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Human Immunodeficiency Virus-1 Protease : Structure and Its Recent Inhibitors

เอนไซม์โปรติเอสของไวรัสเอชไอวี-1 : โครงสร้างและตัวยับยั้ง

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บทความนี้กล่าวถึงแหล่งที่มา โครงสร้าง และหน้าที่ของเอนไซม์โปรติเอสจากไวรัสเอชไอวี-1 และตัวยับยั้งเอนไซม์ดังกล่าว เอนไซม์โปรติเอสเอชไอวี-1สามารถสังเคราะห์โดยวิธี stepwise solid phase หรือผลิตโดยยีนโคลนนิ่งในยีสต์และแบคทีเรีย โครงสร้างของเอนไซม์นี้เป็นแบบไดเมอร์และจัดเป็นเอนไซม์โปรติเอสในกลุ่มของกรดแอสปาติก ทำหน้าที่เปลี่ยนแปลงโครงสร้างของไวรัสไม่เต็มวัย ให้อยู่ในรูปทำงานได้ โดยย่อยพันธะระหว่างกรดอะมิโนในกลุ่มที่มีวงแหวนอะโรมาติก เช่น ไทโรซีนและฟีนิลอะลานีน กับกรดอะมิโนโปรลีน หรือย่อยพันธะระหว่างกรดอะมิโนในกลุ่มไฮโดรโฟบิก ดังนั้นเอนไซม์นี้จึงมีศักยภาพในการใช้เป็นเป้าหมายรักษาโรคเอดส์โดยการพัฒนาตัวยับยั้งเอนไซม์โปรติเอสเอชไอวี-1 เพื่อทำลายไวรัสดังกล่าว

คำสำคัญ : โปรติเอส เอชไอวี-1 ตัวยับยั้งโปรติเอส ยาต้านไวรัสเอชไอวี

Abstract : Worapot Suntornsuk and Leena Suntornsuk. 2001. Human Immunodeficiency Virus-1 Protease: Structure and Its Recent Inhibitors. Thai J Hlth Resch 15(1) : 69-83.

Sources, structure and function of human immunodeficiency virus-1 (HIV-1) protease are overviewed in this article. The enzyme can be synthesized by stepwise solid-phase or produced by gene cloning in yeast and bacteria. Its structure is described as a dimer form which belongs to aspartic protease family. The enzyme plays an essential role in cleaving the HIV protein precursor initially formed by viral mRNA translation into the mature HIV. It normally cleaves the bonds between an aromatic residue (Tyr or Phe) and a Pro residue or cleaves between two hydrophobic residues in viral polypeptide. This enzyme is a potential target for the development of anti-AIDS therapy using a HIV-1 protease inhibitor. The recent examples of its inhibitor are also discussed.

Key words : protease, HIV-1, protease inhibitor, antiretroviral drug

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Introduction

The human immunodeficiency virus (HIV) is an important retrovirus that causes acquired immunodeficiency syndrome (AIDS) by infecting and killing T-helper cells. The HIV type 1 (HIV-1) virion is slightly more than 100 nm. in diameter and on electron microscopy appears as a dense cylindrical core surrounded by a lipid envelope. Like all retroviruses, the HIV-1 virion core contains RNA genome, structural proteins and virally encoded enzymes, including reverse transcriptase and integrase, which are required for efficient viral replication. The RNA genome is roughly 10 kb in length and is characterized by the presence of two flanking long terminal repeat (LTR) sequences as well as genes encoding the structural proteins (*gag*), reverse transcriptase, protease and endonuclease enzymes (*pol*), and envelope glycoproteins (*env*) (5'-LTR-*gag*-*pol*-*env*-LTR-3'). The major structural proteins and enzymes are initially synthesized as large polyprotein precursors (*gag* p55 and *gag*-*pol* p160). The precursors are later processed by HIV-1 protease to yield the mature structural proteins and enzymes including the protease for the successful completion of the retroviral life cycle (Figure 1) and the production of infectious virion.

The HIV-1 protease is a 198-residue protein dimer and classified as an aspartyl protease. It is composed of two identical units, each contributes a single essential D-T-G sequence in the active site, which is essential for viral replication and infectivity. The HIV-1 protease, thus, is considered to be an attractive target of the therapeutic agent for curing the AIDS.

HIV-1 protease sources

Isolation of HIV-1 protease from virus particles yields only small quantities and carries the risks associated with handling a deadly virus. Two possible strategies for generating sufficient quantities of HIV-1 protease for enzymatic and structural characterization are total chemical synthesis (Nutt *et al.*, 1988; Schneider and Kent, 1988) and expression of the cloned gene in an appropriate system.

The chemically synthesized amino acid sequences of HIV-1 protease was prepared by the stepwise solid-phase peptide synthesis on a solid support which is either a polystyrene or Boc-Phe-O-phenylacetamidomethyl resins. The resin support was, then, removed by hydrogen fluoride and dissolved in guandine HCl or 50% acetic acid. After that, the unfolded proteins were refolded to active enzyme under reducing conditions and dialysis against appropriate buffers. The refolded proteases were purified by gel filtration chromatography

using sephadex G-75 medium (40-120 μm beads). The synthetic active HIV-1 protease was demonstrated specific cleavage of the natural substrate *gag* p55 into mature *gag* proteins and cleave oligonucleotides corresponding to the protease processing site in the HIV-1 polyprotein *gag*.

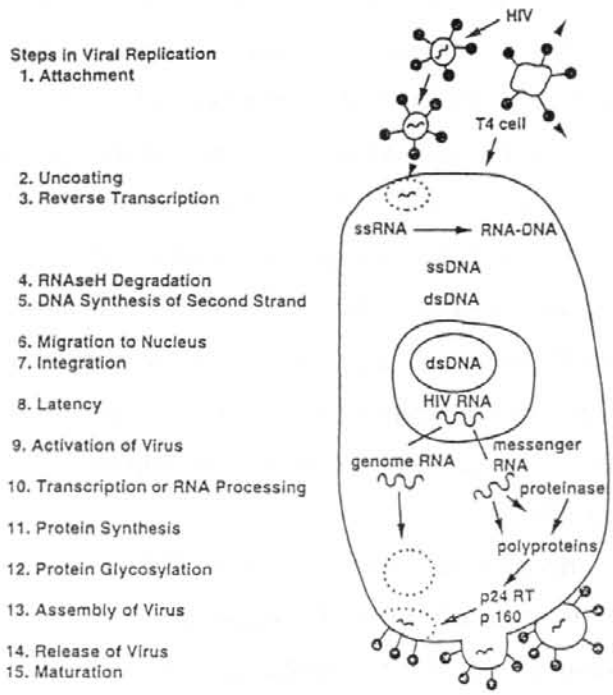


Figure 1 The life cycle of HIV-1 (Schnittman and Pettinelli, 1997)

The HIV-1 protease has been produced by cloning and expression in yeast and *E. coli*. An evidence was shown that an active HIV protease could be expressed in yeast cells transformed with a plasmid containing all the *gag* and a portion of *pol* restriction fragment (Kramer *et al.*, 1986). Debouck *et al.* (1987) reported that the *gag* and parts of the *pol* genes were also cloned and expressed in *E. coli*. The HIV protease gene product was found to process itself from the expressed proteins, resulting in the accumulation of a mature 10-kDa form of the protease as observed on SDS-PAGE. Gene coding HIV protease alone was also cloned and expressed in *E. coli* (Graves *et al.*, 1988; Darke *et al.*, 1989). The active product was obtained. This bypassed the need for autoprocessing. The protease yield could cleave HIV *gag* as well as *pol*-specific polypeptide substrates *in vitro*. This supports the idea that the same processing of *gag-pol* sequences can occur whether the cleavage protease is in intramolecular or intermolecular.

Three dimensional structure determination

The determination of HIV-1 protease structure has been actively performed by X-ray crystallography over the last 10 years. The procedure consists of purification, crystallization and crystal structure determination.

The purification of HIV-1 protease has been successfully achieved from the expression and cloned *E. coli* for structure and function studies, including X-ray crystallographic structure determination. Darke *et al.* (1989) carried out the purification by using DEAE-Sephadex, phosphocellulose and Mono-S column chromatographies to obtain 0.11 mg of HIV-1 protease with 7% overall yield. McKeever *et al.*(1989) purified the HIV-1 protease with DEAE-Sephadex, Baker HiPropyl hydrophobic interaction column and Mono-S column chromatographies. Recently, the purification of homogenous HIV-1 protease was improved the overall yields and rapid production by using 4 column steps: QAE-Sepharose anion exchange chromatography, hexyl agarose hydrophobic interaction chromatography, Mono-S cation exchange chromatography and superose 6 size exclusion chromatography (Margolin *et al.*,1991).

Crystallization was performed by the hanging drop vapor diffusion technique in 24-well Linbro cell culture plates. Two-ten µl of HIV-1 protease at 6 mg protein/ml (diluted with the mixture of 10 mM NaMES/MES, 2-(N-morpholino) ethanesulfonic acid, pH 5.0, 1 mM DTT, dithiothreitol, 1 mM Na_2EDTA and 3 mM NaN_3) were mixed with an equal volume of a precipitating solution consisting of 100 mM imidazole/HCl (or NaMOPS/MOPS, 3-(N-morpholino) propanesulfonic acid), pH 7.0, 250 mM NaCl, 10 mM DTT and 3 mM NaN_3 on a siliconized glass cover slide. The cover slide was inverted and sealed over a reservoir of the precipitating solution with silicone vacuum grease. The plate was stored at $4^{\circ}C$. The tetragonal bipyramid crystals approximately 0.25x0.25x0.50 mm in size were observed within 4-7 days. The crystals were transferred to the precipitating solution and used within 1-2 weeks. Crystals stored for longer periods showed a complete loss of the diffraction pattern even though they physically appeared to remain intact (McKeever *et al.*,1989).

A single crystal was transferred and mounted in a thin-walled glass capillary containing a paper wick wetted with precipitating solution for X-ray diffraction and intensity measurement.

The description of HIV-1 protease structure

The crystal structure of HIV-1 protease was first obtained at 3.0°A resolution from multiple isomorphous replacement (MIR) for the NY5 isolation of HIV-1 cloned from *E. coli* (Navia *et al.*,1989). The protein was proved that it was the active dimer and homologous with microbial aspartyl protease, but regular secondary structure was neither observed in the dimer interface nor in an expected α-helix near the carboxyl terminus, in contrast with the structure reported in these regions for the highly homologous Rous sarcoma virus (RSV) protease. The flap region was observed to be ordered crystallographically, but subjected to intermolecular contacts in the crystal.

Subsequently, Wlodawer *et al.* (1989) solved the crystal structure of chemically synthesized [Aba 67,95] HIV-1 protease, SF2 isolate at 2.7°A resolution by molecular replacement, then confirmed by the MIR method and electron density map and refinement with R-factor 0.184 and an average B of 16.9°A^2. The α-helix region missing in the Navia structure was observed. Other elements of this structure were similar to the earlier result. Comparison of this structure with the proposed models of Pearl and Taylor (1987) obtained a root mean square (rms) deviation 2.6°A for 87 atom pairs, one or two residues shifted the positions, but, in general, well agreement. The rms correspondence with the model structure proposed by Weber *et al.* (1989) was 1.3°A for 82 of the 99 C atoms, excluding the flap region and frameshifting residue 1-5 by two positions.

Lapatto *et al.* (1989) reported the third crystal structure at 2.7°A resolution and the schematic diagrams of the super secondary structure of the HIV-1 protease (Figure 2). It was shown that this protease is a homodimeric molecule of approximately 45x30x25°A, consisting of two monomers related by a crystallographic two-fold axis of symmetry and has 99 amino acids and 758 atoms for each monomer. The monomer contains two similar motifs; each motif comprises anti-parallel strands: a, b, c, and d for the first and a', b', c' and d' for the second. These are organized together in a distorted sheet, with strand c and d' and strands c' and d forming two pairs of parallel strands. Strand b and c and strand b' and c' form antiparallel β-hairpins. The strand d and d' are followed by an irregular region and an α-helix, respectively, which also lie on the same side of the main mixed β-sheet. Strand a of the first motif is displaced from the main mixed sheet and forms an antiparallel four stranded β-sheet with the terminal strand, t, and the two equivalent strands from the other monomer of the dimer. This structural folding confirmed and agreed well with the

Wlodawer's work excepting strand a and t of each monomer in the Wlodawer's were placed at the outer part of the interface sheet but still formed an antiparalled four-stranded β-sheet.

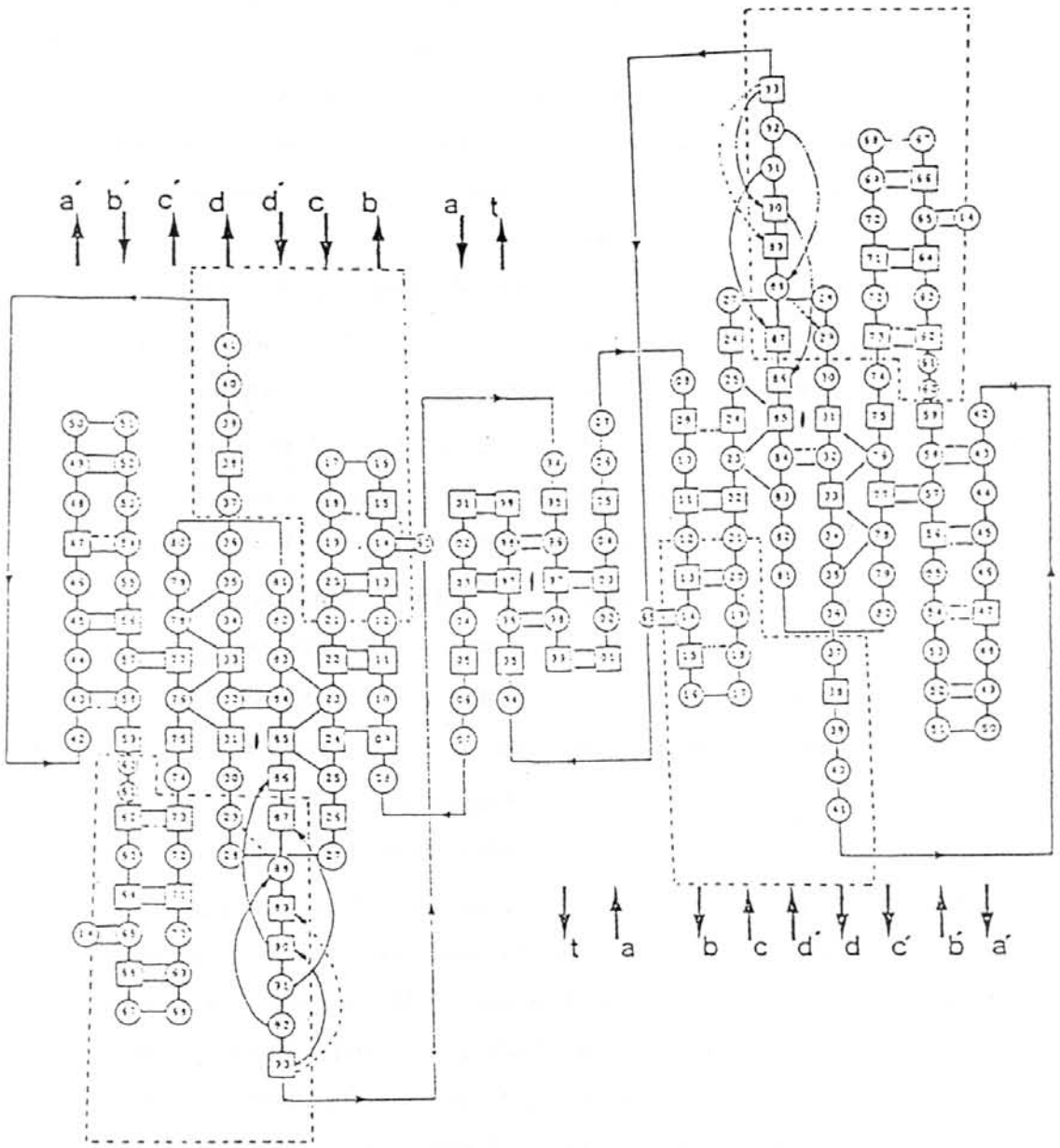


Figure 2 Schematic diagram of the super-secondary structure of HIV-1 protease. Well defined hydrogen bonds are shown by an arrow from the donor to the acceptor; weaker hydrogen bonds with distances less than 3.9°A are donated by a dotted line. Residues indicated by squares provide an extensive hydrophobic core. (Lapatto *et al.*, 1989)

The protease homodimer is stabilized by extensive interactions involving the catalytic residues, D25-T26-G27, and residues at both ends of the polypeptide chain which form an interface β-sheet. As a result of dimerization, the enzyme-active site appears as a tunnel between the two domains (Figure 3), closed at the top by the intrinsically flexible flap residues from both monomers. The hydrophobic core extends through the dimer interface, involving residue P1, I3, L5, V11, L24, L90, C95, L97 and F99. The catalytic polar residue, T26, is completely buried in the interface, forming H-bonds with the equivalent residue from the other subunit, as observed in pepsin-like enzymes. Hydrophobicity of L23, A28, V82 and L84 at the exposed side on the floor and lateral walls of the tunnel seems to play a major role in the HIV-1 protease structure, not only as the driving force in the folding of a single monomer but also in dimer stabilization as well as in substrate binding.

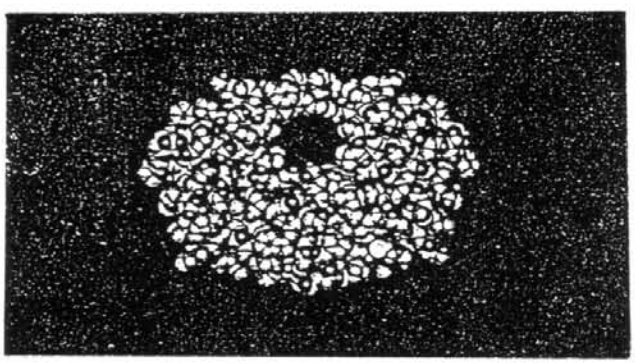


Figure 3 Full sphere representation of the HIV-1 homodimer (Spinelli *et al.*, 1991)

Function of HIV-1 protease

The active site residues, D25-T26-G27, are located in a loop whose structure is stabilized by a network of H bond characteristic of aspartyl proteases. The residues form the substrate binding cleft, including amino acid residue 8-9, 23-32, the "flap" from about 47 to 53, 76, 80-84 and 87 which contains part of a β-sheet and α-helix region near the carboxyl terminus (Weber *et al.*,1989; Wlodawer *et al.*,1989). These regions of the polypeptide chain are sensitive to inactivation by mutagenesis and chemical modification since changes in the component amino acids easily disrupt the active site of the protease (Debouck *et al.*,1987; Seelmeier *et al.*,1988; Loeb *et al.*,1989; Louis *et al.*,1989).

The catalytic mechanism of aspartyl protease is represented in Figure 4. An active site of water molecule is deprotonated by the unprotonated aspartyl carboxylate and adds to the carbonyl oxygen of the scissile amide bond to generate a tetrahedral transition state intermediate.

The HIV-1 protease naturally cleaves at four different positions on the *gag* p55 polyprotein and at least three different locations on the *gag-pol* p160 polyprotein illustrated in Figure 5 (Margolin *et al.*,1990). There is no strict primary sequence conservation among cleavage sites, seem that the determinants of HIV-1 protease cleavage specificity are not simple and/or are not entirely dictated by the primary sequence of the sites. Also, P4 to P4' residues (P1, P2,...are residues numbered from the scissile bond toward the amino terminus and P1', P2',...are residues numbered from the scissile toward the carboxyl terminus) in all HIV-1 sites share an overall hydrophobicity, paricularly in the P1 and P1' positions. Moreover, the amino acid side chain at the P1 position is typically large hydrophobic and unbranched at the β-carbon in all known retroviral cleavage sites (Henderson *et al.*,1988).

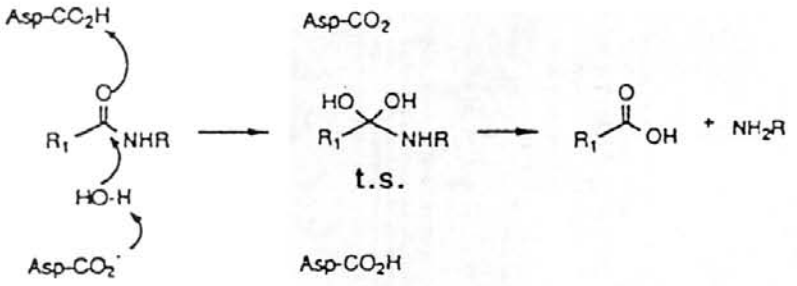
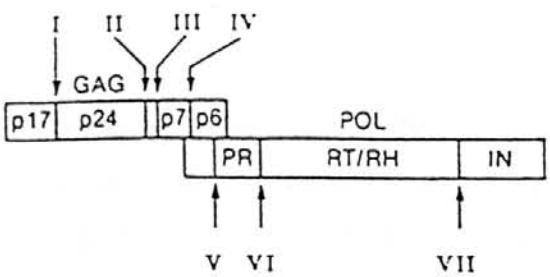


Figure 4 Catalytic mechanism of aspartyl proteases (Debouck, 1992)



I GAG	132-133	S	N	Q	V	S	Q	N	Y—P	I	V	Q	N	I	Q
II GAG	363-364	M	Y	S	K	A	R	V	L—A	E	A	M	S	Q	V
III GAG	377-378	V	T	N	S	A	T	I	M—M	Q	R	G	N	F	R
IV GAG	448-449	Y	K	G	R	P	G	N	F—L	Q	S	R	P	E	P
V POL	56-57	Q	G	T	V	S	F	N	F—P	Q	I	T	L	W	Q
VI POL	155-156	Q	I	G	C	T	L	N	F—P	I	S	P	I	E	T
VII POL	715-716	S	A	G	I	R	K	V	L—F	L	D	G	I	D	K

Figure 5 Protease processing sites in the *gag* and *pol* genes of HIV-1 (Margolin *et al.*, 1990)

Evolution of HIV-1 protease

The HIV-1 protease was shown features of the 3-dimensional structure of aspartyl protease conserved in the retroviral protease by amino acid sequence in a structural alignment of crystal structure and predicted model based on Rous sarcoma virus (RSV) protease represented in Figure 6 (a),(b) (Weber, 1989). The active site triplet, D-T-G, and a sequence, G-R-D/N, near the carboxyl terminus are highly conserved. The basic structural framework of β-strands and the carboxyl terminal helix is also conserved. The R87 of the HIV-1 protease is located in the helix (h') and forms an ion pair with D29 that is highly conserved. However, the 99 residue HIV-1 sequence is shorter than the 124 residue RSV protease sequence, in the alignment, deletions are allowed at the large surface loops. Therefore, divergent evolution is clearly demonstrated.

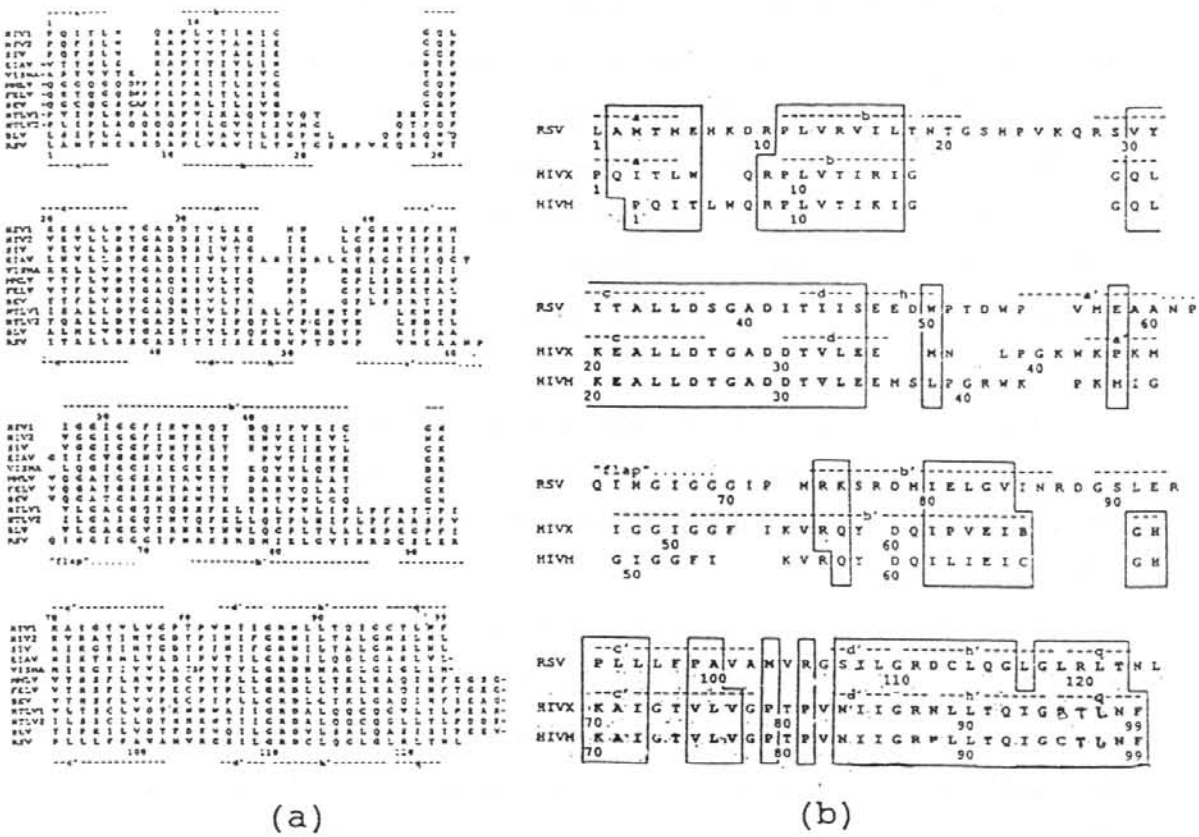


Figure 6 The structural alignment of (a) retroviral proteases (b) RSV and HIV-1 proteases. HIVX indicates the sequence of the synthetic HIV-1 protease, HIVM indicates the sequence of the model based on the RSV protease (Weber, 1989)

There are important differences among aspartyl proteases and the HIV-1 protease, most notably in size. Aspartyl proteases consist of two similar lobes that are thought to have risen by gene duplication, divergence, and gene fusion (Tang *et al.*,1978). Each lobe donates an aspartic acid residue to the active site. The HIV-1 protease is much smaller than the typical aspartyl protease (11 versus 35 kDa), making it unlikely that one molecule of the viral protease could provide both aspartic acid residues to the active site. This has led to the suggestion that the protease must first dimerize to form an active enzyme. The need to dimerize may provide the virus with a mechanism for controlling the activity of its protease. Moreover, the protease corresponded to a single domain of an aspartyl protease (Pearl and Taylor, 1987). It might be a good candidate for the ancestor of aspartyl protease.

Protease inhibitors

As described earlier, HIV proteins require proteolytic cleavage for final assembly of viable virions. This step requires a protease specific to HIV. Therefore, protease inhibitors become one of the most rapid and widespread research areas for new anti HIV agents. Several candidates of protease inhibitors (Figure 7) are active *in vitro*, resulting in the production of defective, incompletely formed, and non-viable viral particles (Volberding, 1994). Unlike other agents, protease inhibitors do not need to be activated inside cells before exerting their antiviral effect. However, most protease inhibitors are expensive, difficult to be synthesized, poor oral bioavailability, highly metabolized, and highly protein bound.

The first protease inhibitor, saquinavir (Ro 31-8959), possessed poor oral bioavailability (< 5%), which was increased when taking with food (Flexer and Hendrix, 1997). This agent is under studying in phase III trial in combination with zidovudine (AZT) and zalcitabine (ddC).

SC-52151 is being studied in phase I trial and data showed the bioavailability was about 20% with a plasma elimination half-life of 2 h (Flexer and Hendrix, 1997). The major route of clearance was through hepatic metabolism. This agent was water and ethanol soluble and highly bound to plasma protein *in vitro* (90%-96.5%). Clinical studies showed that low doses of SC-52151 did not contribute significant antiretroviral activity after 14 days administration (Hao *et al.*, 1990). The larger doses were not investigated due to the long-term ethanol exposure from the formulation and the cost of manufacturing of drug substance.

Other protease inhibitors, MK-639 (formerly L-735,524) and ABT-538 are potent anti-vial agents, which are being studied in clinical phase II/III (Schnittman and Pettinelli, 1997). Data from phase I and II clinical study showed that these agents significantly increased CD4+ T-cell counts and reduced plasma HIV RNA. The maximum activity was achieved in the first weeks of therapy and a slow return toward baseline was obtained by weeks 12 to 24 (Markowitz *et al.*, 1995; Mellors *et al.*, 1995; Schapiro *et al.*, 1995). The loss of the antiviral activity was due to the temporally resistance similar to that of L-735,524 (Condra *et al.*, 1995).

Currently, protease inhibitors are studied in combinations with reverse transcriptase (RT) inhibitors such as AZT and ddC since they are synergistic and could provide multitarget therapy. A phase II study indicated that triple drug combination, Ro31-8959 plus AZT plus ddC, showed higher immunologic and virologic activity than the combination of Ro31-8959 with only one RT inhibitor (AZT or ddC) (Collier *et al.*, 1994). Combinations of MK-639 (Indinavir) and ABT-538 with RT inhibitors are being evaluated. In addition, other protease inhibitors with novel structures, such as KNI-272 (Kynostatin), AG-1343 and VTX-478 are undergoing testing (Mimoto *et al.*, 1992; Quart *et al.*, 1995; Painter *et al.*, 1995).

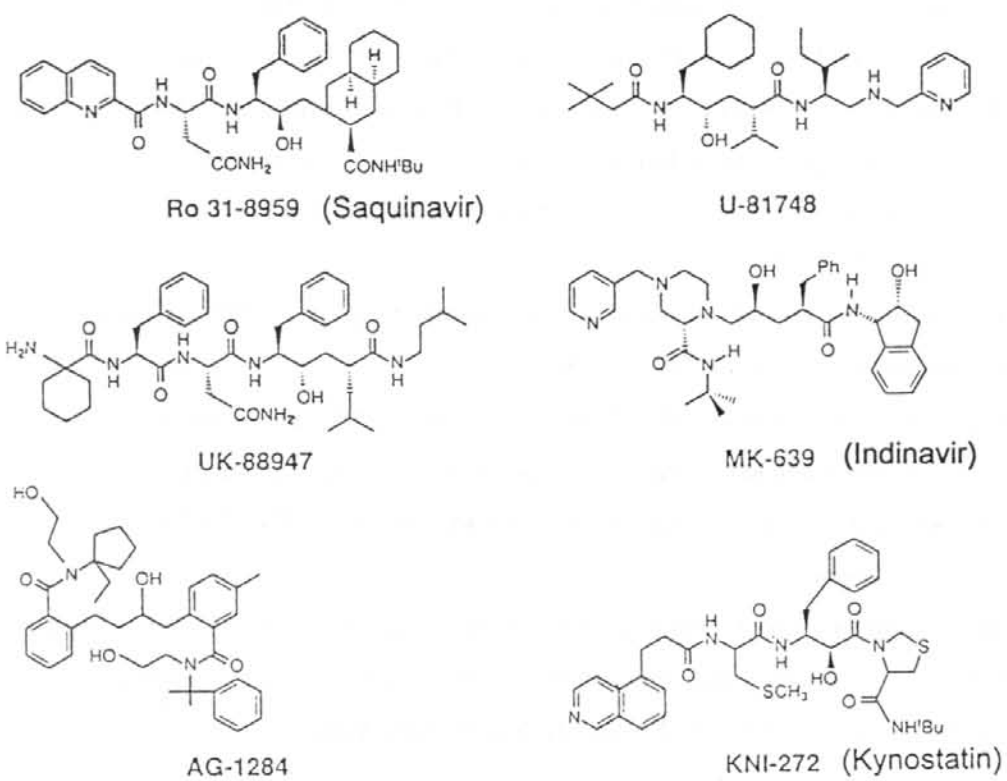


Figure 7 Representative inhibitors of HIV-1 protease (Schnittman and Pettinelli, 1997)

Conclusions

The HIV-1 protease is absolutely essential for the complete infectious HIV-1 virus causing AIDS. It possesses residue D-T-G as an active site and is classified as aspartyl protease family. For understanding structure and function of this protease, the model was first done from a protein of known crystal structure which has similar amino acid sequence. The HIV-1 protease model based on cellular aspartyl protease structure used initially to be proposed. The recent model based on the RSV protease structure is more similar to the crystal structure. The crystal structure at 2.0°A resolution provided more details for understanding the three dimensional structure including substrate binding sites. The specificity of the protease and their interactions with substrates has been also studied by mutagenesis and chemical modification to develop HIV-1 protease inhibitors as valuable anti-AIDS agents. Therefore, the details of HIV-1 protease structure especially active sites and binding sites could contribute to improved drug design and to a better understanding of the mechanisms of drug resistance.

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