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A SYSTEMATIC EVALUATION OF THE INHIBITION OF HIV-1 PROTEASE BY ITS C- AND N-TERMINAL PEPTIDES

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Abstract: C- and N-terminal peptides of varying lengths corresponding to the dimerization interface of the retroviral protease of the human immunodeficiency virus-1 (HIV-1 protease) have been synthesized and evaluated as inhibitors of HIV-1 protease activity.

As more is learned about the molecular events in the replication of human immunodeficiency virus (HIV), more strategies for potential therapeutic intervention have been uncovered. Among those, the virally encoded protease has become a major target in the quest for effective antiviral agents.[1] An essential step in maturation of HIV is the processing of the *gag* and *gag/pol* viral proteins by HIV protease, which is itself encoded in the *pol* gene.[2] The crucial role of this protease in the processing of HIV proteins and the production of infective virus particles makes it a prime target for drug design.

The structure of HIV-1 protease has been elucidated by X-ray crystallography.[3] The protease is composed of 99 amino acid residues and self assembles into a homodimeric structure (Figure 1). Each monomer in the assembly contributes half of the active site (which contains two catalytic aspartate residues) needed for proteolytic activity. Dimerization of HIV-1 protease, therefore, generates the catalytic center of the enzyme and also the substrate binding pocket. The dimeric nature of HIV-1 protease offers a new mode of enzyme inhibition beyond active site inhibition.[4] Blocking the assembly of the HIV-1 protease homodimer or disrupting the dimeric interface would be a unique means of inhibiting protease activity.

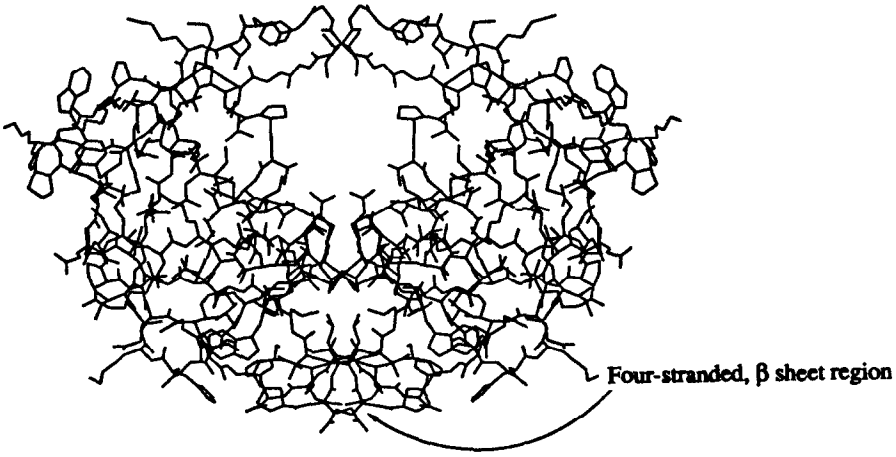

Figure 1. The structure of HIV-1 protease as determined by X-ray crystallography.

When investigating the main areas of monomer overlap in HIV-1 protease for potential dimerization inhibition, we focused on the region centered around the N- and C-termini which accounts for approximately 50% of the interfacial area of the homodimer.[5] In this region the N- and C- terminal residues interdigitate to create a four-stranded, antiparallel β sheet structure (Figure 2). The synthesis of a number of peptide fragments corresponding to C- or N-terminal peptides has been reported from different sources, and some showed a decrease in HIV-1 protease activity or HIV-1 production *in vitro*.[6] In this paper we report a systematic study of the length requirements for the N- and C-terminal peptides of the four-stranded, β sheet dimerization interface needed for optimum HIV-1 protease inhibition. To minimize complications with potential disulfide bond formation, we converted Cys(95) into Ser for our peptide studies. None of the sequences tested for inhibition contain known protease cleavage sites.

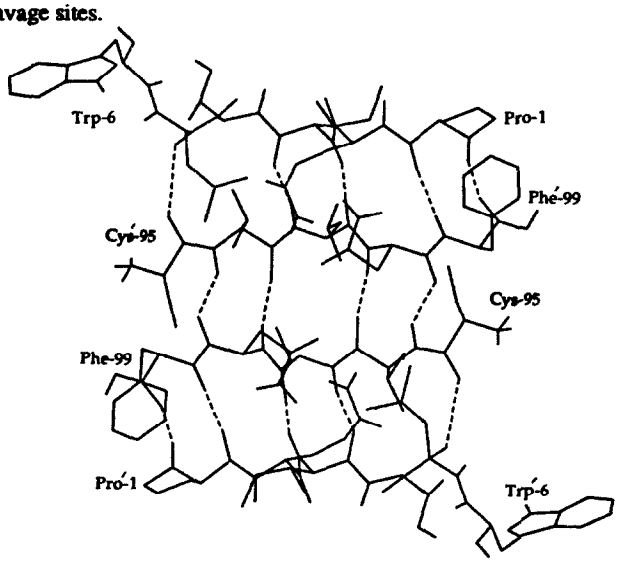

Figure 2. The four-stranded antiparallel β-sheet found at the N- and C-termini of the HIV-1 protease homodimer (dashed lines indicate interstrand hydrogen bonds).

The N- and C-terminal peptides were synthesized by a solid phase procedure using the Merrifield resin modified with a *p*-alkoxybenzylalcohol linker.[7] Fluorenylmethyloxycarbonyl (Fmoc) was used as the semipermanent amine protecting group, and amino acid sidechains were protected with *t*-butyl-based functionality.[8] Amino acid couplings were performed via the amino acid symmetrical anhydrides (3 eq) or via the hydroxybenzotriazole (HOBT) esters for Asn and Gln. The terminal amine was deprotected and acetylated with acetic anhydride and diisopropylethyl amine (DIEA). The peptides were cleaved from the resin with trifluoroacetic acid (TFA) and purified to homogeneity by HPLC. The peptide sequences were confirmed by mass spectrometry and amino acid analysis.

The inhibitory effect of peptides **1-3** on HIV-1 protease activity was evaluated using a fluorogenic substrate assay developed by Toth and Marshall.[9] We found that in the case of the C-terminal peptides maximum inhibition was observed with the pentapeptide **1b** (Table 1).[10] This corresponds well with the C-terminal region involved in the β sheet interface of HIV-1 protease which spans from residue 99 to 95. It is interesting to note

that inhibition with the hexa- and heptapeptides dropped off and was regained with the octapeptide. In the protease a β-turn structure begins at residue Gly(94) extending to Thr(91). Gly(94) has only one H-bond interaction across the β-turn, and Ile(93) makes no H-bonds within the homodimer, but the sidechain fills a hydrophobic pocket created by Phe(99), His(69) and Ile(66). These added residues may make it more difficult for the hexa- and heptapeptides to fit easily into the β-sheet structure at the interface, or they may influence the conformation of the peptides such that they cannot adopt the required β-strand conformation. Gln(92), however, makes a number of H-bonds within the homodimer which may have a positive influence on binding of the peptide and lead to improved inhibition.

Table 1. Inhibition of HIV-1 Protease with C-Terminal Fragments Corresponding to the Dimerization Interface of HIV-1 Protease

	C-terminal Peptides	% Inhibition[a]
1a	Ac-ThrLeuAsnPhe-OH	3.9
1b	Ac-SerThrLeuAsnPhe-OH	37.2
1c	Ac-GlySerThrLeuAsnPhe-OH	4.6
1d	Ac-IleGlySerThrLeuAsnPhe-OH	12.6
1e	Ac-GlnIleGlySerThrLeuAsnPhe-OH	34.6

[a]At 100 μM concentration of peptides.

The N-terminal portion of HIV-1 protease is also involved in the β-sheet structure extending from residues 1 through 6. The hexapeptide corresponding to this sequence is a significantly better inhibitor than the shorter pentapeptide and the longer heptapeptide (Table 2). We have also explored the effect of blocking the N-terminus in these peptides, which is involved in a salt bridge with the C-terminus from the other monomer in the homodimer. As expected there was a small decrease in inhibition with peptides **3a** and **3b**, but the hexapeptide was still quite active. Peptide **3c**, however, had improved inhibition upon acetylation, which may be due to H-bonding interactions between the acetyl-amide and C-terminal carboxylate, in addition to hydrophobic interactions with the Phe(99) sidechain. We have also explored a possible synergistic effect on inhibition with a 1:1 mixture of peptides **1b** and **2b**. No enhanced inhibition was obtained with this mixture, however, over the single peptides, **1b** and **2b**, alone.

Table 2. Inhibition of HIV-1 Protease with N-Terminal Fragments Corresponding to the Dimerization Interface of HIV-1 Protease

	N-terminal Peptides	% Inhibition[a]
2a	H-ProGlnIleThrLeu-OH	5.6
2b	H-ProGlnIleThrLeuTrp-OH	39.9
2c	H-ProGlnIleThrLeuTrpGln-OH	1.3
3a	Ac-ProGlnIleThrLeu-OH	-1.4
3b	Ac-ProGlnIleThrLeuTrp-OH	28.1
3c	Ac-ProGlnIleThrLeuTrpGln-OH	26.0

[a]At 100 μM concentration of peptides.

In conclusion, we have demonstrated that N- and C-terminal peptides of the dimerization interface of HIV-1 protease can act as HIV-1 protease inhibitors. In systematically varying the length of the peptide sequences, definite trends were obtained in which it was determined that peptide **1b** of the C-terminus and peptide **2b** of the N-terminus had the maximum inhibitory activity towards HIV-1 protease. Interestingly these sequences correspond exactly to the full length β-strands in the four-stranded, β-sheet region of the protease, which lends support to the belief that these peptides may be interfering with the dimerization interface. Although the inhibition obtained with these peptides is not large, they may act as good starting points for the design of novel dimerization inhibitors of HIV-1 protease. We are currently incorporating these peptide sequences into new agents to inhibit HIV-1 protease.

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10. The inhibitory effect of the peptides on HIV-1 protease activity was evaluated using a fluorogenic substrate corresponding to a p24/p15 cleavage site, AcNH-Thr-Ile-Nle-Nle-Gln-Arg-NH_2 (Nle = norleucine replaces Met in the natural sequence), with incorporation of 2-amino benzoic acid in place of the acetyl group as the donor, and *p*-nitro-Phe at the P1' position as the acceptor to give an intramolecularly quenched fluorogenic substrate.[9] Cleavage of the substrate by HIV-1 protease released the fluorescent N-terminal tripeptide from the C-terminal peptide resulting in enhanced fluorescence. In a typical experiment the peptides were dissolved in DMSO and incubated for 30 minutes with HIV-1 protease (5% constant DMSO concentration) at a concentration of 100 μM in the peptides. The substrate was added and the increase in fluorescence was monitored with time. The percent inhibition was obtained from a comparison of the increase in fluorescence versus time with and without added inhibitor. MVT-101 was our positive control.[9] Each experiment was performed twice and the error between runs was 10-20%.