Chemistry & Biology, Vol. 11, 1333–1338, October, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.08.010

Combating Susceptibility to Drug Resistance: Lessons from HIV-1 Protease

Brief Communication

Nancy M. King,[1] Moses Prabu-Jeyabalan,[1] Ellen A. Nalivaika, and Celia A. Schiffer*
Department of Biochemistry and Molecular Pharmacology
University of Massachusetts Medical School
364 Plantation Street
Worcester, Massachusetts 01605

Summary

Drug resistance is a major obstacle in modern medicine. However, resistance is rarely considered in drug development and may inadvertently be facilitated, as many designed inhibitors contact residues that can mutate to confer resistance, without significantly impairing function. Contemporary drug design often ignores the detailed atomic basis for function and primarily focuses on disrupting the target's activity, which is necessary but not sufficient for developing a robust drug. In this study, we examine the impact of drug-resistant mutations in HIV-1 protease on substrate recognition and demonstrate that most primary active site mutations do not extensively contact substrates, but are critical to inhibitor binding. We propose a general, structure-based strategy to reduce the probability of drug resistance by designing inhibitors that interact only with those residues that are essential for function.

Introduction

Drug resistance occurs when mutations in the target protein allow the protein to retain function while no longer being inhibited efficiently by the drug. In the case of HIV-1 protease, drug resistance occurs when, even in the presence of protease inhibitors, the enzyme is able to cleave the Gag and Pol polyproteins in at least nine different locations, allowing viral maturation. At first inspection, development of drug resistance for HIV-1 protease would appear to be particularly difficult, as all of the currently prescribed protease inhibitors are competitive inhibitors that bind in the center of the active site [1]. Nevertheless, many viable drug-resistant mutations occur within patients due to the high replicative rate of the virus [2], the infidelity of the reverse transcriptase [3-5], and the selective pressure of protease inhibitor therapy on the evolution of the virus. The accumulation of multiple drug-resistance mutations within HIV-1 protease then renders current therapies ineffective.

To understand how drug resistance can occur while retaining substrate recognition, we have focused on substrate recognition by HIV-1 protease. The nine substrate sequences cleaved by the protease within the viral polyproteins differ significantly, making the determinants of substrate specificity difficult to derive from sequence alignment alone. By analyzing the crystal structures of six substrates [6, 7] in complex with an inactive (D25N) HIV-1 protease variant (Figure 1A), we have developed a "substrate envelope" hypothesis: that substrate specificity for HIV-1 protease is based not on a particular amino acid sequence, but on a conserved shape. This shape, or envelope, is defined by the overlapping volume occupied by the substrates within the active site of HIV-1 protease (Figure 1B). HIV-1 protease likely recognizes a particular sequence as a substrate in part due to the ability of that sequence to adopt this shape by a combination of packing of the substrate's side chains and rearrangements of the substrate's backbone.

All currently prescribed HIV-1 protease inhibitors are competitive active site inhibitors. Thus, for drug resistance to occur, it would seem necessary for the sequences of the substrate cleavage sites to dramatically coevolve with the protease to attain drug resistance while maintaining a replicating and infectious virus. This coevolution would be expected, as the competitive active site inhibitors would likely interact with the same residues that are necessary to recognize and cleave substrates. However, this type of coevolution occurs only within the occasional cleavage site [8-11]; coevolution of the substrates with HIV-1 protease is the exception rather than the rule. The most frequent and well-characterized coevolution of a substrate with a drug resistant mutation in the protease occurs when the P2 residues of the NC-p1 cleavage site mutates from an alanine to a valine in response to the V82A mutation. Although other cases of coevolution between the substrates and protease exist, they appear to be relatively rare; no comprehensive study has been performed on large numbers of viral sequences. Generally, HIV-1 protease manages to evolve drug resistance to active site inhibitors without strongly compromising substrate recognition.

Results and Discussion

The currently prescribed HIV-1 protease inhibitors are all chemically different [12, 13], relatively low molecular weight compounds and can elicit different, yet overlapping, patterns of drug-resistant mutations [14-16]. However, if we superimpose the structures of eight protease-inhibitor complexes, the volumes occupied by the inhibitors overlap, significantly allowing the definition of an "inhibitor envelope" (Figure 1C). The inhibitors are much smaller than the substrates to maintain bioavailability and are, on average, a different shape than the substrates. Similar functional groups within the inhibitors are often positioned at similar locations in the protease active site. This overlap means that many of the inhibitors contact the protease at the same residues (Figure 1D). Overlaying the inhibitor envelope on the substrate envelope [7] (Figure 1E) results in several loca-

*Correspondence: celia.schiffer@umassmed.edu
[1]These authors contributed equally to this work.

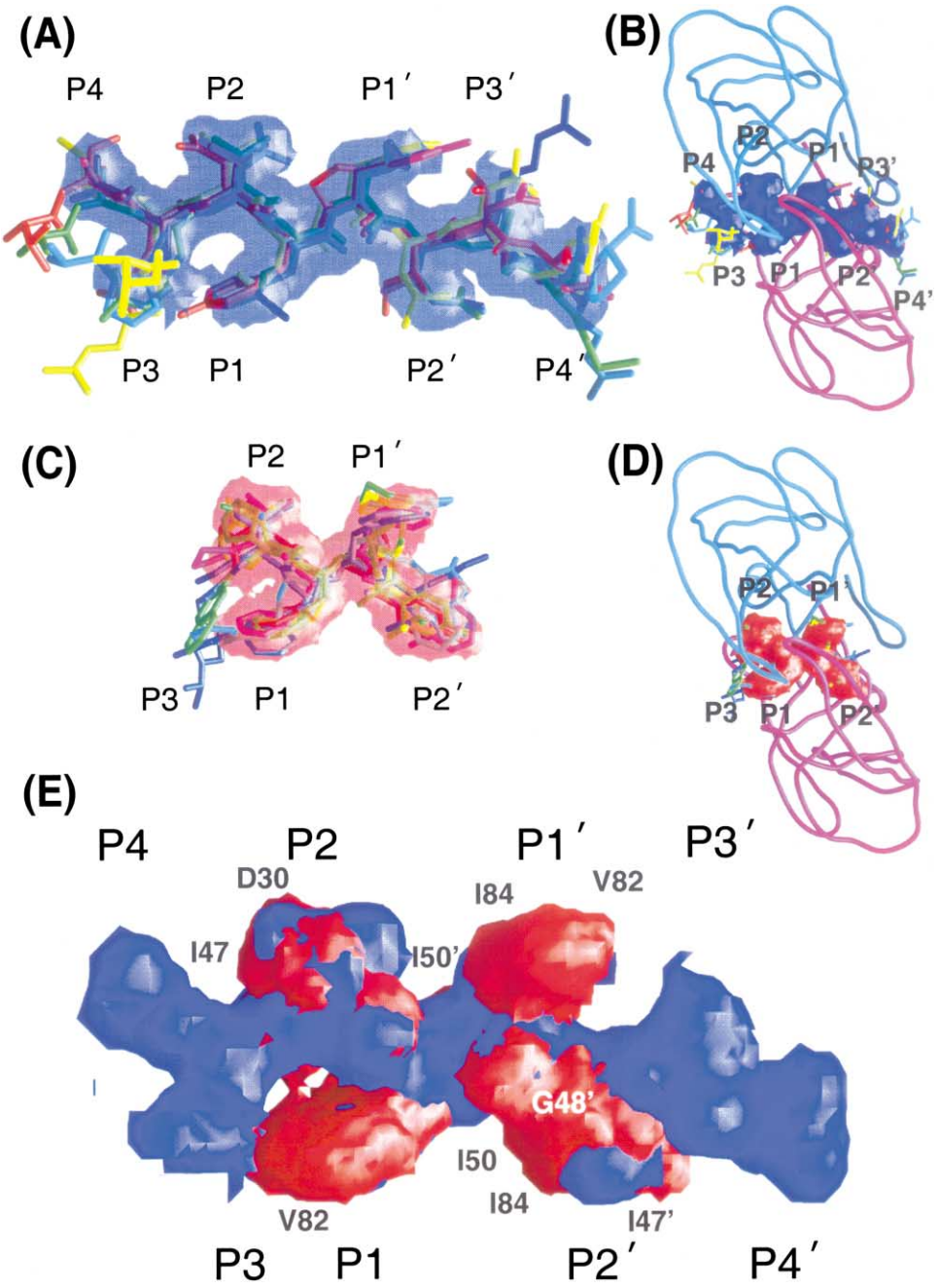

Figure 1. Substrate and Inhibitor Envelopes of HIV-1 Protease

(A) The substrate envelope calculated with GRASP [26] from the overlapping van der Waals volume of four or more substrate peptides. The colors of the substrate peptides are red, matrix-capsid; green, capsid-p2; blue, p2-nucleocapsid; cyan, p1-p6; magenta, reverse-transcriptase-ribonucleaseH; and yellow, ribonucleaseH-integrase.

(B) The substrate envelope as it fits within the active site of HIV-1 protease. The α-carbon trace is of the CA-p2 substrate peptide complex [6].

(C) The inhibitor envelope calculated from overlapping van der Waals volume of five or more of eight inhibitor complexes. The colors of the inhibitors are yellow, Nelfinavir (NFV); gray, Saquinavir (SQV); cyan, Indinavir (IDV); light blue, Ritonavir (RTV); green, Amprenavir (APV); magenta, Lopinavir (LPV); blue, Atazanavir (ATV); and red, (TMC114).

(D) The inhibitor envelope as it fits within the active site of HIV-1 protease.

(E) Superposition of the substrate envelope (blue) with the inhibitor envelope (red). Residues that contact the inhibitors where the inhibitors protrude beyond the substrate envelope and confer drug resistance when they mutate are labeled.

tions, specifically between the P3 and P2′ subsites, where the inhibitor envelope protrudes beyond the substrate envelope. These locations contact specific residues in HIV-1 protease.

We observe that those specific HIV-1 protease residues, which are contacted by the inhibitors where the inhibitors protrude from the substrate envelope, correspond to the residues where most multi-drug-resistant

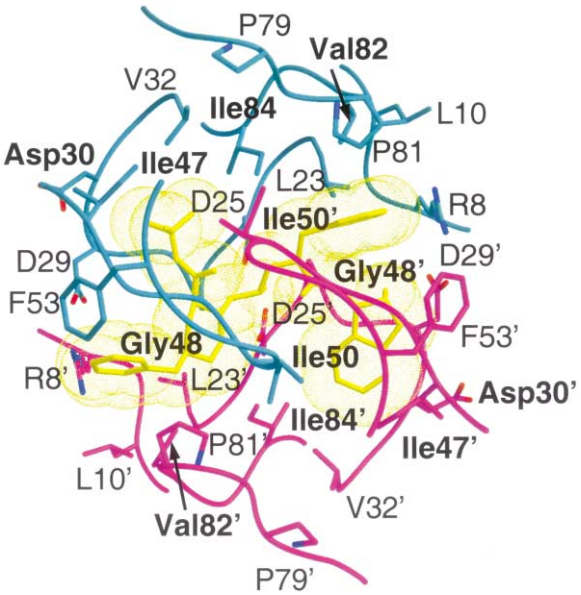

Figure 2. Active Site Region of HIV Protease

Residues that confer at least low-level resistance to three or more inhibitors are highlighted in large bold text. Indinavir [21] is shown in yellow as an example of the interactions of these residues with an inhibitor. The orientation is in a similar orientation as seen in Figure1B. The figure was made with the graphics program MIDAS [25].

mutations occur (Figure 1E). The mutation L90M is the only mutation that confers high levels of drug resistance yet does not make direct contact with the inhibitors, as it is located outside of the active site. Therefore, this mutation must confer resistance through another, or indirect, mechanism. Figure 2 highlights those contacted residues that confer at least low-level drug resistance to three or more inhibitors. For instance, mutation of I84, which is located in the center of the HIV-1 protease active site, to a valine is the worst of the multi-drug-resistant mutations (Table 1), strongly impacting the binding of all of the current protease inhibitors. However, the degree of protrusion of inhibitors from the substrate envelope to contact residue 84 does not appear to account for the site's ability to confer multi-drug resistance. Rather, residue 84's central location likely accounts for its high degree of cross-resistance, since

Table 1. Drug Resistance Conferring Residues which Contact Inhibitors at Positions outside the Substrate Envelope

Inhibitor	High-Level Resistance[1]	Intermediate-Level Resistance[1]	Low-Level Resistance[1]
NFV	D30, I84	G48, V82	
SQV	G48, I84	V82	
RTV	V82, I84	I50	G48
IDV	V82, I84	I47	G48
APV	I50, I84	V82	
LPV	V82	I47, I50, I84	G48
ATV	I50, I84	V82	G48

[1] Level of resistance as defined by the Stanford database [14, 15]. TMC114, which is still in clinical trials, does not yet have a pattern of resistance.

when it mutates to a valine there is a decrease in van der Waals contacts in each of the inhibitor complexes. Nevertheless, the fact that most of the residues where primary drug-resistant mutations occur are at sites where the inhibitors protrude beyond the substrate envelope is not likely fortuitous. These residues are prime sites for the evolution of drug-resistant mutations, as they are at positions that will preferentially impact inhibitor binding over substrate recognition. We previously observed this mechanism of drug resistance by solving and analyzing a series of substrate and inhibitor crystal structures in complex with an HIV-1 protease variant with the multi-drug resistant V82A mutation [17]. The analysis presented here supports the assessment that this mechanism for conferring resistance is a general principle.

The inhibitor atoms that do not overlap with the substrates are highlighted in Figure 3. Each inhibitor has several atoms that are more than 1.4 Å from any substrate atoms (Figure 3A). These atoms are color coded by their average distance from any of the six substrates (Figure 3B), and the protease residues with which they make contact are listed. Atoms within each inhibitor that are more than 2.0 Å from any substrate necessarily protrude from the envelope. The protease residues surrounding the inhibitors can be grouped into two major categories: residues that mutate and confer drug resistance and residues that very rarely mutate. Those that confer resistance are listed in Table 1. At residues G48, I50, V82, and I84 there is clearly a high degree of overlap, although the exact resistance profile varies depending on the particular inhibitor. The remaining residues surrounding the inhibitors rarely mutate and are likely crucial to the protease's structure or ability to recognize and cleave substrates. These residues, with the very small number of HIV-infected patient isolates showing mutations at these sites in parentheses, are R8 (27), G27 (2), A28 (6), D29 (11), G49 (7), T80 (4), and P81 (1) from over 6300 isolates in the Stanford database [14, 15]. Thus, resistance appears to have evolved at those residues where HIV protease can best tolerate change while retaining the protease's function to cleave substrates.

Although the protease residues that mutate and confer drug resistance primarily contact inhibitor atoms, these residues also contact a few substrate atoms. However, for the three drug-resistant mutations [14, 15] I50V, V82A and I84V, where the size of the residue decreases once it mutates, the inhibitors that are compromised due to these mutations lose on average two more van der Waals contacts than do the substrates. Usually, the loss of contact with the substrate is negligible and does not substantially alter its binding, since it represents a relatively small percentage of the total surface area buried on the protease by the substrate. However, in particular cases, a protease mutation may cause a particular substrate to coevolve to preserve substrate recognition. Such a mutation occurs at the rate-determining step in the processing of Gag, the nucleocapsid-p1 cleavage site [8-11]. We have recently discovered [18] that the structural basis for this coevolution occurs when a key contact at the P1′ Phe, which protrudes beyond the substrate envelope, is lost in the HIV protease complex due to the protease mutation V82A. The substrate coevolves when the unusually small alanine at P2 mutates

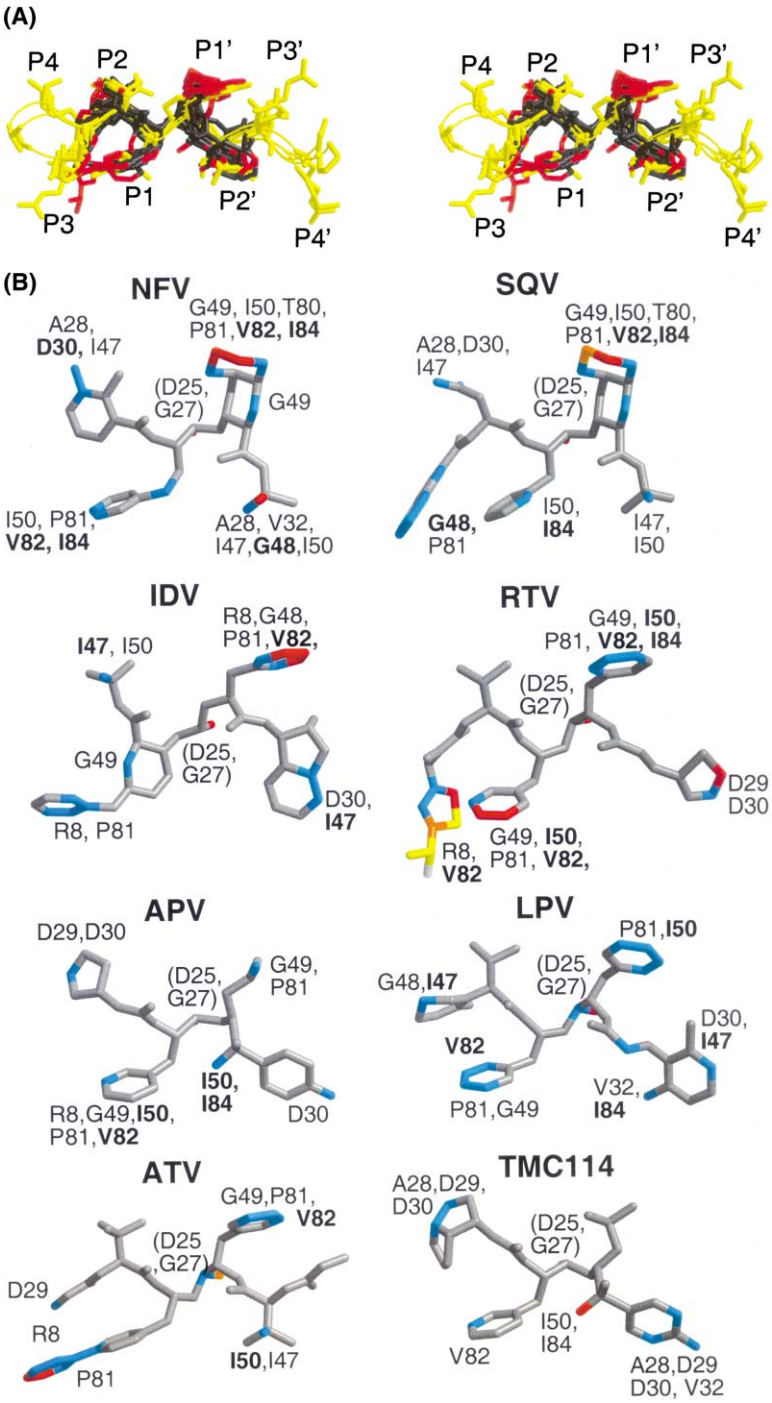

Figure 3. Inhibitor Atoms which Protrude beyond the Substrates

(A) Stereo superposition of six substrates (yellow) and eight inhibitors (black and red). The atoms within the inhibitors that are more than 1.4 Å from substrate atoms are colored in red.

(B) Eight inhibitors (NFV, SQV, IDV, RTV, APV, LPV, ATV, and TMC114) are shown with their atoms colored by their average distance from substrate atoms. The gray (0–1.4 Å), cyan (1.4–2.0 Å), red (2.0–2.5 Å), orange (2.5–3.0 Å), yellow (3.0–3.5 Å), and white (3.5–4.0 Å) show those atoms that are, on average, the furthest from any substrate atom when the inhibitor complexes are superimposed on the substrate complexes of HIV-1 protease. Listed near these atoms are the protease residues within van der Waals contact, and those that confer resistance to a particular inhibitor are in bold (except for TMC114, for which the resistance profile is not yet identified). The figure was made with the graphics program MIDAS [25].

to a more typically branched residue at this site, valine, thereby likely restabilizing the resulting substrate protease complex. Such coevolution can happen if one substrate is impacted by a drug-resistant mutation, but if six or more substrates are affected, it is unlikely that all six substrates could simultaneously coevolve and preserve viral function.

Significance

By exploring how a protein target such as HIV-1 protease functions in atomic detail, we have gained insights into one possible means for circumventing drug resistance. In analyzing the location of the residues of most of the active site drug-resistant mutations in HIV protease, we find that these mutations usually occur where the inhibitors protrude beyond the substrate envelope. Therefore, these residues are more important for inhibitor binding than for substrate recognition. Drug resistance thus occurs in a manner that retains substrate recognition and protease activity. This analysis implies that an inhibitor contained within the substrate envelope, interacting only with the same residues that are necessary to recognize substrate, may be less

susceptible to drug resistance. This should be practical as the picomolar inhibitor TMC114 fits reasonably well within the substrate envelope [19]. Therefore, developing inhibitors in this manner represents a new paradigm for drug design.

This relatively simple description of combating drug resistance can be applied beyond HIV to any molecular target with the potential to evolve drug resistance. Much of modern drug design, either by utilizing high throughput screening and/or with structure-based design, does not focus on the exact molecular interactions by which the target biological macromolecule functions, but rather focuses only on disrupting the target's activity. Disrupting the target's activity is necessary but not sufficient for developing a robust drug. By ignoring the detailed atomic basis for function, many of the inhibitors found by traditional drug design are likely to contact residues within the target protein that could mutate and confer resistance without significantly impairing function. Thus, traditional drug design may inadvertently facilitate the potential for drug resistance to arise. To reduce susceptibility to drug resistance in the design of new inhibitors, a detailed atomic understanding of a target biological macromolecule's molecular interactions with its functionally important partners is required.

Experimental Procedures

To calculate the substrate and inhibitor envelopes, the various crystal structures of HIV-1 protease complexes were superimposed on the capsid-p2 HIV protease (D25N) complex (1F7A). These included five other substrate complexes (1KJ4, 1KJ7, 1KJF, 1KJG, and 1KJH [6, 7, 18]), eight inhibitor complexes (1HPV [20], 1HSG [21], 1HXB, 1HXW [22], 1OHR [23], 1MUI [24]), ATV complex (H.E. Klei and R.J. Colonno, unpublished data), and the TMC114 complex [19]. A van der Waals contact was considered made if two atoms were within 4.2 Å of each other. For each complex, the relatively invariant terminal region, the α carbons of residues 1–9 and 86–99, were used for the superposition, which was performed within the graphics program MIDAS [25]. The inhibitor and substrate envelopes were then calculated within the graphics program GRASP [26]. The van der Waals surfaces of each of the inhibitors and substrates were calculated, and the envelope was defined as a region where four or more van der Waals surfaces intersected for either the substrates or the inhibitors to make their respective envelope.

Acknowledgments

TMC114 was provided by Tibotec, Mechelen, Belgium. The unpublished crystal structure of the ATV complex was provided for this analysis by Bristol Meyers Squibb (H.E. Klei and R.J. Colonno, personal communication). This research was supported by the National Institutes of Health (P01-GM66524-02 and R01-GM64347) and this laboratory receives some funding from Tibotec, Inc.

Received: June 21, 2004
Revised: July 30, 2004
Accepted: August 3, 2004
Published: October 18, 2004

References

1. Erickson, J.W. (1995). The not-so-great escape. Nat. Struct. Biol. *2*, 523–529.
2. Coffin, J.M. (1995). HIV population dynamics in vivo: implications for genetic variation, pathogenesis and therapy. Science *257*, 483–489.
3. Roberts, J.D., Bebenek, K., and Kunkel, T.A. (1988). The accuracy of reverse transcriptase from HIV-1. Science *242*, 1171–1173.
4. Roberts, J.D., Preston, B.D., Johnston, L.A., Soni, A., Loeb, L.A., and Kunkel, T.A. (1989). Fidelity of two retroviral reverse transcriptases during DNA-dependent DNA synthesis in vitro. Mol. Cell. Biol. *9*, 469–476.
5. Ji, J.P., and Loeb, L.A. (1992). Fidelity of HIV-1 reverse transcriptase copying RNA in vitro. Biochemistry *31*, 954–958.
6. Prabu-Jeyabalan, M., Nalivaika, E., and Schiffer, C.A. (2000). How does a symmetric dimer recognize an asymmetric substrate? A substrate complex of HIV-1 protease. J. Mol. Biol. *301*, 1207–1220.
7. Prabu-Jeyabalan, M., Nalivaika, E.A., and Schiffer, C.A. (2002). Substrate shape determines specificity of recognition for HIV-1 protease: Analysis of crystal structures of six substrate complexes. Structure *10*, 369–381.
8. Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L., and Lamarre, D. (1996). Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. J. Virol. *70*, 3763–3769.
9. Zhang, Y.M., Imamichi, H., Imamichi, T., Lane, H.C., Falloon, J., Vasudevachari, M.B., and Salzman, N.P. (1997). Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. J. Virol. *71*, 6662–6670.
10. Bally, F., Martinez, R., Peters, S., Sudre, P., and Telenti, A. (2000). Polymorphism of HIV type 1 Gag p7/p1 and p1/p6 cleavage sites: clinical significance and implications for resitance to protease inhibitors. AIDS Res. Hum. Retroviruses *16*, 1209–1213.
11. La Seta Catamancio, S., De Pasquale, M.P., Citterio, P., Kurtagic, S., Galli, M., and Rusconi, S. (2001). In vitro evolution of the human immunodeficiency virus type 1 gag-protease region and maintenance of reverse transcriptase resistance following prolonged drug exposure. J. Clin. Microbiol. *39*, 1124–1129.
12. Forstenlehner, M. (2000). AIDS: new FDA-approved agents. Pharm. Unserer Zeit *29*, 58.
13. Temesgen, Z. (2001). Current status of antiretroviral therapies. Expert Opin. Pharmacother. *2*, 1239–1246.
14. Shafer, R.W., Stevenson, D., and Chan, B. (1999). Human immunodeficiency virus reverse transcriptase and protease sequence database. Nucleic Acids Res. *27*, 348–352.
15. Shafer, R.W., Hsu, P., Patick, A.K., Craig, C., and Brendel, V. (1999). Identification of biased amino acid substitution patterns in human immunodeficiency virus type 1 isolates from patients treated with protease inhibitors. J. Virol. *73*, 6197–6202.
16. Wu, T.D., Schiffer, C.A., Gonzales, M.J., Taylor, J., Kantor, R., Chou, S., Israelski, D., Zolopa, A.R., Fessel, W.J., and Shafer, R.W. (2003). Mutation patterns and structural correlates in HIV-1 protease following varying degrees of protease inhibitor treatment. J. Virol. *77*, 4836–4847.
17. Prabu-Jeyabalan, M., Nalivaika, E.A., King, N.M., and Schiffer, C.A. (2003). Viability of a drug-resistant HIV-1 protease variant: structural insights for better anti-viral therapy. J. Virol. *77*, 1306–1315.
18. Prabu-Jeyabalan, M., King, N.M., Nalivaika, E., and Schiffer, C.A. (2004). Coevolution of the Nucleocapsid-p1 cleavage site with the V82A mutation in HIV-1 protease preserves substrate recognition. J. Virol., in press.
19. King, N.M., Prabu-Jeyabalan, M., Wigerinck, P., De Bethune, M.-P., and Schiffer, C.A. (2004). The structural and thermodynamic basis for the binding of a next generation HIV-1 protease inhibitor, TMC114. J. Virol., in press.
20. Kim, E.E., Baker, C.T., Dwyer, M.D., Murcko, M.A., Rao, B.G., Tung, R.D., and Navia, M.A. (1995). Crystal structure of HIV-1 protease in complex with VX-478, A potent and orally bioavailable inhibitor of the enzyme. J. Am. Chem. Soc. *117*, 1181.
21. Chen, Z., Li, Y., Chen, E., Hall, D.L., Darke, P.L., Culberson, C., Shafer, J.A., and Kuo, L.C. (1994). Crystal structure at 1.9 Angstrom resolution of human immunodeficiency virus (HIV) II protease complexed with L-735,524, and orally bioavailable inhibitor of the HIV proteases. J. Biol. Chem. *269*, 26344–26348.
22. Krohn, A., Redshaw, S., Ritchie, J.C., Graves, B.J., and Hatada, M.H. (1991). Novel binding mode of highly potent HIV-proteinase inhibitors incorporating the (R)-hydroxyethylamine isostere. J. Med. Chem. *34*, 3340–3342.

23. Kaldor, S., Kalish, V., Davies, J.N., Shetty, B., Fritz, J., Appelt, K., Burgess, J., Campanale, K., Chirgadze, N., Clawson, D., et al. (1997). Viracept (nelfinavir mesylate, AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease. J. Med. Chem. *40*, 3979–3985.
24. Stoll, V., Qin, W., Stewart, K.D., Jakob, C., Park, C., Walter, K., Simmer, R.L., Helfrich, R., Bussiere, D., Kao, J., et al. (2002). X-ray crystallographic structure of ABT-378 (Lopinavir) bound to HIV-1 protease. Bioorg. Med. Chem. *10*, 2803–2806.
25. Ferrin, T.E., Huang, C.C., Jarvis, L.E., and Langridge, R. (1988). The MIDAS display system. J. Mol. Graph. *6*, 13–27.
26. Nicholls, A., Sharp, K., and Honig, B. (1991). Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. Proteins *11*, 281–296.