

INHIBITORS OF HIV-1 PROTEASE: A Major Success of Structure-Assisted Drug Design¹

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KEY WORDS: AIDS, protease, drug design, inhibitors

ABSTRACT

Retroviral protease (PR) from the human immunodeficiency virus type 1 (HIV-1) was identified over a decade ago as a potential target for structure-based drug design. This effort was very successful. Four drugs are already approved, and others are undergoing clinical trials. The techniques utilized in this remarkable example of structure-assisted drug design included crystallography, NMR, computational studies, and advanced chemical synthesis. The development of these drugs is discussed in detail. Other approaches to designing HIV-1 PR inhibitors, based on the concepts of symmetry and on the replacement of a water molecule that had been found tetrahedrally coordinated between the enzyme and the inhibitors, are also discussed. The emergence of drug-induced mutations of HIV-1 PR leads to rapid loss of potency of the existing drugs and to the need to continue the development process. The structural basis of drug resistance and the ways of overcoming this phenomenon are mentioned.

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INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) was the first major epidemic caused by a previously unknown pathogen to appear during the 20th century, the period corresponding to the modern development of pharmaceutical sciences. This disease is caused by human immunodeficiency virus type 1 (HIV-1), a member of the family of retroviruses. At the onset of the epidemic in the early 1980s, no existing drug was known to be useful against AIDS and completely new pharmaceutical agents had to be created. Although azidothymidine (AZT), the first drug shown to counteract the effects of HIV-1 infection, was previously known as a potential anticancer agent, the rapid progress in the understanding of the structure and life cycle of the virus led to unprecedented development of other drugs targeted to a variety of viral proteins. The retroviral enzymes—reverse transcriptase (RT), integrase (IN), and protease (PR)—were the obvious targets for drug discovery.

The first drugs to be identified were inhibitors of RT (23), which were discovered and developed long before the structure of RT itself was solved (48, 60). However, newer RT-targeted drugs, nonnucleoside inhibitors, are being developed bearing the enzyme structure in mind. Even now, only fragmentary structural data have been described for IN (7–9, 27) and no drugs are available. Retroviral protease, however, was identified early as a potential target (59), and the discovery and development of its inhibitors are an unqualified success of modern pharmacology and structural biology.

Analysis of the nucleotide sequence of the HIV-1 genome (88) led to the discovery that the virus encodes an aspartic protease (HIV-1 PR). Inactivation of HIV-1 PR by either mutation or chemical inhibition leads to the production of immature, noninfectious viral particles (59, 72, 106), thus the function of this enzyme was shown to be essential for proper virion assembly and maturation. It is not surprising, then, that HIV-1 PR was identified over a decade ago as the prime target for structure-assisted (sometimes called “rational”) drug design.

It is much more surprising that by now four drugs have already been approved by the US Food and Drug Administration (FDA), and several others are in advanced clinical trials.

The structure-assisted drug design and discovery process (3) utilizes structural biochemical methods, such as protein crystallography, NMR, and computational biochemistry, to guide the synthesis of potential drugs. This information can, in turn, be used to help explain the basis of their activity and to improve the potency and specificity of new lead compounds. Crystallography plays a particularly important role in this process. The past eight years have seen a virtual explosion of crystallographic studies aimed at the characterization of the structures of HIV PR and of HIV PR/inhibitor complexes on an atomic level (note that HIV PR as used here refers to both HIV-1 and HIV-2 PRs). Indeed, the level of involvement of crystallographers in this area is unprecedented in the history of the field, with over 25 laboratories worldwide reporting crystal structures of this enzyme (Table 1). In addition, several more structures were

Table 1 Availability of crystal structures of HIV/SIV PRs (10/97)

Laboratory	Location	#HIV PR database	#in PDB
NCI-ABL	Frederick, MD	17	12
NCI-SAIC	Frederick, MD	9	9
Abbott	Abbott Park, IL	3	3
Merck	Rahway, NJ	8	6
	West Point, PA		
Roche	Nutley, NJ	2	1
	Hertfordshire, UK		
SmithKline	King of Prussia, PA	8	8
Agouron	San Diego, CA	7	0
Upjohn	Kalamazoo, MI	12	8
Birkbeck College	London, UK	2	2
Pasteur Institute	Paris, France	1	1
Glaxo	Greenford, UK	7	3
Boehringer	Ridgefield, CT	4	3
Du Pont-Merck	Wilmington, DE	22	22
Ciba-Geigy	Basel, Switzerland	2	2
Vertex	Cambridge, MA	5	1
Univ. of Brisbane	Brisbane, Australia	2	2
NIDR	Bethesda, MD	2	2
OMRF	Oklahoma City, OK	3	3
UCSF	San Francisco, CA	3	3
Marion Merrell-Dow	Strasbourg, France	7	0
Uppsala University	Uppsala, Sweden	4	2
Bristol-Myers Squibb	Princeton, NJ	2	0
Totals		132	93

solved by NMR. This review will discuss the current structural knowledge of inhibitor complexes of HIV PR and how the use of this information was crucial to the process of drug design. The review will also discuss new and emerging areas of investigation—in particular, the studies of resistance to protease inhibitors.

STRUCTURAL INVESTIGATIONS OF RETROVIRAL PROTEASES

Comparing the genomic sequence of HIV-1 with that of several other retroviruses, Ratner et al (88) postulated that the HIV-1 genome encodes a protease. Subsequently, based on the observation of a signature sequence (Asp-Thr-Gly) and the overall similarity of primary structure between retroviruses and retrotransposons, Toh et al (113) suggested that their proteases might be related to the family of eukaryotic aspartic proteases, exemplified by pepsin. HIV-1 PR and other retroviral proteases also exhibit other characteristics of aspartic proteases, such as inhibition by pepstatin (92, 106) and inactivation by mutation of the putative active-site aspartates (54, 59, 68, 106). Structural studies on HIV-1 PR were initially hampered by the fact that it constituted a minor component of mature virions (45), which therefore necessitated the use of recombinant and synthetic technologies to produce the milligram quantities needed for structural investigations. HIV-1 PR was cloned in a variety of vectors (13, 18, 20) and it was also prepared by total chemical synthesis (101). In contrast, Rous sarcoma virus (RSV) PR was isolated from viral cultures in amounts sufficient for structural studies.

Crystallographic Studies of Retroviral Proteases

The breakthrough that made structure-assisted design of protease-targeted drugs against AIDS possible was the determination in early 1989 of the structures of retroviral proteases, first from RSV (75) and subsequently from HIV-1. The structure of HIV-1 PR was also modeled using the known structures of eukaryotic aspartic proteases as templates (83, 84). In these models, HIV-1 PR was built as a dimer of two identical aspartic protease-like domains. A more accurate model of HIV-1 PR was constructed using the three-dimensional (3D) structure of RSV PR and homology modeling techniques (127). The experimental crystal structures of native HIV-1 PR were reported for the recombinant and synthetic enzymes in several laboratories (66, 79, 110, 132). These studies confirmed that the molecule is a homodimer and that its active site closely resembles the active sites of pepsin-like proteases. Other retroviral proteases for which crystal structures subsequently became available include the enzymes from HIV-2 (77, 116), simian immunodeficiency virus (SIV) (95, 128, 137), feline immunodeficiency

virus (FIV) (131), and equine infectious anemia virus (EIAV) (41). The structures of the latter two enzymes have been reported only for inhibitor complexes; no apoenzyme structures are available at this time. Structures of artificially constructed single-chain enzymes are also available (5).

The crystals of native HIV-1 PR used in all published investigations were isomorphous and belonged to the space group $P4_12_12$. They diffracted to only medium resolution, with the data measured to, at best, only 2.7 Å. The asymmetric unit contained one monomer, and thus the dimer was crystallographically symmetric in the absence of any active-site ligands (other than a water molecule positioned adjacent to the aspartates). In contrast, the RSV PR dimer (50) comprised the asymmetric unit in the space group $P3_121$ crystals, such that the two subunits were in nonequivalent crystal environments. The deviation from exact twofold symmetry for the two subunits was quite small (0.4 Å rms for the $C\alpha$ atoms, with a rotation angle of 178°) and may have been the result of crystal packing forces. The crystals of RSV PR were more highly ordered than those of native HIV-1 PR and diffracted to 2.0 Å (the structure was refined to an R-factor of 0.144). Nonetheless, all the amino acid side chains could be located in the electron density maps for HIV-1 PR, whereas residues 61–70, belonging to the flaps, were crystallographically disordered in both molecules of RSV PR.

The flaps in native HIV-1 PR were about 7 Å distant from the active site and in a very open conformation. This conformation could be considered a consequence of kinetic trapping resulting from the crystallization process and should not necessarily be taken as an indication of a preferred, highly stable conformation in solution (79, 132). Although structures of apparently uninhibited HIV-1 and HIV-2 PRs with flaps closed have been seen (K Watenpaugh and TN Bhat, personal communication), it is likely that, in each of these cases, peptide fragments were still present in the active sites.

A number of crystal forms of the complexes of HIV and SIV PRs with inhibitors have been reported. Some of these crystals diffracted to very high resolution, with at least two structures refined at a resolution of 1.7 Å with R-factors better than 0.2 (108, 115). Most of the crystal forms contained a dimer of the protease and a single inhibitor molecule in the asymmetric unit, although in some cases only a monomer was present (26, 78), whereas two molecules were present for some other structures (108). The availability of multiple crystal forms with vastly different packing should make it possible to differentiate between the intrinsic structural properties of the enzyme and crystal-induced conformational adjustments. Although the inhibitors were clearly ordered in unique orientations in some structures (e.g. 51, 112), they were often present in two superimposed orientations (26, 33, 78). In a particular investigation, only a single orientation was initially claimed (76), whereas two orientations were

detected after careful reanalysis of the data (74). In some cases, especially for the hexagonal crystals of HIV-1 PR, almost completely isomorphous crystals were reported with either a monomer or a dimer in the asymmetric unit. Because some disorder is always present even for completely symmetric inhibitors (26, 41), this difference is more quantitative than qualitative.

Description of the HIV-1 PR Molecule

The initial knowledge of the structures of retroviral proteases came from the crystallographic studies described above. The general topology of the HIV-1 PR monomer is similar to that of a single domain in pepsin-like aspartic proteases, with the main difference being that the dimer interface in the former is made up of four short strands, rather than the six long strands present in the pepsins (Figure 1a). In addition, because the molecular weight of dimeric HIV-1 PR is less than two-thirds that of pepsin, the retroviral enzyme is clearly a parsimonious member of the family (87).

The N-terminal β -strand *a* (residues 1–4) forms the outer part of the interface β -sheet. The β -strand *b* (residues 9–15) continues through a turn into β -strand *c*, which terminates at the active-site triplet (Asp25-Thr26-Gly27). Following the active-site loop is β -strand *d*, containing residues 30–35. In pepsin-like proteases, strand *d* is followed by helix *h*, which also has been seen in EIAV PR (41). In HIV-1 PR, this segment is quite distorted and forms a broad loop (residues 36–42). The second half of the molecule is topologically related to the first half by an approximate intramolecular twofold axis. Residues 43–49 form β -strand *a'*, which, as in pepsin-like proteases, belongs to the flap. The other strand in the flap (residues 52–58) forms a part of the long β -chain *b'* (residues 52–66). The β -chain *c'* comprises residues 69–78 and, after a loop at residues 79–82, continues as strand *d'* (residues 83–85), which leads directly to the well-defined helix *h'* (residues 86–94). The hydrogen-bonding pattern within this helix is intermediate between an α helix and a 3_{10} helix. Helix *h'* is followed by a straight C-terminal strand (residues 95–99), which can be designated as *q* and which forms the inner part of the dimer interface. Four of the β -strands in the molecular core are organized into a Ψ -shaped sheet characteristic of all aspartic proteases. One of the “ Ψ -sheets” comprises chains *c* (residues 23–25), *d*, and *d'*, and the other is made up of strands *c'* (residues 76–78), *d'*, and *d*.

The active-site triad (Asp25-Thr26-Gly27) is located in a loop whose structure is stabilized by a network of hydrogen bonds similar to that observed in eukaryotic enzymes (19). The carboxylate groups of Asp25 from both chains are nearly coplanar and show close contacts. The network is quite rigid due to the interaction (called “fireman’s grip”) in which each Thr26 OG1 accepts a hydrogen bond from the Thr26 main-chain NH of the opposing loop. Thr26 also

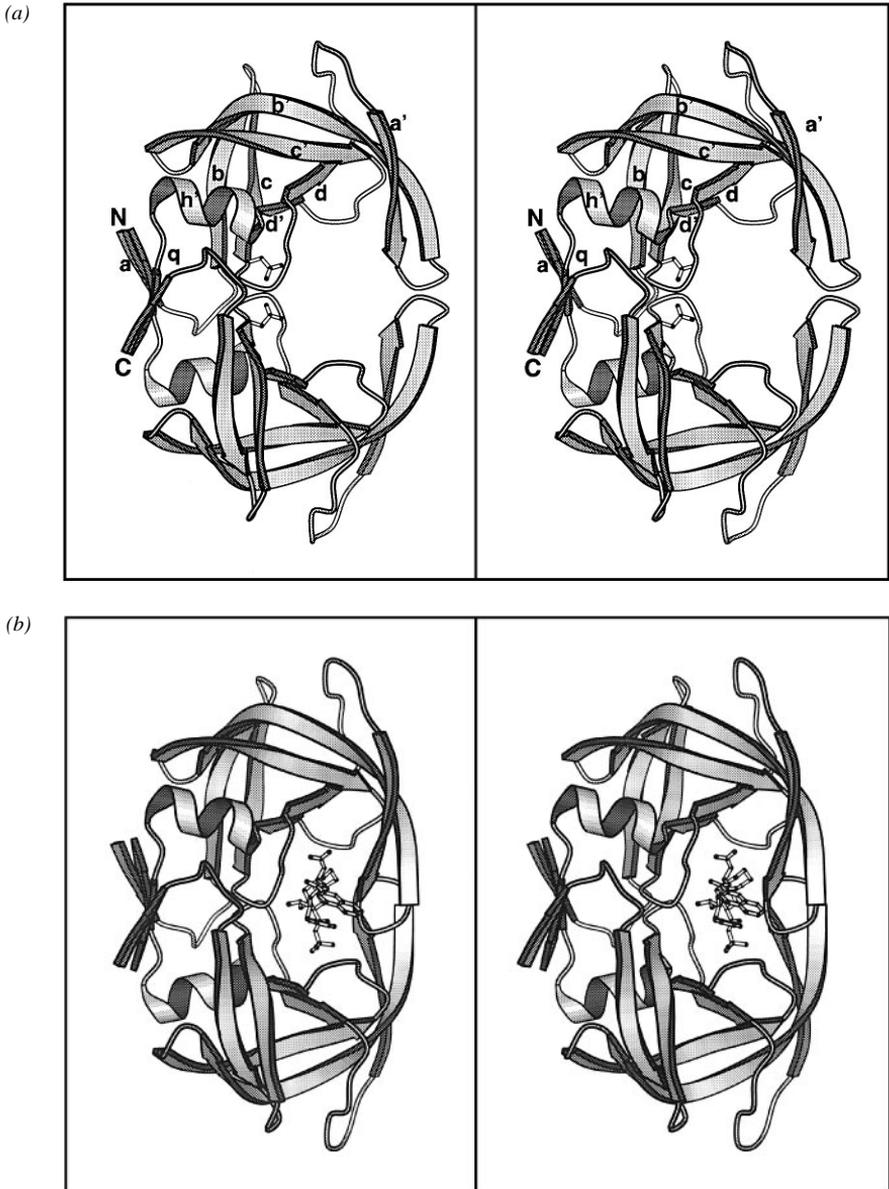


Figure 1 Stereoviews of the chain tracing of HIV-1 PR, prepared with the program Molscript (61). (a) Apoenzyme with the elements of secondary structure marked as discussed in the text; (b) Inhibited enzyme in the complex with Saquinavir (17).

donates a hydrogen bond to the carbonyl O atom of residue 24 on the opposite loop. Although the central features of the catalytic site are very similar between retroviral and cellular aspartic proteases, the residue following the triad differs, with Ala invariably present in retroviral proteases, whereas Ser or Thr are most common in the pepsin-like family of proteases. Another difference is the presence of only one flap in the pepsins, whereas a pair of twofold related flaps is present in HIV-1 PR. The flap is a β hairpin that covers the active site and participates in the binding of inhibitors and substrates (Figure 1*b*).

Common Structural Features of the Inhibitor Complexes of HIV-1 PR

Cocrystals of HIV-1 PR with a variety of inhibitors have been grown in a number of different crystal forms. The structures of these complexes (Figure 1*b*) have been used to investigate the binding of substrate-based modified oligopeptide inhibitors as well as of the inhibitors of non-peptidic nature. Binding of an inhibitor introduces substantial conformational changes to the enzyme. The overall movement of the subunits can be described as a rotation of up to about 2° around a hinge axis located in the subunit β -sheet interface. This motion, which slightly tightens the cavity of the active site, is also accompanied by a very large motion of the flap region—as much as 7 \AA for the tips of the flaps (114). However, the enzyme structure is well conserved among the different complexes, with rms deviations between the $C\alpha$ atoms seldom exceeding 0.6 \AA . Such differences are well within the agreement range for protein structures refined independently or crystallized in different space groups (133).

Most of the inhibitors cocrystallized with HIV PR, including all peptidomimetic inhibitors, are bound in the enzyme active site in an extended conformation so that when they are superimposed upon one another, their functional elements align quite well overall (130). The contacts between the main chain of the peptidomimetic inhibitors and the protease are almost uniform for all the complexes (Figure 2). Following a similar pattern, the hydrogen bonds are made mostly between the main-chain atoms of both the enzyme and the inhibitor. The hydroxyl group at the nonscissile junction, present in inhibitors other than those containing the reduced peptide bond isosteres, is positioned between the Asp25/Asp25' carboxyls of the protease, within hydrogen-bonding distance to at least one carboxylate oxygen of each aspartate. A feature common to almost all complexes of HIV-1 PR is a buried water molecule that bridges the P2 and P1' CO groups of the inhibitor and Ile50 and Ile150 NH groups of the flaps. This water is approximately tetrahedrally coordinated and is completely separated from the bulk solvent (76). The functional substitution of this water has led to the design of urea-based inhibitors (see below).

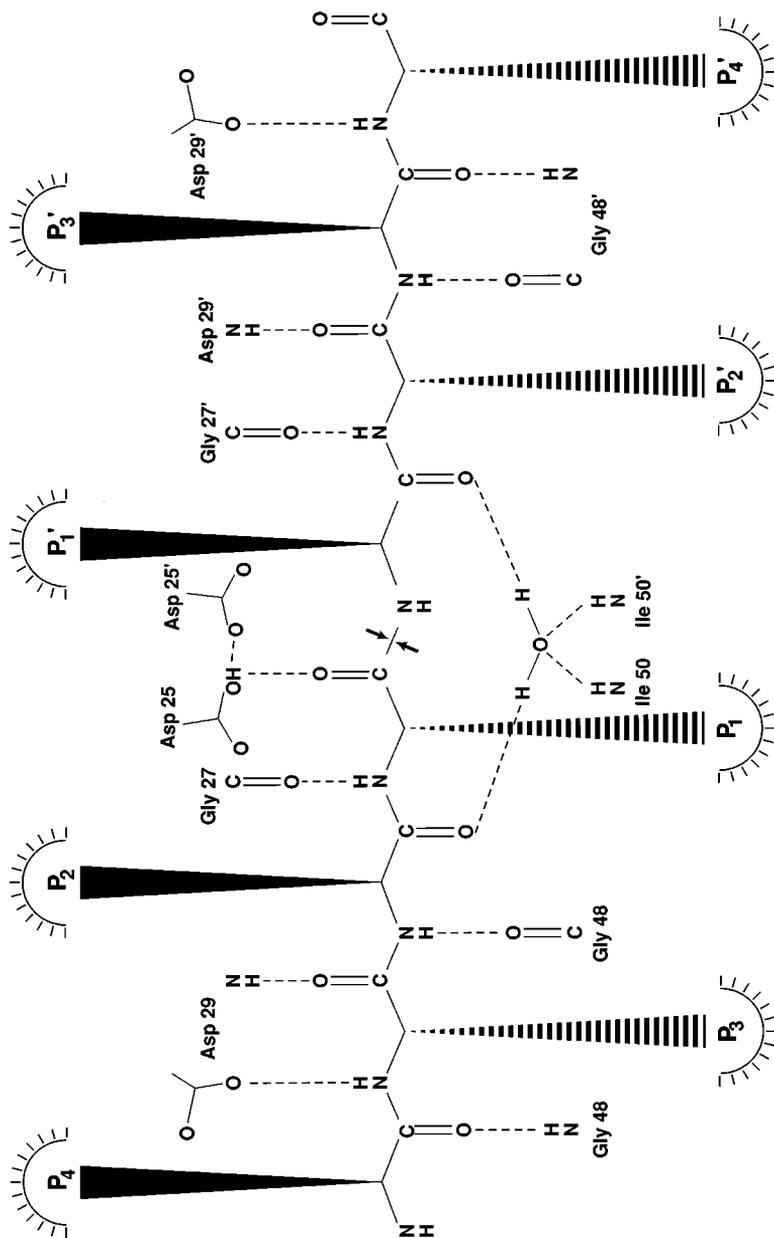


Figure 2 Schematic diagram showing hydrogen bonding between HIV-1 PR and a modeled substrate. The nomenclature of the subsites is that of Schechter & Berger (99). (Reprinted from 130.)

Subsites of the Inhibitor-Binding Pockets

A number of distinct subsites that accommodate side chains of the inhibitors can be identified in HIV PR. Three subsites on each side of the non-scissile bond (S1–S3 and S1'–S3') are very well defined, whereas more distant subsites are not as clear. The protease side chains comprising the pockets S1 and S1', with the exception of the active-site aspartates, are mostly hydrophobic. The side chains of the active-site aspartates and the main-chain hydroxyl of those inhibitors that contain such a central group are involved in polar contacts. Almost all of the documented inhibitors have hydrophobic moieties at P1 and P1' with the exception of the statine- and glycine-containing inhibitors, in which no groups occupy the protease subsite S1'.

Although the S2 and S2' pockets are hydrophobic, both hydrophilic and hydrophobic residues can occupy these sites. The P2 and P2' hydrophobic side chains are observed in different orientations for the different inhibitors, forming contacts with different groups in the enzyme-binding pocket. For inhibitors containing asparagine or glutamine, the amide side chains are also stabilized by polar contact with the carbonyl O atom of the previous residues in the inhibitor. Some polar contacts are also observed between P2/P2' amide groups and polar side chains of the protease.

Distal to S2/S2', the subsites are not as well defined, and diverse side chains are accommodated in subsites S3 and S3'. Only a small number of inhibitors have standard amino acids located in the subsite S4. This subsite does not form an actual binding pocket, and only the carboxyl-most end of the P4 residue is surrounded by protease atoms. Only two inhibitors have been reported with a moiety at P4': AG-4, which has one side of the glutamine fork directed into the S4' subsite, and PS-1, which has a proline at this position. There is really no complementary S5 or S5' pocket on the protease; P5 or P5' residues of the inhibitor extend out of the binding groove of the enzyme, making few contacts with the protease.

NMR Studies

NMR spectroscopy is a technique that, in the last decade, has been successfully used for the determination of 3D structures of a large number of macromolecules, including receptors and ligand/receptor complexes. NMR has also become a useful tool for rapidly determining the conformations of receptor-bound ligands (32). Initial applications of this technique to the studies of HIV-1 PR complexed with different inhibitors were complicated by the limited solubility of the protein, but these difficulties were ultimately overcome. Taken together with data from crystallographic experiments, NMR provides an excellent tool for mapping enzyme-inhibitor interactions in a dynamic state.

The solution-state NMR technique was used to study the symmetric versus asymmetric binding of penicillin-based symmetric inhibitors of HIV-1 PR (134). In this study, the spectra of HIV-1 PR complexed with an inhibitor that contained two symmetrically disposed trifluoro groups indicated the symmetric binding mode. The compounds traced a novel S-shaped path through the active site, with almost no contact with the catalytic aspartates but with full occupancy of the S1/S1' and S2/S2' subsites. This work was later continued in a study of penicillin-based asymmetric inhibitors and revealed that such inhibitors occupied binding subsites in a manner equivalent to half of a dimeric C2-symmetric inhibitor (52).

An interesting attempt to design protease inhibitors utilizing NMR in combination with computational methods was reported by Podlogar et al (85). A novel macrocyclic inhibitor in which the P1 and P3 side chains had been joined was found to be an excellent inhibitor of HIV PR. NMR analysis of the precursor showed that the conformation of the cyclic region was very similar to that observed in the enzyme-bound inhibitor complex as determined by computational approaches. These results confirmed that computational models and simulations (see below), together with NMR data, can provide a basis for further modification and design.

Complexes of HIV-1 PR with cyclic urea-based inhibitors have been subject to extensive NMR studies, starting with a complex with the inhibitor DMP-323 (136). The derived assignments of the resonances opened the way to determining 3D solution structures and suggested methods for reducing the time required for this task (135). DMP-323 was also used for mapping the water molecules bound in such complexes (124), as well as for comprehensive conformational analysis studies (65). Its high affinity made it possible to study the relationship between the flexibility of HIV-1 PR and the function of the enzyme (80). Analysis of ^{15}N spin relaxation parameters of all but 13 backbone amides revealed the presence of significant internal motions of the protein backbone. The flaps covering the protease active site underwent large-amplitude motions on the picosecond to nanosecond time scale, whereas the tips of the flaps underwent a conformational exchange on the microsecond time scale. These studies confirmed the importance of changes in the conformation of the flaps during the catalytic process.

Two NMR studies of KNI-272, a tripeptide inhibitor of HIV-1 PR, have recently been published. In the first one (81), solution conformation of KNI-272 was examined, showing that the backbone of the inhibitor had a fairly rigid conformation. The question of the ionization state of the catalytic aspartyl groups was addressed in the second study (125). The ionization state of the catalytic residues was also examined using chemically synthesized HIV-1 PR in

which Asp25 in each monomer was specifically labeled with ^{13}C (109). In the presence of pepstatin, the catalytic carboxylates did not titrate in the pH range where the enzyme was active. Throughout the pH range of 2.5–6.5, one Asp25 side chain was protonated and the other was deprotonated. In the absence of an inhibitor, the two aspartate side chains were chemically equivalent and both were deprotonated at pH 6, the optimum for enzymatic activity.

Computational Studies

A number of quantum-chemistry based computational techniques can be potentially useful for the description of ligand-enzyme interactions or for the prediction of ligand affinity. Some of these computational approaches have been utilized to probe the binding of various inhibitors and substrates to HIV PR. These methods have ranged from simple molecular mechanics calculations or molecular docking techniques to free-energy perturbation methods using molecular dynamics. In principle, the aims of all these methods have been similar—namely, to find computational techniques for prediction of inhibitor-binding affinity. Molecular dynamics calculations have also been used for studying enzyme dynamics and the influence of certain mutations on structure and stability. A combination of *ab initio*, semiempirical, and empirical calculations has been used for the description of the catalytic mechanism of HIV-1 PR.

An attempt to find a correlation between the experimentally obtained data and the computational results for three inhibitor complexes of HIV-1 PR with known crystal structures (MVT-101, JG-365, and U-85548e) involved energy minimization using molecular mechanics (98). Because of the very different nature of these inhibitors, no correlation between the interaction energy and the binding constants could be found. From the structural point of view, only minor changes of the hydrophobic core and of the inhibitor binding site were reported. The largest change was for the surface loop with the highest B-factors. Improved energy calculations on the same set of inhibitors were later performed, with the aim of determining general rules for inhibitor and substrate binding to the HIV-1 PR (42). A comparison with another 15 published X-ray structures of the complexes of HIV-1 PR with inhibitors was made in order to understand the importance of the hydrogen-bonding interactions between the main-chain atoms of the inhibitor and those of the enzyme. Conserved hydrogen bonds were observed in the subsites ranging from P3 to P3'. The calculated contribution of the main-chain interactions to the total interaction energy ranged from 56% to 68%. It was concluded that the protease-inhibitor interactions with the main chain provided a substantial contribution to the total binding energy. The relative contribution to the total interaction energy of main-chain and side-chain atoms from individual residues was largest for subsite P2.

A study successfully utilizing simple energy minimization of HIV-1 PR inhibitors in the active site in order to predict their activity was performed by a group from Merck (47). Using the MM2X force field and the program OPTIMOL, they found a satisfactorily high correlation between the interaction energy and the experimentally determined IC_{50} constants for almost 50 inhibitor-enzyme complexes. Thirty-four of the complexes were used as a training set and the others were examined as a set for prediction. The interaction energy corresponded to the sum of van der Waals and electrostatic interactions between the inhibitor and the enzyme when the inhibitor was minimized in the rigid active site of the enzyme. The proposed correlation was premised on the assumptions that the interaction energy (E_{inter}) might be proportional to the enthalpy of binding (ΔH_{bind}), while the entropy of binding (ΔS) might be small or, more likely, constant. The use of this simple model is limited to inhibitors that were neutral and of approximately the same size, and it obviously neglected some factors which were key to binding. For example, the enzyme active-site flexibility and the difference in energy between the solution and bound conformations of the inhibitor were not taken into account.

Weber & Harrison (126) used molecular mechanics in their calculations of enzyme-substrate interactions and found correlations between the interaction energy and the kinetic characteristics for 21 peptide substrates and their reaction intermediates. They provided a statistical mechanics interpretation of the molecular mechanics energy and discussed its justification. Another study involved molecular dynamics (MD) calculations on HIV-1 PR with a bound peptide substrate (43). A stable minimum energy position for a proton artificially placed between the two aspartates and the carboxyl oxygen of the cleaved peptide bond was determined, and this study served as a basis for the later runs of energy minimization.

Calculations utilizing molecular dynamics coupled with the free energy perturbation method have served as relatively reliable tools for the prediction of the free energy of binding by computational methods. The early utilization of free-energy perturbation theory (FPT) was reviewed by McCarrick & Kollman (71). The computational studies by Ferguson et al (31) and Tropsha & Hermans (117), focusing on the difference in free energy of binding between the *S*- and *R*-hydroxy stereoisomers of the inhibitor JG-365, have shown that two independent experiments could lead to similar results. The FPT method was recently used in several cases to explain the differences between the binding constants of similar inhibitors (53, 86), to analyze HIV-1 PR mutants and their affinity to different inhibitors (102), or to serve as a tool for molecular modeling and drug design (123). Activated molecular dynamics calculations were used to model flap opening (14, 15), a crucial step during the binding of a substrate or

an inhibitor. These studies compared the wild-type protease with the M46I mutant associated with drug resistance and indicated that this mutation stabilizes the flaps in a closed conformation.

The knowledge of the ionization state of the two catalytic aspartates is extremely important in determining the correct binding mode of the substrate and the free energy, so considerable efforts have been spent on this problem (11, 31, 37, 44), as well as on theoretical modeling of the catalytic mechanism. Different steps of the reaction were modeled using *ab initio* and MD calculations (108). Another recent approach to that subject utilized a combination of quantum and classical molecular dynamics (67). Both of these studies reported protonation of only one aspartic acid during catalysis. It has been indicated that the nucleophilic water molecule bound at a position different from the positions of the hydroxyl groups observed in various aspartic protease-inhibitor complexes. The carboxyl group of the scissile peptide bond also adopted a different orientation. During the approach to this bond, the reaction center changed gradually to a conformation close to that derived from X-ray structures of HIV-1 PR with various inhibitors.

The interactions of HIV-1 PR with different inhibitors, determined from their crystal structures, have served as a starting point for tests of various docking techniques. The first of such docking studies of HIV-1 PR was performed by the Kuntz laboratory (21), who discovered that haloperidol could be an inhibitor of this enzyme. However, subsequent crystal structure determination revealed a different orientation for haloperidol than the one predicted (97). Docking methods and algorithms were tested using the known structural data and experimental characteristics by Monte Carlo docking (10) or by comparison with *de novo* constructed inhibitors by a fragment-based method (96), in which the inhibitors were constructed entirely from individual functional groups chosen from a predefined library. A method of continual energy minimization implemented in the program SCULP was a new paradigm for modeling proteins in interactive computer graphic systems (111). This physically realistic attempt made possible the modeling of very large changes and aided the understanding of how different energy terms interact to stabilize a given conformation. Other recent studies examined empirical free energy as a target function in docking and design, showing the advantages of this approach over studies using the calculation of interaction energy (58).

Database of Three-Dimensional Structures of HIV-1 PR

With the rapid progress in determining the structures of HIV PRs, it had been postulated as early as 1993 that a collection of such structures in a single database would be beneficial (130). The HIV PR database was established

three years later at the National Cancer Institute (122). It is an Internet-based service that now provides direct access to more than 130 crystal structures. Almost 40 of these structures of HIV-1, HIV-2 or SIV PRs in complexes with various inhibitors are not publicly available elsewhere (Table 1). There are two main reasons why this database was created. First, with a number of inhibitors having reached approval by the FDA as anti-AIDS drugs and with others in advanced clinical trials, there was some danger that many of the structures with no direct clinical relevance would ultimately be lost. Second, the database provides a unique source of information about ligand-enzyme interactions in a well-characterized system, which could be essential for formulation of new drug-design principles and synthetic strategies.

A detailed description of the database was provided elsewhere (122). Its shell contains structural files that had been previously deposited in the Protein Data Bank (PDB) or were directly placed into the HIV PR database. The information about these complexes is placed in the chronological order of deposition in the main table of contents. Branched links provide more detailed information about the inhibitors, their chemical formulas, literature references, and 3D structures visualized by a Web-based browser applet. Special attention was paid to the kinetic parameters for the inhibitors, including the conditions of such measurements. Information contained in this part of the database can be easily accessed using a simple search engine. The core of the database consists of the Analytical Part, designed as a source of various tools for the statistical analysis of the archived complexes. Some results of this analysis are immediately accessible. The more complicated calculations or analytical programs are run in batch mode and the results are returned by e-mail. This part also contains coordinates of the complexes transformed into a common frame of reference, as well as the separate files of the proteases, inhibitors, and solvent. For those who perform molecular modeling and are interested in different modes of superimposing the structures, a fit based on the user's definition is provided using the program ProFit.

Particular attention was paid to volume and surface calculations for the protein and the inhibitors. These characteristics are very important when combined with reasonable template structure and docking algorithms in searching directly for novel inhibitors and in formulating new rules for docking. An initial example of the analysis of structures in the database involved construction of minimal and maximal binding volumes in the active site of HIV PR. The volume calculations were performed for all the stored structures from which the inhibitors and the water molecules were removed. These volumes spanned a surprisingly wide range of magnitudes, although no correlation between the volume of the inhibitor and the volume of the binding cavity could be found.

Difference-distance matrix calculations were used to correlate some structural features of the complexes with crystal packing. The basic assumption was that, for isomorphous structures, any differences would have to be caused by inhibitors rather than by crystal packing forces. On the other hand, comparison of complexes crystallized in different space groups should also show the changes caused by different packing forces. Such calculations have shown that the most rigid main-chain regions of HIV PR consist of residues 1–4, 25–28, 49–52 and 94–99, possibly indicating the importance of these areas to the process of enzyme folding.

DESIGN, STRUCTURE, AND PROPERTIES OF SELECTED INHIBITORS

Saquinavir (Ro 31-8959, Invirase): The First Approved Protease Drug

The discovery and development of the Hoffmann–La Roche drug Saquinavir (Figure 3), the first inhibitor of HIV-1 PR to be approved by the FDA, proved to be a classic example of serendipity coupled with hard work. Initial inhibitors created in that study were peptide derivatives utilizing transition-state mimetic concepts (94). The basic design criterion relied on the observation that HIV-1 PR, unlike other proteases, is able to cleave Tyr-Pro or Phe-Pro sequences in the viral polyprotein. Because the amide bonds of proline residues are not susceptible to cleavage by mammalian endopeptidases, the design of HIV-1 PR inhibitors based on this criterion was expected to bring potential advantages of higher selectivity. Reduced amides and hydroxyethylamine isosteres most readily accommodate the imino acid moiety characteristic of a Phe-Pro or Tyr-Pro retroviral substrate and, therefore, were chosen for further studies. As was shown later, the reduced amide isosteres were relatively poor inhibitors but, in contrast, the compounds incorporating the hydroxyethylamine moiety were very potent and highly selective inhibitors of HIV PR.

A minimum sequence required for potent inhibition, as well as the unexpected preference for *R* stereochemistry at the hydroxyl-bearing carbon, was determined by enzymatic studies of a series of related compounds. The minimum length inhibitor included three residues on the N-terminal side of the isostere and two residues on the C-terminal side. Varying the side chains of the residues in all subsites did not lead to dramatic improvement of the potency of inhibitors. The most marked improvements in potency were achieved by varying the amino acid at subsite P1', via replacement of proline by (S, S, S)-decahydroisoquinoline-3-carbonyl (DIQ). The resulting compound (later designated as Ro 31-8959) had a K_i value of 0.12 at pH 5.5 against HIV-1 PR and an even

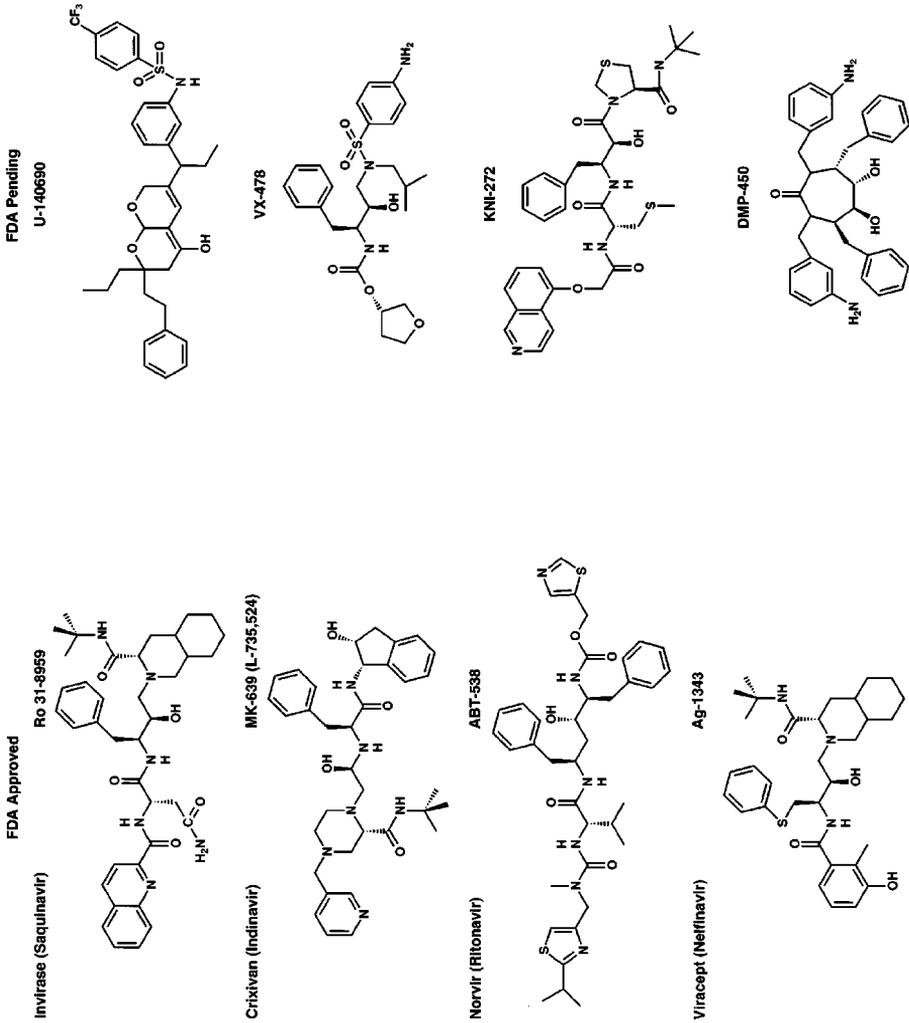


Figure 3 Chemical structures of the HIV PR inhibitors which have either been approved as anti-AIDS drugs by the FDA or are known to be in advanced clinical trials in late 1997.

better inhibition constant against HIV-2 PR ($K_i < 0.1$) (94). It was also shown to be highly selective, causing less than 50% inhibition of the human aspartic proteases. The compound was subsequently used for further clinical trials and was finally approved by the FDA in 1995 under the name Saquinavir (Invirase).

This design effort was accompanied by only limited structural studies. The first crystal structure to be solved was of a complex with another peptidic inhibitor, Ro 31-8588 (Figure 4), which exhibited the expected *S*-configuration of the carbon bearing the central hydroxyl group (38). The subsequent crystallographic study of Ro 31-8959 showed that, as predicted, this inhibitor bound in an extended conformation, forming a characteristic set of hydrogen bonds with the enzyme (62). With the exception of the flap, the twofold symmetry of the enzyme was preserved, allowing the *S* and *S'* subsites to be essentially equivalent. A comparison of the HIV-1 PR/Ro 31-8959 structure with the structure of HIV-1 PR complexed with a longer inhibitor, JG-365 (112), showed that the conformation of the inhibitor in the binding cavity critically depended on the nature of the P1' residue and on the presence or absence of the extension beyond subsite S2'. It was also established that short inhibitors preferred the *R*-configuration of the central carbon. Moreover, the carbonyl O atom of the DIQ group was able to maintain the hydrogen bond between the water molecule connecting the inhibitor with the flap regions (Wat301).

Ritonavir (ABT-538, Norvir)

The development of the Abbott drug Ritonavir is an interesting case showing how the thinking of the designers shifted from the creation of symmetric inhibitors of this inherently symmetric enzyme to their ultimate conversion to asymmetric compounds. The concept was first tested with the synthesis and characterization of symmetric inhibitors of HIV-1 PR (28, 29, 95), which were designed to match the *C*₂ symmetry of the homodimeric HIV PR. Although symmetry was not thought to be an absolute requirement for the design of HIV PR inhibitors, it was expected to be useful in tightly constraining the rather rigid ligands (40). It was also expected that the less peptidic nature of the inhibitors might enhance their stability *in vivo*. Such symmetric inhibitors were expected to confer higher specificity for retroviral proteases over the related mammalian proteases, whose substrate binding sites are less symmetric. A hypothetical tetrahedral intermediate of the cleaved peptide divided by the *C*₂ axis of the enzyme was taken as the template for the development of inhibitors. This hypothetical axis was placed on the carbonyl O atom or between the C-N atoms of the cleaved peptide bond respectively, and one half of the template was deleted. The *C*₂ operation was then implemented on the remaining template, generating symmetric inhibitors in either the mono-ol or diol form.

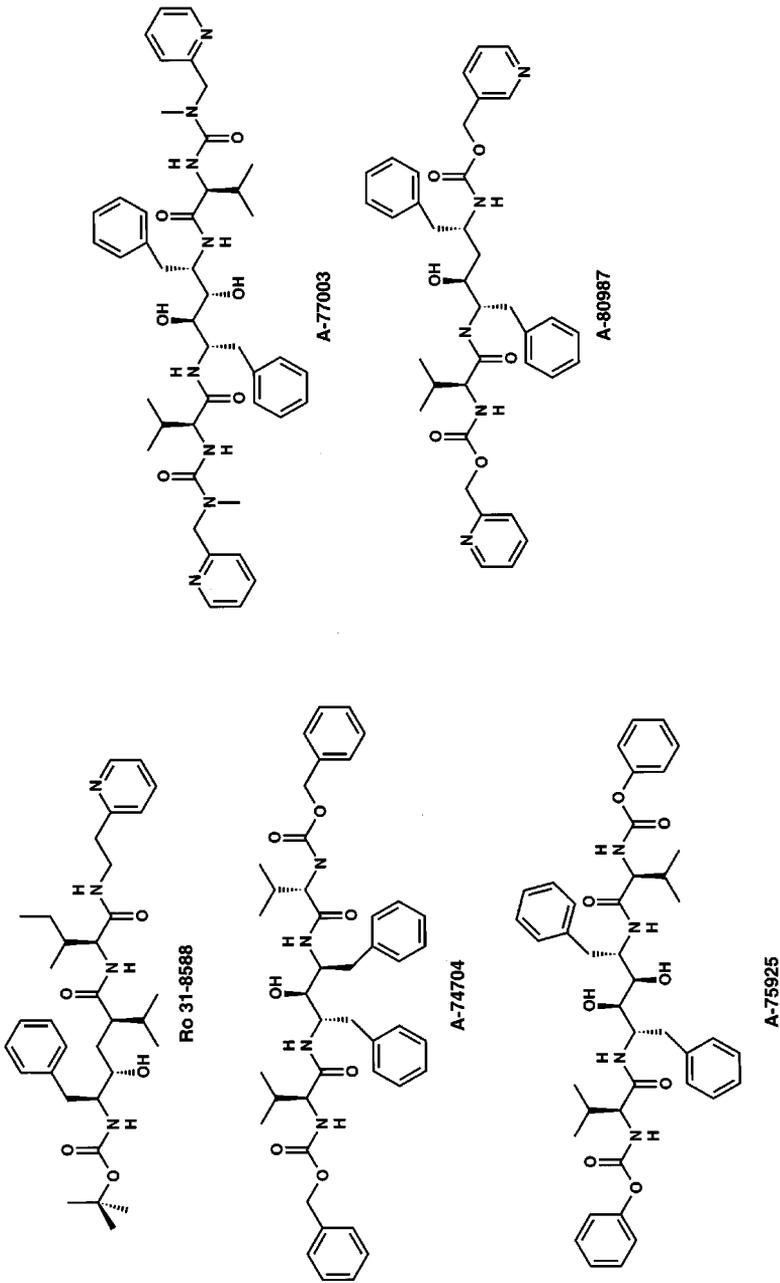


Figure 4 Chemical structures of compounds that were intermediates in the design of Saquinavir and Ritonavir.

The first of these symmetric inhibitors showed good kinetic profiles, and the concept of symmetry seemed to work remarkably well in cell cultures. The antiviral activity against HIV-1 in H9 cells determined by IC_{50} varied from 0.4 μ M to 20 nM. However, good performance in vitro did not result in acceptable bioavailability. The early inhibitors, designated A-74704 and A-75925 (Figure 4), had high lipophilicity and poor aqueous solubility and therefore presented difficulties for evaluation in vivo. In order to enhance their aqueous solubility, the terminal phenyl residues were modified to pyridyl groups (57). The diol inhibitors were substantially more active than the corresponding mono-ol inhibitors, although their aqueous solubility was at least one order of magnitude lower. These studies led to the creation of a new compound, A-77003 (Figure 4), which became a candidate for further in vitro kinetic evaluations based on a good combination of solubility and antiviral activity. Further examination of the inhibitor revealed broad-spectrum activity against both HIV types in a variety of transformed and primary human cell lines. Moderate oral bioavailability was observed after administration of the inhibitors to rats. The concept of symmetry was also successfully used for pseudosymmetric difluoroketones, highly potent HIV-1 PR inhibitors; these compounds also demonstrated good antiviral activity via inhibition of the cytopathic effects (107).

Dreyer et al (25) obtained the surprising result that even symmetric inhibitors may bind to HIV-1 PR in an asymmetric fashion. It was shown that crystal packing forces were not responsible for the observed asymmetry, but that, more probably, the asymmetric binding mode represented a lower energy complex in solution. In addition, some of the examined mono-ol and diol inhibitors had the unexpected ability to inhibit a prototypical cellular aspartic protease, porcine pepsin. These observations were important in guiding some groups, including the one from Abbott, in changing the direction of the development of new clinical candidates. It was also more difficult to make symmetric compounds with good oral bioavailability, which is often influenced by the termini of the inhibitor.

For these reasons, the process that began by designing symmetric or pseudosymmetric inhibitors resulted in compounds with significant asymmetry. The aqueous solubility of A-77003 was further improved by introducing moderately polar, heterocyclic groups at one or both ends of the inhibitors while maintaining inhibition activity against HIV PR. Introduction of more polar groups for the attenuation of lipophilicity drastically reduced the antiviral activity, apparently owing to insufficient cellular penetration (55). Pharmacokinetic studies in rats indicated a substantial difference in absorption properties of mono-ol and diol inhibitors, probably because of unfavorable desolvation effects of the diol. The discovery that the deshydroxy diols had superior potency led to the development of A-80987 (Figure 4), a shorter, orally bioavailable analog of

A-78791 (Figure 4), and its use in clinical trials. A-80987 was the penultimate compound in the synthesis of ABT-538 (Ritonavir) (56). The latter compound was designed by utilizing data from a study of a series of analogs of A-80987, which yielded valuable insight into the relationship of chemical structure to antiviral activity, aqueous solubility and hepatic metabolism. In this form the inhibitor reached clinical trials and was approved by the FDA in early 1996 under the name Norvir (Figure 3).

Indinavir (MK-639, L-735,524, Crixivan)

The discovery and development of the Merck drug Indinavir (Figure 3) was a long and complicated research project. Similarly to the approach taken at Roche, the original design strategy began with a compound based on the transition-state mimetic concept (91). This approach was successfully implemented in an earlier design of renin inhibitors, although no approved drugs resulted from the project (39). One important structural feature present in most tight-binding aspartic protease inhibitors is a critical hydroxyl group that hydrogen bonds to the carboxyl groups of the catalytically active aspartic acids. Incorporation of a hydroxyethylene isostere as a dipeptide mimic resulted in compounds that were potent and selective inhibitors of HIV PR. The available structure-activity data indicated that an *S*-hydroxyl was the preferred configuration. The constituent hydroxyethylene isostere was extensively examined in a series of peptidomimetic inhibitors of different length and with particular residues occupying subsites on both sides of the nonscissile isostere. Residues in these positions were systematically changed to establish the relationships between particular modifications and the efficiency of the inhibitors. The important questions of bioavailability were also addressed by these modifications. Some of the useful directions of synthesis were based on the results of molecular modeling and calculations.

Further approach to the successful design of HIV PR inhibitors with nanomolar inhibition constants led to a series of tetrapeptide analogs of a pentapeptide, L-682,679 (Figure 5), in which the C terminus had been shortened and modified (22). The inhibitory properties of these compounds were measured in peptide cleavage assays, with selected inhibitors also tested for their antiviral properties in cell culture. The first studies addressed the influence of the C terminus, with particular emphasis on the variation of the P2' amino acid and elimination/replacement of the P3' amino acid. The compounds that were systematically varied in P2' were almost exclusively aliphatic. Only one amino acid with a hydroxyl group (serine) was examined, without any indication of an improvement in the inhibitory activity. Substitution by (aminomethyl) benzimidazole provided the most potent compounds in this series, as the imidazole portion appeared to be mimicking a carboxamide, whereas the phenyl portion

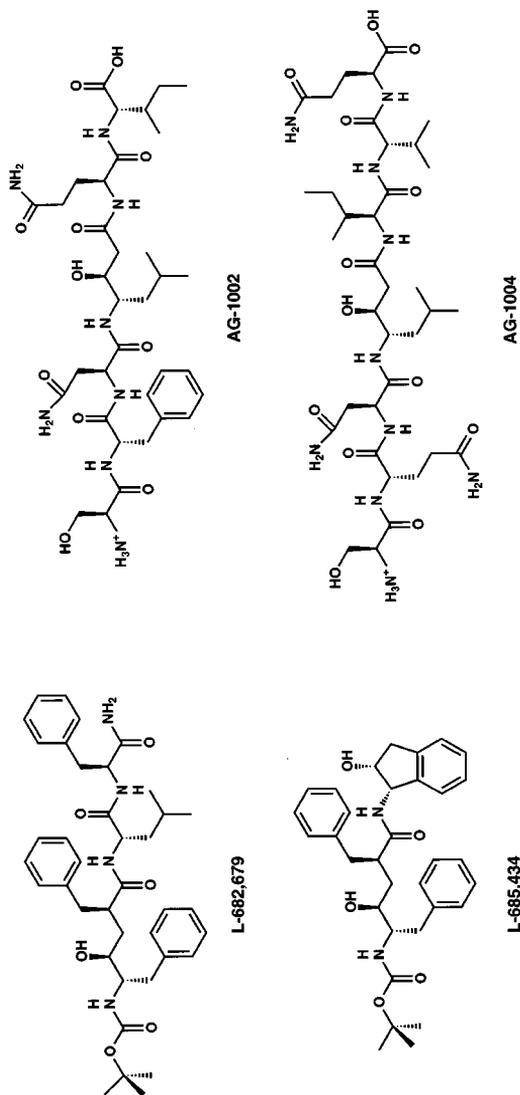


Figure 5 Chemical structures of compounds that were intermediates in the design of Indinavir and Nelfinavir.

was probably contributing additional hydrophobic binding, essentially in the P4' site. Important observations were made by testing the ability to inhibit HIV-1 infection in cell culture. Some of the inhibitors were considerably less potent in cell culture than would have been predicted by their IC₅₀ values, potentially reflecting their inability to penetrate the hydrophobic cell membrane. The important conclusion was that as the terminal amide increased in size or polarity, the intrinsic potency improved but the minimum inhibitory concentration did not improve. Inhibitors with benzimidazole were the most potent compounds in this series.

More than 150 compounds were described in several papers as intermediate steps in the design of Indinavir. The drug lead compound was L-685,434 (70, 120), a hydroxyethylene-based inhibitor with a benzocycloalkyl amine at the C terminus (Figure 5). The properties of this compound were improved by variation at the N terminus. Although very potent, the molecule lacked solubility in aqueous media and did not show an acceptable pharmacokinetic profile. Incorporation of a basic amine into the backbone of the L-685,434 series provided antiviral potency combined with highly improved pharmacokinetic profiles in animal models. The design of L-735,524 (Figure 3) was guided by molecular modeling and X-ray crystal structure determination of the inhibited enzyme complex (24, 119). The inhibitor was potent and competitively inhibited both HIV-1 and HIV-2 PRs with K_i values of 0.52 and 3.3 nM, respectively. This inhibitor, highly selective for retroviral proteases, showed no inhibition against a variety of mammalian proteases including human renin and cathepsin D, porcine pepsin, and bovine chymosin. It also was capable of preventing the growth of HIV-1-infected MT4 lymphoid cells at concentrations of 25–50 nM. L-735,524 was orally bioavailable in three animal models and therefore was tested as a promising drug in clinical trials. In early 1996, the compound (under the name Crixivan) was approved by the FDA as a drug against HIV infection.

Nelfinavir (AG-1343, Viracept)

The pathway of the discovery and development of Viracept (Figure 3) by Agouron Pharmaceuticals is the least completely described in the literature of the four FDA-approved protease inhibitor drugs. Little is known about the basis of its synthesis, and no crystal structure of the complex with HIV-1 PR has been released. This inhibitor is the only compound among these four that does not utilize a peptidomimetic concept, although its development pathway may have utilized some peptidomimetics. Two inhibitors developed by Agouron, AG-1002 and AG-1004 (Figure 5), had a statine isostere instead of a normal peptide bond (2). These compounds were peptidomimetic inhibitors with the flanking amino acid sequences naturally recognized by HIV-1 PR and were

bound to the active site in an extended conformation. They could form 16 and 18 hydrogen bonds, respectively, due to the presence of hydrophilic side chains. Despite the large number of hydrogen bonds, the inhibitors had relatively low potency, with binding constants of $0.55 \mu\text{M}$ for AG-1002 and $0.32 \mu\text{M}$ for AG-1004. A possible explanation for this low potency was the absence of a P1' group, as well as the large free energy required for desolvating of the hydrophilic side chains.

In another study, the structure of HIV-1 PR complexed with MVT-101 was utilized for modeling and synthesis of a lead compound, still based on the peptidomimetic concept (121). The best inhibitor in this series had an inhibition constant of about 30 nM and was characterized by the determination of its crystal structure, as well as by the calculations based on the free-energy perturbations method. The C terminus of the lead compound was replaced by an indole ring occupying the P3' pocket and replacing the phenyl group and was still able to maintain the hydrogen bond with Gly48. Iterative protein cocrystal structure analysis of peptidic inhibitors and the replacement of parts of the inhibitors by other substituents of nonpeptidic character (89) were used in order to achieve the goal of designing orally bioavailable, nonpeptidic inhibitors. A Monte Carlo program that could generate ligands was used for filling in subsite S1 (35), resulting in the placement of a large cyclopentylethyl group in this position. Combining the 5-chloro with the dimethylbenzyl and cyclopentyl amides resulted in the best compound in this series, with a K_i value of about 2 nM (comparable to the efficiency of Saquinavir).

Little information about the design of Agouron's approved drug, AG-1343 (Nelfinavir), can be found, although clearly this compound is the logical endpoint of a well-conceived design effort. One terminus of Nelfinavir contains the same DIQ group as Saquinavir. Studies examining the physicochemical properties of the former utilized the unpublished crystal structure of this inhibitor complexed with HIV-1 PR for testing of conformationally flexible docking by evolutionary programming (36, 69). This work was followed by further developments in the design strategy of nonpeptidic inhibitors (73, 90). Nelfinavir was rapidly approved by the FDA (under the name Viracept) and was the first of the protease inhibitors to be approved for the treatment of pediatric AIDS.

Inhibitors Designed to Replace Wat301

One of the completely unexpected features of the structures of complexes between HIV-1 PR and the inhibitors was the finding of a conserved water molecule mediating the contacts between the P2/P1' carbonyl O atoms of the peptidic inhibitors and the amide groups of Ile50/Ile50' in the enzyme. This tetrahedrally coordinated water, usually denoted Wat301 [but originally listed as Wat511 (76)], has been reported in practically all structures of not only the

complexes of HIV-1 PR, but also HIV-2 and SIV PRs. Not surprisingly, incorporation of its replacement into the ligands has long been postulated as a possible way of making highly specific protease inhibitors (112), and this suggestion has been implemented by several groups. Although no approved drugs have emerged as yet, it is likely that this approach will ultimately succeed and thus we will discuss it here.

One of the approaches to finding the starting points for the design of such inhibitors was to search databases of small-molecule compounds for molecules that contain two appropriately spaced O atoms, one capable of replacing Wat301, and the other able to bind to one or both of the active site aspartates. Chenera et al (12) identified core structures containing *trans*-1, 4-cyclohexanediol or hydroquinone by using the program DOCK (63) and showed that six-membered rings with *para*-related oxygens could make the desired interactions. With phenyl rings placed adjacent to one of these oxygens and the other replaced by a better-fitting sulfoxide group, it was possible to create inhibitors with K_i values as low as 7 μM . The molecules created in this experiment, however, lacked hydrophobic moieties that could occupy the S2 and S2' pockets of the enzyme, and thus it was not possible to enhance their binding further.

A much more extensive and successful effort by the scientists from Du Pont Merck led to the development of a number of very potent inhibitors of HIV-1 PR and to the creation of a candidate drug compound, DMP-450 (Figure 3). The crucial difference in this approach was the utilization of a seven-membered cyclic urea ring as the starting pharmacophore. This design step was accomplished by an analysis of the existing experimental structures of the enzyme-inhibitor complexes as well as by modeling those structures that were not directly available (64). Cyclic ureas derived from L- and D-phenylalanine showed inhibition constants of about 4 μM , in good agreement with the data presented above. The ring nitrogens, however, offered the opportunity of further modifications—in particular, by the addition of large hydrophobic groups expected to bind in the S2/S2' pockets. The use of groups such as *para*-hydroxymethylbenzyl led to the development of DMP-323 (Figure 6), an inhibitor having a subnanomolar inhibition constant. This compound showed significant oral bioavailability in dogs and rats, indicating its possible usefulness in humans. It inhibited SIV PR only weakly, however, suggesting that it would not be a broad-spectrum drug. Although for a while DMP-323 was a clinical candidate (64), it was ultimately abandoned because of its poor oral bioavailability in humans.

Because this poor bioavailability was most likely due to low solubility in aqueous or lipid solvents, the next design step involved the addition of charged groups to the benzyl rings. Although the attempts to introduce highly basic groups did not succeed, it was possible to replace the hydroxymethyl

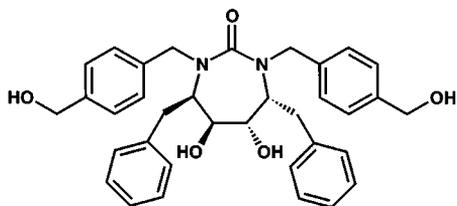
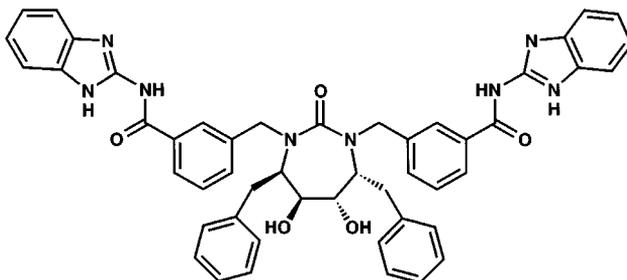
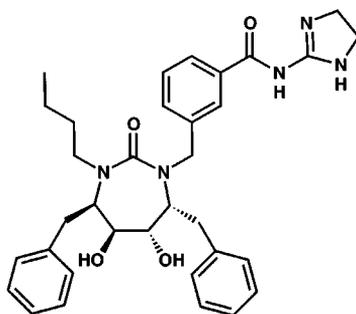
**DMP-323****SD-146****SD-152**

Figure 6 Chemical structures of several inhibitors of HIV-1 PR in which an oxygen atom replaces Wat301.

substituents by *meta*-amino groups. The inhibitory properties of the resulting compound, DMP-450, were very similar to those of DMP-323 ($K_i = 0.3$ nM), yet DMP-450 was very potent against the virus in cell culture and was shown to be orally available in humans (46). Like most other clinically useful protease inhibitors, DMP-450 would lose potency against the HIV-1 PR double V82F/I84V mutant by as much as two orders of magnitude (1). Nevertheless, at the time of this writing, this inhibitor is still in active clinical trials, conducted by Avid Therapeutics, Inc.

All of the above-mentioned inhibitors with six- or seven-membered rings in their centers are symmetric or quasisymmetric. This design recalls the initial approaches taken at Abbott (28) (see above). The presence of strict symmetry has not, however, correlated with the usefulness of the Abbott compounds as drugs, and the design rules have ultimately been relaxed. An analogous observation was also relevant to the cyclic urea-based compounds. Further development of these inhibitors, aimed at increasing their effectiveness against drug-resistant mutants of the virus, often utilized asymmetric design, in which the two ring nitrogens were substituted by different groups. Some of them, such as SD-152 (Figure 6), showed good activity profiles against most of the protease mutants, although the best new inhibitors in this series, such as SD-146 (Figure 6), were again symmetric (49). The ring substituents of the latter compounds were much larger than in DMP-450 and were capable of making more hydrogen bonds with the polar groups of the enzyme, especially with the main-chain N and O atoms of Gly48, as well as with the amide N and side-chain carboxyl of Asp30. Although these newer compounds had high antiviral activity and very good resistance profiles, their poor solubility in both water and oils prevented their formulation as drugs. Further developments in this elegantly designed series are still needed.

A parallel effort in designing this class of inhibitors yielded a puzzling result: When the seven-member cyclic urea ring was modified to a cyclic sulfamide and all the side chains contained phenyl groups, the pockets occupied by the S1' and S2' side chains were reversed. The unexpected presence of the putative S2' residue in the P1' pocket, and vice versa, was attributed to the more rigid and predefined structure of the seven-member ring utilized in this case (4).

DEVELOPMENT OF DRUG RESISTANCE

In common with other retroviral polymerases, HIV RT is unable to edit transcription errors during nucleic acid replication, and thus enhances the mutational rates of the virus (93). As one of the results, divergent viral populations are present during infection and the sequences of the proteases from these different strains differ, sometimes substantially. Many of these differences do not affect the activity of the enzyme, and the structures of fully active proteases that

differ in a number of positions in their sequences, from strains such as NY5 or SF2, have been reported. However, high rates of viral turnover in HIV infection and the inability of HIV reverse transcriptase to correct transcriptional errors also mean that populations of resistant virus will eventually emerge during any antiviral therapy as a result of drug selection of viral strains (6). It has previously been shown that the clinical use of RT inhibitors has led to the rapid development of resistance and cross-resistance against different RT inhibitors (100). Similarly, drug-induced mutations in HIV PR alter the susceptibility of the enzyme to inhibition by specific inhibitors.

A recent survey of the mutations associated with drug resistance has shown the modifications of 45 residues in HIV-1 PR, almost half of the total, for enzyme isolated from samples obtained with the help of 21 drugs (100). An obvious and expected mechanism for the development of resistance to HIV PR inhibitors is caused by the changes of specificity-determining residues that can directly interfere with the binding of the inhibitor to the enzyme. The mutations that do not directly change the shape or character of the binding cavity can indirectly influence inhibitor binding via long-range structural perturbations of the active site, or they can change the efficiency of catalysis and the stability of the enzyme. Other possible pathways in the development of resistance to the protease have been discussed in an earlier review (30).

A characterization of resistant variants isolated from patients undergoing therapy with the protease inhibitor MK-639 (Indinavir) was published in 1995 (16). Some of these variants exhibited cross-resistance to all members of a panel of six structurally diverse protease inhibitors. This study provided the first evidence that inhibition of HIV-1 PR can also lead to the emergence of drug-resistant mutants *in vivo* and that combination therapy with multiple protease inhibitors may not preclude the loss of antiviral activity resulting from resistance selection. A similar study using clinical isolates was also reported by Winslow et al (129). Five noncontiguous regions of HIV-1 PR, as described originally by Fontenot et al (34), were found to be conserved across all examined isolates. These regions include residues 1–9 (N terminus), 21–32 (the sequence surrounding the catalytic aspartate found in the active site), 47–56 (flap region), 78–88 (substrate-binding region), and 94–99 (C terminus and the dimerization region). The mutational analyses have shown that diversity is allowed at some positions, whereas mutations in these conserved regions often result in abnormal *gag* processing *in vitro*.

CONCLUSIONS AND PERSPECTIVES

The numerous studies leading to the discovery and development of HIV PR inhibitors that are now approved as anti-AIDS drugs are a unique source of

information that could, in the future, be used in other attempts of structure-based drug design. Obviously, structural studies are only one part of this complicated process. Detailed analysis of a large ensemble of crystal structures (such as, for example, the HIV PR database) can provide a surprising perspective to the problem of drug-target interactions and lead to the design of more efficient drugs. The coupling of such data with other *in vitro* and *in vivo* studies makes it possible to enhance the process of design. It is not always possible to correlate such data directly with inhibition activities toward a particular system, but in some respects they can be used for a qualitative estimation of their influence.

Some other approaches to designing HIV PR inhibitors were not reviewed here, since they have not resulted in the creation of successful drugs. One such approach is the design of dimerization inhibitors, based on the observation that, at higher pH, HIV PR is present in a monomeric form. Dimerization could be prevented by low-molecular-weight ligands with an affinity to the dimer interface regions. Short peptides from the termini of HIV-1 PR were first documented to be weak inhibitors of the protease and even better inhibitors in *in vitro* studies (103–105). The follow-up efforts met with only partial success. Some of the new inhibitors had better K_i values, but it is still unclear whether they were indeed bound to the dimer interface region. No X-ray structures, and only indirect proofs of the proposed inhibitory mechanism, were published.

Uhlikova et al (118) published a study showing the design of new, modular inhibitors which combined in one molecule the activities towards both the active site and dimerization domain. These inhibitors, longer than the longest inhibitors which bind only in the active site, showed good inhibition activity and relatively stable pH profiles. Obvious disadvantages of such a design is the length and the peptidic character of the inhibitors, because it is not clear how to prevent their degradation by cellular enzymes.

All of the inhibitors reviewed here are reversible, although some of them bind very tightly. Another promising concept is to design irreversible inhibitors of HIV PR, able to bind covalently to the catalytic aspartates of the enzyme. Epoxide-containing, symmetric, irreversible inhibitors with high potency were a result (82). The principle of the design was to enable the attack by the catalytic water molecule on the epoxide ring and to create a covalent bond between the inhibitor and one of the catalytic aspartates. Because it is necessary to maintain the right orientation of the epoxide so that it could make favorable contacts with the two active-site aspartates, such a design is quite challenging.

It is difficult to provide general principles of inhibitor design at this time. Structural properties of the compounds are not the only consideration; ease of chemical synthesis, low molecular weight, bioavailability, and stability are also of crucial importance. The development of protease inhibitors as antiviral drugs has showed that it was necessary to keep in mind the conditions under

which the inhibition takes place, both in vitro and in vivo. The phenomenon of drug resistance will require preparation of new generations of drugs. Although the knowledge of structural principles of inhibition has been very important, it is still not completely clear why some of the compounds have been successful, while others have failed. Obviously, this field will continue its development, resulting in new, more powerful drugs.

ACKNOWLEDGMENTS

We wish to thank all the present and former members of the Macromolecular Structure Laboratory at the National Cancer Institute–Frederick Cancer Research and Development Center who contributed to these studies. We are indebted to Dr. Clare Sansom and Ms. Anne Arthur for their editorial comments. Research was sponsored in part by the National Cancer Institute, DHHS, under contract with ABL. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

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