Counteracting HIV-1 Protease Drug Resistance: Structural Analysis of Mutant Proteases Complexed with XV638 and SD146, Cyclic Urea Amides with Broad Specificities

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ABSTRACT: The long-term therapeutic benefit of HIV antiretroviral therapy is still threatened by drug-resistant variants. Mutations in the S1 subsite of the protease are the primary cause for the loss of sensitivity toward many HIV protease inhibitors, including our first-generation cyclic urea-based inhibitors DMP323 and DMP450. We now report the structures of the three active-site mutant proteases V82F, I84V, and V82F/I84V in complex with XV638 and SD146, two P2 analogues of DMP323 that are 8-fold more potent against the wild type and are able to inhibit a broad panel of drug-resistant variants [Jadhav, P. K., et al. (1997) *J. Med. Chem. 40*, 181−191]. The increased efficacy of XV638 and SD146 is due primarily to an increase in P2−S2 interactions: 30−40% more van der Waals contacts and two to four additional hydrogen bonds. Furthermore, because these new interactions do not perturb other subsites in the protease, it appears that the large complementary surface areas of their P2 substituents compensate for the loss of P1−S1 interactions and reduce the probability of selecting for drug-resistant variants.

The human immunodeficiency virus (HIV)[1] encodes an aspartic protease (PR) that cleaves the *gag* and *gag-pol* viral polyproteins into four replicative enzymes and four structural proteins (*1*). The finding that incomplete processing of these precursors produces immature, noninfectious viral particles (*2*, *3*) elicited an intense search for synthetic inhibitors of the protease (*4*, *5*). Recently, the FDA has approved the use of four protease inhibitors—saquinavir, ritonavir, indinavir, and nelfinavir—in preventing the progression of HIV infection (*6*−*8*). Unfortunately, their therapeutic benefit is short-lived because they do not completely suppress viral replication. A virus that evolves under the selective pressure of these inhibitors often becomes drug-resistant, during the first year of monotherapy, by encoding mutant proteases that have a reduced affinity for the inhibitors but retain enough enzymatic activity to process the viral precursors (*9*−*12*). Efforts to delay the onset of drug resistance have led to the use of a combination of protease and reverse transcriptase antiretroviral agents. Although multidrug therapy has maintained the viral load in some patients at undetectable levels for 2 years, recent studies have reported the isolation of a replication-competent virus from their resting $CD4^+$ T cells (*13*). The existence of this latently infected reservoir of HIV coupled with the daunting ability of the virus to mutate suggests there is an ongoing need for new HIV PR inhibitors with superior efficacy against mutant proteases.

Designing broadly active protease inhibitors requires detailed structural knowledge of mutant proteases. Obtaining such information, however, is complicated by the fact that more than 20% of the protease residues are associated with resistance to various inhibitors (*14*) and most resistance profiles consist of multiple mutations in and around the active site. Deconvoluting the structure−activity relationships of resistant mutants is therefore complicated by the presence of multiple active-site and compensatory changes. Although both types of mutations are commonly associated with resistance, we believe that active-site changes play a leading role in modulating the affinity of the protease because mutations appear to accumulate in a stepwise fashion, appearing first in the active site and then in compensatory regions (*10*), and because they interfere with inhibitor binding; e.g., the K_i values of most active-site mutants increase by 100−1000-fold (*15*−*21*).

The two mutations that confer resistance to HIV grown in the presence of our first cyclic urea-based development candidate, DMP323 (*22*, *23*), are Val82 to Phe and Ile84 to Val (*24*); these two substitutions increase the K_i value by 1000-fold (*19*). Because these mutations are located in the active site of the protease, we have an excellent opportunity to study how changes in the active site lead to drug resistance. In a previous study, we showed that a loss of vdw interactions was in part responsible for the reduced sensitivity toward DMP323 (*19*). We have now extended our understanding of resistance by identifying the interactions

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[1] Abbreviations: CU, cyclic urea; E_{vdw}, energy associated with vdw interactions; FDA, Food and Drug Administration; HIV, human immunodeficiency virus; IC_{90}, concentration of inhibitor required to inhibit viral replication by 90%; Ile50′, symmetry-related residue of Ile50; K_i, inhibition constant; PDB, Protein Data Bank; PR, protease; rms, root-mean-square; SA, simulated annealing; vdw, van der Waals.

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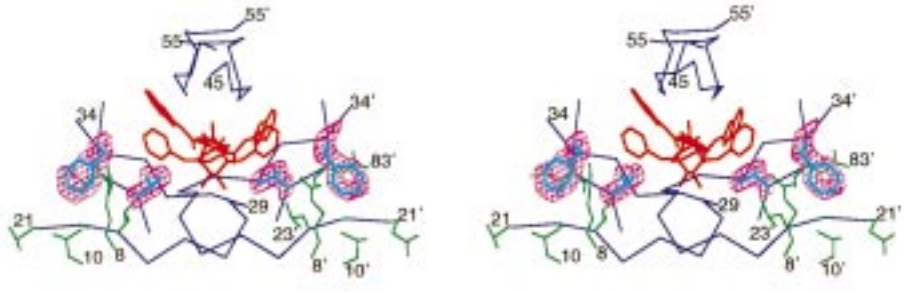


FIGURE 1: Conformation of Phe82 in V82F/I84V−SD146. The side chain of Phe82 (blue) binds in a hydrophobic cleft lined by Leu10, Leu21′, and Leu23. The electron density (pink) around Phe82 (Phe82′) and Val84 (Val84′) was extracted from a $(2F_{obs} - F_{calc})\alpha_{calc}$ 1.8 Å simulated annealed omit map with the F_{calc} terms and phases calculated from a model excluding the side chains of residues 82 (82′) and 84 (84′); the map was contoured at the 1.0σ level. Note that the mutations reduce the number of P1−S1 vdw interactions by enlarging the S1 subsite.

Table 1: Data Collection and Refinement Statistics

	HIV PR−XV638			HIV PR−SD146
	V82F	I84V	V82F/I84V	V82F/I84V
data collection statistics with all the data				
resolution (Å)	2.0	1.9	1.9	1.8
no. of total reflections	36 747	55 099	61 415	99 302
no. of unique reflections	12 347	13 817	14 433	17 416
completeness (%)	97	94	98	99
R_{sym} (%)[a]	9.2	10.5	9.2	7.9
refinement statistics with $F_{obs} > 2\sigma F_{obs}$				
R_{factor} (%)[b]	19.6	19.8	19.9	19.9
completeness (%)	79	80	83	81
no. of protein atoms	1850	1840	1848	1848
no. of inhibitor atoms	54	54	54	62
no. of solvent atoms	76	81	72	73
rms deviations, angles (deg)	3.1	3.1	3.1	2.9
rms deviations, bonds (Å)	0.012	0.012	0.013	0.012

[a] $R_{sym} = \sum|\langle I\rangle - I|/\sum I$. [b] $R_{factor} = \sum|F_{obs} - kF_{calc}|/\sum F_{obs}$.

responsible for the high potency and broad specificity of two second-generation inhibitors, XV638 and SD146 (thiazolylbenzamide and benzimidazolylbenzamide P2 derivatives of DMP323, respectively) (*25*, *26*).

MATERIALS AND METHODS

Mutant Preparation, Crystallization, and Data Collection. The HIV-1 protease mutants V82F (Val82 → Phe), I84V (Ile84 → Val), and V82F/I84V (Val82 → Phe and Ile84 → Val) were constructed as reported previously (*19*). The mutant genes were inserted into the translation vector pET11C and expressed in *Escherichia coli* BL21(DE3) cells. The protein was purified from inclusion bodies and refolded using a hydrophobic interaction column (*27*). The mutant complexes crystallized as hexagonal rods (0.06 mm × 0.06 mm × 1.2 mm) after 5 days at 18 °C by vapor diffusion in 4 μL hanging drops, containing 1 mg/mL protein, 250 mM acetate buffer (pH 5.0−5.6), 80−240 mM ammonium sulfate, and a 1000-fold molar excess of inhibitor over its K_i value (*28*).

Diffraction data were collected at room temperature on a RAXIS IIc imaging plate mounted on a Rigaku RU-H2R rotating anode generator equipped with a copper anode, a 0.3 mm cathode, and Yale double-focusing mirrors. The unit cell parameters were determined from four still frames, taken at 15° intervals, using the RAXIS processing software. Full data sets were obtained from a single crystal by collecting 30−60 oscillation images at 2° intervals for 60 min at a detector distance of 70 mm. All mutant complexes crystallized in the space group $P6_1$ with a dimer in the asymmetric unit and the following cell dimensions: $a = b = 62.8$ Å and $c = 83.5$ Å.

Structure Refinement. The atomic coordinates for the protein in the HIV PR−XK216 complex (XK216 is a symmetric cyclic urea with benzyl and allyl groups at P1 and P2, respectively; PDB code 1HWR) were used as the starting model in refinement, except Val82 and Ile84 were substituted with alanines. The data were refined by performing several cycles of simulated annealing (SA) followed by positional and restrained *B*-factor refinements (*29*). The conformations of the inhibitors and mutated residues were clearly defined in the electron density; e.g., a SA omit map of Phe82 and -82′ and Val84 and -84′ agrees with the refined model of the double mutant (Figure 1). Refinement reduced the starting R_{factor} of ∼40% to final values of 19−20%. The data collection and refinement statistics are listed in Table 1. Coordinates for the recombinant HIV-1 protease mutants V82F, I84V, and V82F/I84V in complex with XV638 have been deposited at the Brookhaven Protein Data Bank under file names 1bv7, 1bv9, and 1bwa, respectively; the corresponding file for V82F/I84V−SD146 is 1bwb.

K_i Measurements and E_{vdw} Calculations. All K_i measurements were recorded as reported previously (*30*). Briefly, HIV-1 PR was incubated with an inhibitor and a fluorescent cationic peptide substrate for 60 min at 20 °C. The enzymatic hydrolysis of the substrate yielded a fluorescent

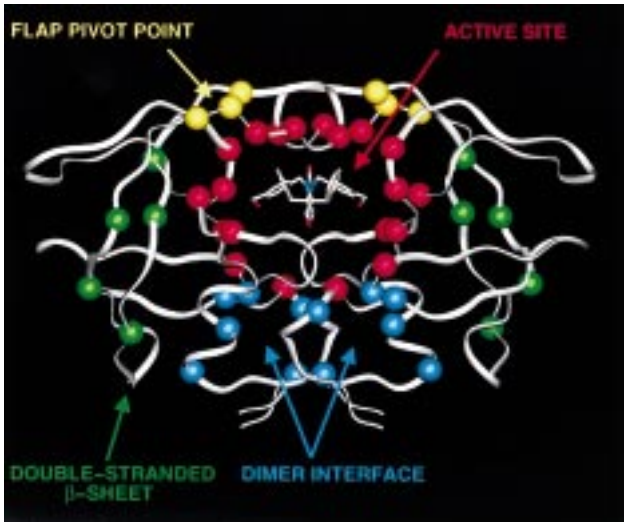


FIGURE 2: Residues associated with HIV-1 protease drug resistance. The color-coded spheres represent point mutations in different regions of the protein: red, active site; yellow, flap pivot point; blue, dimer interface; and green, double-stranded β-sheet (Val56−Gly78). The C_α atoms of the protease are drawn as white ribbons, and DMP323 is bound in the active site.

anionic product, which was purified using a mono-Q FPLC column (Pharmacia); the cleavage product was quantified by measuring its fluorescence at emission and excitation wavelengths of 430 and 330 nm, respectively. The K_i values for each inhibitor were estimated with the equation $K_i = I/\{[[K_m + S - (f_aS)]/(f_aK_m)] - 1\}$, where I is the inhibitor concentration, S is the substrate concentration, f_a is the fractional activity (V_i/V_o), and K_m is the Michaelis constant. The K_i values reported are averages of two or three measurements.

The energetic contributions of the vdw interactions to the binding energies between mutants and cyclic urea (CU) inhibitors were approximated by the Lennard-Jones potential energy function using the *X-PLOR* program (*31*).

RESULTS AND DISCUSSION

Molecular Basis of HIV PR Drug Resistance. Sequence variation in the protease is primarily caused by the high error rate of reverse transcriptase (*32*) and the rapid rate of HIV replication (*33*). More than 20 mutations—clustered in the active site, flap pivot point, dimer interface, and double-stranded β-sheet (Val56−Gly78) of the protease—have been identified in clinical and in vitro isolates exposed to protease inhibitors (Figure 2). Approximately half of the mutations are located in the active site, where they reduce the number of vdw contacts, increase steric hindrance, or increase the number of unfavorable electrostatic interactions between the protease and inhibitors (*15*−*19*). Mutations outside of the active site, on the other hand, are thought to produce conformational changes that compensate for the impaired activity of active-site mutants by preferentially increasing the protease's affinity for substrates over inhibitors or by increasing the mutant enzyme's rate of catalysis (*34*, *35*). Unfortunately, very little is known about compensatory changes because the crystal structures of proteases containing these mutations do not reveal any significant structural perturbations (*15*, *18*). A superposition of the complexed and uncomplexed structures of the protease, however, reveals

Table 2: K_i Values of Mutant HIV-1 PRs

	K_i (nM)		
HIV-1 PR	DMP323	XV638	SD146
wild type	0.8	0.1	0.1
fold resistance[a]			
V82F	0.5	0.5	0.5
I84V	20	1.1	2.6
V82F/I84V	1000	25	38

[a] Ratio of K_i values (mutant/wild type).

that inhibitor binding induces two large conformational changes: (1) the well-documented movement (7 Å) of the flaps, which close over the active site (*36*), and (2) the rotation of each monomer into the active site. The latter movement is best viewed when two monomers, one from each state, are superimposed; the relative shift between the other two monomers emphasizes the rigid-body movement that occurs during complex formation. In the case of DMP323 binding to HIV PR, the rms deviation of the C_α atoms for the superimposed monomers is only 0.7 Å, but the C_α atoms of Gly17 in the other two monomers are 6 Å apart. These two conformational changes suggest that binding occurs at least as a two-step process, similar to that proposed for peptidomimetics; inhibitors bind to the open state of the protease and form a loose complex, which becomes more compact as the flaps close over the active site (*37*). Because the flap is an extension of a large double-stranded β-sheet (Val56−Gly78), its movement might pull on the β-sheet which in turn pulls the rest of the monomer toward the active site. An important consequence of this rearrangement is the movement of a loop (Pro79−Val82) into the active site, creating part of the S1 subsite. This proposed mechanism is consistent with our assumption that mutations in the flap pivot point, in the hydrophobic pockets near the C and N termini, and along the β-sheet disrupt the coordinated movement that occurs upon complex formation. For example, mutating Lys45, Met46, or Ile54 in the flap pivot point may enable the flap to open and close independently of the β-sheet movement; mutations along the β-sheet may allow the latter to slide more freely over the rest of the monomer, and mutating Leu24 and Leu97′ (two residues involved in vdw contacts) to valines might weaken the dimer interface and allow the monomers to move toward the active site independently of the flap and β-sheet movements. The function of compensatory mutations would therefore be to allow the flap to close without forming the S1 subsite, and vice versa. This type of flexibility might enhance the ability of active-site mutants to discriminate against inhibitors; interfering with these conformational changes would therefore provide an alternate strategy for inhibiting the protease.

DMP323 Resistance Problem. Two active-site substitutions in HIV PR, Val82 to Phe and Ile84 to Val, reduce the protease's affinity for DMP323 by 1000-fold (*19*). These mutations are apparently sufficient to tip the binding equilibrium in favor of the substrates, for the K_m of the double mutant only decreases by 6-fold (*21*). The structure of the recombinant double mutant in complex with DMP323 reveals that the substitutions enlarge the S1 subsite and reduce the number of vdw contacts between Val82 and Ile84 and DMP323 by 70% (*19*). We have now extended our structural analysis to include XV638 and SD146, which are more active against the wild-type and recombinant mutant proteins (Table

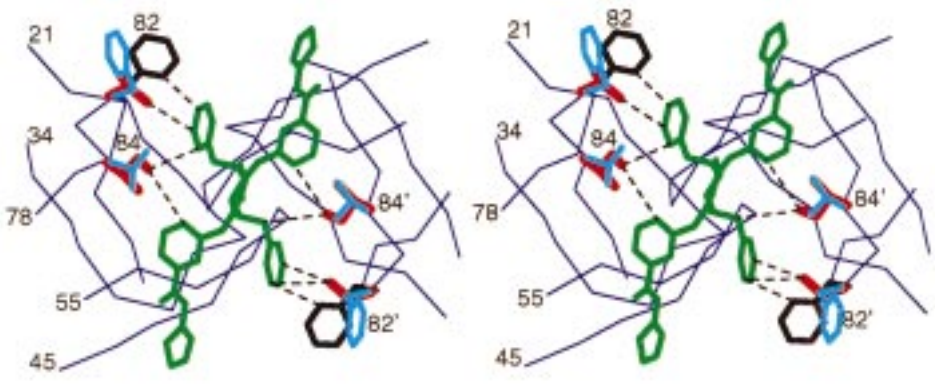


FIGURE 3: Conformations of the mutant residues when the protein is complexed with XV638. Mutant residues are superimposed onto the C_α trace of V82F/I84V−XV638: Val82 and Ile84 from the wild type (red), Phe82 from V82F (black), and Phe82 and Val84 from the double mutant (blue). Note the vdw contacts (dashed lines, distances of less than 4.1 Å) to XV638 (green) in the native and V82F complexes. Equivalent interactions are not present in the double mutant because Val84 (Val84′) is too small to contact the inhibitor and the side chain rotamer of Phe82 (Phe82′) rotates out of the S1 (S1′) subsite; the side chains of the phenylalanines in V82F and in the double mutant pack as the $\chi^1 = -60°$ and $\chi^2 = 125°$ and $\chi^1 = -150°$ and $\chi^2 = 90°$ rotamers, respectively.

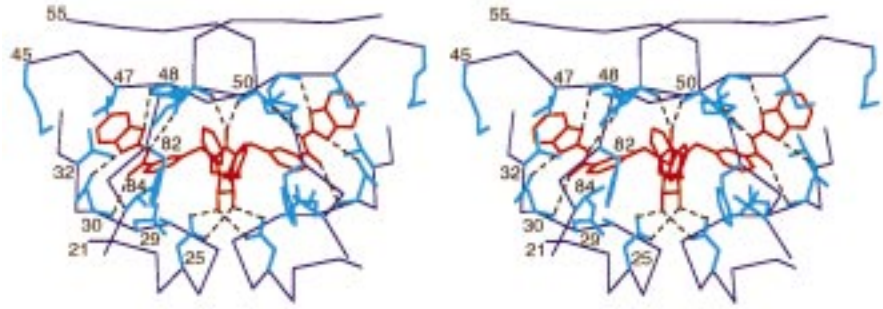


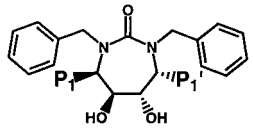
FIGURE 4: Interactions between SD146 and the double mutant. SD146 inhibits the double mutant because it interacts extensively with S2 subsite residues. In total, SD146 (red) interacts with 31 residues (blue) and participates in 177 vdw contacts and 14 hydrogen bonds (dashed lines).

2). In addition, SD146 has a long-lasting antiviral effect, as we were unable to raise a resistant virus after several months of selection pressure in vitro (S. Garber, DuPont Pharmaceuticals, personal communication). To determine how these CUs retain their high potency against the active-site mutants, we have solved the crystal structures of the three mutant proteases (V82F, I84V, and V82F/I84V) in complex with XV638 and SD146.

Mutant Proteases Complexed with XV638 and SD146. Mutating the active-site residues Val82 and Ile84 does not alter the overall conformation of the protein; the rms deviation of the C_α atoms between any two CU complexes is <0.35 Å. Small structural perturbations, however, are observed near the mutated sites. Substituting Val82 with Phe causes the backbone atoms of Pro79−Phe82 to shift away from the inhibitor by up to 0.5 Å to accommodate the bulky side chain; in the symmetry-related monomer, P1′ provides space by moving out of the S1′ subsite by ∼0.5 Å. In both monomers, the side chain of Phe82 exhibits torsion angles similar to that of the preferred conformer (*38*) (Figure 3) and packs close enough to form vdw contacts with the P1 benzyl moiety of XV638. The substitution of Ile84 with Val is nearly isosteric; the χ^1 angle of Val84 deviates by only 15° from the most favorable χ^1 of 173°, and the backbone atoms around it shift into the active site by ∼0.5 Å. The loss of the two CD1 atoms, one in each monomer, therefore enlarges the active site and reduces the vdw contact surface area between the protease and the P1 and P1′ substituents of the inhibitor. In the double mutant complexes, Val84 and Val84′ pack just like those in I84V−XV638, but the rotamers of Phe82 and Phe82′ differ significantly from those in V82F−XV638; the χ^1 angles of the phenylalanines differ by 90°, a rotation that, in the double mutant, swings the side chain out of the S1 (S1′) subsite and into a hydrophobic cleft lined by Leu10 (Leu10′), Leu21 (Leu21′), and Leu 23 (Leu23′) (Figures 1 and 3). In this orientation, the side chains of Phe82 and Phe82′ are not close enough to form vdw contacts with the inhibitor; we are currently trying to identify the structural features responsible for triggering the rotamer switch by solving the structures of the uncomplexed mutants.

In general, the mutations do not disrupt hydrogen bonds to the inhibitors; instead, they alter the extent of vdw interactions with the P1 and P1′ substituents. In V82F, the substitution increases E_{vdw} by ∼0.1 kcal/mol, which is consistent with the slight decrease in its K_i values (Table 2). In I84V, the substitution decreases E_{vdw} by ∼0.5 kcal/mol, which corresponds only to a 2-fold increase in the K_i value. Although this might appear to be consistent with the K_i values for XV638 and SD146, such a small increase is not predictive for DMP323. Finally, in the double mutant complexes, E_{vdw} decreases by 1.5 kcal/mol, which corresponds to a 13-fold increase in K_i, a value too small to account for the 1000-, 38-, and 25-fold losses for DMP323, SD146, and XV638, respectively. These results suggest that, although many vdw contacts (<4.1 Å) to P1 are lost in the I84V and double

Table 3: K_i Values for P1-Substituted CUs



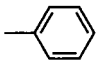
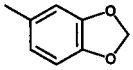
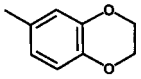
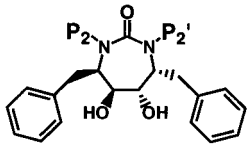
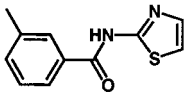
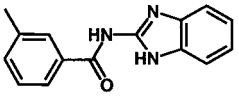
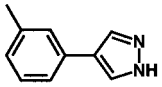
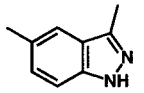
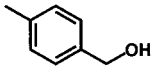
Cyclic Urea	P1(P1')	K_i (nM)	K_i(82F/84V) / K_i (WT)
XN127		2047	2
XR808		220	3
XV076		38	92
XL075		5	146
SC120		2	656
SC133		1	687

Table 4: K_i Values for P2-Substituted CUs



Cyclic Urea	P2(P2')	K_i (nM)	K_i(82F/84V) / K_i (WT)
XV638		0.1	25
SD146		0.1	38
XN974		0.1	114
XZ885		0.1	298
DMP323		0.8	1016
XK234		6.0	1645

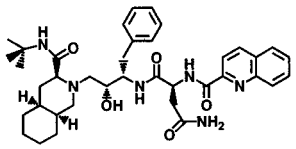
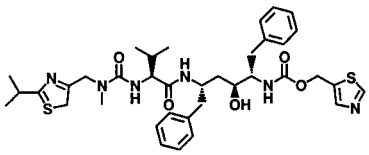
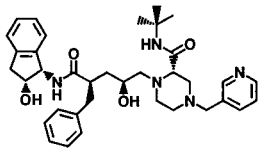
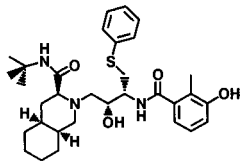
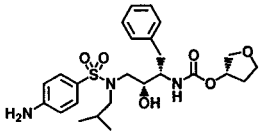
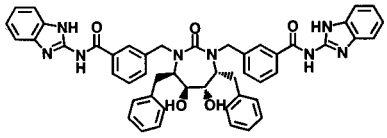
mutant complexes, the loss of energy associated with vdw interactions is too small to account for the entire loss of protease affinity. The effects of the mutations must therefore be more complex, and further studies will be needed to assess how mutations affect other energetic terms of binding. Nevertheless, because the structures of the first- and second-generation CU complexes are very similar, we can conclude that the P2 substituent does not influence how the inhibitors interact with the S1 subsite; i.e., the same interactions are lost whether the mutants are complexed to DMP323, XV638, or SD146 (Figure 4). The high potency of the second-generation inhibitors against the double mutant and a panel of HIV PR-selected strains is therefore not a result of any unique structural perturbations in the protein but rather a result of an increase in the number of P2−S2 vdw contacts and hydrogen bonds (Figure 4).

Counteracting Cyclic Urea Resistance. To further investigate the CU resistance problem, we measured the K_i values for a series of P1 and P2 analogues (Tables 3 and 4). In Table 3, the K_is range from 2000 to 1 nM, decreasing as the size of the P1 substituents increases. XN127, for example, is the least potent inhibitor because it contains an isobutyl group, which is too small to fill the S1 subsite, whereas SC133, the most potent inhibitor, contains a bulky benzodioxan. Apparently, the K_i values decrease as the extent of P1−S1 interactions increases. The size of the P1 group also influences the probability of selecting for the DMP323-selected double mutant, for the resistance values (the ratio of the double mutant and wild-type K_i values) increase from 2 to 687 for XN127 and SC133, respectively (Table 3). Inhibitors with small P1 groups have low resistance values because they do not contact Val82 and Ile84; mutating these residues would thus not significantly affect protease affinity. In contrast, inhibitors that contain large P1 substituents, which interact extensively with Val82 and Ile84, have high resistance values and will probably select for the drug-resistant double mutant. This analysis illustrates the complexity of the resistance problem in that an increase in the potency of a small inhibitor is often accompanied by a significant increase in the risk of selecting for resistant mutants.

To determine the effects of the P2 substituents on the resistance values, we measured the K_i values for a series of P2 analogues of XL075. The latter inhibitor was chosen to test P2−S2 compatibility because it possesses the traditional phenyl substituents at P1 and P1′ and it has a moderate resistance value, which will either increase or decrease as we modify P2. The K_is in Table 4 range from 6 to 0.1 nM, decreasing as the size of P2 increases. As in Table 3, we believe the K_i values decrease as the total number of interactions increases, but this time, they level off at 0.1 nM (the detection limit for the assay is 0.01 nM); the resistance values, however, continued to drop to a value of 25 for XV638. This suggests that the additional P2−S2 interactions observed with the large inhibitors, XN974, SD146, and XV638, do not significantly affect the wild-type K_i values but instead play an important role in maintaining potency against the double mutant. Conversely, the small inhibitors, XK234 and DMP323, tend to lose a larger percentage of the total interactions and have higher resistance values. This analysis indicates that large inhibitors are more likely to have a longer therapeutic benefit than smaller ones because their total contact surface area with respect to the protease is not significantly reduced by one or two mutations; a more complex set of mutations will therefore be needed to overcome their inhibition.

Combination Therapy. Until more broadly active HIV PR inhibitors become available, the most effective therapy is to combine antiretroviral agents; the best blend of inhibitors,

Table 5: Selected Characteristics of HIV PR Inhibitors

HIV PR inhibitors	K_i[a] (nM)	vdw[b]	h-bonds[b]	mutations[c]	$\frac{K_i(82F/84V)}{K_i(WT)}$
FDA APPROVED					
SAQUINAVIR 	0.15	135	7	[1]G48V L90M	19
RITONAVIR 	0.17	ND[d]	ND	[2]K20R L33F M36I,L E35D M46I,L I54V,L L63P A71V,T V82F I84V L90M	700
INDINAVIR 	0.14	117	6	[3]L10I,R,V M46I,L L63P V82T I84V	79
NELFINAVIR 	0.28	ND	ND	[4]D30N M46I L63P A71V V77I I84A,V	86
EXPERIMENTAL					
VX478 	0.11	97	5	[5]L10F M46I I47V I50V	100
SD146 	0.10	191	14	K41I A71T	38

[a] Reference *21*. [b] Total number of contacts of <4.1 Å and hydrogen bonds between the wild-type protease and inhibitors. [c] Mutations in the protease associated with drug resistance as reported in refs *12* and *39* (1), *10* and *40* (2), *9* (3), *11* (4), and *41* (5). [d] Not determined.

however, can only be determined by improving our understanding of cross resistance. Ideally, the drug candidates should not have overlapping toxicity profiles and should bind to different targets. Using multiple protease inhibitors is therefore particularly worrisome because they have similar chemical structures and bind to the same target. For example, all the inhibitors in Table 5 are mono-ols except for SD146 which is a diol; one-half of nelfinavir is identical to saquinavir, and most inhibitors possess a benzyl group at P1 or P1′. Furthermore, the bound structures of indinavir, VX478, saquinavir, A77003, and SD146 are very similar, only deviating at P2 (P2′) and P3 (P3′). Fortunately, despite their similarities, inhibitors select for similar but not identical sets of resistance mutations. For example, the key mutations associated with saquinavir resistance are Val48 in the active site and Met90 at the dimer interface. Ritonavir and indinavir select for Phe82 and Val84, and Thr82 and Val84, respectively; nelfinavir and SD146 select for Asn30 in the active site and Thr71 in the double-stranded β-sheet, respectively, and VX478 selects for Val47 and Val50 in the active site (Table 5). Although we are currently unable to predict resistance profiles, some general concepts of resistance can be formulated by studying how these inhibitors interact with the protease.

In general, at least one or two residues involved in inhibitor binding are mutated in a resistant variant; direct contact, however, is not the only criterion for becoming a resistance mutation. The residue must be able to modulate the affinity of the protease. This is best illustrated for small inhibitors, which interact with only a few residues. For example, DMP323 and VX478 select for mutations at Val82 and Ile84, and Ile47 and Ile50, respectively, because these residues are involved in a significant percentage (~13%) of the total number of vdw contacts between the inhibitors and the

protease; mutating other residues in the active site may not sufficiently reduce protease affinity. Other than DMP323, ritonavir is the only inhibitor that interacts extensively with Val82 and Ile84, since it also contains benzyl groups at P1 and P1′. On the basis of the structural similarities between ritonavir and the reported structure of A747004, ritonavir's P1 and P1′ benzyls probably occupy the same sites as those in DMP323, and thus, a loss of P1−S1 and P1′−S1′ interactions probably accounts for the selection of the double mutant and the 700-fold increase in the K_i value (*21*). The other inhibitors have smaller resistance values and do not interact sufficiently with the S1 subsite to warrant the selection of the double mutant. All of this suggests that inhibitors must interact extensively with Val82 and Ile84 as well as with their symmetry-related residues to select for the double mutant.

Although ritonavir and DMP323 are the only inhibitors in Table 5 that select for the double mutant, the efficiency of the other inhibitors against the double mutant is also reduced; the degree of cross resistance apparently depends on the overall extent of interactions lost due to the mutations (Table 5). For example, indinavir, nelfinavir, and VX478 have moderate resistance values of 79, 86, and 100, respectively, because they possess a benzyl group at P1 that interacts with Val82 and Ile84; however, saquinavir, which also contains a benzyl group at P1, has a much lower resistance value because it compensates for the loss of contacts by interacting extensively in other subsites of the protease by participating in ∼135 vdw interactions and seven hydrogen bonds. SD146 also has a very low resistance value because of extensive P2−S2 interactions, but its value is slightly higher than that for saquinavir because of its C_2 symmetry.

Our structural analysis therefore suggests that the primary determinant of active-site resistance profiles is the structure of the inhibitor, and that the extent of interactions lost due to mutations plays an important role in determining whether a mutant will be selected by an inhibitor or simply be cross resistant. In either situation, the loss of affinity can be overcome by increasing interactions in other subsites of the protease. Further examination of mutant structures will no doubt improve our understanding of resistance and enhance our ability to predict resistance profiles, two key pieces of information that will help to produce more diverse pools of inhibitors and extend the therapeutic life span of combination therapy.

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