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INHIBITORS OF HIV-1 PROTEASE

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The human immunodeficiency virus (HIV), the etiological agent for the acquired immune deficiency syndrome (AIDS), is a retrovirus which makes use of a virally-encoded aspartic protease to perform specific proteolytic processing of two of its gene products in order to form active enzymes and structural proteins within the mature virion. Accordingly, specific, exogenous inhibition of the HIV-1 protease is thought to be a viable approach for the development of novel therapeutics for the treatment of AIDS. Indeed, this hypothesis has been validated in virally-infected cell culture with synthetic inhibitors of HIV-1 protease. This chapter reviews the current status of the development of inhibitors of this enzyme.

KEY WORDS: retroviral protease, human immunodeficiency virus (HIV), inhibitor, transition state analogue, peptide, anti-viral agent, isostere, polyprotein.

1. INTRODUCTION

The rapid emergence in the last decade of the acquired immune deficiency syndrome (AIDS) as a health threat of global significance has fostered extensive research of its etiological agent, the human immunodeficiency virus (HIV),[1] in order to develop effective therapies. Since its discovery in the mid-1980's, the current understanding of the expression and function of the various genes of HIV, its life cycle in infected human blood cells, and its pathological course in infected animals has very quickly reached a high level of sophistication. From this, several of the gene products of HIV, especially reverse transcriptase, have become popular targets for chemotherapeutic intervention, for which inhibitors or inactivators have been discovered and investigated to variable extents as potential drugs. The most conspicuously successful of these is azidothymidine, a pro-drug form of a potent inactivator of reverse transcriptase, which is presently in clinical use for the treatment of AIDS.[2-4] Despite its current widespread use, azidothymidine should be thought of as a "first generation" drug for the treatment of AIDS, in that its benefits may be limited to increasing the survival of AIDS patients, improving their quality of life, and delaying the onset of disease in asymptomatic patients.[5-7] At present, strains of HIV resistant to azidothymidine are now emerging in a clinical setting.[8] Armed with a more complete understanding of the biochemistry of HIV replication, one can envision targetting facets of the viral life cycle, other than reverse transcription, with the hope of developing new chemotherapeutic agents which would augment available therapies.[9]

By its nature, HIV infection and its pathological consequences provide extraordinary challenges for the development of effective therapies for the treatment of AIDS: (1) Since the virus becomes stably integrated into the genome of its human host and can apparently be expressed in an infectious form from stricken cells in a continuous manner (as described below), HIV infection constitutes a chronic disease requiring that drugs be safe and effective enough for a life-long course of treatment.

(2) In addition, the virus's heavy reliance on the normal replicative functions of the cell for its own expression and assembly underscores the paucity of exploitable and isolable molecular targets that are unique to the virus. (3) The opportunistic diseases which ultimately result from HIV-induced cytopathology of the cells of the immune system, such as *Pneumocystis carinii* pneumonia, are a secondary consequence of HIV infection and require independent treatment. Despite these strictures, a detailed examination of the replication cycle of HIV as briefly outlined below reveals new and unique viral molecular targets, other than reverse transcriptase, for which specific chemical agents can be designed to block their action, and the effects of this blockade on the infectivity of HIV in infected cell culture may be assessed. One such molecular target is the retroviral protease of HIV.[10]

2. ROLE OF HIV-1 PROTEASE IN THE VIRAL LIFE CYCLE

HIV, a lentivirus, is a member of the family of retroviruses, *Retroviridae*.[1,11–14] Two related and pathogenic strains of the virus have now been identified, denoted HIV-1[11–14] and HIV-2,[15] with the former being the pathogen of greater consequence. The genome of the retroviruses is contained within a dimer of single-stranded RNA chains, and in the case of HIV, each chain is composed of 9.7-kilobases.[11–14] The replication cycle of HIV and other retroviruses within infected host cells can be divided into two phases: the pre-integration and post-integration phases. The pre-integration phase is carried out almost exclusively by enzymes and proteins encoded within the infecting virion, while a number of steps in the post-integration phase, in which viral genes are expressed and new virions are assembled, are performed by cellular enzymes.

The primary cell type in which HIV-1 replicates is the human $T4^+$-lymphocyte.[16–18] (1) The pre-integration phase is initiated when a virion particle attaches to the surface of the lymphocyte by binding of its viral envelope protein gp120 to the extracellular portion of the receptor protein T4.[19–21] The membrane portion of the envelope then fuses to the cellular membrane, followed by uptake of the virion within the cell and its subsequent uncoating. (2) Disintegration of the virion capsid releases the viral RNA genome, the nucleocapsid proteins, and retroviral enzymes into the cytoplasm. These viral components, which comprise three enzymes which are products of the HIV-1 *pol* reading frame (Figure 1), are by themselves sufficient to permanently install the HIV-1 genome into the host cell without apparent assistance from cellular enzymes.[22] A double-stranded cDNA copy of the viral RNA genome is made by the action of two retroviral enzymes, reverse transcriptase ($RT^{[54]}$) and ribonuclease H (RN), which are contained within a single, heterodimeric, bifunctional protein. (3) The viral cDNA then translocates to the nucleus, and the action of a third viral enzyme, endonuclease (integrase; IN) results in the stable integration of the cDNA viral genome into chromosomal DNA to establish the provirus, a permanent presence of the retrovirus in the lymphocyte, thereby completing the pre-integration phase. Subsequent activation and expression of these proviral genes may occur repetitively during the lifespan of this chronically-infected cell.

In the post-integration phase of the HIV-1 replication cycle, cellular enzymes perform the expression of the stably-integrated proviral genes. (1) Transcription and translation of HIV-1 provirus is subject to complex regulation by both *cis*-acting elements within the DNA genome and by several *trans*-acting viral proteins, such as *tat*[23–25] and *rev*,[26,27] which are unique to HIV. (2) An RNA transcript of the provirus

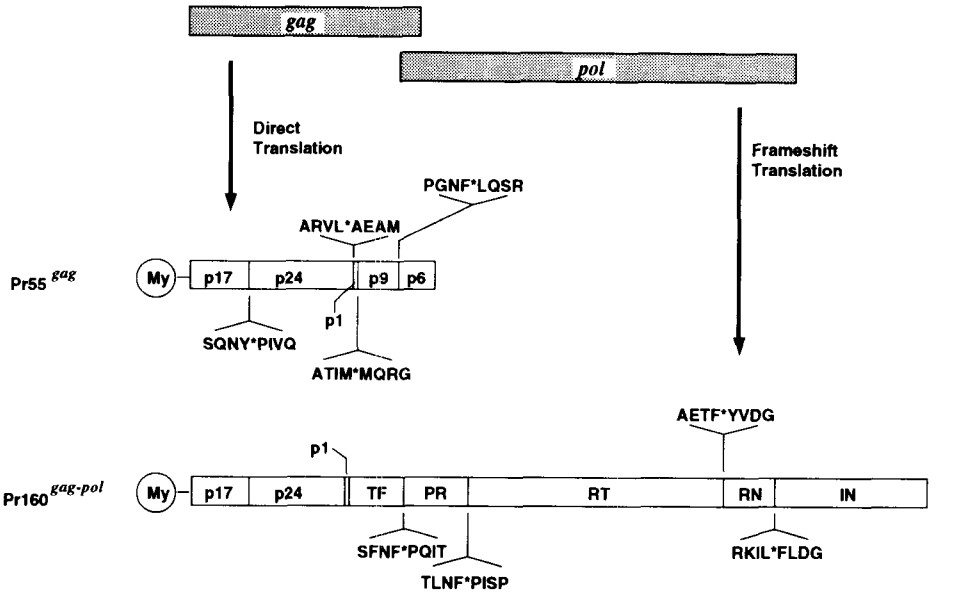


FIGURE 1 The overlapping *gag* and *pol* open reading frames of HIV-1 (stippled boxes), their polyprotein translation products $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$, and the proteolytic cleavage sites are indicated.[11-14,34-44] The sequences within the initial translation products which are processed by HIV-1 protease are shown, in which the cleavage is represented by a vertical line in the polyprotein and by * in the amino acid sequence in one-letter code. The *gag* protein occurring between the L*A and M*M cleavage sites consists of 14 amino acids and is designated "p1" in the text. The amino termini of both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ are myristoylated (My). The 66-kDa and 51-kDa subunits of reverse transcriptase are both within the region designated, the associated ribonuclease H (RN)[154] activity is contained within the carboxyl-terminal region of the 66-kDa subunit.

is synthesized by the cellular RNA polymerase II, followed by its post-transcriptional processing. This primary transcript is not only the source of mRNA from which viral proteins are made, but also serves as the genomic RNA which is ultimately packaged into new virions. Translation of the proviral mRNA results in the synthesis of viral structural proteins and enzymes which exist in immature form within polyproteins.[28] (3) Post-translational modifications of the proviral gene products, as performed by cellular enzymes, include glycosylation of the envelope proteins and myristoylation[29] of the N-termini of the viral polyproteins. (4) Virions are initially assembled proximal to the cell membrane as "immature" particles, composed of the external glycoprotein envelope, the genomic RNA and viral polyproteins found beneath the cell membrane, and begin to form and bud from the cell. (5) Maturation of fully-formed "immature" virion particles which have budded from the cell is effected by the action of the fourth virially-encoded enzyme, the retroviral protease (PR).[10,30-33] The protease specifically cleaves the encapsulated viral polyproteins into the functional enzymes and structural proteins of the virion core. The processing of the polyproteins inside the virion particle completes the replication cycle of HIV-1. The resulting mature virion particles are now able to promote a new infection in an adjacent T-lymphocyte.

Although more complicated than most retroviruses, the overall genomic organization of HIV-1 is common to that of other retroviruses, 5′-*gag-pol-env*-3′.[11-14] The

proteins which ultimately comprise the virion core and the enzymes essential to viral replication are the respective products of the *gag* and *pol* genes. HIV-1 protease catalyzes the processing of these proteins from two polyprotein substrates which are the initial products of these genes, $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ (Figure 1). The latter protein is a fusion product resulting from a frameshift translation within the overlapping *gag* and *pol* genes.[34] $Pr55^{gag}$ is a 55-kD polyprotein which contains the unprocessed forms of the virion core proteins in a single polypeptide chain, arranged as H_2N-p17-p24-p1-p9(p7)-p6-COOH (Figure 1).[11,12,35–38] The eventual proteolytic processing of $Pr55^{gag}$ generates the *gag* proteins, each of which has a specific role in the fully-formed virion. The p17, or matrix (MA) protein, constitutes the membrane-associated external shell of the virion core; the virion capsid itself is composed of the capsid protein p24 (CA); and in the capsid, the nucleocapsid protein, NC, (p9 or p7) and possibly p6 is associated with the genomic RNA. Within the $Pr55^{gag}$ precursor, p9 (NC) is presumably responsible for sequestering the RNA into the forming virion.[28]

The second polyprotein substrate of HIV-1 protease, $Pr160^{gag\text{-}pol}$, is a 160 kD polypeptide fusion product which results from an occasional translational frameshift from the 3′-end of the *gag* reading frame into the overlapping -1 reading frame of *pol*.[34] The frameshift results in the evasion of the termination codon of *gag*. $Pr160^{gag\text{-}pol}$ not only contains the precursor forms of p17, p24, and p9, but also the protease (PR), reverse transcriptase-ribonuclease (RT-RN) and endonuclease (integrase, IN) in the sequence, H_2N-p17-p24-p9-TF-PR-RT-RN-IN-COOH in which TF denotes a "transframe" protein of unknown function (Figure 1).[39–42] This frameshifting occurs with an efficiency of 11%, resulting in a ratio of $Pr55^{gag}$ to $Pr160^{gag\text{-}pol}$ of 8 : 1.[34]

The assembly of new virions from the expressed proviral genes within an infected cell proceeds at the cellular membrane following the synthesis of the two polyprotein substrates (Figure 2). By virtue of the translation strategy, $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ both have glycinyl residues at their N-termini. This amino acid is recognized as a specific site for myristoylation, and so both polyproteins are post-translationally modified to contain a covalent myristoyl (tetradecanoyl) substituent at their N-termini.[29,43,44] Myristolylation of the retroviral polyproteins is essential for their proper assembly into virion particles,[43–45] presumably because this lipid substituent directs the *gag* and *gag-pol* polyproteins to the cellular membrane, and allows these polyproteins to "anchor" their N-termini into the lipid bilayer (Figure 2).[46] As they concentrate, these lipid-embedded polyproteins collect into a crescent shape and begin to bud from the cell beneath the viral envelope,[46] eventually forming a complete, spherical virion particle which contains an annular core composed of the polyproteins. Retroviral particles of this type are of "immature" morphology[46] and are unable to infect cells (Figure 2).[47–50]

At some point during the detachment of the immature, assembled virion, HIV-1 protease becomes activated and catalyzes its own cleavage from $Pr160^{gag\text{-}pol}$. The protease then specifically cleaves $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ at discrete sites (Figure 1) to release and activate the structural proteins and enzymes, thereby rendering the virion replication-competent. The attending change in virion morphology upon its maturation is the conversion of the annular virion to one containing a condensed cone-shaped core,[46] the virion capsid, which is composed of p24. Virions of this more familiar "mature" morphology are infectious and replication-competent (Figure 2). The virion capsid houses the genomic RNA, the nucleocapsid protein p9 and p6, and the retroviral enzymes reverse transcriptase and endonuclease. It would appear from the above discussion that the role of the retroviral proteases is limited to post-

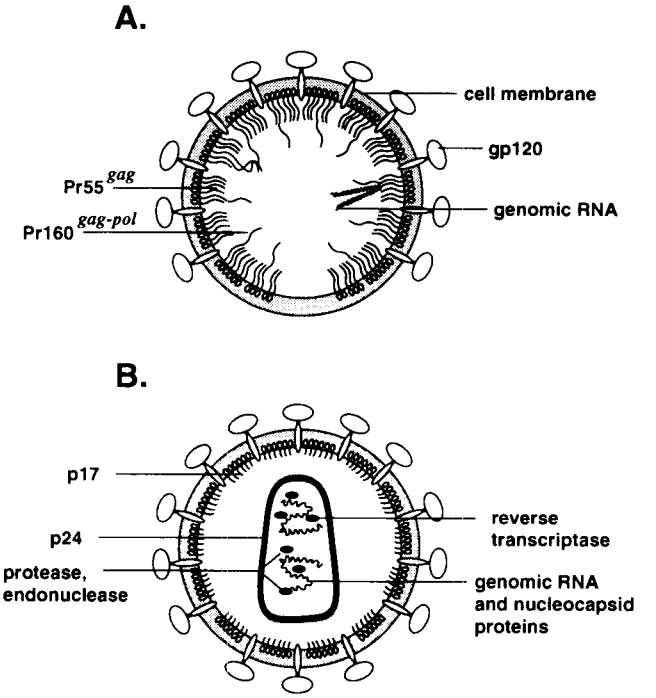


FIGURE 2 Schematic depiction of immature (A) and mature (B) morphologies of HIV-1 as indicated by electron microscopy.[46] (A): The budded, immature virion contains the unprocessed *gag* and *gag-pol* polyproteins which are embedded into the viral membrane by their N-terminal myristoyl groups. Following cleavage from gp160, the envelope glycoprotein gp120 is situated on the outside of the membrane. The genomic RNA is attached to the nucleocapsid protein domain of $Pr55^{gag}$. The immature virion appears to contain an annular core of unprocessed polyproteins. Processing is probably initiated within the immature particle upon the association of three protease domains within the tethered $Pr160^{gag\text{-}pol}$. (B): Morphology of the mature virion core following proteolytic processing. The cone-shaped core, composed of p24, houses the retroviral enzymes, the nucleocapsid proteins and the RNA genome.

integrational steps in the viral replication cycle, such that blockade of the action of this enzyme can do nothing for stably infected cells, but should inhibit the spread of infection to other cells. However, recent studies with the equine infectious anemia virus indicate that active protease exists within the mature capsid and that a proteolytic event occurs after capsid formation.[51] Retroviral proteases may therefore have an important role in a pre-integrational event of the retroviral life cycle, such as destabilization of the virion capsid or perhaps, activation of the endonuclease, and as such, inhibition of the protease may prevent proviral integration.

Given the indispensible function of the retroviral proteases in the assembly, maturation, and replication-competence of the virion, the evolutionary necessity of a highly conserved, virally-encoded enzyme becomes apparent. The vital role of these enzymes has been demonstrated in studies[45,48–53] in which specific, lethal mutation of the protease gene within clones of infectious proviruses, including that of HIV-1, resulted in the formation of replication-incompetent, non-infectious virions. The resulting virions were characterized by the presence of unprocessed *gag* polyproteins,

by the diminution or absence of reverse transcriptase activity, and where examined, by aberrant morphology in which the virions appeared to be of the "immature" form. Such studies promote the hypothesis that specific inhibitors of HIV-1 protease should prevent the formation of infectious virions from chronically-infected T-lymphocytes, and should ultimately result in a therapeutically significant anti-viral effect. Given that HIV-1 protease is a self-generating enzyme, the impact of its inhibition on virion maturation should be all the more dramatic.

3. STRUCTURAL PROPERTIES OF HIV-1 PROTEASE

The retroviral proteases belong to the family of the aspartic proteases, so named because a pair of highly conserved aspartyl residues constitute the catalytic groups at their active sites. The non-viral aspartic proteases, such as pepsin, the fungal pepsins, renin, and cathepsin D, are monomeric, "pseudosymmetric", bi-lobal proteins, composed of >300 amino acids, in which each lobe contributes one of the catalytic aspartyl residues to an active site which is found at the bottom of a deep cleft formed at the interface of the lobes.[54,55] In contrast, the primary structures of the retroviral proteases consist of less than 130 amino acids. The retroviral proteases display minimal sequence homology among themselves and with the non-viral aspartic proteases except in two conserved domains: Leu-Leu(Val)-Asp-Thr(Ser)-Gly-Ala (domain I) and Ile(Leu)-Leu-Gly-Arg-Asp (domain II).[56] Domain I contains the triad Asp-Thr(Ser)-Gly which is identical in sequence to both of the catalytic aspartyl residues in the non-viral aspartic proteases. In HIV-1, these domains are located at the 5′-end of the *pol* reading frame, upstream from the reverse transcriptase coding region. The domains are flanked at either end by peptide sequences which are commonly recognized as cleavage sites by the retroviral proteases, Ser(Thr)-Xaa-Yaa-Phe*Pro-Zaa[57] (scissile bond is denoted by asterisk throughout) (Figure 1). Both the conserved domains and the consensus cleavage sequences are encoded within the *pol* open reading frame. Autocatalytic cleavage at the Phe*Pro bonds by the protease domain within $Pr160^{gag\text{-}pol}$ would release a 99-amino acid polypeptide containing domains I and II.

To date, the expression of HIV-1 protease activity in bacteria[49,58–68,111] or yeast[69–71] has been demonstrated in a large number of laboratories. By comparison with the primary sequence of the HIV-1 protease purified from virions[41], active protease resulting from autoprocessing in bacteria yields, where studied,[66,72] the authentic viral protease. These findings were confirmed by the demonstration of HIV-1 protease activity within synthetic polypeptides composed of this 99-amino acid sequence.[73–75] Expression in *Escherichia coli*[58] of recombinant constructs containing the coding region of HIV-1 protease demonstrated that the enzyme autoprocesses at the two cleavage sites shown in Figure 1 to generate a 99-amino acid polypeptide found within Pro-69 and Phe-167 of the translated *pol* coding region.[11] The recombinant HIV-1 protease has been subsequently purified to apparent homogeneity from bacterial extracts,[72] allowing biochemical characterization.

As proposed by Pearl and Taylor[76] from computer modelling studies, the retroviral proteases could achieve structures which are equivalent to the much larger non-viral aspartic proteases by dimerization of their smaller subunits, such that each monomer contributes a Asp-Thr-Gly triad to an active site formed at the subunit interface. For

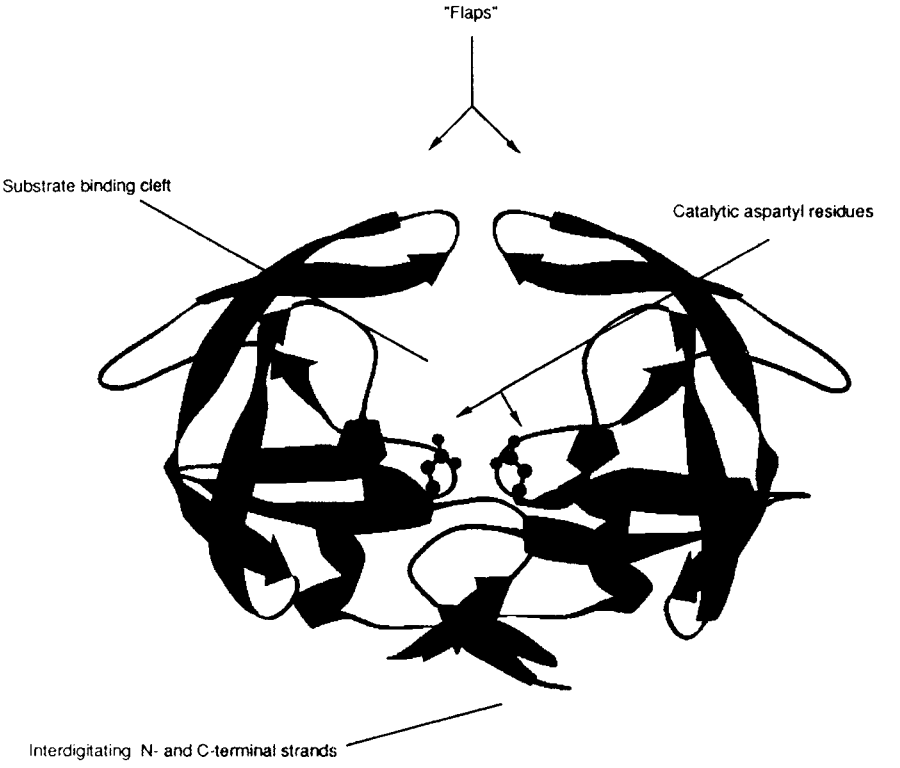


FIGURE 3 Crystal Structure of HIV-1 Protease. The ribbon diagram of the HIV-1 protease homodimer, taken from the data of Wlodawer *et al.*,[80] depicts the glycine-rich, substrate-binding "flaps" in their unbound, or "open" conformation, which move by 7 Ångströms into the substrate binding cleft upon inhibitor binding.[89] The active site aspartyl groups, Asp-25 and Asp-25′, are shown. The homodimer is associated by β-sheet interactions between the "interdigitating" N- and C-terminal chains of each monomer.

HIV-1 protease, this prediction has been borne out both by biophysical studies[77] and by crystallographic studies[78–81] of the enzyme.

Recently, the solution of the three-dimensional structure of HIV-1 protease of both synthetic and recombinant sources,[78–81] as well as the protease from Rous sarcoma virus[82] (RSV) has confirmed the proposed homodimeric structure of these enzymes, and revealed that their overall structure is quite similar to the bi-lobal structures of the monomeric, non-viral aspartic proteases.[83–88] The orientation of the catalytic aspartyl groups in the active sites of the retroviral proteases is indistinguishable from those of the monomeric aspartic proteases of known three-dimensional structure, a likeness which is revealed emphatically by the recently-solved structures of HIV-1 protease containing peptide analogue inhibitors within the active site (Figure 3).[89–93]

As described above, the active sites of retroviral proteases are formed at the interface of the monomers in which each monomer contributes one of the two aspartyl residues within the conserved Asp-Thr(Ser)-Gly sequences. As a result, the unbound forms of the homodimer possess a C-2 axis of symmetry, which is perturbed upon binding of an unsymmetrical inhibitor or substrate.[89] The HIV-1 and RSV retroviral

proteases contain two identical "flap" regions, analogous to the single flap found in the non-viral aspartic proteases, which apparently form in part the substrate/inhibitor binding sites. These flaps move by as much as 7 Å upon binding of an inhibitor.[89]

In addition to the homodimeric nature of the retroviral proteases, two other structural features of these enzymes distinguish them from their non-viral aspartic protease counterparts (Figure 3). In the structures of all HIV-1 protease-inhibitor complexes solved to date, within the "flaps" is a water molecule which is hydrogen-bonded in a tetrahedral fashion to the amide hydrogens of the Ile-50 and Ile-50′ residues in the flaps,[89-93] and to the carbonyl oxygens between the P1–P2 and P1′–P2′[153] residues of the inhibitors. This water molecule is clearly crucial in bringing elements of the flaps together with the inhibitor, and most likely assists in ensuring that the inhibitor binds in its now familiar extended, β-sheet-like conformation. The second unique structural feature of the retroviral proteases is the arrangement of the terminal chains to form the homodimeric interactions. The amino- and carboxylic termini of both monomers are arranged in an "interdigitating" network of anti-parallel β-sheets which hold the protease dimer together (Figure 3). Since these rigidly-held terminal strands are remote from the active site and are themselves the remnants of the cleavage sites for the autoprocessing of the protease, the most likely mechanism for the self-cleavage of the dimeric protease from $Pr160^{gag\text{-}pol}$ would be intermolecular, involving at least three protease domains.

4. FEATURES OF THE SUBSTRATE SPECIFICITY AND MECHANISM OF ACTION OF HIV-1 PROTEASE WHICH CONTRIBUTE TO INHIBITOR DESIGN

Prior to the availability of structural data of HIV-1 protease-inhibitor complexes, the quest for rationally-designed inhibitors of HIV-1 protease began with a study of the substrate requirements for the enzyme as well as biochemical characterization of its peptidolytic reaction to elucidate details of its mechanism of action. In this way, inhibitors could be derived from the replacement of the scissile dipeptide of a known peptide substrate of the protease with non-hydrolyzable dipeptide isosteres, which would bind tightly to the enzyme due to structural mimicry of an enzymatic transition state or reaction intermediate. The implementation of these tasks was greatly facilitated by the early recognition that HIV-1 protease was an aspartic protease, and that much was already known about the structure, mechanism of action, and substrate preferences of the non-viral aspartic proteases. More importantly, the design of peptide analog inhibitors of the monomeric aspartic proteases has been extremely successful, particularly with renin,[94] a therapeutically relevant enzyme integrally involved in the regulation of blood pressure in mammals, such that these proven inhibitory concepts served as a point of departure for a "first generation" of inhibitors of HIV-1 protease.

The complete proteolytic processing of the $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ substrates by HIV-1 protease apparently requires only eight discrete cleavages (Figure 1).[35-42,95] This rather limited digestion of these large polyproteins misleadingly suggests that HIV-1 protease is highly specific towards the primary sequence of its substrates. Three of these cleavage sites, including the p17–p24 cleavage sites of $Pr55^{gag}$ and the termini of the protease, are of the sequence Ser(Thr)-Xaa-Yaa-Phe(Tyr)*Pro. While unusual for the known endopeptidases, this type of cleavage site is thematic to the retroviral proteases.[57] The other cleavages are less well conserved and reflect a general pattern

for substrates for the retroviral proteases and monomeric aspartic proteases, i.e., hydrophobic residues in the P1 and P1′ positions. From the solved three-dimensional structures of HIV-1 protease complexed to a variety of peptide analog inhibitors, it is evident that the active site of HIV-1 protease may accommodate the equivalent of a heptapeptide, spanning the sites P4–P3′ residues. A somewhat diverse collection of residues is found at these positions within the polyprotein substrates of HIV-1 protease:

P4 = Ser (2), Thr, Ala (3), Pro, Arg
P3 = Gln, Arg, Thr, Gly, Phe, Leu, Glu, Lys
P2 = Asn (4), Val, Ile (2), Thr
P1 = Tyr, Leu (2), Phe (4), Met
P1′ = Pro (3), Ala, Met, Leu, Tyr, Phe
P2′ = Ile (2), Glu, Gln (3), Asp, Thr
P3′ = Val, Ala, Arg, Ser (2), Ile, Asp (2)

This highly variable array of residues in the proteolytic cleavage sites suggests that primary sequence beyond the P1 and P1′ positions is of limited importance for the substrate, and that features of secondary and tertiary structure within the polyprotein substrates contribute significantly to their recognition by HIV-1 protease.

The substrate specificity of HIV-1 protease has been investigated in more detail in a number of laboratories using oligopeptides which constitute the cleavage sites identified within the viral polyproteins.[73,95–102] To date, oligopeptides containing all eight of the cleavage sites within the polyprotein substrates have been shown to be competent substrates for HIV-1 protease.[42,95,97] Typically, these peptide substrates consist of six or more amino acids, and depending on the assay conditions, exhibit Michaelis constants in the range of 0.1–10 mM. As expected from structural analysis, the best of these peptide substrates of this sequence type contain residues at positions P4 to P3′. The most complete set of data, including kinetic studies of the protease, exist for peptide substrates which contain a form of the cleavage sites at the *gag* p17/p24 and *gag* p24/p1 junctions: Ser-Gln-Asn-Tyr*Pro-Ile-Val and Lys-Ala-Arg-Val-Leu*Ala-Glu-Ala. A series of studies in which residues at the P3,[100] P2,[100] P1,[100,101] and P2′[102] positions have been systematically substituted within peptide substrates of otherwise invariant sequence reveals that the following residues are suitable (+) or unsuitable (−) at these positions:

P3: Gln (+), Cys (+), Pro (−), Tyr (+), Arg (+), Val (+), Asn (+), Asp (+), Glu (+)

P2: Val (+), Ile (+), Leu (+), Phe (+), Ala (+), Asp (+), Asn (+), Gly (−), Pro (−)

P1: Tyr (+), Phe (+), Met (+), Glu (−), Arg (−), Leu (+), norleucine (+), Ile (−), Val (−)

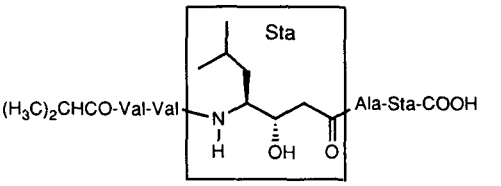
P2′: Ile (+), Ala (+), Leu (+), Trp (−), Phe (−), Gly (−).

Unsurprisingly, the amino acid preferences for peptide substrates of HIV-1 protease reflect those found within the proteolytic cleavage sites. One could conclude

from these results that an optimal oligopeptide "template" for inhibitor design would be a heptapeptide spanning the P4–P3′ residues, in which P4 is a small, hydrophilic residue, P3 and P3′ are larger residues of a highly variable polarity, at P2, Asn or a hydrophobic residue is favored, P2′ is a hydrophobic or anionic group and the inhibitory dipeptide "cassette" which would comprise the P1 and P1′ positions would be hydrophobic residues and in the case of P1′.

From a broad panel of structural and kinetic studies of the monomeric aspartic proteases,[54,55,103–109] it is now widely believed that the two active site aspartyl residues assume opposite roles in general acid-general base catalysis: the protonated aspartyl group effects protonation of, or hydrogen-bonding to, the carbonyl oxygen of the scissile amide bond, while the unprotonated aspartyl residue deprotonates the lytic water molecule which subsequently attacks the scissile carbonyl as a hydroxide ion. By virtue of its structural and biochemical similarities to this class of proteases, one would expect HIV-1 protease to operate by a similar chemical mechanism.

Details of the catalytic mechanism of HIV-1 protease have been investigated by kinetic studies in numerous laboratories.[66,77,111–114] Among these results are kinetic characteristics of HIV-1 protease which are common to the monomeric aspartic proteases, such as inhibition, albeit weak, by pepstatin A(**1**; $K_i \leqslant 2\,\mu M$),[66,110] an acidic pH optimum (4.5–6.0),[66,77,112,114] and time-dependent inactivation by 1,2-epoxy-(4-nitrophenoxy)propane (EPNP),[77] which esterifies one or both of the active-site aspartyl residues in porcine pepsin.[115–117]



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The pH dependence of the kinetic constant $\log k_{cat}/K_m$ for several oligopeptide substrates of HIV-1 protease constitutes a "bell-shaped" curve over a pH range of 3.4–6.5, indicating that an unprotonated group of pK = 3.3 and a protonated group of pK = 5.5–6.1 are required for catalysis.[114] These results indicate that both Asp-25 and Asp-25′ are involved, and that one is protonated in the reaction mechanism while the other is not. The analysis of recent structural data of complexes of HIV-1 protease and peptide analogue inhibitors are in support of the mechanistic involvement of Asp-25 and Asp-25, most likely in opposite states of protonation.[89–93] From these results, as well as from solvent deuterium isotope effects,[114] proton inventory,[114] and the partitioning of ^{18}O from solvent into product or re-formed substrate,[113] the chemical mechanism that is most consistent is that shown in Figure 4. In this mechanism, the sole enzyme-bound intermediate is the amide hydrate **2**, which would be formed and disassembled through reaction transition states of varying degrees of tetrahedral character. In accordance with theory regarding the enzymatic stabilization of transition states, one would expect these transition states and the amide hydrate intermediate to be very tightly bound by the enzyme.[118]

Accordingly, successful structural mimicry of this amide hydrate intermediate by non-hydrolyzable dipeptide isosteres with, in kind, tetrahedrally-substituted moieties should lead to compounds with high affinity for the enzyme, thereby yielding potent

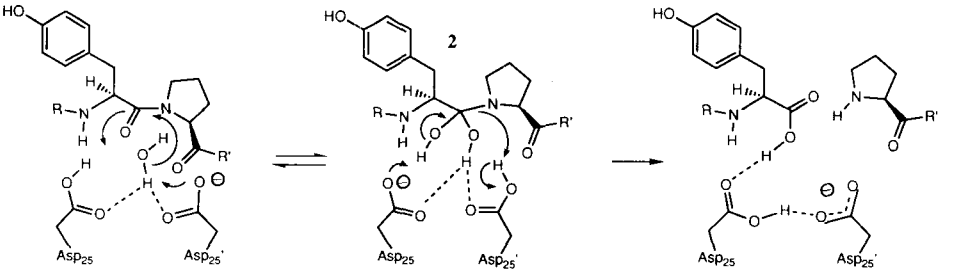


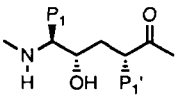
FIGURE 4 Proposed chemical mechanism for HIV-1 protease. The depicted chemical mechanism involving the cleavage of a Tyr-Pro peptide bond is based on kinetic[113,114] and structural data.[89-93] A water molecule is hydrogen-bonded to the Asp-25 and Asp-25′ residues found in opposite states of protonation. Binding of the peptide substrate elicits general-acid protonation of its carbonyl from Asp-25 and general-base deprotonation of lytic water by Asp-25′ to form the initial tetrahedral intermediate, the amide hydrate **2**. Simultaneous deprotonation of a hydroxy group of **2** by Asp-25 and protonation of the departing prolyl peptide by Asp-25′ renders the carboxylic acid and amine hydrolysis products, and restores the aspartyl residues to their initial protonation states.

inhibitors.[118,119] It remains therefore to design and synthesize a panel of potential "transition-state analogs" on these mechanistic concepts within the framework of an acceptable P1-P1′ dipeptide, and then to incorporate this synthetic dipeptide surrogate into a peptide or peptide analog template which comprises a substrate of HIV-1 protease. In this way, a number of potential inhibitory dipeptides can be evaluated with a variety of peptide templates of varying length and composition, in the hopes that the resulting inhibitors could readily be optimized for potency, brevity, and specificity.

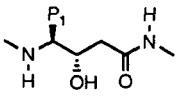
5. PEPTIDE ANALOG INHIBITORS CONTAINING DIPEPTIDE ISOSTERES

The design of HIV-1 protease inhibitors by the "transition state analog" approach has rapidly generated a number of very successful results, owing in large part to existing synthetic inhibitors of the monomeric aspartic proteases, in particular renin. Greenlee has recently reviewed peptide analogue inhibitors of renin in which origins of many of the transition state analog isosteres found in the present review are detailed.[94] These inhibitory concepts are in some way predicated on the natural product inhibitor of the aspartic proteases, pepstatin A. Pepstatin A (N-isovaleryl-Val-Val-Sta-Ala-Sta; Sta, statine = 4*S*-amino-3*S*-hydroxy-6-methylheptanoic acid; **1**), a microbial product,[120] is an extremely potent inhibitor of the monomeric aspartic proteases (K_i < 1 nM).[54,55] While it is only a moderately potent inhibitor of HIV-1 protease (K_i = 1.4 μM),[66,110] pepstatin A indicates an important direction that the development of more potent inhibitors should lead. The tetrahedral hydroxyl-bearing carbon in the unusual amino acid statine is the inhibitory moiety of pepstatin A, which presumably resembles to some degree the amide hydrate intermediate or a transition state leading to its formation.[121] Another interpretation is that the statine group may act as a "bisubstrate" analog of peptide and the lytic H_2O molecule.[108,122] As Rich has emphasized, the statine moiety, which generically can be referred to as a hydroxymethylene isostere (Table I), is a suitable mimic for a dipeptide even though

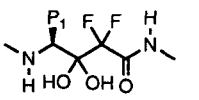
TABLE I
Dipeptide isosteres which effect inhibition of the aspartic proteases



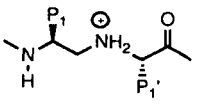
Hydroxyethylene



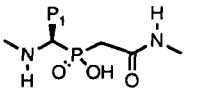
Hydroxymethylene

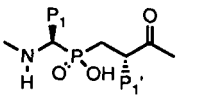


Difluoroketomethylene

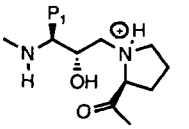


Reduced amide





Phosphinates



Hydroxyethylamine

it does not constitute a full dipeptide isostere and lacks the P1′ residue.[108,119] In general, in the design of synthetic dipeptide isosteres, both the P1 and P1′ residues have been retained with the atomic spacing of a dipeptide, although examples exist in which additional atoms have been substituted between the P1 and P1′ groups.

It should be noted here that the inhibition constants (K_i or IC_{50} values) tabulated in Tables I–V were obtained in numerous laboratories under highly variable assay conditions. The pH, ionic strength, and assay temperature employed for a given inhibition study, for which HIV-1 protease activity is very sensitive,[112-114] vary significantly from study to study. In our studies,[77,97,110,113-114,131] we have adhered to assay conditions which we feel are likely to reflect the actual physiological environment of the maturing HIV-1 virion; namely, human physiological temperature (37°C), moderately high ionic strength (0.2 M NaCl), and slightly acidic pH (6.0), even though these conditions are not optimal for enzyme activity. The ionic strength and pH are deliberately slightly different from true physiological values in view of the high local concentration of the polyanionic RNA in the developing virion. In addition, the recombinant or synthetic forms of HIV-1 protease used in these analyses are by no means uniform in terms of either their primary sequences or methods of preparation. As a result, a direct comparison of inhibitory potency of the cited inhibitors is, strictly speaking, inappropriate. However, for purposes of clarity in this review we quantitatively compare the inhibitors by their reported inhibition constants without qualifying the values themselves.

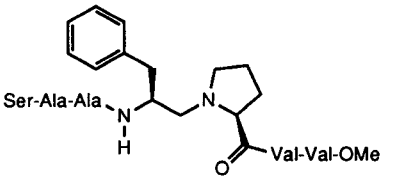
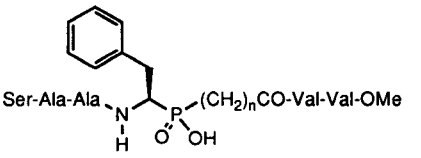
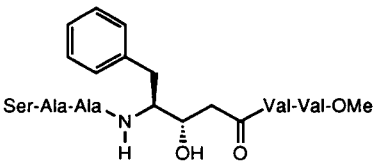
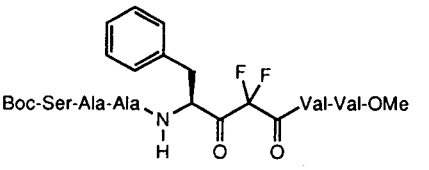
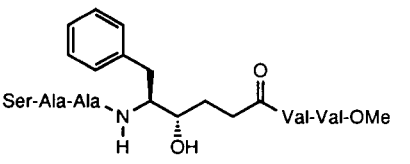
A variety of non-hydrolyzable, dipeptide isosteres have been developed to mimic

the putative transition state of the aspartic protease-catalyzed reaction, and those which have been utilized to date for peptide analogue inhibitors of HIV-1 protease are shown in Table I. These isosteres all possess sp^3 hybridization at the carbon which poses for the scissile carbonyl of the substrate, thereby providing structural mimicry of the presumed transition state or the enzyme-bound reaction intermediate (Figure 4, **2**). The hydroxyethylene isostere, first used in the inhibition of renin by Szelke and co-workers,[123] contains a secondary alcohol substitution for the scissile carbonyl, as does pepstatin A, but also maintains the correct atomic spacing of the scissile dipeptide, which allows for substitution of residues germane to the specificity of the target protease at both the P1 and P1′ positions. While the hydroxyethylene and hydroxymethylene isosteres contain but one of the two hydroxyl groups of the reaction intermediate, Gelb *et al.*,[124] have used an α,α-difluoroketomethylene analog of a "statine-like" (hydroxymethylene) inhibitor to achieve a new class of potent pepsin inhibitors which contain a stable ketone-hydrate to better mimic the amide hydrate reaction intermediate **2**. The reduced amide isostere, also developed by Szelke and co-workers,[125] contains no heteroatom substitution for the scissile carbonyl which enables the resulting amine to be protonated. This isostere has led to the development of potent renin inhibitors presumably due to an ionic interaction between the protonated secondary amine of the inhibitor and the unprotonated catalytic aspartyl residue. Bartlett and Kezer[126] first made use of phosphinate analogs of the hydroxymethylene class of inhibitors to develop potent inhibitors of pepsin. The phosphorus atom provides a tetrahedral surrogate for the scissile carbonyl which is amenable to suitable substitution by oxygen atoms in order to mimic the amide hydrate. Phosphinate-containing analogues of both the hydroxyethylene and hydroxymethylene type are possible. The spatial separation of the nitrogen of the reaction intermediate **2** from the scissile carbonyl by a methylene unit gives rise to the hydroxyethylamine (amino alcohol)[127,128] isostere, which combines structural features of both the hydroxy(m)ethylene and reduced amide isosteres in that it preserves the secondary alcohol function as well as a protonated tertiary amine.

For HIV-1 protease, Dreyer *et al.*,[110] have placed Phe-Pro or Phe-Gly dipeptide isostere forms of those found in Table I (except for the hydroxyethylamine isostere) with the context of a single oligopeptide substrate in order to compare the relative inhibitory potency of the isosteres themselves (Table II). The peptide template used was of the sequence Ser-Ala-Ala-Phe*Pro(Gly)-Val-Val-OMe, and the inhibition constants found in Table II may be compared with the Michaelis constant of 9.8 mM obtained for the corresponding peptide substrate, Ac-Ser-Ala-Ala-Tyr*Pro-Val-Val-NH_2.[110] The sequence of the chosen peptide template arose from the recognition of three similar sequences as proteolytic processing sites in the polyprotein substrates of HIV-1 protease.

The inhibitors in Table II were found to be linear competitive inhibitors of recombinant HIV-1 protease when assessed in a peptidolytic assay, and exhibited a wide range of inhibition constants (apparent K_i = 62–19 000 nM).[110] These inhibitors could be ranked by potency in the order: reduced amide (**3**, least potent) < phosphinic acid "hydroxymethylene" (**4**) and "hydroxyethylene" (**5**) < statine analogue (**6**) < α,α-difluoroketomethylene (**7**) < hydroxyethylene isostere (most potent, **8**). The heptapeptide Phe-Gly hydroxyethylene isosteric inhibitor (**8**) was a reasonably potent competitive inhibitor of HIV-1 protease with an apparent inhibition constant (K_i = 62 nM) five orders of magnitude lower than the Michaelis constant of the substrate from which it was derived.[110] The rank order of inhibitory potency of the dipeptide isosteres shown above has been in part demonstrated by other research

TABLE II
Comparison of dipeptide isosteres within single peptide template

Compound	Structure	K_i(nM)
3		19,000
4, 5 (n = 1,2)		4500, 4400
6		810
7		160
8		62

groups,[96,99,111] in which peptide analogues of different overall sequence containing reduced amide, statine or "statine-like" moieties, or hydroxyethylene replacements for the scissile dipeptide have been studied.

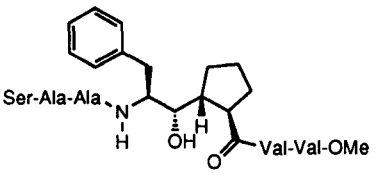
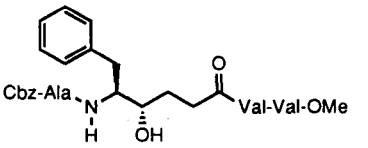
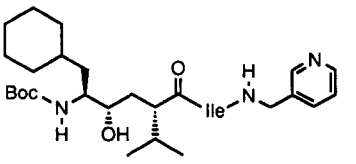
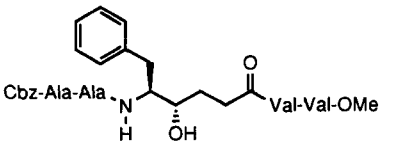
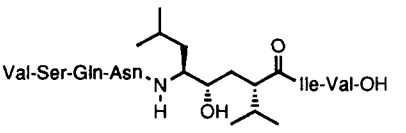
5.1. *Hydroxyethylene Isosteric Inhibitors*

From this survey, one could conclude that the hydroxyethylene isostere provides the most potent inhibition of HIV-1 protease within a single type of peptide template. To date, the hydroxyethylene isostere has been the most extensively utilized moiety in the design of HIV-1 protease inhibitors, and a significant amount of structure-activity relationship data has now been compiled. A variety of these inhibitors is shown in Table III. For reasons of metabolic stability, synthetic facility, and favorable pharmacological properties, it is a prevailing goal of this research to minimize both the overall size and peptide character of such HIV-1 protease inhibitors, and several of the inhibitors shown in Table III reflect the desirability of these features. The (4*S*)-stereochemical configuration of the secondary alcohol in both the hydroxyethylene and hydroxymethylene isosteres is crucial for potent inhibition, and analogues containing a (4*R*)-hydroxyl group are poorer inhibitors than their (4*S*)-diastereomers.[110] Compound **9**, prepared by Dreyer *et al.*,[110] represents a Phe-Pro isostere which is a modestly potent inhibitor of the protease. The *trans*-cyclopentyl ring possesses a configuration like that of L-proline, and may mimic, albeit not optimally, the bound reaction intermediate of a Phe-Pro peptide substrate. The pH dependence of inhibition by the neutral, N-Boc analogue of compound **9** indicates that this inhibitor binds best between pH 3.1–5.3, and to a form of the protease in which the catalytic aspartyl residues are in opposite forms of protonation, identical to the enzyme form which binds substrates.[114]

The Phe-Gly-containing inhibitors **10** and **12** which constitute penta- and hexapeptide analogues, respectively,[129] indicate that diminishing peptide length attentuates inhibitory potency in this series of inhibitors although the hexapeptide **12** is surprisingly a slightly better inhibitor than its heptapeptide congener **8**. However, the Phe-Gly hydroxyethylene isostere, while an obvious improvement over the corresponding hydroxymethylene isostere, does not make optimal use of the P1′-residue. By analogy to that which has been observed for hydroxyethylene isosteric inhibitors of renin[94,123] and pepsin,[130] the Phe-Gly dipeptide isostere should provide the poorest inhibition of HIV-1 protease, and by elaborating P1′ to residues of increasing steric size and bulk, more potent inhibitors may be achieved. This supposition is borne out by numerous recently-disclosed examples of hydroxyethylene isosteric inhibitors of HIV-1 protease as shown in Table III. By the single alteration of the Gly residue at P1′ to that of Ala to produce the Phe-Ala isostere (**15**),[131] the potency of **8** is enhanced nearly 70-fold, yielding a hexapeptide analogue possessing a sub-nanomolar inhibition constant.

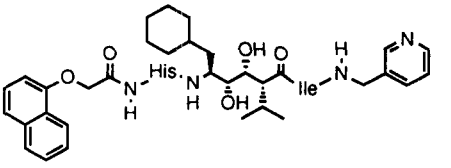
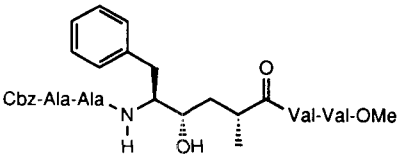
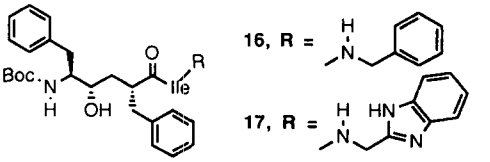
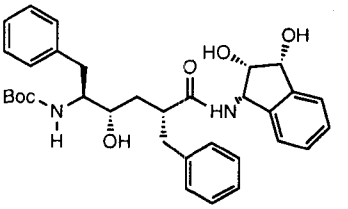
This, and the other examples in Table III, suggest that by the proper substitution of residues at the P1 and P1′ positions, sufficiently inhibitory isosteres will result, which would possibly obviate the need for a lengthy peptide analogue to effect potent inhibition. While the cyclohexylalanyl-Val tripeptide isostere **11** is a good inhibitor for its size (K_i = 70 nM),[132] the Phe-Phe isostere within the tripeptide analogues **16**[133] and **17**[133] undoubtedly contributes favourably to the significant potency of these inhibitors. Note that in the cited examples the aminomethylpyridyl, benzylamino and aminomethylbenzimidazole moieties provide suitable, and presumably proteolytically

TABLE III
Hydroxyethylene isostere inhibitors of HIV-1 protease

Compound	Structure	K_i(nM)
9		500
10		120
11		70
12		48
13		<10

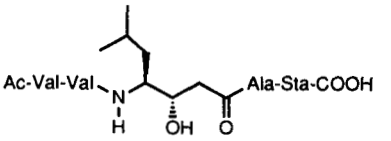
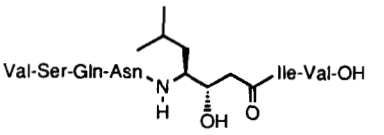
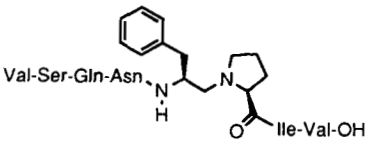
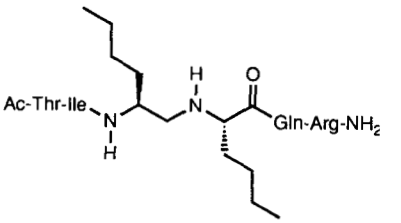
stable, C-termini for these inhibitors, and may in fact bind favorably to the S3′ binding site on the protease. Inhibitor **13**[111] demonstrates that a slightly different substitution pattern at P1-P1′ namely Leu-Val, within an octapeptide analogue also affords a good protease inhibitor. Thaisrivongs and colleagues[134] have adapted a 1,2-diol analogue of the hydroxyethylene isostere from known renin inhibitors to produce the cyclohexylalanyl-Val tetrapeptide analogue **14**. It remains to be

TABLE III (Continued)

Compound	Structure	K_i(nM)
14		<1
15		0.7
16, 17		IC_{50} = 0.71, 0.07
18		IC_{50} = 0.1

determined from structural analysis whether or not this second hydroxyl group actively forms an additional hydrogen bond to an enzymatic group. Lyle and colleagues[135] have combined classical medicinal chemical strategies with molecular modelling of structures of HIV-1 protease-inhibitor complexes to produce the extremely potent Phe-Phe dipeptide analogue inhibitor **18**, in which the substituted aminoindan group at the terminus imparts to the compound significant binding affinity for the enzyme. The examples in Table III amply demonstrate that small, but potent, HIV-1 protease inhibitors of minimal peptide character are achievable.

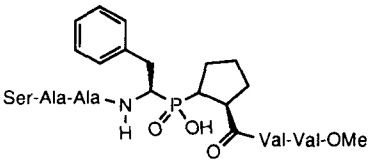
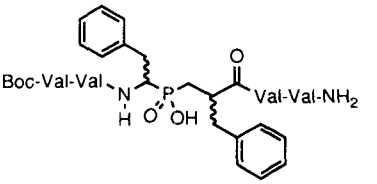
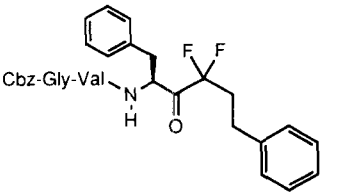
TABLE IV
Other dipeptide isostere inhibitors of HIV-1 protease

Compound	Structure	*Ki* (nM)
Hydroxymethylene isosteres		
19		20
20		3690
Reduced amide isosteres		
21		3520
22		780

5.2. *Hydroxymethylene and Reduced Amide Isosteres*

In addition to the weakly-binding pepstatin A and compound **6**, two other examples of the hydroxymethylene isostere class are shown in Table IV. Incredibly, replacement of the isoamyl N-terminal group of pepstatin A with an acetyl group to yield acetyl-pepstatin (**19**) results in a 70-fold increase in activity,[99] even though structural data[93] clearly indicate that the acetyl group is far removed from the catalytic aspartyl groups, which are hydrogen-bonded to the secondary alcohol of the statine residue.

TABLE IV (Continued)

Compound	Structure	*Ki* (nM)
Phosphinate isosteres		
23		4000
24		0.4
Difluoroketomethylene isostere		
25		IC_{50} = 1.0

Such affinity is not evident in the statine-containing analogue **20**,[111] in which the statine residue is "housed" in a heptapeptide template which is ostensibly more conducive to HIV-1 protease than the flanking residues found in the pepstatins. Compound **20** is less inhibitory than its phenylalanyl-containing congener **6**.

The additional examples of the reduced amide isostere, compounds **21**[97,111] and **22**[89] in Table IV, support the earlier findings that this class of compound is, relatively speaking, poorly inhibitory for HIV-1 protease although one would expect that the secondary amine, which would be protonated at the germane pH range (3–7), would form a suitable ionic interaction with the unprotonated catalytic aspartyl residue in the active site of the protease. However, Hyland *et al.*[114] have recently shown that the pH dependence of inhibition by a close analogue of compound **3** is consistent with binding of this cationic inhibitor to the protease only when *both* of the catalytic aspartyl residues are unprotonated. Results from the pH dependence of catalysis have shown that substrates do not bind to this "di-anionic" form of the protease, which

exists at pH > 5.3, so that the modest inhibition of the reduced amide isosteres is due in part to its binding to an enzyme form which is in a different protonation state than the one which binds substrates. Compound **21**,[111] a Phe-Pro isostere within an octapeptide analogue, is five-fold more potent than its heptapeptide homolog **3**. The hexapeptide analogue **22** contains a sequence analogous to the cleavage site between the p1–p7 *gag* proteins in $Pr55^{gag}$, in which the norleucyl residue at P1 and P1′ are substituted for the Met residues found in the polyprotein substrate. This cleavage site has been found to provide an oligopeptide which, in terms of its relative value of k_{cat}/K_m, is among the most efficiently processed substrates of the enzyme.[95] Correspondingly, the reduced amide isostere **22** which comprises this cleavage sequence is, at K_i = 780 nM, the most potent of the reduced amide isosteres reported to date.

5.3. *Phosphinate and Difluoroketomethylene Isosteres*

As indicated in Table III, phosphinate-containing inhibitors of the hydroxymethylene-type isostere and Phe-Gly hydroxyethylene-type isostere varieties are equally poor inhibitors of HIV-1 protease. Dreyer *et al.*[110] have used a *trans*-cyclopentyl to prepare the Phe-Pro dipeptide hydroxyethylene-type phosphinate isostere **23**, which evinces no apparent improvement in binding affinity for the protease over its congeners in Table III which possess no corresponding steric substitution at their P1′ positions. The poor inhibition exhibited by these inhibitors may be due to the anionic nature of the phosphinates at the assay pH (6.0), at which at least one of the catalytic aspartyl groups is also anionic and presumably repellant to the phosphinate. The phosphinate isosteres are quite potent inhibitors of both porcine pepsin[126] and penicillopepsin,[136] which characteristically exhibit maximal enzymatic activity at much lower values of pH (less than 4.0) than does HIV-1 protease, and at which, the neutral, protonated forms of the phosphinate inhibitors may in fact be the true inhibitory species. In kind, the K_i value for compound **23** at pH 3.5 is 30-fold lower than that obtained at pH 6.0.[110] A somewhat surprising result is presented by compound **24**, a Phe-Phe phosphinate inhibitor which at pH 6.5 is an extremely potent inhibitor (K_i = 0.4 nM).[137] Compared to the other phosphinate examples, this Phe-Phe isostere clearly underscores the importance of proper substitution at the P1′ position. As expected, inhibition by **24** is enhanced ten-fold at pH 4.5.

Compound **25** represents the use of the α,α-difluoroketomethylene isostere in a tripeptide analogue.[138] Presumably, the compound exists in aqueous solution exclusively as the ketone hydrate. This compound exerts significantly more potent inhibition than does the larger inhibitor **7**. The reason for this may well reside in the phenylethyl substituent which is appended to the difluoromethylene group, in that this moiety may act as a Phe residue at the P1′ position. Thus the potency of **25** may be due in large part to more faithful mimicry of a hydroxyethylene-type inhibitor than the difluoroketomethylene inhibitor **7**.

6. C-2 SYMMETRIC INHIBITORS

Upon the discovery of the homodimeric nature of HIV-1 protease and the solution of its three-dimensional structure, it was recognized that the uncomplexed enzyme possesses a C-2 axis of symmetry which is perpendicular to the linear binding orientation of the inhibitors[80,89] (in Figure 3, this C-2 axis is in the plane of the page, and runs

from top to bottom, bi-secting the flaps and the catalytic aspartyl residues). The active site constitutes the center of this C-2 axis, such that the S_n and S'_n substrate binding pockets are disposed on either side of the axis. Erickson,[90] Kempf and colleagues[139] (as well as others)[140] have used this concept to design different types of "C-2 symmetric" inhibitors of HIV-1 protease, some of which are shown in Table V. It was recognized that the C-2 axes of the potential inhibitor had to be co-incident with that of the enzyme, and that the equivalents of the P_n and P'_n positions be appropriately substituted with "residues" that would be in duplicate for each pair of positions.

For the first class of C-2 symmetric inhibitors (**26–28** in Table V),[90,139] the C–O bond of the central secondary alcohol comprises the C-2 axis, and presumably, this hydroxyl group becomes centrally interposed between the aspartyl groups (see discussion on structure below) to effect inhibition. This would require that the benzyl groups are equivalent to the now familiar Phe residues in the P1 and P1′ positions of the other inhibitors, although the atomic spacing between these benzyl groups is one carbon unit less than that of the hydroxyethylene isosteres. The groups at Xaa become the P2 and P2′ "residues". Note that while the P_n groups are arranged in a peptide backbone, the symmetrical nature of these inhibitors demands that the P'_n groups exist as "inverso" peptide bonds; that is, two peptide "chains" meet at the secondary alcohol in a head-to-head fashion. While several of these mono-hydroxy C-2 symmetric inhibitors exert potent inhibition of HIV-1 protease (**26**, **27**; Xaa = Ile or Val), the absence of good activity with compound **28** (Xaa = Leu) attests to the importance of the substitution of the P2/P2′ positions of this type of inhibitor.[139] "Dipeptide" analogues of **26–28** are poor inhibitors.[139]

A second example of C-2 symmetric inhibitor is found in compounds **29–33**, in which the C-2 axis cuts through the middle of the carbon–carbon bond of the 3,4-diol.[139] These homologues maintain the atomic spacing of the hydroxyethylene isosteres, and in fact, are anticipated by the diol **14**. Typically, these C-2 symmetric diols are ten-fold more inhibitory than their mono-hydroxy congeners, some of which effect inhibition of HIV-1 protease at sub-nanomolar concentrations. As above, the dipeptide forms of the C-2 symmetric diols (**29**, **30**) are considerably less potent than the tetrapeptide analogues (**31–33**), although these tetrapeptide analogues equal or exceed in inhibitory potency typical hydroxyethylene isosteric inhibitors of equivalent size. Interestingly, the inhibitory activity of **31–33** is not nearly as dependent on the stereochemistry of the hydroxyl groups as one would expect.

The C-2 symmetric inhibitors of HIV-1 protease represent an important advance in the development of inhibitors of this enzyme for several reasons: (1) the greatly diminished peptide character of these compounds should prove inhibitors or drugs which are decidedly less susceptible to metabolic degradation *in vivo*, and will possibly show improved pharmacological properties over the existing inhibitors, (2) the symmetrical nature of these compounds should afford them enhanced selectivity for the homodimeric retroviral proteases over their monomeric counterparts, thereby minimizing potential toxicity (pepsin and renin activities are both refractory to micromolar concentrations of compound **26**), (3) and again due to their symmetrical structures, these inhibitors may ultimately prove to be synthetically more accessible than the other isosteres.[140] This type of inhibitor also makes other isosteric replacements for the scissile dipeptide possible which are otherwise synthetically intractable for the hydroxyethylene isosteric inhibitors.

7. HYDROXYETHYLAMINE ISOSTERES

The hydroxyethylamine, or amino alcohol, isostere (Table I) has been successfully employed in renin inhibitors.[127,128] This isostere combines elements of both the hydroxy(m)ethylene and reduced amide isosteres, in that a secondary alcohol is in place to interact with the active site aspartyl residues while the protonated secondary amine is adjacent, and could possibly interact with the enzyme in an ionic interaction. This class of isosteres contains an additional methylene group relative to that of the hydroxyethylene isosteres, such that three atoms in the "peptide" backbone separate the α-carbons of the P1 and P1′ residues.

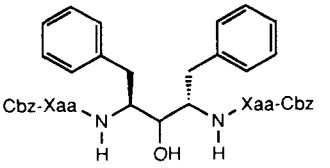
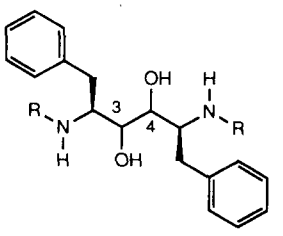
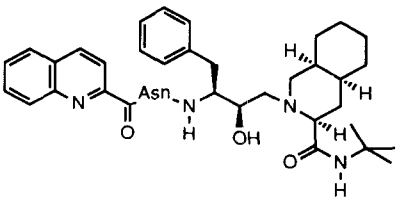
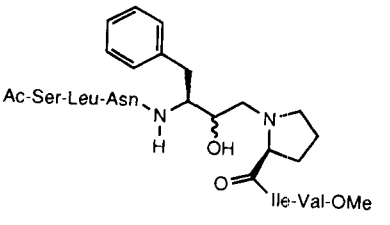
Roberts and co-workers[141] have reported several examples of this class of isostere which are potent inhibitors of HIV-1 protease, one of which (**34**) is shown in Table V. This compound is in effect a tripeptide analogue containing a Phe-Pro hydroxyethylamine isostere; in this case a decahydro-isoquinoline group substitutes for the prolyl residue. In contrast to all aforementioned inhibitors, the *R*-stereoisomer of the secondary alcohol provides the more potent inhibitor. The decahydro-isoquinoline moiety contributes considerably to the potency of this compound; the corresponding derivative containing a prolyl group at this position is more than 50-fold less potent. This suggests that this bicyclic structure may be arresting the pipecolic ring, the surrogate of proline, in a conformation which faithfully reflects the conformer of proline which is recognized by the enzyme.

Rich and colleagues[142,143] have further investigated the hydroxyethylamine isostere for inhibition of HIV-1 protease in a series of peptide analogues. Two examples (**35**, **36**) are mentioned here. Interestingly, it is the *S*-hydroxyl stereoisomer (**35**) which is the better inhibitor; the *R*-isomer is about 80-fold less potent.[143] This stereochemical discrimination is identical in magnitude to that found for the hydroxyethylene isosters,[110] suggesting that the binding of **34** to the enzyme may be unlike that of **35**. Rich attributes these differences in stereochemical preferences to the presence or absence of a residue at the P3′ positions of these inhibitors.[143]

8. STRUCTURAL ASPECTS OF COMPLEXES OF HIV-1 PROTEASE AND ITS INHIBITORS

To date, eight crystal structures of HIV-1 protease have been reported,[78–80,89–93] five of which contain inhibitors in the active site of the enzyme.[89–93] We will not discuss these structural data in any detail here, but instead we will point out common features of these structures which contribute to the binding of peptide analogue inhibitors. A broad sampling of inhibitors, of widely variable potency, are found in these structures: the reduced amide isostere **22**,[89] the hydroxymethylene analogue, acetyl-pepstatin (**19**),[93] the hydroxyethylene isostere **13**,[92] the C-2 symmetric analogue **26**,[90] and the hydroxyethylamine **35**.[91] This compendium encompasses four types of inhibitor bearing a secondary alcohol substituent (**13**, **19**, **26** and **35**), and in all structures this hydroxyl group is positioned equally (within < 3 Ångströms) among the four oxygen atoms of the catalytic aspartyl residues. This suggests that the interactions of other substituents of these compounds with the enzyme may be of secondary importance to this interaction. In fact, of these four inhibitors, the separation of the α-carbons of the P1 and P1′ residues varies from 1–3 carbon atoms, and the interactions of the remainder of the molecules with the enzyme appear to be conserved. The enzyme therefore may

TABLE V
C-2 symmetric and hydroxyethylamine isostere inhibitors of HIV-1

Compound	Structure	K_i(nM)
C-2 Symmetric inhibitors.		
26-28		**26**, Xaa = Val IC_{50} = 3.0 **27**, Xaa = Ile IC_{50} = 4.9 **28**, Xaa = Leu IC_{50} = > 100
29-33		**29**, R = Boc; 3*R*, 4*R* IC_{50} = 40 **30**, R = Boc; 3*S*, 4*S* IC_{50} = 280 **31**, R = Cbz-Val; 3*R*, 4*R* IC_{50} = 0.22 **32**, R = Cbz-Val; 3*S*, 4*S* IC_{50} = 0.38 **33**, R = Cbz-Val; 3*R*, 4*S* IC_{50} = 0.22
Hydroxyethylamine isosteres.		
34		IC_{50} < 0.4
35-36		**35**, *S*-OH 0.24 **36**, *R*-OH 20

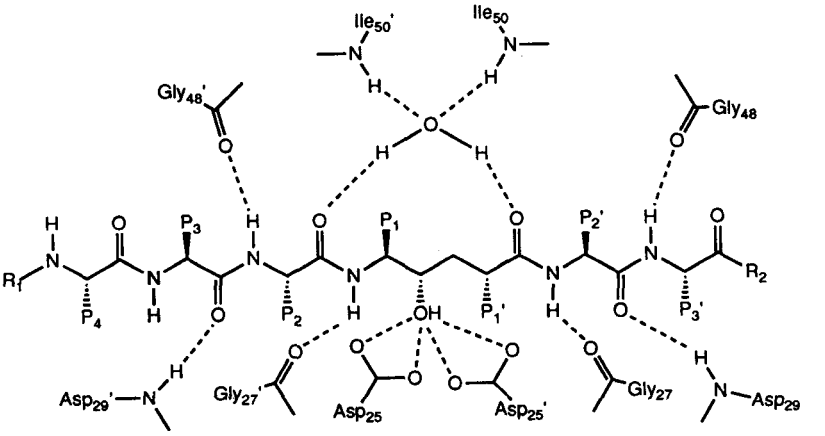


FIGURE 5 Schematic depiction of hydrogen-bonding interactions of a hydroxyethylene isostere inhibitor in the active site of HIV-1 protease. The scheme is based on data found in reference 92. The secondary hydroxyl group of the inhibitor is within hydrogen-bonding distance to all four oxygens of Asp-25 and Asp-25′. Other important hydrogen bonds include those from the inhibitor to residues in the flaps (Ile-50, Ile-50′, Gly-48, Gly-48′), in the active site triad (Asp-Thr-Gly; Gly-27, Gly-27′), and Asp-29 and Asp-29′.

accommodate dipeptide isosteres of various sizes so long as the hydrogen-binding contacts between the inhibitory group and the aspartyl residues, as well as those of the peptide backbone of the inhibitor and groups on the enzyme are maintained.

The most conspicuous differences between the free and complexed forms of HIV-1 protease are found in the flap region, which moves by as much as 7 Ångströms upon binding of the inhibitors. All of the inhibitors are in an extended, β-sheet conformation, which is similar to that of the monomeric aspartic proteases. Shown in Figure 5 is a schematic representation of a hydroxyethylene isosteric inhibitor in the active site of HIV-1 protease, which was constructed from data obtained for compound **13**.[92] The binding of the inhibitor involves a number of crucial hydrogen bonding interactions which are strongly conserved among of the protease-inhibitor structures: (1) those of the inhibitory second alcohol which is centered between the two aspartyl residues, (2) the hydrogen bonds formed between the carbonyl groups between the P2/P1 and P1′/P2′ residues with a conserved water molecule which bridges the carbonyl oxygens of the inhibitor with the amide hydrogens of residues Ile-50 and Ile-50′ in the flap and, (3) hydrogen bonding of the P2/P1 and P1′/P2′ amide hydrogens of the inhibitors to the carbonyl groups of Gly-27 and Gly-27′.

The single, striking feature of these structures is the conserved bridging water molecule, which is unique to the retroviral proteases. This feature, as well as the homodimeric structure of the enzyme, are two novel structural elements which have yet to be exploited for the design of specific inhibitors. Compounds which interact with or dislodge this water molecule would provide inhibitors of high specificity for this enzyme, as would compounds which could specifically disrupt the protease dimer.

9. EFFECTS OF PEPTIDE ANALOGUE INHIBITORS OF HIV-1 INFECTED CELL CULTURE

The ultimate goal of the inhibition of HIV-1 protease is, of course, the development of potent and selective anti-AIDS therapeutic agents. In view of the discussion above, one would expect that if an inhibitor of this enzyme could access the enzyme during virion assembly of an infected T-lymphocyte, the resulting virion would be arrested in an immature morphology, and should be incapable of advancing subsequent infection.

Two types of studies are conducted to assess the anti-viral properties of HIV-1 protease inhibitors:[129,144] (1) In the first, the direct observation of inhibition of HIV-1 protease in chronically-infected T-lymphocytes is realized by performing Western blots of cell lysates following treatment of the infected cell culture with inhibitors for a 24–48 h period.[129] The extent of processing of the viral polyproteins is determined by use of monoclonal antibodies which react with both the protein precursors as well as their products. In a variation of this assay, cells are labelled with ^{35}S-methionine by use of the "pulse-chase" protocol, at the same time of inhibitor addition.[129] Viral proteins are isolated by immunoprecipitation and polyprotein processing is determined by gel electrophoresis. Since virions are continuously assembling and maturing in the chronically-infected T-lymphocyte, these two assays provide an ideal vantage point for observing inhibition of HIV-1 protease in cell culture. (2) The second type of study involves acute infection of virus-naive T-lymphocytes. Inhibitors and infectious virus are added simultaneously, and after several days, the extent of viral infectivity is determined by quantification of three parameters: p24 *gag* antigen and reverse transcriptase activity released into the medium, and the population of syncytia, the multi-nuclear giant T-lymphocytes that form during the infection period.[145] This acute infection, or "viral infectivity", assay reflects the lytic phase of viral infection, and diminution of infectivity by protease inhibitors would indicate that either multiple rounds of infection, which follows the initial infection of a small sub-population of the cells, or the primary infection of even this subpopulation of cells, is being prevented or retarded.

Both the chronic and acute infection assays were first conducted on hydroxyethylene isosteric inhibitors (Compounds **10** and **12**).[129] From the western blot assays, both of these compounds inhibited the conversion of both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ into their mature protein products at micromolar concentrations. Use of the pulse-chase assay confirmed these results, and demonstrated that, at concentrations of 2–5 μM, 50% inhibition of the protease was observed. As anticipated, reverse transcriptase enzymatic activity was also substantially reduced in the lysates of the inhibitor-treated cells. These efficacious concentrations are considerably higher than the K_i values of these compounds, which may reflect poor uptake by the cells, precipitation of the compounds into the medium, or metabolic degradation of the inhibitors. The more potent inhibitor **15** was correspondingly more active in this assay.[146]

Pepstatin A and hydroxyethylene isosteres larger than compounds **10** and **12** at similar concentrations had no effect in our studies.[129] Immunoblot analysis verified that purified virus harvested from chronically-infected H9/IIIB cells treated with compound **12** contained only incompletely processed *gag* proteins ($Pr55^{gag}$, p47, and p40), and no mature p17 or p24.[146] Electron microscopy revealed that the virion particles produced by the inhibitor-treated lymphocytes contained apparently defective core structures; virions possessing "crescent-shaped" cores were preponderant while virions containing mature, cone-shaped cores or annular, immature cores

represented smaller populations.[144,146] Given the composition of *gag* proteins found within the inhibitor-treated virions, this aberrant morphology is suggestive of a stage of polyprotein processing which is intermediate between the annular cores of the immature virions and the cone-shaped cores of the mature virions.

These results demonstrated that, as expected, the exogenous inhibition of HIV-1 protease within actively infected cultures of lymphocytes was sufficient to significantly reduce the proteolytic processing of both the $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ products. Moreover, perturbations in the levels of these polyproteins and their processing intermediates were accurately reflected in the composition of proteins packaged within virions.

It remained to be shown whether replication-competent virions could establish an acute infection in T-lymphocytes in the presence of a protease inhibitor. After a seven-day acute infection of Molt4 cells compounds **10** and **12** at low micromolar concentrations produced commensurate reductions of p24 antigen, particle-associated reverse transcriptase activity, and syncytia in a dose-dependent manner.[129] The more potent inhibitor **15** was effective in the acute infection assay at sub-micromolar concentrations.[146] There was no evidence of cytotoxicity by these compounds. Clearly, protease inhibitors block the enzyme in both the chronically and acutely infected T-lymphocyte, which may indicate that the inhibition of protease activity affects both pre-integrational as well as post-integrational steps in the viral replication cycle.

By now, many peptide analogue inhibitors of HIV-1 protease have demonstrated anti-viral effects in T-lymphocyte culture. Active protease inhibitors represent many of the classes of inhibitors shown in Tables I–V. Of the hydroxyethylene isosteres, compounds **10–12**[129,132] and **14–18**[133–135,146] are all effective in infected cell culture, with IC_{50} values obtained in viral infectivity studies ranging from 40–3000 nM. The IC_{50} values measured for the viral infectivity assay of these compounds are generally considerably higher than their corresponding K_i or IC_{50} values of enzyme inhibition.

Similarly, other types of protease inhibitors demonstrated anti-viral properties. Of the C-2 symmetric inhibitors, compounds **26** and **31–33** potently inhibited viral infectivity with IC_{50} = 20–400 nM.[90,139] The hydroxyethylamine **34**, is among the most potent inhibitors of viral infectivity reported to date (IC_{50} = 2 nM),[140] and is currently being evaluated in a clinical setting. Of all the protease inhibitors which are active in viral assays as reported here, none has proven to be either potently inhibitory of the mammalian aspartic proteases (renin, pepsin, cathepsin D), nor are they appreciably cytotoxic. These findings are quite encouraging for the eventual development of an HIV-1 protease inhibitor as an AIDS therapeutic agent.

10. NON-PEPTIDE INHIBITORS OF HIV-1 PROTEASE

As first shown for porcine pepsin by Tang,[115] the aspartic proteases may be irreversibly inactivated by epoxy-containing compounds, such as 1,2-epoxy-3-(4-nitrophenoxy)propane (EPNP; **37**). It has been shown from crystallographic studies of EPNP-treated penicillopepsin[83] that this epoxy compound inactivates the enzyme by esterification of one or both of the catalytic aspartyl residues. In our hands, EPNP was found to be a time-dependent, irreversible inactivator of HIV-1 protease, but only at pH values above 3.5 at which, presumably, the nucleophilic catalytic aspartyl residue is unprotonated.[77] A proposed chemical mechanism for inactivation by EPNP is shown in Figure 6. The nitrophenoxy group of EPNP could reside in the S1 pocket

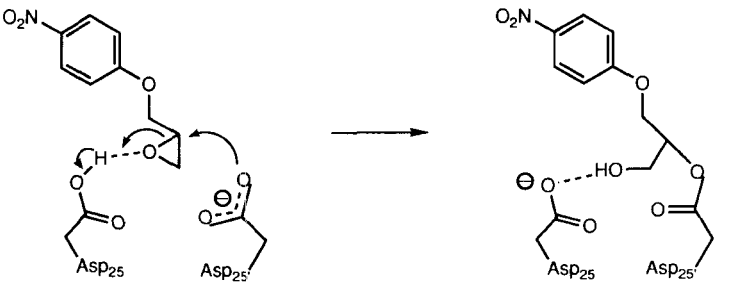
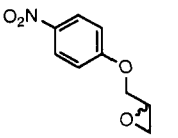


FIGURE 6 Proposed mechanism of inactivation of HIV-1 protease by 1,2-epoxy-3-(4-nitrophenoxy)-propane (EPNP, **37**), based on data in references 77 and 83. The nitrophenoxy group of EPNP presumably occupies the S1 site of the protease. Hydrogen-bonding to, or protonation of, the ring oxygen of the epoxide by the protonated Asp-25 labilizes the C-2 of the epoxide toward esterification by the unprotonated Asp-25.

of the active site, allowing the epoxy group to interpose the aspartyl residues. The attack of the unprotonated Asp-25′ upon the C-2 carbon of the epoxide is facilitated by protonation of the ring oxygen by Asp-25, leading to the esterified form of the protease. Attack of the carboxylate at C-2 of the epoxypropane was indicated from the crystal structure of EPNP-penicillopepsin.[83]

HIV-1 protease is also inhibited (or inactivated) at millimolar concentrations by other epoxide compounds, such as cerulenin (**38**[147]) and **39**.[148] The chemical mechanisms of inactivation of HIV-1 protease by these compounds has yet to be elucidated. Interestingly, Pal *et al.*[149] have reported that cerulenin apparently blocks polyprotein processing in HIV-infected Molt 3 cells.

DesJarlais *et al.*[150] have used a structure-based, molecular modelling approach to discover novel inhibitors of HIV-1 protease. In this method, a data base containing crystallographic data of thousands of known compounds (some of which are clinically useful drugs) is searched for steric complementarity with the three-dimensional structure of the active site of HIV-1 protease. One such compound which was identified



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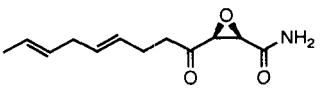

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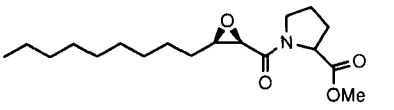

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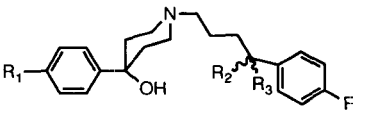

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by this method and which proved to be an inhibitor of HIV-1 protease is haloperidol (**40**; R_1 = Br, R_2 = R_3 = O; K_i = 100 μM). Based on its presumed orientation in the active site of the protease, the hydroxy derivative of haloperidol was synthesized (**40**, R_2 = H, R_3 = OH) in the hope that the secondary alcohol would have high binding affinity for the aspartyl residues. However, this derivative afforded no improvement over haloperidol. As well-resolved structures of HIV-1 protease are widely available, this technique should prove very useful in uncovering novel inhibitors of the enzyme, or at the least, "template" structures which to some extent structurally complement the active site cavity from which inhibitors may be rationally designed and synthesized.

11. PROSPECTS FOR ANTI-AIDS THERAPY BY HIV-1 PROTEASE INHIBITORS

Since it has been recently demonstrated that $T4^+$ T-lymphocytes comprise perhaps the largest and most important reservoir of HIV-1 in the peripheral blood of AIDS patients at various stages of the disease,[151] the demonstration that certain inhibitors of HIV-1 protease can potently block the action of this enzyme within infected T-lymphocytes validates this enzyme as a suitable target for the development of novel, rationally-designed therapeutic agents for the treatments of AIDS. The apparent ability of these protease inhibitors to block maturation of virions shed from chronically-infected T-lymphocytes, as well as to attenuate acute infection of uninfected T-lymphocytes, suggest that inhibition of this enzyme may have an impact on both pre- and post-integrational steps in the HIV-1 replication cycle. This may offer a therapeutic advantage over inhibitors of reverse transcriptase such as azidothymidine, which has been recently shown to be more effective against an acute infection than in reducing the population of active virus in chronically-infected T-lymphocytes.[152] Inhibitors of HIV-1 protease may also prove less toxic than azidothymidine, possibly resulting from their specificity for the retroviral proteases. For many of the inhibitors cited in this review, profound and selective inhibition of HIV-1 maturation and infectivity have been demonstrated at concentrations that might be therapeutically relevant. Moreover, most of these inhibitors have proven to be selective for HIV-1 protease from among the relevant aspartyl proteases, and cytotoxicity is only observed at concentrations which greatly exceed efficacious levels.

Having defined the necessary elements for the design of potent, small-molecule inhibitors of HIV-1 protease and shown that these inhibitors can function as anti-HIV agents, the remaining task is to identify one of these inhibitors which will have pharmacological and toxicological properties suitable for its use as a clinical agent. As the lesson of renin inhibitors has shown, the delivery of peptide analogues as drugs is extemely problematic, due to poor aqueous solubility, poor oral bioavailability, and rapid clearance from the serum by hepato-biliary extraction.[94] Undoubtedly, these complications will be common to the peptide analogue inhibitors of HIV-1 protease. For these reasons, it is perhaps best to regard the present examples of HIV-1 protease inhibitors as "prototypes" from which novel derivatives can be achieved, guided in part by molecular modelling, which are totally devoid of peptide character yet still retain potent inhibition of the enzyme. While myriad practical issues remain to be addressed, the compounds cited here provide an encouraging foundation for the development of protease inhibitors as anti-retroviral agents.

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