JOURNAL OF
MEDICINAL
CHEMISTRY

Volume 34, Number 8 August 1991

Perspective

HIV Protease: A Novel Chemotherapeutic Target for AIDS

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Received January 14, 1991

The pandemic spread of acquired immunodeficiency syndrome (AIDS) has promoted an unprecedented scientific and clinical effort to understand and combat this lethal disease. The etiological agent of AIDS has been identified as a retrovirus of the *Lentiviridae* family.[1,2] Originally referred to as HTLV-III or LAV, this enveloped, single-stranded RNA virus is now designated human immunodeficiency virus (HIV),[3,4] and two genetically distinct subtypes, HIV-1 and HIV-2, have been characterized.[5-7] Infection by the virus, which targets monocytes expressing surface CD4 receptors, eventually produces profound defects in cell-mediated immunity.[8] Over time infection leads to severe depletion of $CD4^+$ T-lymphocytes resulting in opportunistic infections, neurologic and neoplastic disease, and ultimately death. Identification of the molecular events critical to virus replication has enabled the selection of several strategies for potential chemotherapeutic intervention.[9,10] Among those, blockade of the virally encoded protease has become a major target in the quest for an effective antiviral agent.

Identification as an Aspartyl Protease

On the basis of the virus' genomic sequence, Ratner et al. postulated that the second open reading frame of HIV-1 encoded a protease analogous to those of other retroviruses.[11] Toh[12] subsequently recognized that the highly conserved triad, Asp-Thr(Ser)-Gly, found in putative retroviral protease sequences was homologous to the catalytic site of proteases belonging to the aspartic acid family and proposed that the viral enzymes were of this class. Despite this notable sequence conservation, significant structural differences were nevertheless observed between retroviral and classical aspartyl proteases. Whereas fungal and mammalian enzymes of this class were generally comprised of more than 200 amino acids and consisted of two homologous domains,[13] retroviral proteases contained approximately half that number of residues. Furthermore, the key catalytic triad which occurs twice in archetypal aspartic acid proteases like pepsin and renin appears only once in the proposed retroviral proteases. These observations led Pearl and Taylor[14] to suggest that the catalytically competent form of retroviral proteases existed as a homodimer, with each monomer contributing one of the two conserved aspartates to the active site. They further

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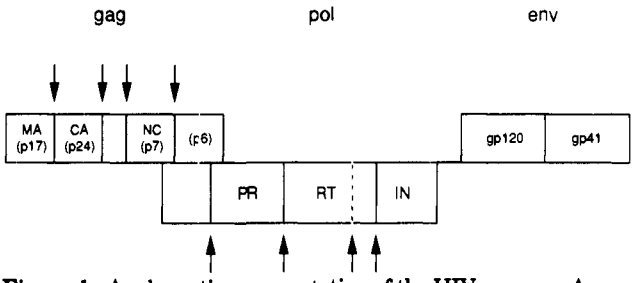

Figure 1. A schematic representation of the HIV genome. Arrows indicate junctions within the *gag-pol* region that are processed by the retroviral protease. The nomenclature of proteins is from ref 32.

proposed a three-dimensional model for HIV protease based on additional limited sequence homology and on known secondary structural features of classical aspartic proteases.

Three independent lines of evidence subsequently confirmed both the mechanistic classification and the dimeric nature of the enzyme. Site-directed mutagenesis of the putative active site aspartate completely abolished enzymatic activity.[15-17a] Moreover, HIV-1 protease was inhibited by the prototypical aspartyl protease inhibitor pepstatin A.[18-21] Ultimately, elucidation of the crystal structure of both native HIV protease[22-24] and HIV protease complexed with inhibitors[25-28] corroborated both the symmetrical dimeric architecture of HIV protease and its mechanistic class.

Proteolytic Processing in Viral Replication

The critical role of proteolytic processing in retroviral replication has been recently reviewed.[29-31] Although genetically more complex than other retroviruses, HIV expresses three genes common to all replication-competent members of the Retroviradae family (Figure 1).[11,29] The *gag* gene is translated as a 55 kDa fusion protein, $p55^{gag}$, from which the core structural proteins of the virion, MA (p17), CA (p24), NC (p7), and p6, are derived.[32] The *pol* gene contains the transcript for viral enzymes and is translated as a high molecular weight *gag-pol* polyprotein resulting from a ribosomal frameshift within *gag*.[33] The *gag-pol* precursor, $p160^{gag\text{-}pol}$, is processed to produce mature *gag* proteins, reverse transcriptase (RT), integrase (IN), and the protease (PR) itself.[34] Finally, the *env* gene encodes the surface glycoproteins of the virion, gp41 and gp120, which are responsible for binding to the target cell CD4 receptor. The virally encoded protease is responsible for the highly specific proteolytic processing of fusion proteins which arise from translation of the *gag* and *pol* genes. Prior to processing, the viral polyproteins are myristylated and aggregate at the cell membrane.[35-37] Concentration and alignment of precursor proteins at the membrane surface promote self-assembly and result in budding of virus particles. Newly budded virions appear to lack assembled nucleocapsid cores, suggesting that *gag* and *gag-pol* processing occurs during or subsequent to virion formation.[1,2,38,39]

Site-directed mutagenesis played an essential role in both characterizing HIV protease and in establishing its vital function in the maturation of virus particles. Earlier studies with murine lukemia virus showed that major deletions in the protease region of the *gag* gene resulted in noninfectious virus.[40] Kohl et al.[16] transfected SW480 human colon carcinoma cells with HIV-1 proviral DNA which incorporated a single-point mutation of the putative active site Asp25 to Asn25 in the protease coding sequence. This substitution abolished enzymatic activity of the protease in vitro and yielded mutant virions that contained unprocessed *gag* polyprotein and were unable to infect MT-4 lymphoid cells. The results of this and similar experiments[37,41] established the obligatory role of the viral

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protease in replication and demonstrated the inability of mammalian host cell proteases to subserve this essential task.

Preparation and Purification of HIV Protease

Isolation of HIV protease directly from virus particles afforded only small quantities of the pure enzyme and required manipulating large amounts of the deadly virus.[42] To provide sufficient enzyme for further biochemical characterization and inhibition studies, both total synthesis and recombinant techniques have been employed.

Schneider and Kent[43] synthesized an enzymatically active 99-residue protease based on the sequence from the SF2 strain of HIV-1. Nutt et al.[21] also prepared HIV protease by total synthesis, reproducing the sequence of the NY5 strain and demonstrated that refolding of the polypeptide yielded active enzyme that behaved as the expected dimer on size exclusion gels. Other syntheses of both HIV-1[44] and HIV-2[44,45] proteases have also been reported.

Several laboratories have cloned and expressed constructs containing the protease coding sequence within a larger transcript.[24,46-53] Expression of these extended constructs resulted in autoprocessing and accumulation of the mature 11-kDa form of the enzyme as characterized by SDS-PAGE. Constructs containing only the HIV-1[20,48,54] or HIV-2[55] protease sequence have also been cloned and expressed in yeast and insect cell culture.[56]

A variety of methods for purifying the enzyme have been reported; affinity chromatography appears to be particularly facile and efficient.[27,55,57]

Substrate Specificity

The substrate specificity of HIV protease represents a paradoxical situation in which the enzyme is required to make a number of highly specific cleavages with the *gag* and *gag-pol* polyproteins at sites spanning remarkably heterogeneous amino acid sequences. Despite considerable study, determinants of the observed substrate specificity remains undefined. Conformational factors as well as sequence appear to be important.[45,49] In an effort to further characterize the enzyme as well as to provide insight for inhibitor design, studies concerning specificity have been carried out with use of both protein substrates and smaller oligopeptides.

Although no consensus sequence for HIV protease has been deduced, the common occurrence of an AromaticPro line cleavage site in 19 retroviruses has been noted with particular interest since hydrolysis N-terminal to proline is unusual for mammalian endopeptidases.[58] Tyr-Pro or Phe-Pro comprise the P1-P1′ residues (notation of Schecter and Berger[59]) in three of the processing sites of HIV-1; however, the remaining cleavage sites show a remarkable variety of amino acids constituting the scissile bond: Leu-Ala, Leu-Phe, Met-Met, and Phe-Leu dipeptides are all found in HIV-1 cleavage sites.[60] Analysis of the proteolytic processing of HIV-1, HIV-2, and simian immunodeficiency virus led to a proposal that sequences flanking the cleavage site could be assigned to one of three classes.[61] Class 1 contains Phe-Pro or Tyr-Pro at P1 and P1′; class 2 sites have Arg at P4 and Phe-Leu at P1′-P2′; and class 3 sequences contain Gln or Glu at P2′.

Several studies have examined the issue of substrate specificity by using oligopeptides. Darke et al.[62] showed that both synthetic and recombinant HIV-1 protease was able to specifically cleave oligopeptides corresponding to all of the proposed processing sites in the *gag* and *pol* gene products. Seven residues spanning P4-P3′ were required for efficient and specific cleavage of the P1-P1′ amide bond.[46,62,63] These findings are in concert with crystallographic data suggesting multiple hydrogen bonds to the backbone of inhibitors spanning these subsites and close van der Waals contact for the P3-P3′ side chains.[25-27] The relative rates of cleavage for polypeptides corresponding

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to the natural cleavage sites may reflect a regulatory role.[64]

Results from studies of polypeptide hydrolysis have been recently reviewed.[65] Branched-chain amino acids such as Ile are preferred at the P2′ position in the peptide Ser-Gln-Asn-Tyr*Pro-X-Val (asterisk shows the position of scissile bond), followed by Leu and Ala.[66] Substitution with Phe and Gly resulted in poor substrates, and peptides containing Trp at P2′ were not cleaved. On the other hand, incorporation of Ile or Val at P1 in the substrate Lys-Ala-Arg-Val-X*Nph-Glu-Ala-Nle-NH_2 (Nph: 4-nitrophenylalanine) resulted in peptides refractory to hydrolysis.[67] Incorporation of Leu, Nle, Met, Phe, or Tyr gave rapidly processed substrates. Variation of P3, P2, and P1 residues in the chromogenic substrate Ala-Thr-His-Gln-Val-Tyr*Nph-Val-Arg-Lys-Ala was examined by Konvalinka et al.[68] Position P3 tolerated all substitutions except proline; P2 showed a distinct preference for β-branched amino acids (Val, Ile); and Met, Phe, or Tyr were preferred at P1. Despite the lack of an obvious consensus sequence, the nature and identity of residues flanking the P1-P1′ site clearly have considerable influence on a substrate's susceptibility toward hydrolysis.

The specificity of HIV protease has also been studied in recombinant proteins.[69] Random mutagenesis of the P1 and P1′ positions at three of the *gag-pol* cleavage sites produced proteins which varied widely in their ability to be processed.[70] Few or no changes were tolerated at the PR-RT and RT-IN junctions; however, the p6-PR site accepted a variety of changes. Replacement of the natural Phe-Pro with sequences as disparate as Gly-His and Ala-Ile resulted in processing equivalent to that of the wild-type sequence. Substitution of the HIV-2 protease coding sequence into the HIV-1 genome or similar substitution of HIV-1 protease into HIV-2 produced viral proteins which were processed in the expected manner.[71] The rate of proteolytic cleavage for these heterologous systems was reduced, however. Selective proteolysis of nonviral proteins by HIV-1 protease has also been demonstrated recently. Tomasselli observed enzymatically mediated hydrolysis of a truncated *Pseudomonas* exotoxin protein.[72] Proteolytic cleavage did not occur within the expected Leu-Glu-Arg-Asn-Tyr*Pro-Thr-Gly sequence, but rather at the nearby sequence of Ser-Gly-Asp-Ala-Leu-*Leu-Glu-Arg-Asn located within the interdomain region and at a Leu*Ala site near the N-terminus. Hydrolysis of oligopeptides spanning these sequences showed the same hydrolytic susceptibility as the corresponding protein

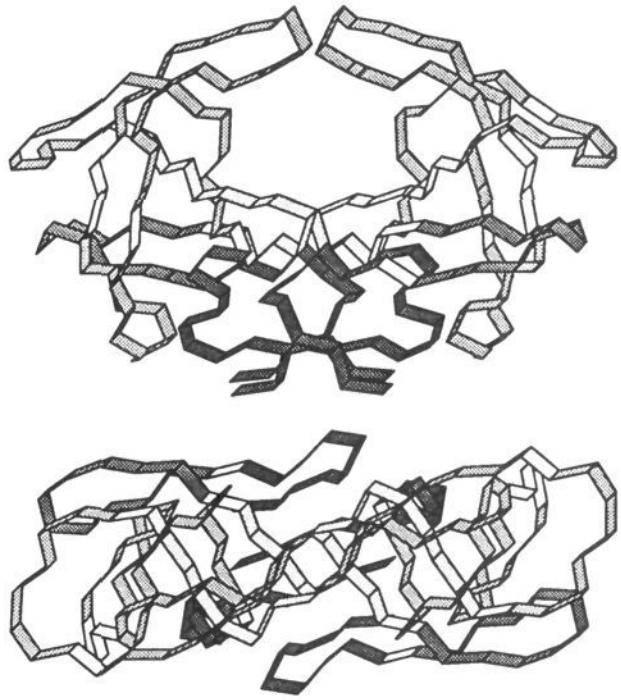

Figure 2. The structure of native HIV-1 protease drawn as a ribbon connecting the positions of α-carbons. The upper structure, in which the pseudo-2-fold axis relating one monomer to the other is vertical and in the plane of the page, represents a view along the substrate binding cleft. The lower structure is a topview with the pseudo-2-fold axis perpendicular to the page.

substrate, suggesting that sequence and accessibility play a dominant role in enzyme specificity in this case. The intermediate filament proteins vimentin, desmin, and glial fibrillary acidic protein were also cleaved by HIV-1 protease in vitro.[73] One of the vimentin cleavages occurred within the sequence Ser-Ser-Leu-Asn-Leu*Arg-Glu-Thr-Asn-Leu, constituting the first report of a charged amino acid at P1 or P1′. Denatured protein was processed less efficiently, in agreement with reports by Hansen et al.[49] that denatured proteins are generally poor substrates for retroviral proteases. Comparison of HIV-1 and HIV-2 protease specificity toward protein and oligopeptide substrates[74] suggested a preference by HIV-2 protease for smaller P1′ residues. Synthetic HIV-2 protease specifically cleaved HIV-1 $p55^{gag}$ at the predicted sites.[45] Recombinant HIV-2 protease processed myristylated $p55^{gag}$ and oligopeptides representing HIV-$1^{gag\text{-}pol}$ junctions with kinetics comparable to those of HIV-1 protease.[75] Similar kinetics and specificity by HIV-1 and HIV-2 proteases for a series of chromogenic substrates has also been reported.[76]

A variety of assays have been developed to permit analysis of substrate hydrolysis and inhibitor binding. Immunoblot assays have provided a highly sensitive, but qualitative, method for following the cleavage of protein

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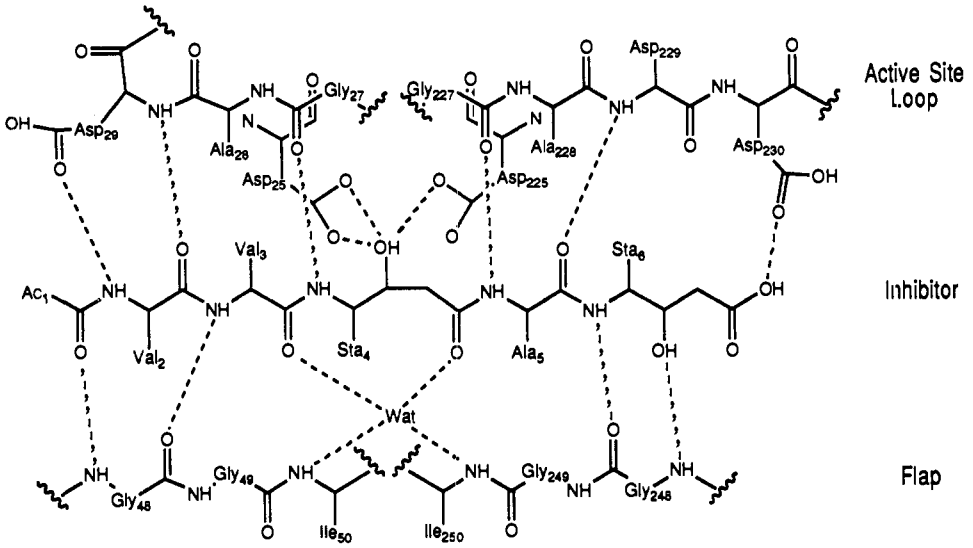

Figure 3. Hydrogen bonds between acetyl pepstatin and HIV-1 protease. The residues are labeled at the C-β position (C-α for glycine). The residues labeled 25–50 are from monomer A; those labeled 225–250 are from monomer B; and those labeled 1–6 are associated with acetyl pepstatin.

substrates.[17,43,47-49] Product separation and detection by HPLC has been employed in several studies. Both UV[21,62,64,77,78] and fluorometric[79] detection methods have been described. Although discontinuous and sometimes labor intensive, this method can provide extremely high sensitivity. Continuous spectrophotometric[67,80] and fluorescence-based[81,82] assays have also been reported.

Structure and Function of HIV Protease

Crystallography. The structure and function of retroviral proteases have been reviewed recently.[83] Determination of the crystal structure of native HIV-1 protease[22-24] confirmed the homodimeric architecture of the enzyme, with each monomer related to the other by a crytallographic 2-fold rotation (Figure 2). The protein contains the typical β-sheet structure found in both mammalian and fungal aspartic proteases,[84] although the viral protease has four rather than six interdigitated strands.[22] The active site triad Asp25-Thr26-Gly27 occurs within a loop that forms a number of hydrogen bonds with the corresponding loop of the opposite monomer, comprising the characteristic "fireman's grip" of aspartic proteases.[85] Close structural similarities were found between HIV-1, Rocus sarcoma virus (RSV), and archetypal fungal proteases.[86] The similarity was particularly striking when the active sites of the enzymes were superimposed. Modeling studies[87] based on the crystal structure of RSV protease correctly predicted features of the HIV-1 protease structure.[23]

A prominent β-hairpin loop from each monomer projects over the active site, enclosing the substrate binding cleft. By contrast, monomeric mammalian and fungal aspartic proteases have only one such "flap". The binding site of HIV protease is large enough to accommodate six or seven residues of the substrate, confirming previous predictions based on modeling studies.[87] Charged residues are observed at the ends of the binding cleft and appear to stabilize the dimeric form of the enzyme through coulombic interactions between opposing monomers.[23,24]

The crystal structures of enzyme–inhibitor complexes are of particular interest with respect to inhibitor design. Structures of the enzyme complexed with four structurally distinct inhibitors (**2**, **19**, **20**, **26**) have been reported.[25-28] Despite the diversity of inhibitor structures and differences in crystal symmetry, several common attributes of the inhibited complex have emerged. One feature is the close similarity of the protease structure in all four complexes. Only a slight reorganization of the enzyme's core is observed upon binding to an inhibitor. For example, comparison of the native structure with that of the acetyl pepstatin complex shows that 44 Cα core atoms of each monomer can be aligned with a root mean square deviation of less than 0.4 Å.[26] The portion of the enzyme which does show significant movement is the flap region, with the tip of the loops moving 7 Å. The observed root mean square difference for all protein Cα positions in the complexes with **20** and **26** is only 0.65 Å, despite the striking disparity of inhibitor structures.[27]

In their contracted conformation, the flaps form the top half of a pocketed hydrophobic tube extending approximately from P3 to P3′, thereby shielding about 80% of the bound inhibitor from surrounding solvent.[25] Unlike mammalian or fungal aspartic proteases which promote contact between bound inhibitors and residues extending

(77) Moore, M. L.; Bryan, W. M.; Fakhoury, S. A.; Maagard, V. W.; Huffman, W. F.; Dayton, B. D.; Meek, T. D.; Hyland, L.; Dreyer, G. B.; Metcalf, B. M.; Strickler, J. E.; Gorniak, J. G.; Debouck, C. *Biochem. Biophys. Res. Commun.* **1989**, *159*, 420–425.
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(87) Weber, I. T.; Miller, M.; Jaskólski, M.; Leis, J.; Skalka, A. M.; Wlodawer, A. *Science* **1989**, *243*, 928–931.

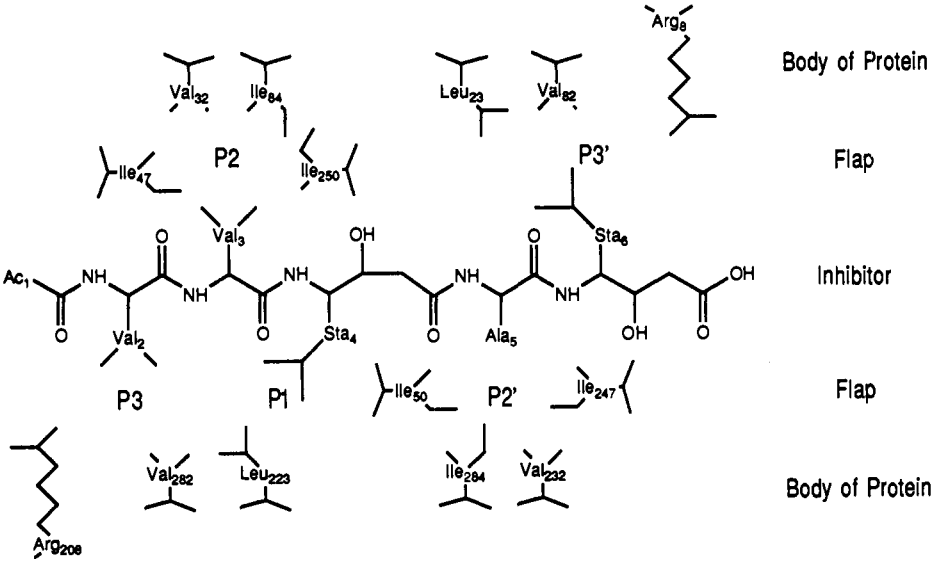

Figure 4. Nonbonded contacts between acetyl pepstatin and HIV-1 protease are comprised of hydrophobic residues from the core of the protein and flaps. The residues labeled 8–84 are from monomer A; those labeled 208–284 are from monomer B; and those labeled 1–6 are associated with acetyl pepstatin.

from the face of their single flap,[88-92] HIV protease makes contact with inhibitors along the edge of each of its two flaps.[25-28] One striking feature of all four inhibitor complexes is a tightly bound water molecule that bridges the two enzyme flaps to the inhibitor through hydrogen bonds formed by the Ile^{50} and Ile^{250} amide hydrogens and the P2 and P1′ carbonyl oxygens of the inhibitor (Figure 3). The regular occurrence of this structural detail in all characterized enzyme–inhibitor complexes has provocative implications for inhibitor design.

All four inhibitors are bound in an extended conformation, spanning P4-P3′. An extensive network of hydrogen bonds is observed between the enzyme and polar atoms in the inhibitor (Figure 3). These hydrogen bonds are formed primarily with backbone atoms of the floor and flap region of HIV protease. No apparent interaction is observed between the active site aspartic acid residues and the nearby amine incorporated into reduced dipeptide or hydroxyethylamine isostere inhibitors.[25,28,93] The hydrophobic walls of the binding cleft show extensive van der Waals contacts with the side chains of inhibitors. The binding pockets discernable from P2 through P2′ are comprised almost entirely of hydrophobic residues in the enzyme[25-27] (Figure 4).

Mutagenesis Studies. Site-directed mutagenesis has provided complementary information regarding the structure and function of HIV-1 protease. Substitution of the putative catalytic aspartic acid residue by Ala,[15] Asn,[16] or Thr[17a] rendered the enzyme inactive, supporting the classification of HIV protease as a member of the aspartic acid protease family. Each residue within the enzyme was substituted by using recombinant techniques, and the effects were related to the protein's structure. Loeb et al. examined the effects of 330 separate single-site mutations in HIV-1 protease.[70,94] Three regions proved to be particularly sensitive to sequence changes.[94] Ala22-Leu33 comprised the sequence surrounding the active site and includes Thr26 which contributes to dimer stabilization. The second region included the flap residues Ile47-Gly52. Residues in these first two regions form an extensive network of hydrogen bonds with bound inhibitors and presumably with substrates. The third region included residues Thr74-Arg87. Many of these residues participate in shaping the substrate binding sites. Molecular modeling studies[95] and analysis of X-ray diffraction data[24] suggested that Arg87 also plays a key role in dimer stabilization. Selective modification of Arg87 and Asn88 destabilized the dimeric form of the protease and led to inactivation of the enzyme.[37,95-97]

Inhibitors

Following Kramer's proposal that inhibition of HIV protease represented a viable strategy for developing antiviral agents,[98] a significant effort to identify potent inhibitors has ensued. One of the classical strategies for designing enzyme inhibitors relies on incorporating a transition-state mimic into substrate analogues. Refinements of this ploy, in which nonhydrolyzable dipeptide isosteres were substituted for the scissile amide bond in an appropriate sequence context, proved highly successful for producing potent renin inhibitors[99] (Figure 5). Dreyer et al.[100] compared the effectiveness of five such dipeptide

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(100) Dreyer, G. B.; Metcalf, B. W.; Tomaszek, T. A., Jr.; Carr, T. J.; Chandler, A. C., III; Hyland, L.; Fakhoury, S. A.; Magaard, V. W.; Moore, M. L.; Strickler, J. E.; DeBouck, C.; Meek, T. D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9752–9756.

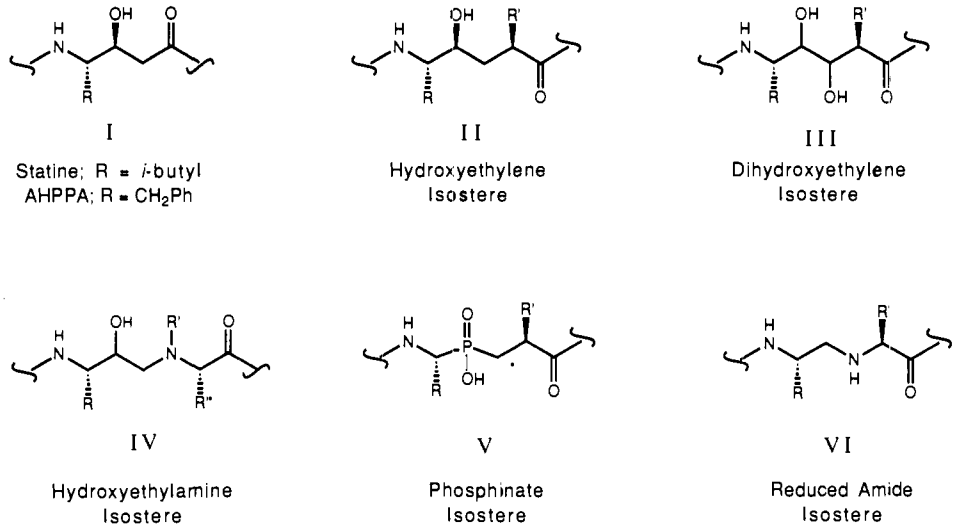


Figure 5. Hydrolytically stable dipeptide isostere replacements for the scissile bond in aspartyl protease substrates. The analogues mimic the geometry of the tetrahedral intermediate in peptide hydrolysis.

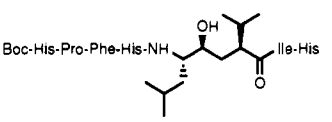
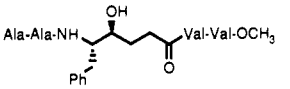
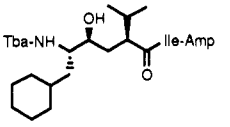
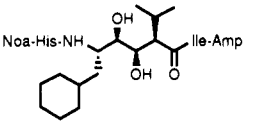
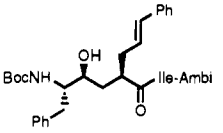
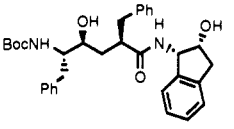
Table I. Inhibitors Based on Statine-like Dipeptide Isosteres

no.	compound	K_i, nM	ref
1	Iva-Val-Val-Sta-Ala-Sta (pepstatin A)	400–1400	21, 64, 79, 100, 20
2	Ac-Val-Val-Sta-Ala-Sta (acetyl pepstatin)	20	19
3	Ser-Ala-Ala-AHPPA-Val-Val-OCH_3	1200	100
4	Ac-Ser-Gln-Asn-AHPPA-Val-Val-NH_2	39000	77

isosteres inserted into a common heptapeptide template spanning P4-P3′. Inhibitors incorporating the hydroxyethylene dipeptide isostere (II) showed the greatest affinity for HIV-1 protease; difluoroketones and statine-based inhibitors (I) showed moderate to low potency; and phosphinate (V) isosteres exhibited only modest affinity. Inhibitors incorporating reduced-peptide (VI) transition-state mimics were weak inhibitors. These trends are consistent with similar comparisons.[46,77,101]

The natural product pepstatin A (1)[102] is considered a typecasting inhibitor of aspartic acid proteases. The unusual amino acid, statine, which occurs twice within this inhibitor, embodies transition-state analogue I (Figure 5). Katoh[103] demonstrated that 1 blocked the proteolytic action of several retroviral proteases, and subsequent studies showed that 1 also inhibited hydrolysis of both polyprotein and oligopeptide substrates by HIV-1 protease.[18-21] Inhibition of viral replication in cell culture by high concentrations of 1 (100 μM) has also been reported.[104] Concentrations of 1 required to inhibit HIV-1 protease were significantly higher than those required to block many of the monomeric aspartic acid proteases; IC_{50} values ranging from 0.4 to 1.4 μM have been reported.[20,21,64,79] Richards et al. observed that acetyl pepstatin (2) is significantly more potent (K_i = 20 nM) against HIV-1 protease than 1.[19] Moreover, 2 inhibited HIV-2 protease with a K_i value of 5 nM.[105] Some representative examples of inhibitors incorporating transition-state mimic I are listed in Table I.

Table II. Inhibitors Based on Hydroxyethylene Dipeptide Isosteres

no.	compound	K_i, nM	ref
5		5	19
6		18	100
7		70	108
8		<1	109
9		0.03 (IC_{50})	110
10		0.3 (IC_{50})	111

Introduction of hydroxyethylene transition-state analogues (II) into inhibitors provided compounds with high affinity for the enzyme (Table II). H-261 (5) is a nonspecific aspartic acid protease inhibitor[106] that mimics the cleavage sequence of the renin substrate angiotensinogen (Leu-Val). Inhibition of HIV-1 (K_i = 5 nM[19]) and HIV-2 (K_i = 35 nM[105]) protease was observed in vitro with this pseudo-nonapeptide. Hexa- and heptapeptide analogues containing a Phe-Gly hydroxyethylene transition-state mimic, e.g., 6,[100] effectively inhibited HIV-1 protease in vitro (K_i = 18–180 nM). These compounds also blocked

(101) Tomaselli, A. G.; Olsen, M. K.; Hui, J. O.; Staples, D. J.; Sawyer, T. K.; Heinrikson, R. L.; Tomich, C.-S. C. *Biochemistry* **1990**, *29*, 264.
(102) Umezawa, H.; Aoyagi, T.; Morishima, H.; Matsuzaki, M.; Hamada, M.; Takeuchi, T. *J. Antibiot.* **1970**, *23*, 259–262.
(103) Katoh, I.; Yasunaga, T.; Ikawa, Y.; Yoshinaka, Y. *Nature* **1987**, *329*, 654–656.
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(105) Richards, A. D.; Broadhurst, A.; Ritchie, A. J.; Dunn, B. M.; Kay, J. *FEBS Lett.* **1989**, *253*, 214–216.
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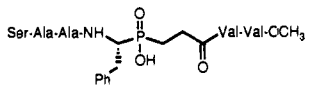
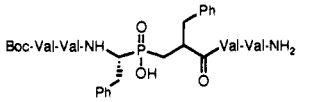
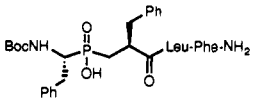
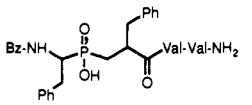
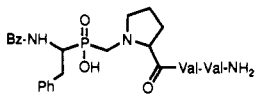
$p55^{gag}$ and $p160^{gag\text{-}pol}$ processing in chronically infected T-lymphocyte cultures and prevented the spread of HIV infection in susceptible lymphoid cell lines at 25–100 μM.[107] McQuade et al.[108] described a pentapeptide analogue incorporating the cyclohexylalanine-Val hydroxyethylene isostere, U-81749 (7), which inhibited HIV-1 protease in vitro with a K_i of 70 nM. Antiviral activity of this compound was assessed in primary cultures of peripheral blood lymphocytes (PBL). Addition of 1 μM 7, a level determined to be nontoxic to PBL proliferation, to the culture medium immediately after virus innoculation reduced the levels of mature p24 by 70% at three or four days post infection (determined by ELISA) compared with p24 levels in the supernatants of control, infected cells.

A similar series of hexapeptide analogues containing the dihydroxyethylene isostere (III) of cyclohexylalanine-Val was reported to potently inhibit HIV-1 protease.[109] One member of that series, U-75875 (8), (K_i < 1 nM), was examined for antiviral activity in cells. Concentrations of 1 μM 8 completely blocked the spread of $HIV\text{-}1_{LAV}$ infection in primary cultures of peripheral blood mononuclear cells for six days as judged by supernatant levels of RT and p24. The potency of 8 in these cells was equivalent to that of 3′-azido-3′-deoxythymidine (AZT). Longer term experiments were carried out in CEXx174 cells infected with $HIV\text{-}1_{LAV}$. In the presence of 1 μM 8, no spread of viral infection within the culture occurred over a 4-week period. With regard to antiviral mechanism, the studies demonstrated that 1 μM 8 blocked processing of $p55^{gag}$ to p24 in H9 cells chronically infected with $HIV\text{-}1_{IIIB}$. The virus-like particles found in supernatants of cells cultured with 8 exhibited an immature morphology by electron microscopy and were noninfectious.

The hydroxyethylene isostere was also used successfully by Vacca et al.[110] to generate highly potent inhibitors of HIV-1 protease. The markedly hydrophobic inhibitor 9 exhibited an IC_{50} value of 30 pM against the enzyme in vitro and completely blocked the spread of $HIV\text{-}1_{IIIb}$ in H-9 cells for 14 days at a concentration of 12 nM (determined by immunofluorescence). β-Branched residues which were expected to stabilize an extended conformation, enhanced potency when incorporated at the P2′ position. Further modification in this series by Lyle et al.[111] resulted in the development of 1-amino-2-hydroxyindan as an effective surrogate for the P2′ residue and allowed development of subnanomolar inhibitors that span only the P2-P2′ subsites. The hydroxyl group in the indan appears to replace the P2′ carbonyl oxygen in a critical hydrogen bond to the enzyme. One analogue from this series, 10, showed high intrinsic potency (IC_{50} = 0.3 nM) and blocked the spread of $HIV\text{-}1_{IIIb}$ in H-9 cells (IC_{100} = 200 nM by immunofluorescence).

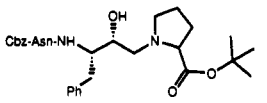
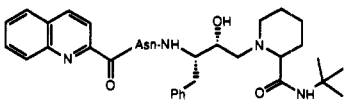
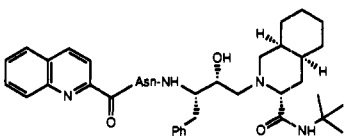
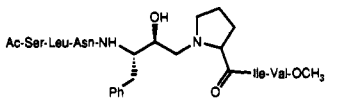
Phosphinic acid dipeptide isosteres (V) have also been adapted to serve as transition-state analogues (Table III).

Table III. Inhibitors Based on Phosphinate Dipeptide Isosteres

no.	compound	K_i, nM	ref
11		4400	100
12		0.04[a]	112
13		9 (IC_{50})	122
14		0.6[a]	112
15		450[a]	112

[a] Assayed at pH 4.5.

Table IV. Inhibitors Based on the Hydroxytheylamine Dipeptide Isostere

no.	compound	K_i, nM	ref
16		140 (IC_{50})	113
17		2 (IC_{50})	113
18		<0.4 (IC_{50})	113
19		0.66	115

Grobelny et al.[112] demonstrated that tetrahedral phosphinates incorporated into peptides capable of minimally spanning P2-P3′ function effectively in vitro as highly potent inhibitors of HIV-1 and HIV-2 proteases. Affinity for the enzyme was quite sensitive to substitution at the P1 and P1′ sites with the Phe-Phe isostere 14 providing superiod binding (K_i = 0.6 nM). Phe-Pro isosteres were poor inhibitors, confirming earlier reports.[100] The binding affinity of phosphinate-based inhibitors was also sensitive to pH; a 500-fold increase in affinity was observed for some compounds as the pH was lowered from 6.5 to 4.5, thereby neutralizing the charged phosphinate.

Consideration of the unusual ability of HIV protease to cleave substrates N-terminal to proline led Roberts et al.[113] to design a series of inhibitors adapting the hydroxyethylamine dipeptide isostere (IV) to mimic the Phe-Pro site (Table IV). Selectivity for retroviral proteases was predicted for inhibitors incorporating this feature. As previously noted for renin inhibitors containing the hydroxyethylamine isostere,[114] the preferred absolute con-

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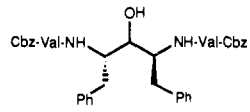
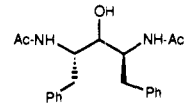
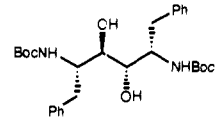
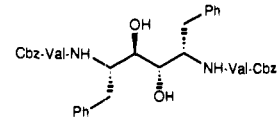
Table V. Symmetrical Inhibitors

no.	compound	IC_{50}, nM	ref
20		3.0	27, 116
21		>10,000	116
22		12	116
23		0.2	116

figuration at the hydroxyl-bearing carbon is (*R*). One of these inhibitors, Ro 31-8959 (18), blocked both HIV-1 and HIV-2 enzymes with a K_i value ≤ 0.12 nM and exhibited antiviral activity in HIV-1_{RF} infected C8166 cells (IC_{50} = 2 nM by p24 determination) and in HIV-1_{GB8} infected JM cells (IC_{50} = 2.5 nM by syncitia formation). A significant increase in potency was realized by converting the initial proline substructure to a decahydroisoquinoline nucleus which may be regarded as a conformationally constrained hydroxyethylamine mimic of Phe-cyclohexylalanine. Expectations of high selectivity for retroviral proteases by inhibitors of this class were realized. Less than 50% inhibition was observed for the human aspartic acid proteases renin, pepsin, gastricsin, cathepsin D, and cathepsin E at concentrations of 10 μM. Hydroxyethylamine dipeptide mimics of Phe-Pro also were exploited by Rich et al.[115] in the design of potent inhibitors of HIV-1 protease. Maximum affinity was achieved with peptide analogues spanning P4-P3′, with one inhibitor, **19**, exhibiting a K_i value of 0.66 nM.

In contrast to inhibitors embodying traditional transition-state analogues, two novel classes of protease inhibitors were reported by Kempf et al.[27,116] that capitalized on the unique C_2 symmetry of the homodimeric enzyme (Table V). Pseudosymmetric inhibitor **20**, spanning P3-P3′, blocked the enzyme with an IC_{50} value of 3 nM. Symmetric inhibitor **23** was more potent with IC_{50} values of <0.4 nM. Surprisingly, the affinity of **23** was not highly sensitive to the stereochemistry of the two hydroxyl groups, in dramatic contrast to inhibitors based on traditional transition-state analogues.[99] These inhibitors blocked replication of HIV-1_{IIIB} in H9 and MT4 cells as judged by either p24 levels or cytopathic effects (IC_{50} = 20–150 nM). The compounds showed little or no toxicity (TC_{50} = 10 to >100 μM) in these cell lines.

Table VI. Nonpeptide Inhibitors of HIV Protease

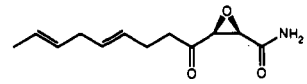
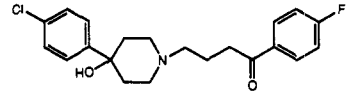
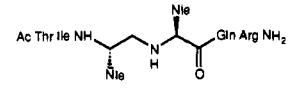
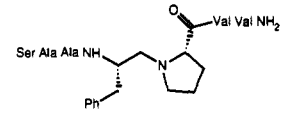
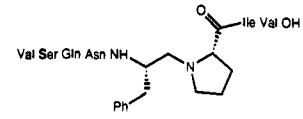
no.	compound	K_i, μM	ref
24		2,500 (IC_{50})	119
25		100	120

Table VII. Inhibitors Based on the Reduced Peptide Isostere

no.	compound	IC_{50}, μM	ref
26		0.78	25
27		19	100
28		3.5	101

Few nonpeptide inhibitors of HIV protease have been reported (Table VI). The antifungal antibiotic cerulenin (24) did effect weak inhibition of HIV-1 and HIV-2 proteases in vitro (IC_{50} = 2.5 mM).[117-119] Data from preincubation experiments supported the proposal that the epoxy amide slowly inactivated the enzyme, presumably by esterification of a catalytic aspartate residue through epoxide opening.[119] More recently, DesJarlais et al.[120] reported that haloperidol (**25**) identified by means of a structure-based, computer-assisted search of the Cambridge Crystallographic Database, inhibited HIV-1 and HIV-2 proteases in a concentration dependent manner with a K_i = 100 μM. The search algorithm employed quantitatively evaluated the goodness of fit for a series of structures docked into the active site of the enzyme. The active site, in turn, was based on crystallographically determined coordinates. Analysis of X-ray data regarding subunit interactions that stabilize the dimeric form of the enzyme prompted the suggestion of dimer disruption as a novel strategy for protease inhibition.[95]

Conclusion

Remarkable progress has been made with respect to establishing the HIV-encoded protease as viable target for chemotherapeutic intervention and toward developing potent inhibitors. Hydrophobic interactions appear to dominate inhibitor–enzyme binding, a conclusion consistent with structural data from crystallographic studies of enzyme–inhibitor complexes. Sequence appears less crucial, and the optimum residues for an inhibitor need not resemble substrate sequences. Of the classical tran-

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sition-state mimics for aspartic acid proteases, the hydroxyethylene, dihydroxyethylene, and hydroxyethylamine isosteres appear to provide the greatest intrinsic affinity for the enzyme. A number of inhibitors from these structural classes show high specificity for HIV protease in comparison to other aspartic acid proteases.

A variety of highly potent and specific inhibitors of the viral protease are therefore available. Moreover, several of these molecules strongly inhibit viral replication in cell culture at nanomolar concentrations. The chief remaining challenge in transforming such molecules into effective therapeutic agents lies in overcoming deficiencies in a metabolism, distribution, and oral bioavailability. The fact that the more potent inhibitors are largely peptide in character presents a significant obstacle to this goal.[121]

One of the intriguing circumstances in this area has been the relatively early availability of high resolution structural information. The possibility of leapfrogging classical inhibitor design and the resulting peptoid class of inhibitors is appealing. Some efforts are already apparent. Although still peptide in nature, the symmetrical inhibitors reported by Kempf et al. were based on analysis of the enzyme's unique architecture. The discovery that haloperidol weakly inhibits HIV protease resulted directly from computer-assisted analysis of potential protein–ligand interactions. Strong efforts have been mounted in several laboratories to exploit this rare opportunity to test current strategies for de novo drug design and to develop novel classes of inhibitors.

Acknowledgment. The valuable assistance of Beverly Schaefer in the preparation of this manuscript is gratefully acknowledged, as is the provision of artwork by Paula M. D. Fitzgerald.

Registry No. Aspartic proteinase, 78169-47-8.

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Articles

Cholinergic Activity of Acetylenic Imidazoles and Related Compounds

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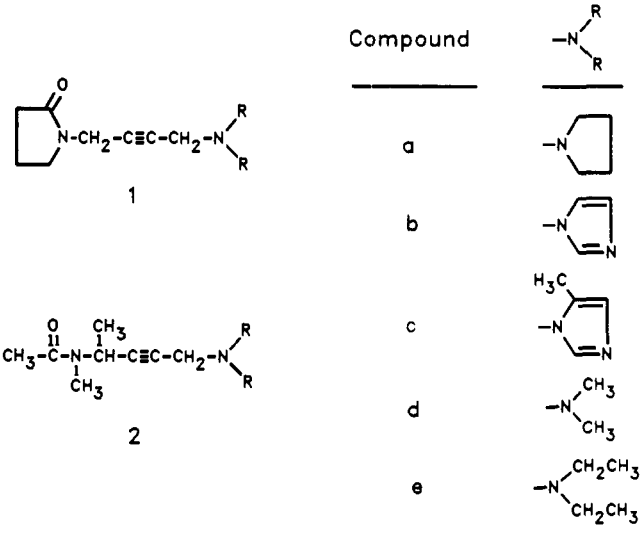
A series of acetylenic imidazoles related to oxotremorine (**1a**) were prepared and evaluated as cholinergic agents with in vitro binding assays and in vivo pharmacological tests in mice. 1-[4-(1*H*-Imidazol-1-yl)-2-butynyl]-2-pyrrolidinone (**1b**) was a cholinergic agonist with one-half the potency of oxotremorine. Analogues of **1b** with a 5- or 2-methyl substituent in the imidazole ring (compounds **1c** and **1g**) were cholinergic partial agonists. Analogues of **1b** with a methyl substituent at the 5-position in the pyrrolidinone ring (**7b**) or at the α-position in the acetylenic chain (**8b**) were antagonists. Various analogues of these imidazole acetylenes where the pyrrolidinone ring was replaced by an amide, carbamate, or urea residue were prepared. Several compounds which contained 5-methylimidazole as the amine substituent were partial agonists. The activities of the imidazole compounds are compared with those of the related pyrrolidine and dimethylamine analogues. Agonist and antagonist conformations for these compounds at muscarinic receptors are proposed.

Introduction

There has been considerable interest recently in the development of cholinergic agonists and partial agonists for the treatment of Alzheimer's disease or as cognition activators.[1] As part of a program in this area we have prepared a series of compounds related to oxotremorine (**1a**). Our major interest in this work was the preparation of cholinergic partial agonists in the belief that such a compound would selectively ameliorate the cholinergic deficits characteristic of Alzheimer's disease without producing excessive cholinergic side effects such as tremor, salivation, or lachrimation which may be associated with a full cholinergic agonist. In this paper we describe the synthesis of various acetylenic amines and their evaluation as cholinergic agents in binding assays and in in vivo pharmacological tests in mice.

Oxotremorine is a potent, centrally active, cholinergic agonist. Since its discovery in 1961,[2] many related acetylenic amines have been described. It has been shown



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