

Molecular Recognition of Cyclic Urea HIV-1 Protease Inhibitors*

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As long as the threat of human immunodeficiency virus (HIV) protease drug resistance still exists, there will be a need for more potent antiretroviral agents. We have therefore determined the crystal structures of HIV-1 protease in complex with six cyclic urea inhibitors: XK216, XK263, DMP323, DMP450, XV638, and SD146, in an attempt to identify 1) the key interactions responsible for their high potency and 2) new interactions that might improve their therapeutic benefit. The structures reveal that the preorganized, C₂ symmetric scaffolds of the inhibitors are anchored in the active site of the protease by six hydrogen bonds and that their P1 and P2 substituents participate in extensive van der Waals interactions and hydrogen bonds. Because all of our inhibitors possess benzyl groups at P1 and P1', their relative binding affinities are modulated by the extent of their P2 interactions, e.g. XK216, the least potent inhibitor (K_i (inhibition constant) = 4.70 nM), possesses the smallest P2 and the lowest number of P2-S2 interactions; whereas SD146, the most potent inhibitor (K_i = 0.02 nM), contains a benzimidazolylbenzamide at P2 and participates in fourteen hydrogen bonds and ~200 van der Waals interactions. This analysis identifies the strongest interactions between the protease and the inhibitors, suggests ways to improve potency by building into the S2 subsite, and reveals how conformational changes and unique features of the viral protease increase the binding affinity of HIV protease inhibitors.

An essential step in the life cycle of the human immunodeficiency virus (HIV)¹ is the proteolytic cleavage of the viral polyprotein gene products of *gag* and *gag-pol* into active structural and replicative proteins (1, 2). The finding that a viral-encoded protease is responsible for processing these precursors, and that its inactivation produces immature, noninfectious viral particles, elicited an intense search for synthetic inhibitors. The first competitive inhibitors of HIV protease (PR) were transition-state analogs (peptidomimetics) in which the scissile bonds were replaced with nonhydrolyzable isosteres such as a reduced amide, phosphinate, hydroxyethylene, dihydroxyethylene, statine, and hydroxyethylamine (3–5). Recently, the Food and Drug Administration (FDA) has approved the use of four peptidomimetic protease inhibitors (saquinavir, zidovudine, zalcitabine, and didanosine) to treat HIV infection. Although these compounds are potent inhibitors of the wild-type protease, their therapeutic benefit is, in most cases, short-lived because they select for variants of HIV that have a reduced sensitivity toward inhibitors, as a result of mutations within the HIV protease sequence (6–10). In an attempt to delay the onset of drug resistance, the FDA approved the use of combination therapy, i.e. a mixture of protease and reverse transcriptase antiretroviral agents. Although multidrug therapy has reduced the plasma viral load of some HIV-infected individuals to undetectable levels (11), the daunting ability of the virus to rapidly mutate suggests an ongoing need for new antiretroviral drugs.

In order to design new and more potent inhibitors of HIV protease, we must improve our understanding of the principles of molecular recognition for the protease. So far researchers have identified two unique features of the viral protease that distinguish it from the human aspartic proteases pepsin and renin: 1) the active form of the viral enzyme is a homodimer, in which each monomer contributes equally to the active site and 2) the presence of a structural water molecule that bridges linear inhibitors to the flap of the protein via hydrogen bonds. Although hydroxyethylene isosteres and phosphinates were among the first C₂ symmetric molecules reported to bind HIV PR (12, 13), C₂ symmetric cyclic urea-based inhibitors were one of the first molecules capable of displacing the structural water (14). The cyclic urea (CU) scaffold is therefore well suited to interact with the viral protease and to discriminate against human proteases. Since these inhibitors were first reported, the number of CU mimics has rapidly increased, and this class of cyclic compounds may soon become a viable alternative to the currently available antiretroviral agents (15–18). In a continuing effort to identify new interactions that might increase the potency of our inhibitors, and other members of the cyclic family, we have performed a structural analysis of HIV-1 protease in complex with a series of CUs, which have IC₅₀ (concentration of inhibitor required to inhibit viral replication by 95%) values ranging from 5.1 to 4700 nM.

EXPERIMENTAL PROCEDURES

Inhibitors and K_i Measurements—The inhibitors XK216, XK263, DMP323, DMP450, XV638, and SD146 were synthesized as reported (14, 19–22), and their K_i values were measured as described previously (23).

HIV-1 Protease Preparation and Purification—The protease was mutated at a single position (Cys⁹⁵ → Ala), expressed in *Escherichia coli* BL21 (DE-3) (24), purified from inclusion bodies, and refolded using a hydrophobic interaction column (25).

Crystallization and Data Collection—Frozen aliquots (~120 ml) of the protease (32 µg/ml) in 50 mM sodium acetate buffer (pH 5.5), 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 5% ethylene glycol, and 350 mM NaCl were thawed and immediately mixed with one of the six inhibitors at a concentration equal to a 1000–5000-fold molar excess over its K_i value. The protein was then concentrated to 150 µg/ml, using an Amicon-stirred cell equipped with a YM3 membrane, and exchanged

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¹ The abbreviations used are: HIV, human immunodeficiency virus; CU, cyclic urea; Ile^{50'}, symmetry related residue of Ile⁵⁰; PR, protease; r.m.s., root mean square; vdW, van der Waals; WT, wild type.

TABLE I
Data collection and refinement statistics for HIV-1 protease-CU complexes

	HIV PR-CU					
	XK216	XK263	DMP323	DMP450	XV638	SD146
Data collection statistics with all the data						
Resolution (Å)	1.8	1.8	1.8	1.9	1.8	1.8
Total reflections	54,363	55,263	43,285	45,866	39,076	38,275
Unique reflections	13,881	15,417	15,720	12,478	16,197	15,429
Completeness (%)	80	88	90	84	92	89
R_{sym} (%) ^a	9.4	6.0	6.4	10.5	11.0	10.4
Refinement statistics with $ F_o > 2\sigma F_o $						
R_{factor} (%) ^b	19.0	19.3	19.1	19.8	19.5	19.8
No. of protein atoms	1840	1844	1844	1844	1840	1840
No. of inhibitor atoms	30	46	42	40	54	62
No. of solvent atoms	84	124	122	81	89	86
r.m.s. deviations, angle (°)	3.5	3.3	3.4	3.8	2.9	2.9
r.m.s. deviations, bonds (Å)	0.017	0.016	0.017	0.018	0.012	0.012

$$^a R_{\text{sym}} = \frac{\sum |I - \langle I \rangle|}{\sum I}$$

$$^b R_{\text{factor}} = \frac{\sum ||F_o| - k|F_c||}{\sum |F_o|}$$

TABLE II
Properties of P2 analogs of cyclic urea inhibitors

Cyclic urea	P2/P2'	K_i (nM)	E_{VDW}^a (kcal/mol)	E_{HBOND}^b (kcal/mol)
XK216		4.70	-28.7	-2.3
XK263		0.31	-47.0	-2.3
DMP323		0.33	-40.8	-3.8
DMP450		0.34	-38.3	-5.5
XV638		0.03	-50.5	-8.5
SD146		0.02	-54.8	-9.1

^a Interaction energy associated with vdw contacts between the inhibitor and the protein was calculated using X-PLOR.

^b Interaction energy associated with hydrogen bonds between the inhibitor and the protein (excluding the catalytic aspartates) was calculated using X-PLOR (27).

by diafiltration into 17.4 mM acetic acid, 5 mM dithiothreitol, and an inhibitor concentration equal to a 1000–5000-fold molar excess over its K_i value. Finally, the protein was concentrated to 5 mg/ml, using a YM10 membrane, and crystallized at 18 °C in hanging drops by vapor diffusion (12). Hexagonal rods (0.35 × 0.08 × 0.08 mm) grew within 7 days in 4-μl drops, which contained 1 mg/ml protease, 250 mM acetate buffer (pH 4.8–5.6) and 80–240 mM ammonium sulfate.

Diffraction data were collected at room temperature with a R-AXIS II imaging plate mounted on a Rigaku RU200 rotating anode generator operating at 50 kV/100 mA (CuK α radiation), equipped with a 0.3-mm cathode and a graphite crystal monochromator. Full data sets were obtained from a single crystal by collecting 30–50 oscillation images, at 2° intervals for 60 min. The unit cell parameters were determined from four still frames (15° intervals) using the RAXIS processing software. All protease-CU complexes crystallized in the space group P6 $_1$ with a dimer in the asymmetry unit and the following unit cell parameters: $a = b = 62.9$ Å and $c = 83.5$ Å.

Structure Refinement—The protein model from the HIV PR-A74704 complex (12) (Protein Data Bank file 9HVP) was used as the

starting model to refine the first CU complex, HIV PR-XK216, which was then used as the protein model in all subsequent refinements. The structures were refined by performing several cycles of simulated annealing followed by positional and restrained B-factor refinements (26). The conformations of the protein and inhibitors were adjusted using simulated annealing omit maps. The data collection and refinement statistics are tabulated in Table I. The refined coordinates for HIV-1 protease in complex with XK216, XK263, DMP323, DMP450, XV638, and SD146 have been deposited in the Protein Data Bank under the file names 1HWR, 1HVR, 1QBS, 1DMP, 1QBR, and 1QBT, respectively.

Binding Energies—The energetic contributions of the vdw interactions and hydrogen bonds to the binding energies of CU inhibitors were calculated using the X-PLOR program: the vdw energy was approximated by the Lennard-Jones potential energy function and the hydrogen bond energy was calculated based on the donor-acceptor distance and the donor-hydrogen-acceptor angle (27). Although the two energy terms, by themselves, do not provide an estimate of the total binding energy, we believe they can help us gain some insights into the relative binding energies of the inhibitors. Because the inhibitors presented in this report have very similar structures (only differing by their P2 substituents) and bound conformations, variations in their P2 vdw interactions and hydrogen bonds to the protease must be in part responsible for the differences in their K_i values. In addition, we believe these two terms can be used to help identify which moieties of our inhibitors interact favorably with the protease.

RESULTS

To study the interactions between CU inhibitors and HIV-1 PR, we have solved the crystal structures of the protease in complex with six inhibitors: XK216, XK263, DMP323, DMP450, XV638, and SD146. These CUs are symmetric molecules that possess a common central structural unit: a seven-membered heterocyclic ring, a urea moiety, and diols; and their P1(P1') and P2(P2') substituents are attached to C3(C6) (atoms adjacent to the diols) and the urea nitrogen atoms of the ring, respectively (Table II).

CU Conformations: Unbound Versus Bound—Several design features of CUs were confirmed by determining the small molecule crystal structures of XK263 and DMP323. For example, the seven-membered ring adopts a twisted chair conformation, the urea group is planar, and the configurations of the chiral ring atoms C3, C4, C5, and C6 are *R*, *S*, *S*, and *R*, respectively. Furthermore, the C_2 symmetry of these inhibitors was confirmed by the presence of a noncrystallographic 2-fold axis that passes through the urea carbonyl bond and bisects the bond between the hydroxyl-bearing carbon atoms, C4 and C5. The only deviations from C_2 symmetry were caused by the different crystal packing environments around P2 and P2', e.g. the torsion angles of the P2 and P2' groups of XK263 differ by 25°, and those of the P2 and P2' hydroxyls of DMP323 differ by 145° (Fig. 1).

FIG. 1. Induced fit of cyclic urea inhibitors. Stereo drawings of the bound (dark blue) and unbound (red) states of XK263 (top) and DMP323 (bottom). The superpositions of the inhibitors were performed by minimizing the r.m.s. deviations between the scaffolds in the two states; note the different P2 conformations. The protease is drawn with light blue bonds, and dashed lines correspond to hydrogen bonds between the P2/P2' (OH) and amides of Asp²⁹(30)(29')(30'). The crystal structures of XK263 and DMP323 were determined by J. Calabrese (unpublished data).

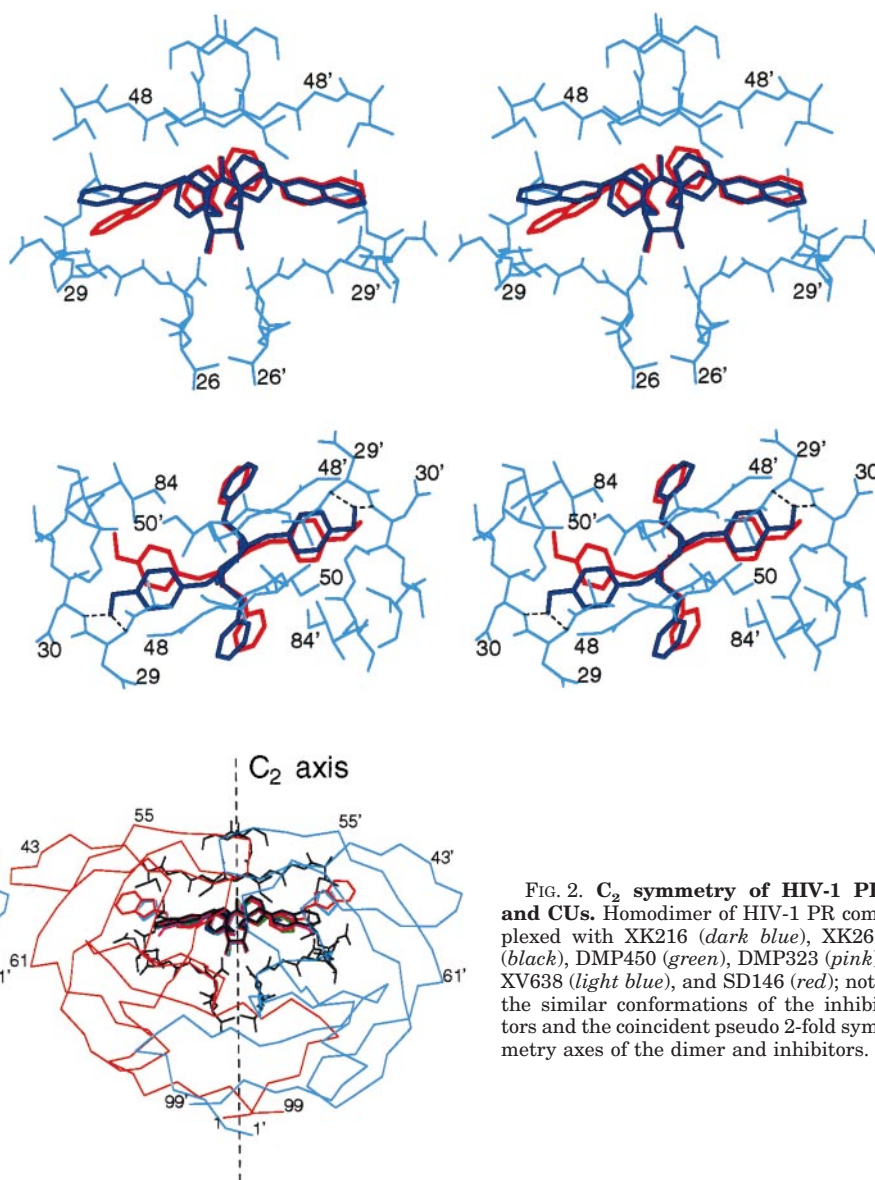
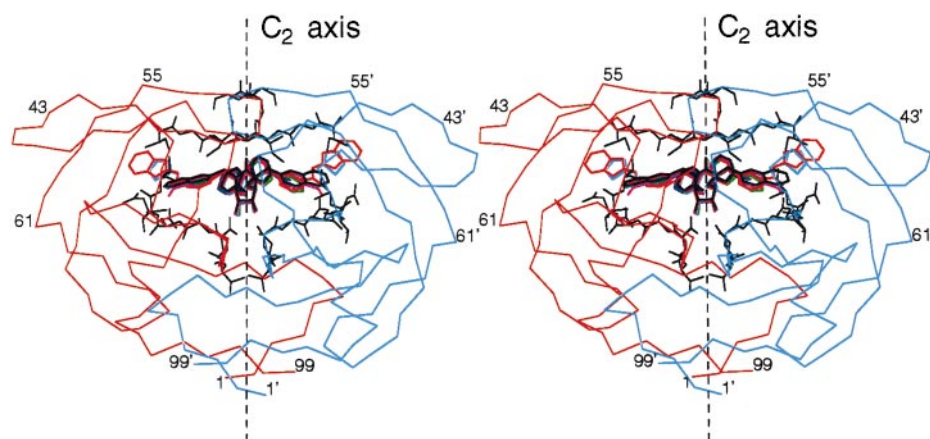


FIG. 2. C₂ symmetry of HIV-1 PR and CUs. Homodimer of HIV-1 PR complexed with XK216 (dark blue), XK263 (black), DMP450 (green), DMP323 (pink), XV638 (light blue), and SD146 (red); note the similar conformations of the inhibitors and the coincident pseudo 2-fold symmetry axes of the dimer and inhibitors.



When XK263 and DMP323 are complexed to the protease, the conformations of their scaffolds and P1, P1', and P2' groups are very similar to their uncomplexed states; only P2 reorganizes to alleviate steric strain, *e.g.* the P2 naphthyl of XK263 rotates 20° toward the flap to avoid steric interactions with Ala²⁸, Asp²⁹, and Asp³⁰; and the hydroxymethylbenzyl and P2-hydroxyl of DMP323 rotate 20 and 85°, respectively, away from Ile⁸⁴ (Fig. 1). Overall, the similarities between the bound and unbound structures of the CUs indicate that the inhibitors are preorganized for binding. The structures also confirm that the size and C₂ symmetry of the scaffold and the stereochemical arrangement of the chiral ring atoms are responsible for placing the urea oxygen near the flaps of the protease, the diols between the catalytic aspartates, and correctly projecting the substituents into the subsites. Finally, the 2-fold axis of each inhibitor coincides with that of the homodimer (Fig. 2), which means that CUs interact with symmetry related residues, *e.g.* the P2 and P2' hydroxyls of DMP323 hydrogen bond to the amides of Asp²⁹ and Asp³⁰ and their symmetry-related residues (Fig. 1 and Table III).¹

CU-induced Changes in HIV-1 PR—All CUs induce very

similar conformational changes in the protease; the r.m.s. (root mean square) deviations between the C_α atoms of any two complexes is less than 0.40 Å. The most notable change induced by CU binding is the well documented 7-Å shift in the position of the flap, which closes over the active site in the complexes (28). Although cyclic ureas induce flap closure by directly interacting with the backbone atoms of the flaps, peptidomimetics induce closure with the aid of a bridging water molecule (Fig. 3). A superposition of CU and linear inhibitor complexes confirms that the urea oxygen displaces the structural water, as the distances between O1 of XK216 and the structural water molecules in HIV PR-P9941,² PR-A77003, PR-MK639, PR-VX478, and PR-A74704 (Protein Data Bank files 1HSG, 1HVI, 1HPV, and 9HVP), are only 0.16, 0.34, 0.54, 0.56, and 0.64 Å, respectively. Other conformational changes are more subtle and unique for each inhibitor. For example, Asp²⁹, Asp³⁰, and the C-terminal helix move out of the active site by ~0.5 Å to accommodate the P2-hydroxyl of DMP323 (29), and Gly⁴⁸ of the flap moves deeper into the active site by ~0.5 Å when complexed with SD146 and XV638 (Fig. 4). Although these structural shifts are relatively small compared with flap closure, they appear to have a significant impact on the binding affinity of the

² P. J. Ala and C.-H. Chang, unpublished data.

TABLE III
Hydrogen bonds between HIV-1 PR and cyclic urea inhibitors

Hydrogen bonds were identified using the following cutoff criteria: 2.5–3.6 Å for the donor-acceptor distance and a maximum angular deviation of 60° from donor-hydrogen-acceptor angle of 180°.

H-bonding groups		Distance between atoms					
HIV-1 PR	CU	XK216	XK263	DMP323	DMP450	XV638	SD146
Å							
I50/50'-N	O1	3.3/3.0	3.4/3.4	3.3/3.2	3.3/3.4	3.4/3.0	3.0/3.2
D25/25'-OD	O4	3.0/3.0	2.8/2.8	2.9/3.1	2.9/3.1	2.8/3.0	2.9/2.8
D25/25'-OD	O5	3.1/3.2	2.9/3.2	3.1/3.1	2.7/3.2	2.8/3.0	2.9/3.1
D29/29'-N	O26/O26'			3.2/3.2			
D30/30'-N	O26/O26'			2.9/2.9			
D29/29'-N	N-Aniline				3.5/3.6		
D30/30'-OD	N-Aniline				2.9/2.8		
D30/30'-N	O25/O25'					3.2/3.1	3.1/3.0
G48/48'-O	N25/N25'					3.1/3.1	3.2/3.1
G48/48'-N	N28/N28'					3.0/3.1	3.1/3.2
D30/30'-OD	N27/N27'						2.9/2.9
Total no. of H bonds		6	6	10	10	12	14

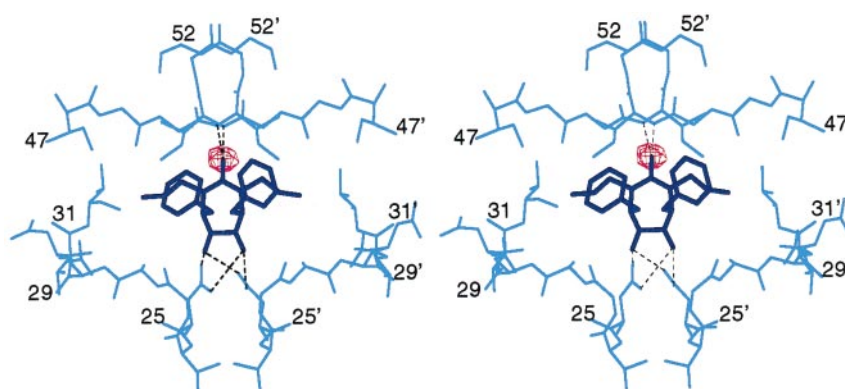


FIG. 3. **Displacing the structural water molecule.** Overlay of HIV-1 protease (light blue) complexed with XK216 (dark blue) and the electron density (pink) corresponding to the structural water molecule found in the linear inhibitor complex HIV PR-P9941. Note the urea oxygen (O1) of XK216 displaces the structural water molecule and hydrogen bonds directly to the flaps. Electron density ($F_o - F_c$) was contoured at 1.5 σ ; dashed lines correspond to hydrogen bonds between the urea oxygen and the amides of Ile⁵⁰(50') and between the diols and carboxylates of Asp²⁵(25').

FIG. 4. **CU-induced changes in the protease.** Overlay of the C α atoms of HIV PR-DMP323 (red) and HIV PR-SD146 (blue). Note the contraction of the active site at Asp³⁰ and Gly⁴⁸ by ~ 0.5 Å when complexed with SD146 (green); dashed lines indicate hydrogen bonds to Asp³⁰(30') and Gly⁴⁸(48'). DMP323 was omitted for clarity.

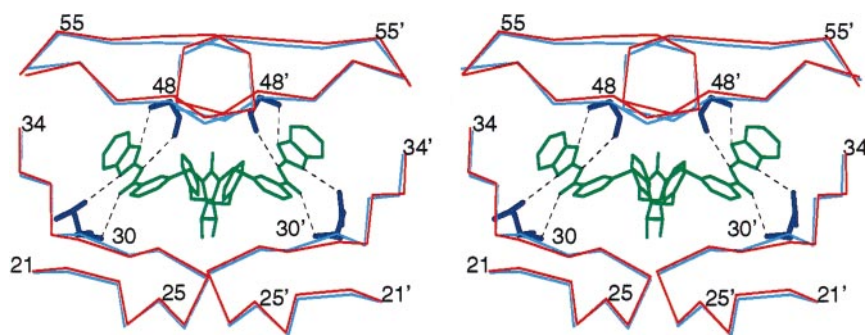


FIG. 5. **CU specificity.** Stereoview of pepsin (light blue) complexed with pepstatin (dark blue) (Ref. 34; Protein Data Bank file 1PSO). DMP323 (red) was positioned in the active site by optimizing the interactions between its diols and the catalytic aspartates; note the steric strain between the urea moiety and the flap: the O1 of CU is only ~ 1 Å away from Gly⁷⁶. Hydrogen bonds are indicated as dashed lines.

protease. For example, the shift at Asp³⁰ reduces steric contacts to DMP323 and permits the formation of a hydrogen bond between Asp³⁰ (NH) and O26 of the inhibitor, and the

shift at Gly⁴⁸ strengthens the hydrogen bonds between Gly⁴⁸ (CO and NH) and SD146 (NH₂5 and N28), respectively, by reducing the donor-acceptor distance by ~ 0.5 Å.

Finally, CU binding reduces the crystallographic 2-fold symmetry of the uncomplexed enzyme to pseudo symmetry; the r.m.s. deviations for the backbone atoms of the symmetry-related monomers in the XK216, DMP450, XV638, XK263, SD146, and DMP323 complexes are 0.45, 0.36, 0.30, 0.28, 0.26, and 0.23 Å, respectively, and only 0.16 Å for the core (excluding the surface loops) C_α atoms of HIV PR-DMP323. Unfortunately, the induced asymmetry cannot be directly linked to inhibitor binding, because the observed structural deviations from C₂ symmetry are located in regions involved in crystal packing, *i.e.* the flap, C and N termini, and surface loops.

Interactions between CUs and HIV-1 PR—The interactions between CUs and the protease can be divided into two groups: those that anchor the scaffold in the active site and those that fix the substituents in their target subsites. The most important interactions in the first group are 1) two hydrogen bonds between the urea oxygen and the amides of Ile⁵⁰ and Ile^{50'}, which contribute a total of 2.3–3.2 kcal/mol to binding, and 2) four hydrogen bonds between the diols and the carboxylates of the catalytic aspartates (Fig. 3). Although we did not attempt to estimate the energetic contributions of the later interactions because the catalytic aspartates are protonated and probably form several nearly isoenergetic hydrogen bonding networks with the diols (30), we believe the existence of alternate networks should provide even greater stability. The scaffold is therefore firmly held in place by ~six hydrogen bonds, as evidenced by the lack of lateral movement in the active site when CUs with different P2 substituents are superimposed (Fig. 2).

The most important P2-S2 interactions are vdw contacts and hydrogen bonds. XK216 is the least potent inhibitor ($K_i = 4.7$ nM) in this series and its P2(P2') allyls participate in only 10 vdw interactions. The fact that XK216 possesses the lowest number of P2-S2 vdw contacts and no P2 hydrogen bonds is consistent with its K_i rank-order in this series (Tables II and III). By building larger P2 groups such as hydroxymethylbenzyl, aniline, and naphthyl, the K_i values decreased by 15-fold. The higher relative affinities for DMP323 and DMP450 are consistent with the formation of 35 new vdw contacts, which increase E_{vdw} (energy associated with vdw interactions) by ~10 kcal/mol, and to a lesser extent, four additional hydrogen bonds, the strongest of which are between the backbone amides of Asp³⁰(Asp^{30'}) and O26(O26') of DMP323 and between the carboxylates of Asp³⁰(Asp^{30'}) and *N*-anilines of DMP450, contributing a total of ~0.5 and 2.0 kcal/mol, respectively. The 15-fold decrease for XK263, however, appears to be in part due to a greater increase in vdw interactions (Table II).

The K_i values for SD146 and XV638 are 0.03 and 0.02 nM, respectively, which correspond to approximately a 200-fold decrease compared with XK216 and a 10-fold reduction relative to DMP323 and DMP450. The greater potency of these CUs was achieved by further increasing the number of P2 hydrogen bonds and vdw interactions (Tables II and III). Not only do the latter inhibitors have the highest E_{vdw} (50 and 55 kcal/mol, respectively), they participate in 12 and 14 hydrogen bonds, respectively. In both inhibitor complexes, the strongest hydrogen bonds are between Gly⁴⁸(48') (CO) and NH²⁵(25'), contributing a total of 3.6 kcal/mol to binding. The number of interactions is so extensive that all of the inhibitors' electronegative atoms (except for the sulfur atom in XV638) are hydrogen-bonded, and the only accessible protein atoms that are not hydrogen-bonded to the inhibitors are Gly²⁷ (CO), Asp²⁹ (OD and NH), Asp³⁰ (CO), and the guanidino group of Arg⁸, but these functional groups interact with other protein residues, *e.g.* Asp^{29'} forms a salt bridge with Arg⁸. Therefore, XV638 and

SD146 are the most potent inhibitors in this series due in part to the following factors: 1) a large buried surface area, in which no unpaired polar/charged atoms (protein or inhibitor) are trapped in a hydrophobic environment, 2) strong P2 hydrogen bonds, and 3) extensive vdw interactions.

DISCUSSION

CUs are nanomolar competitive inhibitors of HIV-1 PR (Table II). Their high potency can be attributed primarily to the design of their scaffold and the high degree of complementary surfaces between their P1 and P2 substituents and the corresponding S1 and S2 subsites. The CU scaffold evolved from a methoxyphenyl ring in which the methoxy group was first replaced with a ketone and then with a urea moiety to improve the hydrogen bonding character of the carbonyl oxygen, and the ring was enlarged to seven members to position diols between the catalytic aspartates (14). These modifications created two hydrogen bonds to the flaps and four to the catalytic aspartates, interactions needed to anchor the ring in the active site (Fig. 3 and Table III). A cyclic scaffold was also chosen in an attempt to reduce the entropy penalty associated with complex formation of flexible ligands. Fortunately, the CU scaffold exists predominantly in a single pseudo chair conformation, whether it is bound to the protease or not, because of steric strain between its P1 and P2 substituents (19). An important consequence of the ring's lack of flexibility is the requirement for *RR*, derived from *D*-phenylalanines, and *SS* configurations of the chiral ring atoms C3 C6 and C4 C5, respectively, to optimally project the substituents into the subsites.

The P1 and P2 substituents of CUs interact extensively with the protease through a combination of vdw contacts and hydrogen bonds. While the S1 subsite clearly prefers hydrophobic substituents, the specificity of S2 is much broader, accommodating the naphthyl group of XK263 and the electronegative atoms of XV638 and SD146. Because the scaffolds and P1(P1') benzyls participate in a similar number of interactions (~30 vdw contacts) in every complex, the observed K_i values for the different CUs are in part modulated by the extent of P2 interactions (Table II). The K_i values for DMP323 (DMP450) and SD146 (XV638), for example, are 15- and 200-fold lower than that for XK216, which is consistent with the fact that an allyl group is too small to fill the S2 subsite (E_{vdw} is only -29 kcal/mol). The most potent inhibitor, SD146 ($K_i = 0.02$ nM), participates in ~200 vdw interactions and 14 hydrogen bonds, which are evenly distributed around the binding pocket: six to the flaps, four to the catalytic aspartates, and four more to the base of the pocket. Hydrogen bonds, however, are not always required for high potency. For example, XK263, which does not hydrogen bond to the S2 subsite, is just as potent as DMP323 and DMP450 because it has a larger E_{dvw} (-47 kcal/mol) (Table II).

Another important feature of CUs is the displacement of the intervening water molecule by the urea oxygen atom. In the XK263 complex, the urea oxygen (O1) clearly occupies the same site as the structural water found in the linear inhibitor complex HIV PR-P9941 (Fig. 3). An independent NMR study also concludes that DMP323 displaces the long-lived water molecule (31). Displacing a conformationally restrained water increases the entropy of the system, and direct interaction with the flaps may help to stabilize the closed state of the protease. Many other cyclic compounds now displace the structural water (15–18).

CUs Versus Linear Inhibitors—The four FDA-approved inhibitors, saquinavir, ritonavir, indinavir, and nelfinavir, are linear, asymmetric compounds with mono-ol functionality (32–34), whereas CUs are cyclic, C₂ symmetric diols. Because both classes of compounds are potent inhibitors of the wild-type

protease, it is not evident which features are more desirable. Further studies are needed to quantitate the gain in binding energy associated with the rigid CU scaffold; the use of diols, which form a network of hydrogen bonds with the catalytic aspartates, and C_2 symmetry, which allows CUs to interact symmetrically with both monomers. The benefit of displacing the structural water molecule, however, is more evident, because human proteases do not use an intervening water to bind substrates or inhibitors, *i.e.* their flaps interact directly with ligands. This different binding requirement allows CUs to easily discriminate between human and viral proteases. For example, the large CU scaffold can only fit in pepsin's active site if the flap opens by ~ 2.0 Å, such a displacement would enlarge the binding pocket and reduce the overall number of interactions to the inhibitor (Fig. 5). This is consistent with the fact that a concentration of DMP323 that inhibits HIV-1 PR by 50% only inhibits pepsin by 1% (23). The templates of linear inhibitors, on the other hand, are more flexible and can probably more easily reorganize to fit into the active sites of several different proteases. Therefore, in practice, most linear inhibitors discriminate against human proteases based only on their P1-S1 and P2-S2 interactions, whereas CUs discriminate with their substituents as well as their scaffolds. The rigid CU scaffold therefore not only reduces the entropy penalty associated with complex formation but also plays an important role in discriminating against human proteases.

Implications for Drug Design—The design of effective anti-retroviral agents requires a detailed understanding of the target, for inhibitors that do not interact directly with residues involved in substrate binding or catalytic activity will likely select for drug-resistant variants. The CU scaffold addresses this issue by displacing the structural water molecule and interacting with the catalytic aspartates. These are important design elements, for mutants that displace the structural water with a large side chain will not bind substrates as efficiently, and those that replace the catalytic aspartates with any other residue will be inactive (35). The CU scaffold is therefore well suited to inhibit mutant proteases.

To investigate the structure-activity relationships of the P2 substituents, we built various P2-substituted inhibitors (Table II). In general, the small allyl group of XK216 simply does not interact sufficiently with the subsite to achieve a subnanomolar K_i value. A significant improvement was only observed when we increased the size and the hydrogen bonding character of the substituent, *e.g.* XK263, DMP323, and DMP450. Further attempts at increasing E_{HBOND} (energy associated with hydrogen bonds) generated XV638, one of the first CUs to hydrogen bond to Gly⁴⁸ of the flap. The thiazole ring of XV638 was later replaced with a benzimidazole to pick up an extra hydrogen bond to Asp³⁰ (OD) (Table III). Apparently, the S2 subsite can accommodate a large variety of substituents, which rely in part on vdw interactions and hydrogen bonds for tight binding. Unfortunately, our most potent inhibitor (20 pM), SD146, is not a development candidate because it is too lipophilic. Based on our structures, however, we might be able to improve the solubility of SD146 by replacing the benzyl moiety of the benzimidazole with a hydrophilic group or simply removing it altogether; either modification should not significantly affect its potency because the benzyl is partially exposed to the solvent and thus does not interact extensively with the protease. Finally, further attempts to increase CU potency might include building off the P1 benzyl to hydrogen bond with the carbonyl oxygen of Gly²⁷ and the guanidino group of Arg⁸¹, and off P2, to interact with Asp²⁹ (NH and COO).

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