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Viral proteases as targets for chemotherapeutic intervention

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Many viruses encode proteinases that are essential for infectivity, and are consequently attractive chemotherapeutic targets. The biochemistry and structure of the human immunodeficiency virus proteinase have been characterized extensively, and potent peptide-mimetic inhibitors have been developed. Techniques and strategies used to improve the efficiency of these compounds are likely to be applicable to other viral proteinases.

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Introduction

Many human and animal viruses encode proteinases that play important roles at different stages in the infection cycle, including separation of functionally different domains from a precursor polyprotein (enabling cleavage products to be transported to different cellular compartments) and regulation of a variety of events in viral replication, such as uncoating, activation of replicative enzymes and morphogenesis [1]. These proteinases are essential for virus infectivity, and have therefore come to be seen as attractive targets for chemotherapeutic intervention, particularly because they have unusual cleavage specificities that differ from those of host proteases.

Proteinases are encoded by all retroviruses, including HIV and human T-cell leukemia virus and have been identified in a growing number of DNA and positive-sense RNA viruses. These include adenoviruses and herpesviruses [2•,3•] as well as picornaviruses, flaviviruses (such as Dengue and yellow fever viruses), pestiviruses and the related hepatitis C virus. A number of these viruses cause diseases of medical or veterinary importance that are not amenable to conventional preventive or prophylactic measures, and proteinase inhibition therefore represents a valid alternative therapeutic approach. Many viral proteinases have only been identified recently, and characterization is consequently in its early stages. To illustrate potential strategies in the analysis of viral proteinases, and in the design and development of inhibitors we shall therefore focus on the picornavirus and retrovirus proteinases, as they have elicited the greatest academic and industrial interest, and as a result, have been characterized in detail.

Development of HIV proteinase inhibitors

HIV-1 has the genetic organization 5′-*gag-pol-env*-3′ that is typical of retroviruses. The *gag* and *pol* genes encode inner structural, and replicative proteins respectively, and are translated as polyproteins that are cleaved at eight sites by the proteinase (PR) (Fig. 1 and Table 1). These polyproteins are transported to the plasma membrane and cleavage occurs after budding of immature particles, resulting in morphological changes associated with virion maturation. The substrate specificity of HIV-1 PR is puzzling in that PR catalyzes specific cleavage at a small number of polyprotein sites that show no apparent sequence conservation. Analysis of viral and non-viral substrates suggests that no subsite has absolute specificity, and that a combination of moderate interactions may be sufficient to confer catalytic specificity [4•]. Heterogeneity in the composition of viral polyprotein cleavage sites probably plays a role in determining the rate, and consequently the order, of cleavage at different sites.

A proteinase-deficient HIV mutant produced non-infectious immature virions containing unprocessed polyprotein [5], an observation that is crucial to the consideration of PR as a therapeutic target. HIV-1 PR is a C_2 symmetric homodimeric aspartyl proteinase that consists of two identical 99-amino-acid subunits. Their termini interdigitate at the dimer interface, but otherwise the topology of HIV-1 PR is similar to that of pepsin-like aspartyl proteinases [6••].

Techniques for the large scale purification of recombinant HIV-1 PR, and for the routine assay of its proteolytic activity are fundamental prerequisites for the development of inhibitors, and various methods have been reported [7••]. The successful design of substrate-based inhibitors of other aspartyl proteinases, such as

Abbreviation
PR—proteinase.

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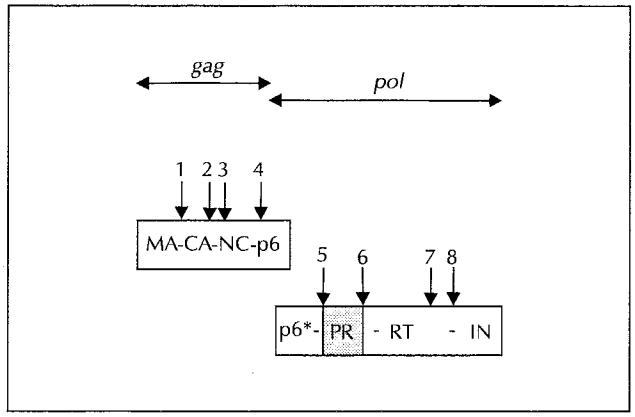

Fig. 1. Gene organization, processing scheme and cleavage sites of the HIV-1 *gag-pol* polyprotein. Cleavage sites are indicated by arrows. The position of the proteinase is indicated by a shaded box.

pepsin and renin, suggested the strategy of designing analogous peptide-mimetic inhibitors of HIV-1 PR by incorporating non-hydrolyzable 'transition-state' mimics into substrate analogues [7••,8•,9••]. This approach is based on a key step in aspartyl proteinase catalysis: generation of a tetrahedral diol by hydration of a trigonal amide (Fig. 2a). The high (millimolar) K_m values of peptide substrates indicate that potent (i.e. nanomolar) PR inhibitors must incorporate structural features that significantly increase their binding affinity. Peptide-based inhibitors have several disadvantages, including vulnerability to degradative enzymes, rapid clearance, and poor oral absorption. These problems are commonly addressed by minimizing size and peptide-like character of promising lead compounds.

The first reported PR inhibitor was pepstatin [10], a diagnostic inhibitor of aspartyl proteinases. This weak inhibitor contains two statine residues that embody transition state analogue I (Fig. 2b). To identify more potent inhibitors, Dreyer *et al.* [11] compared the effectiveness as PR inhibitors of five different classes of dipeptide isosteres inserted into a consensus heptapeptide template. Heptapeptides (P_4–P_3' or P_3– P_4') are the shortest substrates that are cleaved efficiently by PR. Statine-based (I), reduced amide (II) and phosphinate (III) transition state analogues exhibited modest potency, but placement of Phe–Gly hydroxyethylene dipeptide isosteres (IV) into the consensus template yielded compounds that inhibited HIV PR at nanomolar concentrations *in vitro* and prevented polyprotein processing, virion maturation and viral spread at 25–100 μM in cell culture. Truncation and extensive structure–activity analysis at the P_1, P_1' and P_2' positions led to the identification of highly potent (subnanomolar) PR inhibitors based on dihydroxyethylene (V) [12•] and hydroxyethylene (IV) [13•] isostere transition state analogues. Potency was enhanced by incorporating residues that stabilize the extended inhibitor structure, presumably due to optimized hydrogen-bonding in the substrate binding cleft. For example, the P_2' and P_3' residues (Leu–Phe) can effectively be replaced by various substituted aminobenzocycloalkanes [14•]. The P_1' position can accept side-chains unrelated to natural amino acids, allowing modifications to be made that enhance solubility and thus cell penetration [15•,16••]. Such substitutions may reduce binding affinity (K_i) but the enhanced solubility may nevertheless result in a net increase in antiviral activity.

The ability to cleave the amino terminus to proline distinguishes HIV PR from non-viral aspartyl proteinases. Hydroxyethylamine (VI) structures that readily accomodate the prolyl imino acid have been incorporated into a number of potent inhibitors [17,18•,19,20•]. Modification of a protected tripeptide incorporating this structure by substituting the imino residue decahydroisoquinoline at the P_1' position yielded highly potent inhibitors of PR *in vitro* and in cell culture, such as the compound Ro 31-8959 [19,21••]. This inhibitor was expected to have considerable selectivity, and indeed it inhibited human aspartyl proteinases such as gastricsin, renin and pepsin by less than 50% at a concentration of 10 μM. Typical IC_{90} values for Ro 31-8959 (5–30 nM) are 1000-fold below its cytotoxic concentration in uninfected host cells. Recent reports indicate that a 600 mg oral dose every 8 hours is sufficient to maintain the mean human plasma concentration at about 70 nM. The potency of Ro 31-8959 is strongly dependent on tight binding by the P_2 and particularly the P_3 substituents, whereas binding of a second class of hydroxyethylamine inhibitors (which contain a noncyclic, secondary amine in place of the decahydroisoquinoline residue) [22•] is less dependent on these

Table 1. Cleavage sites of HIV-1 PR.

PR site	P4	P3	P2	P1	P1′	P2′	P3′	P4′
1	Ser	Gln	Asn	Tyr	Pro	Ile	Val	Gln
2	Ala	Arg	Val	Leu	Ala	Glu	Ala	Met
3	Ala	Thr	Ile	Met	Met	Gln	Arg	Gly
4	Pro	Gly	Asn	Phe	Leu	Gln	Ser	Arg
5	Ser	Phe	Asn	Phe	Pro	Gln	Ile	Thr
6	Thr	Leu	Asn	Phe	Pro	Ile	Ser	Pro
7	Ala	Glu	Thr	Phe	Tyr	Val	Gly	Asp
8	Arg	Lys	Ile	Leu	Phe	Leu	Asp	Gly

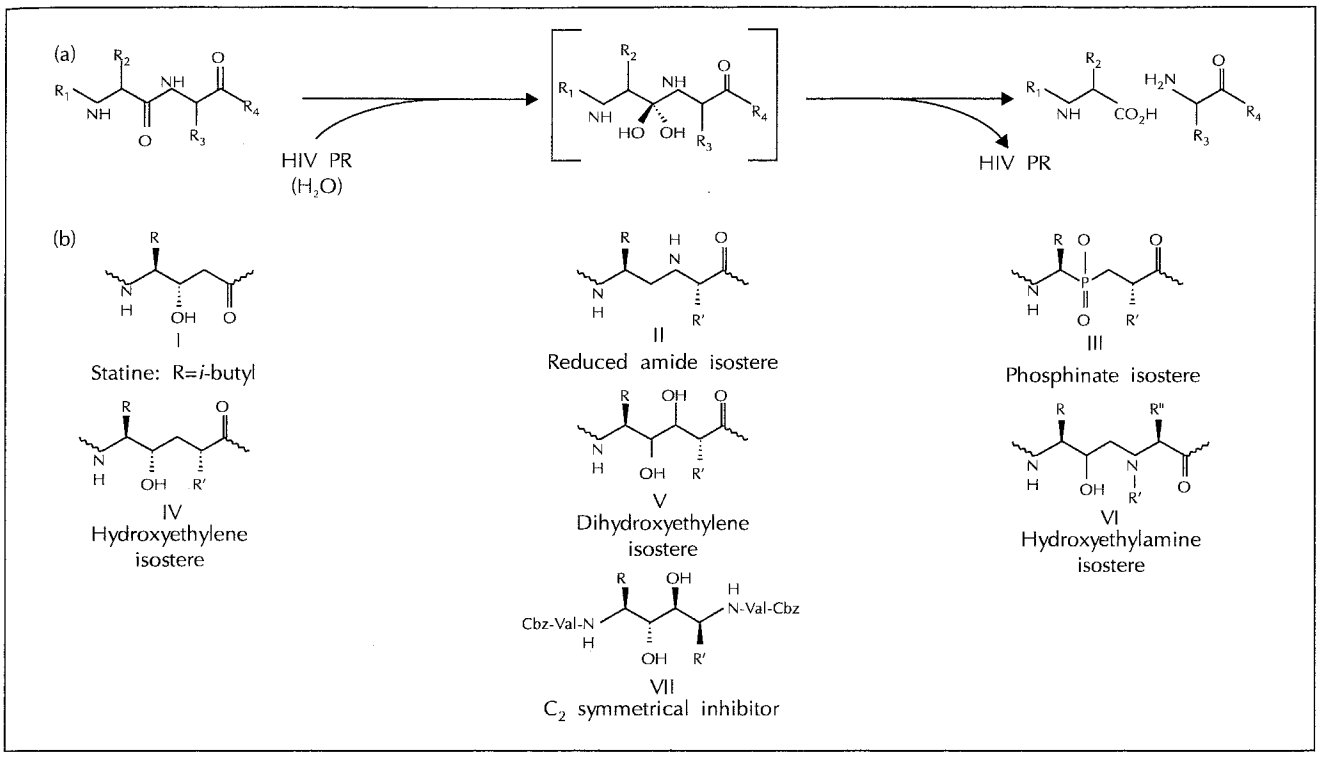

Fig. 2. Mode of function of HIV proteinases and structure of a number of proteinase inhibitors. (a) General scheme of substrate hydrolysis by HIV-1 proteinase (PR). (b) Hydrolytically stable P_1–P_1' dipeptidemimetic replacements of the scissile bond in HIV-1 PR substrates. These analogues mimic the geometry of the tetrahedral intermediate in peptide hydrolysis.

interactions, and this second class binds more tightly in the P_1'–P_2' region.

Peptide substrates are inherently asymmetric and the PR dimer must therefore lose its perfect C_2 symmetry during catalysis. Symmetry is permissible for inhibitors, however, and might even improve binding affinity and selectivity over endogenous aspartyl proteinases. These considerations have led to the design of a series of diaminoalcohol- and diaminodiol-based inhibitors with C_2 (VII) or pseudo-C_2 symmetry [23,24•–26•]. These inhibitors are potent even at subnanomolar concentrations and highly selective *in vitro*, but most have suffered from poor solubility, leading to modest potency in cell culture. Strategies to circumvent this deficiency and thus enhance activity in cell culture have included modification of terminal residues and their linkage groups to increase solubility.

The X-ray crystallographic structures of over 100 HIV PR–inhibitor complexes have been analyzed to assist in the design of improved inhibitors. Complexes of PR with five different classes of inhibitors (I, II, IV, VI and VII) have been reported [16••,20•,24•,25•,27,28••]. Inhibitor binding induces a slight 'hinge' closure of the interface between subunits, and extensive movement of both flaps, tightening the active-site cavity and shielding P_3–P_3' residues of the bound inhibitor from solvent contact. Despite the diversity of inhibitor structures, they all adopt generally similar extended conformations and make very similar contacts with the proteinase, binding of the hydroxyethylamine (VI) inhibitor Ro 31-8959 being a notable exception [20•]. These interactions include extensive Van der Waal's contacts with residues that define the hydrophobic S_2–S_2' binding pockets, and a hydrogen bonding system that sandwiches the inhibitor strand between the catalytic cleft and the flaps. The hydroxyl groups of type V, VI and VII inhibitors form hydrogen bonds with both catalytic aspartates. Significantly, all complexes contain a tetrahedrally coordinated active-site water molecule, which bridges two flap residues and two inhibitor carbonyl groups, prompting suggestions that an improved inhibitor would contain a functional replacement for the water [24•,27]. The similarities in the extended conformation of all inhibitors, as well as in the induced conformational changes that they cause in the enzyme, indicate that it is possible to model and improve peptidic inhibitors on the basis of these known structures. An alternative approach to discover novel templates for the design of non-peptide inhibitors is to search three-dimensional structure databases for molecules with a shape that is complementary to the active-site cleft. To date, this approach has led to identification of the antipsychotic agent haloperidol as a weak PR inhibitor [29].

Proteolysis in picornavirus protein expression

The *Picornaviridae* are a family of small icosahedral viruses that includes the etiological agents of several important human and animal diseases. It consists of

five genera, including rhinovirus (the common cold virus) and enterovirus (e.g. poliovirus and hepatitis A virus).

Picornaviruses have a positive-sense monopartite RNA genome that encodes a single large polyprotein. It is processed by three different proteolytic activities which can each be regarded as serving a distinct function (Fig. 3 and Table 2) [30•]. The initial event in this cascade is cleavage by $2A^{pro}$ at its own amino terminus, separating the P_1 structural protein precursor from the nascent polyprotein. Secondly, functional proteins are released from the P_1 and P_2–P_3 (non-structural) protein precursors by $3C^{pro}$ or its precursors. Finally, maturation cleavage of the VP0 capsid protein occurs on encapsidation of viral RNA to yield infectious virus particles. In addition to their role in viral replication, the 2A and 3C proteinases of poliovirus (and by implication, of other picornaviruses) are responsible for aspects of the dramatic inhibition of host cell RNA and protein synthesis that occurs on infection. The 2A proteinase is involved in degradation of the eukaryotic initiation factor eIF–4Fγ, which is correlated with shut-off of cap-dependent translation [31], and $3C^{pro}$ inactivates transcription factor IIIC, inhibiting polymerase III transcription [32•].

Sequence alignment and inhibitor studies suggested that both 2A and 3C proteinases are related structurally to trypsin-like serine proteinases, with the notable difference of their having a nucleophilic Cys residue within the catalytic triad. These proposals are supported by recent mutagenesis studies [33•–36•]. All picornavirus 3C proteinases are closely related, but there is no similarity between the entero- and rhinovirus 2A proteinases and their counterparts in other genera. Aphthoviruses encode a third (L) papain-like thiol proteinase [37•].

Cleavage-site recognition by polio $3C^{pro}$ is unusually stringent, occuring exclusively at Gln–Gly dipeptides

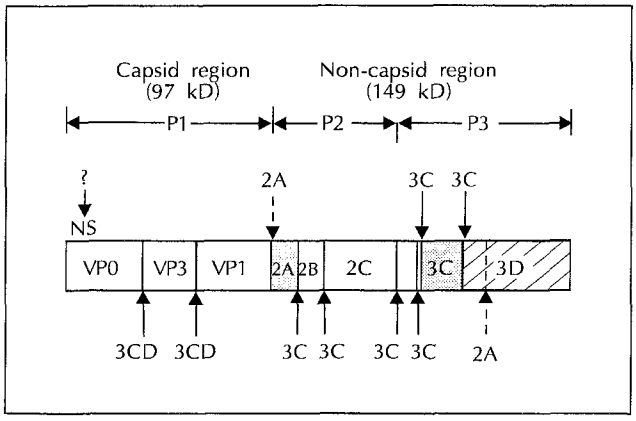

Fig. 3. Gene organization, processing scheme and cleavage sites of the poliovirus polyprotein. Cleavage sites are indicated by arrows and the appropriate proteinases. The positions of proteinases are indicated by shaded boxes.

at all eight sites within the polyprotein (Fig. 3). Sites in other picornaviruses are slightly more heterogenous. Poliovirus $2A^{pro}$ cleaves Tyr–Gly dipeptides at the P_1–2A junction and within the three-dimensional polymerase, but although all corresponding sites in other picornaviruses have a Gly residue at the P_1' position, various residues occur at the P_1 position. Aliphatic residues occur at the P_4 positions of most $2A^{pro}$ and $3C^{pro}$ sites. Mutagenesis and peptide cleavage experiments indicate that cleavage site recognition depends on a minimum substrate length (six residues for $3C^{pro}$) and the presence of specific residues at positions that differ according to both the virus and the proteinase [38,39•,40••,41•–43•]. There are additional conformational determinants of recognition of cleavage sites within polyproteins, so the large (millimolar) K_m values of peptide substrates may reflect their greater conformational freedom. Potential peptidemimetic in-

Table 2. Cleavage sites of poliovirus 2A and 3C proteinases.

	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′
3C site										
VP0/VP3	Leu	Pro	Arg	Leu	Gln	Gly	Leu	Pro	Val	Met
VP3/VP1	Lys	Ala	Leu	Ala	Gln	Gly	Leu	Gly	Gln	Met
2A/2B	Glu	Ala	Met	Glu	Gln	Gly	Ile	Thr	Asn	Tyr
2B/2C	Tyr	Val	Ile	Lys	Gln	Gly	Asp	Ser	Trp	Leu
2C/3A	Glu	Ala	Leu	Phe	Gln	Gly	Pro	Leu	Gln	Tyr
3A/3B	Phe	Ala	Gly	His	Gln	Gly	Ala	Tyr	Thr	Gly
3B/3C	Thr	Ala	Lys	Val	Gln	Gly	Pro	Gly	Phe	Asp
3C/3D	Phe	Thr	Gln	Ser	Gln	Gly	Glu	Ile	Gln	Trp
2A site										
VP1/2A	Asp	Leu	Thr	Thr	Tyr	Gly	Phe	Gly	His	Gln
3C′/3D′	Leu	Leu	Asp	Thr	Tyr	Gly	Ile	Asn	Leu	Pro

hibitors are likely to exhibit similar flexibility, and must therefore be conformationally constrained and incorporate structural features that increase their binding affinity. The lack of absolute specificity at most subsites, and the requirement for peptide substrates to extend to the P_4 position indicates that the substrate binding clefts of $3C^{pro}$ and probably $2A^{pro}$ are capable of extensive hydrogen bond interactions with such inhibitors. However, only a few inhibitors of $3C^{pro}$ have been reported [44•,45].

Conclusions

Proteases are encoded by several DNA viruses and numerous RNA viruses in addition to the picornaviruses and retroviruses discussed above. Although they are all potential targets for chemotherapeutic intervention, significant progress in inhibitor development has only been reported for HIV-1 PR. In the few years since its identification, the structure of PR and numerous inhibitor complexes have been determined, and highly potent peptidemimetic inhibitors have been developed. Knowledge of the strategies used in enhancing the potency and specificity of PR inhibitors, and in overcoming the inherent limitations of peptide-based inhibitors is likely to prove invaluable in the development of peptidemimetic inhibitors of other viral proteinases.

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