT1 cells were infected with HIV-1 expressing either PR_{wt} or PR_{6X} protease variants at an MOI of 0.001–0.005 and cultured with protease inhibitors **1** or **2** at concentrations from 1 to 4000 nM. On days 5–6, 50 μL of cell-free supernatants were added to 10⁴ TZM-bl cells in 96-well plates. After 48 h, 100 μL culture supernatant was removed and luciferase activity was quantified by the addition of 100 μL Brite-Lite Plus reagent (Perkin-Elmer) to each well and measured with a Clarity luminometer (BioTek, VT). EC₅₀ values were determined by nonlinear regression of normalized luciferase activity (Graphpad Software, San Diego, CA).

Crystallization. HIV-1 PR_{6X} was mixed with a 4- to 10-fold molar excess of **2** and incubated on ice for 30–60 min. Insoluble material was removed by centrifugation at 14000g for 15 min. The PR₆–compound **2** complex was crystallized by mixing 1 μ L of complex solution with 1 μ L of well solution containing 0.1 M sodium acetate, pH 4.5, 3.5 M NaBr, by sitting drop vapor diffusion at 6 °C.

Data Collection. Diffraction data for PR_{6X}–**2** crystals were collected at 100 K by the rotation method (120 frames, 1° oscillation per frame) to 1.3 Å resolution on an Saturn 92 CCD system mounted on a FR-E X-ray generator equipped with VariMax optics ($\lambda = 1.54$ Å). The data were processed with d*TREK (Table 2).³⁴

Structure Refinement. The structure of PR_{6X} was solved by molecular replacement at 3 Å using a monomer of the PR_{6X}–compound **1** complex structure (PDB 2AZC) as a search model in Molrep.³⁵ Five percent of randomly selected reflections were designated as test reflections for use in the Free-*R* cross-validation method³⁶ and used throughout the refinement. The correlation coefficient and *R*-factor from the molecular replacement solutions indicated that the correct space group was *P*4₃. Rigid body and restrained refinement were performed in REFMAC at 3 and 2.0 Å, respectively.³⁷ Simulated annealing, Powell minimization, and individual temperature factor refinements were performed using CNS.³⁸ The model was further refined to 1.3 Å in SHELXL³⁹ using a bulk solvent correction and isotropic *B*-factors, followed by several rounds of model adjustment using the $\sum A$ -weighted $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ electron density maps using Coot.⁴¹ Subsequently, Br ions from the crystallization medium and 89 water molecules were added, anisotropic temperature factors were refined (SHELXL), and 1 residue was modeled as having an alternate side chain conformation. Statistically disordered copies A and B of compound **2** were refined with 0.5 occupancy each, and two Br ions present in the active site, mutually exclusive with the alternate copies of compound **2**, were refined at 0.5 occupancy. The final $R_{\text{cryst}}/R_{\text{free}}$ was 15.6/18.9% (Table 2). Coordinates have been deposited with RCSB, accession code 2HC0.

Structural Analysis. Structural analysis and plots of protease–inhibitor interactions were completed using LIGPLOT.⁴² Protease–inhibitor intersurface distances and active site shape correlation were calculated using GRASP⁴³ and sc.⁴⁴ Structural superpositions were performed using C α atoms from monomers A and B of the protease dimer with CCP4. Difference–distance matrix plots were produced using the program DDMP from the Center for Structural Biology at Yale University, New Haven, CT. Structure figures were generated with PyMOL.⁴⁵

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Supporting Information Available: Difference distance matrix plot showing changes in relative distances between C α atoms in PR_{wt} and PR_{6X} proteases in complex with compound **2**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) del Rio, C. AIDS: the second wave. *Arch. Med. Res.* **2005**, *36*, 682–688.
- (2) Piacenti, F. J. An update and review of antiretroviral therapy. *Pharmacotherapy* **2006**, *26*, 1111–1133.
- (3) Kutilek, V. D.; Sheeter, D. A.; Elder, J. H.; Torbett, B. E. Is resistance futile? *Curr. Drug Targets Infect. Disord.* **2003**, *3*, 295–309.

- (4) Machado, D. M.; Fernandes, S. C.; Succi, R. C.; Freire, W. S.; Pannuti, C. S.; Gouveia, A. B.; Levi, J. E.; Diaz, R. S. Analysis of HIV- type 1 protease and reverse transcriptase in Brazilian children failing highly active antiretroviral therapy (HAART). *Rev. Inst. Med. Trop. Sao Paulo* **2005**, *47*, 1–5.
- (5) Muzammil, S.; Ross, P.; Freire, E. A major role for a set of non-active site mutations in the development of HIV-1 protease drug resistance. *Biochemistry* **2003**, *42*, 631–638.
- (6) Clemente, J. C.; Moose, R. E.; Hemrajani, R.; Whitford, L. R.; Govindasamy, L.; Reutzel, R.; McKenna, R.; Agbandje-McKenna, M.; Goodenow, M. M.; Dunn, B. M. Comparing the accumulation of active- and nonactive-site mutations in the HIV-1 protease. *Biochemistry* **2004**, *43*, 12141–12151.
- (7) Tie, Y.; Boross, P. I.; Wang, Y. F.; Gaddis, L.; Liu, F.; Chen, X.; Tozser, J.; Harrison, R. W.; Weber, I. T. Molecular basis for substrate recognition and drug resistance from 1.1 to 1.6 angstroms resolution crystal structures of HIV-1 protease mutants with substrate analogs. *FEBS J.* **2005**, *272*, 5265–5277.
- (8) Ohtaka, H.; Schon, A.; Freire, E. Multidrug resistance to HIV-1 protease inhibition requires cooperative coupling between distal mutations. *Biochemistry* **2003**, *42*, 13659–13666.
- (9) Rhee, S. Y.; Taylor, J.; Wadhwa, G.; Ben-Hur, A.; Brutlag, D. L.; Shafer, R. W. Genotypic predictors of human immunodeficiency virus type 1 drug resistance. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 17355–17360.
- (10) Buhler, B.; Lin, Y. C.; Morris, G.; Olson, A. J.; Wong, C. H.; Richman, D. D.; Elder, J. H.; Torbett, B. E. Viral evolution in response to the broad-based retroviral protease inhibitor TL-3. *J. Virol.* **2001**, *75*, 9502–9508.
- (11) Lee, T.; Laco, G. S.; Torbett, B. E.; Fox, H. S.; Lerner, D. L.; Elder, J. H.; Wong, C. H. Analysis of the S3 and S3' subsite specificities of feline immunodeficiency virus (FIV) protease: development of a broad-based protease inhibitor efficacious against FIV, SIV, and HIV in vitro and ex vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 939–944.
- (12) Heaslet, H.; Kutilek, V.; Morris, G. M.; Lin, Y. C.; Elder, J. H.; Torbett, B. E.; Stout, C. D. Structural insights into the mechanisms of drug resistance in HIV-1 protease NL4–3. *J. Mol. Biol.* **2006**, *356*, 967–981.
- (13) Brik, A.; Muldoon, J.; Lin, Y. C.; Elder, J. H.; Goodsell, D. S.; Olson, A. J.; Fokin, V. V.; Sharpless, K. B.; Wong, C. H. Rapid diversity-oriented synthesis in microtiter plates for in situ screening of HIV protease inhibitors. *ChemBioChem* **2003**, *4*, 1246–1248.
- (14) Ghosh, A. K.; Chapsal, B. D.; Weber, I. T.; Mitsuya, H. Design of HIV Protease Inhibitors Targeting Protein Backbone: An Effective Strategy for Combating Drug Resistance. *Acc. Chem. Res.* **2008**, *41*, 78–86.
- (15) Ghosh, A. K.; Sridhar, P. R.; Leshchenko, S.; Hussain, A. K.; Li, J.; Kovalevsky, A. Y.; Walters, D. E.; Wedekind, J. E.; Grum-Tokars, V.; Das, D.; Koh, Y.; Maeda, K.; Gatanaga, H.; Weber, I. T.; Mitsuya, H. Structure-based design of novel HIV-1 protease inhibitors to combat drug resistance. *J. Med. Chem.* **2006**, *49*, 5252–5261.
- (16) Brik, A.; Alexandratos, J.; Lin, Y. C.; Elder, J. H.; Olson, A. J.; Wlodawer, A.; Goodsell, D. S.; Wong, C. H. 1,2,3-Triazole as a peptide surrogate in the rapid synthesis of HIV-1 protease inhibitors. *ChemBioChem* **2005**, *6*, 1167–1169.
- (17) Kim, E. E.; Baker, C. T.; Dwyer, M. D.; Murcko, M. A.; Rao, B. G.; Tung, R. D.; Navia, M. A. Crystal Structure of HIV-1 Protease in Complex with VX-478, a Potent and Orally Bioavailable Inhibitor of the Enzyme. *J. Am. Chem. Soc.* **1995**, *117*, 1181–1182.
- (18) Ghosh, S.; Rosenthal, R.; Zajac, P.; Weber, W. P.; Oertli, D.; Heberer, M.; Martin, I.; Spagnoli, G. C.; Reschner, A. Culture of melanoma cells in 3-dimensional architectures results in impaired immunorecognition by cytotoxic T lymphocytes specific for Melan-A/MART-1 tumor-associated antigen. *Ann. Surg.* **2005**, *242*, 851–857.
- (19) King, N. M.; Prabu-Jeyabalan, M.; Nalivaika, E. A.; Wigerinck, P.; de Bethune, M. P.; Schiffer, C. A. Structural and thermodynamic basis for the binding of TMC114, a next-generation human immunodeficiency virus type 1 protease inhibitor. *J. Virol.* **2004**, *78*, 12012–12021.
- (20) Prabu-Jeyabalan, M.; Nalivaika, E.; Schiffer, C. A. How does a symmetric dimer recognize an asymmetric substrate? A substrate complex of HIV-1 protease. *J. Mol. Biol.* **2000**, *301*, 1207–1220.
- (21) Prabu-Jeyabalan, M.; Nalivaika, E.; Schiffer, C. A. Substrate shape determines specificity of recognition for HIV-1 protease: analysis of crystal structures of six substrate complexes. *Structure* **2002**, *10*, 369–381.
- (22) Foulkes-Murzycki, J. E.; Scott, W. R.; Schiffer, C. A. Hydrophobic sliding: a possible mechanism for drug resistance in human immunodeficiency virus type 1 protease. *Structure* **2007**, *15*, 225–233.
- (23) Heaslet, H.; Rosenfeld, R.; Giffin, M.; Lin, Y. C.; Tam, K.; Torbett, B. E.; Elder, J. H.; McRee, D. E.; Stout, C. D. Conformational flexibility in the flap domains of ligand-free HIV protease. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2007**, *63*, 866–875.

- (24) Liu, F.; Boross, P. I.; Wang, Y. F.; Tozser, J.; Louis, J. M.; Harrison, R. W.; Weber, I. T. Kinetic, stability, and structural changes in high-resolution crystal structures of HIV-1 protease with drug-resistant mutations L24I, I50V, and G73S. *J. Mol. Biol.* **2005**, *354*, 789–800.
- (25) Liu, F.; Kovalevsky, A. Y.; Louis, J. M.; Boross, P. I.; Wang, Y. F.; Harrison, R. W.; Weber, I. T. Mechanism of drug resistance revealed by the crystal structure of the unliganded HIV-1 protease with F53L mutation. *J. Mol. Biol.* **2006**, *358*, 1191–1199.
- (26) King, N. M.; Prabu-Jeyabalan, M.; Nalivaika, E. A.; Schiffer, C. A. Combating susceptibility to drug resistance: lessons from HIV-1 protease. *Chem. Biol.* **2004**, *11*, 1333–1338.
- (27) Logsdon, B. C.; Vickrey, J. F.; Martin, P.; Proteasa, G.; Koepke, J. I.; Terlecky, S. R.; Wawrzak, Z.; Winters, M. A.; Merigan, T. C.; Kovari, L. C. Crystal structures of a multidrug-resistant human immunodeficiency virus type 1 protease reveal an expanded active-site cavity. *J. Virol.* **2004**, *78*, 3123–3132.
- (28) Ghosh, A. K.; Ramu Sridhar, P.; Kumaragurubaran, N.; Koh, Y.; Weber, I. T.; Mitsuya, H. Bis-tetrahydrofuran: a privileged ligand for darunavir and a new generation of hiv protease inhibitors that combat drug resistance. *ChemMedChem* **2006**, *1*, 939–950.
- (29) Amano, M.; Koh, Y.; Das, D.; Li, J.; Leschenko, S.; Wang, Y. F.; Boross, P. I.; Weber, I. T.; Ghosh, A. K.; Mitsuya, H. A Novel Bis-tetrahydrofuranylurethane-Containing Nonpeptidic Protease Inhibitor (PI), GRL-98065, Is Potent against Multiple-PI-Resistant Human Immunodeficiency Virus In Vitro. *Antimicrob. Agents Chemother.* **2007**, *51*, 2143–2155.
- (30) Wang, Y. F.; Tie, Y.; Boross, P. I.; Tozser, J.; Ghosh, A. K.; Harrison, R. W.; Weber, I. T. Potent new antiviral compound shows similar inhibition and structural interactions with drug resistant mutants and wild type HIV-1 protease. *J. Med. Chem.* **2007**, *50*, 4509–4515.
- (31) Toth, M. V.; Marshall, G. R. A simple, continuous fluorometric assay for HIV protease. *Int. J. Pept. Protein Res.* **1990**, *36*, 544–550.
- (32) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50% inhibition (I₅₀) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.
- (33) Platt, E. J.; Wehrly, K.; Kuhmann, S. E.; Chesebro, B.; Kabat, D. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol.* **1998**, *72*, 2855–2864.
- (34) Pflugrath, J. W. The finer things in X-ray diffraction data collection. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1999**, *55*, 1718–1725.
- (35) Vagin, A.; Teplyakov, A. An approach to multicopy search in molecular replacement. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2000**, *56*, 1622–1624.
- (36) Brunger, A. T. Assessment of phase accuracy by cross validation: the free R value. Methods and applications. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1993**, *49*, 24–36.
- (37) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1997**, *53*, 240–255.
- (38) Brunger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1998**, *54*, 905–921.
- (39) Sheldrick, G. M. A short history of SHELX. *Acta Crystallogr., Sect. A: Found. Crystallogr.* **2008**, *64*, 112–122.
- (40) Read, R. J. Improved Fourier coefficients for maps using phases from partial structures with errors. *Acta Crystallogr., Sect. A: Found. Crystallogr.* **1986**, *42*, 140–149.
- (41) Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2004**, *60*, 2126–2132.
- (42) Wallace, A. C.; Laskowski, R. A.; Thornton, J. M. LIGPLOT: a program to generate schematic diagrams of protein–ligand interactions. *Protein Eng.* **1995**, *8*, 127–134.
- (43) Nicholls, A.; Sharp, K. A.; Honig, B. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **1991**, *11*, 281–296.
- (44) Collaborative Computational Project, Number 4The CCP4 suite: programs for protein crystallography. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1994**, *50*, 760–763.
- (45) DeLano, W. L. *The PyMOL Molecular Graphics System*; DeLano Scientific LLC: San Carlos, CA, 2006.
- (46) Cruickshank, D. W. Remarks about protein structure precision. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1999**, *55*, 583–601.

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