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Aspartic Peptidase Inhibitors: Implications in Drug Development

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ABSTRACT: The last decade has witnessed an effervescence of research interest in the development of potent inhibitors of various aspartic peptidases. As an enzyme family, aspartic peptidases are relatively a small group that has received enormous interest because of their significant roles in human diseases like involvement of renin in hypertension, cathepsin D in metastasis of breast cancer, β-Secretase in Alzheimer's Disease, plasmepsins in malaria, HIV-1 peptidase in acquired immune deficiency syndrome, and secreted aspartic peptidases in candidal infections. There have been developments on clinically active inhibitors of HIV-1 peptidase, which have been licensed for the treatment of AIDS. The inhibitors of plasmepsins and renin are considered a viable therapeutic strategy for the treatment of malaria and hypertension. Relatively few inhibitors of cathepsin D have been reported, partly because of its uncertain role as a viable target for therapeutic intervention. The β-secretase inhibitors OM99-2 and OM003 were designed based on the substrate specificity information. The present article is a comprehensive state-of-the-art review describing the aspartic peptidase inhibitors illustrating the recent developments in the area. In addition, the homologies between the reported inhibitor sequences have been analyzed. The understanding of the structure-function relationships of aspartic peptidases and inhibitors will have a direct impact on the design of new inhibitor drugs.

KEY WORDS: aspartic peptidases, inhibitors, drugs, sequence homology.

I. INTRODUCTION

The diversity and selectivity of peptidases contribute the basis for their quintessential nature and multifaceted physiological activities. Peptidases, once considered primarily as "enzymes of digestion", are one of the largest and most diverse families of enzymes known and are involved in every aspect of organismal function. They play a critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins and transport of secretory proteins across membranes. Their activity, if uncontrolled, would be destructive to the cell or organism and therefore must be precisely regulated. The most difficult and arguably most important aspect of peptidase action is the control of peptidase activity to limit cleavage to intended substrates without the general destruction of functional proteins within and outside otherwise normal tissue. Thus, inhibitors of such peptidases are emerging with promising therapeutic uses (Shaw, 1990; Craik et al., 1995; Seife, 1997), in the treatment of diseases such as cancers (Beckett et al., 1996; Johnson et al., 1998; Yan et al., 1998), parasitic, fungal, and viral infections (Wlodawer et al., 1993; Darke and Huff, 1994; Li et al., 1994; Becker et al., 1995; West and Fairlei, 1995; Abad-Zapatero et al., 1996; Kim et al., 1996; Love et al., 1996; Shieh, 1996; Silva et al., 1996; Brindley et al., 1997;

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Abad-Zapatero et al., 1998; Gibson and Hall, 1997;), and inflammatory, immunological, respiratory, cardiovascular, and neurodegenerative disorders (Stubbs and Bode, 1993; Bernstein et al., 1994; Tanak et al., 1995; Hugli, 1996; Fath et al., 1998; Vassar et al., 1999). The present review article describes the recent developments in the area of aspartic protease inhibitors, with special reference to their role in the development of drugs.

II. CLASSIFICATION OF PEPTIDASES

Peptidases catalyze the addition of water across amide (and ester) bonds to effect cleavage using a reaction involving nucleophilic attack on the carbonyl carbon of the scissile bond. The exact mechanisms of cleavage and the active site substituents vary widely among different peptidase subtypes. This provides the basis for the classification of peptidases into the serine peptidases, cysteine peptidases, metallo peptidases, and aspartic peptidases (Barett et al., 1998). There are a few miscellaneous peptidases that do not precisely fit into the standard classification, for example, ATP-dependent peptidases, which require ATP for activity (Menon and Goldberg, 1987).

A. The Aspartic Peptidases

Aspartic peptidases are a group of proteolytic enzymes of the pepsin family that share the same catalytic apparatus and usually function in acidic conditions. This latter aspect limits the function of aspartic peptidases to some specific locations in different organisms, thus, the occurrence of aspartic peptidases is less abundant than other groups of peptidases, such as serine peptidases. However, aspartic peptidases have been isolated and studied from a wide range of organisms, varying from vertebrates to plants, fungi, parasites, retroviruses, and very recently bacteria (Hill and Phylip, 1997; James et al., 1998). Of the five currently documented from the human body, three (pepsin, gastricsin, and renin) are secretory and have well-defined physiological roles. The fourth peptidase, cathepsin D, is found ubiquitously in the lysosomes of most cells (Saftig et al., 1995), while the fifth, cathepsin E, is neither secretory nor lysosomal, but it is located within the endoplasmic reticulum/trans-Golgi network/endosomal compartments of cells (Kageyama, 1995). The cathepsin E molecule is readily distinguished from the other aspartic peptidases not only by this cytomorphological compartmentation but also by its unique molecular architecture (Rao-Naik et al., 1995) and its limited tissue distribution. Aspartic peptidases have been studied extensively for their structure and function relationships and have been the topics of several reviews or monographs (Tang, 1977; Tang, 1979; Kay, 1985; James et al., 1987; Dunn, 1991; Dunn, 1992; Dunn et al., 1995; Fusek et al., 1995; Takahasi, 1995; James, 1998; Rao et al., 1998; Dunn, 2002; Fruton, 2002).

Aspartic peptidases are directly dependent on aspartic acid residues for their catalytic activity and represent the simplest sub-subclass of peptidases comprising the three families. Among the three families, two families known to be related are those of pepsin (A1) and retropepsin (A2). The enzymes from pararetroviruses such as the cauliflower mosaic virus that form family A3 shows signs of a relationship to retropepsins. Crystallographic studies have shown that the enzymes of the pepsin family are bilobed molecules with the active-site cleft located between the lobes, and each lobe contributes an aspartate residue of the catalytically active diad of aspartates. These two aspartyl residues are in close geometric proximity in the active molecule and one aspartate is ionized, whereas the second one is unionized at the optimum pH range of 2 to 3 (Blundell et al., 1991; Sielicki et al., 1991). The lobes are homologous to one another, having arisen by gene duplication. Retropepsins are monomeric, that is, they carry only one catalytic aspartate, and thus dimerization is required to form an active enzyme (Miller et al., 1989; Blundell et al., 1991). In contrast to serine and cysteine peptidases, catalysis by aspartic peptidases does not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers, one from a water molecule to the diad of the two-carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a noncovalent neutral tetrahedral intermediate (Holm et al., 1984; Blundell et al., 1991; Veerapandian et al., 1992; Northrop, 2001; Dunn, 2002).

In family A1 of pepsin (clan AA), the catalytic Asp residues occur within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr. The presence of this motif in a protein has often been taken as evidence for

an aspartic peptidase, which is rather unsound. The Asp residue in the catalytic triad of the serine peptidases of the subtilisin family (S8) occurs in such a motif, and the motif is present in many proteins that are not peptidases. Members of clan AA are all more or less strongly inhibited by pepstatin by a mechanism that is known to be similar at least for families A1 and A2 (Fitzgerald et al., 1990). Leader peptidase II (family U11) and thermopsin (family U16) also are sensitive to this inhibitor. Covalently reacting inhibitors of pepsin, namely, diazoacetylnorleucine methyl ester (DANLME), 1,2-epox-3-(*p*-nitrophenoxy) propane (EPNE), and *p*-bromophenacyl bromide, have also been used as diagnostic reagents for aspartic endopeptidases. An acidic pH optimum is certainly not the sole criterion of a relationship to pepsin. Unrelated peptidases that are maximally active at acidic pH include lysosomal cysteine endopeptidases (family C1), serine carboxypeptidases (S10), thermopsin (U16), and the enzymes of the scytalidopepsin (U18) and pseudomonapepsin (U25) families.

1. Pepsin Family

All members of the pepsin family have been found in eukaryotes, which includes animal enzymes from the digestive tract, such as pepsin and chymosin, lysosomal enzymes such as cathepsin D, and enzymes involved in posttranslational processing such as renin and yeast aspartic peptidase 3. There are also examples from protozoa (e.g., Eimeria, Plasmodium), fungi, and plants. Family A1 contains many enzymes that enter the secretory pathway, and it is probable that all the proteins are synthesized with signal peptides and propeptides. Unusually, barrier pepsin from yeast has a long C-terminal extension that can be excised without affecting enzymatic activity (Mackay et al., 1988).

2. Retropepsin Family

Retropepsin is required for processing of all three viral polyproteins, although cellular enzymes perform initial stages of envelope polyprotein cleavage. Processing occurs at a very late stage in virion assembly, usually after budding of virus particles from the cell membrane; inactive virions containing only gag polyprotein can be formed. Processing seems to be essential for RNA dimerization within the virion, and hence for infectivity. Therefore, there have been intense research interests in the development of inhibitors of retropepsins as antiretroviral agents (Hellen and Wimmer, 1992). A subset of the retropepsins from oncoviruses and avian retroviruses are larger proteins with N-terminal domain homologous to dUTPases.

3. Cauliflower Mosaic Virus Peptidase Family

Cauliflower mosaic virus belongs to a group of plant viruses known as pararetroviruses. Although the viral genome is double-stranded DNA, it contains an open reading frame (ORF V) analogous to the pol gene of retroviruses. ORF V encodes a polyprotein, including a reverse transcriptase, that is homologous to that of retroviruses and, based on an Asp-Thr-Gly triplet near the N terminus, was suggested to be included as an aspartic peptidase as well (Fuetterer and Hohn, 1987). The existence of an endopeptidase was confirmed by mutational studies that implicated the involvement of Asp-45 in catalysis. There was also weak inhibition by pepstatin. The peptidase is larger than retropepsin, however, because it contains only one Asp-Thr-Gly sequence, it is assumed to be active only as a dimer (Torruella et al., 1989). Other pararetroviruses contain sequences homologous to the cauliflower mosaic virus peptidases.

4. Plant Aspartic Peptidases

Aspartic peptidases reported from plants are mostly confined to seeds and are involved in the processing of storage proteins during ripening and germination (Runeberg-Roos et al., 1994; Takahasi, 1995; Hiraiwa et al., 1997; James, 1998; Mutlu et al., 1998). Aspartic peptidases in plant seeds have been purified from barley wheat, rice (Doi et al., 1980; Runeberg-Roos., 1991; Asakura et al., 1995), castor bean (Hiraiwa et al., 1997), and buckwheat, and their enzymatic properties have been investigated. Many plant aspartic peptidases are synthesized as the preproform. cDNA cloning of plant aspartic peptidases has demonstrated the presence of an insert of approximately 100 amino acids at the C-terminal region that is not found in animal or microbial aspartic peptidases. This plant aspartic peptidase-specific insert (PSI) may thus characterize aspartic peptidases of plant origin (Runeberg-Roos et al., 1994; Asakura et al., 1995; D'Arcy-Lameta et al., 1996; Verissimo

et al., 1996; Faro et al., 1999). The phytophysical role of the PSI has been inferred from several findings. Secondary structural analysis of the phytepsin PSI showed it to resemble saposin, which exists in the lysosome, indicating that this PSI may act as a vacuole-targeting signal (Guruprasad et al., 1994). Although the function of PSI (Ramalho-Santos et al., 1998) is not completely understood, it does share considerable sequence identity with sapposins, the group of mammalian sphingolipid activator proteins.

Saposins have been postulated to bind selectively to certain lipids and thus direct the precursor form of the aspartic peptidase into the appropriate cytomorphological compartment in the plant cell (Guruprasad et al., 1994). Investigations of the crystal structures of phytepsin (Kervinen et al., 1999) and cardosin A (Frazao et al., 1999) also showed that the PSI should be located in the surface of the molecule, as each of these proAPs binds to the plasma membrane at the PSI site prior to being transported into the vacuole, where the PSI is eventually removed by processing (Kervinen et al., 1999). The gene for cyprosin obtained from the cardoon, *Cyanara cardunculus* was expressed in the yeast *Pischia pastoris*. The resulting protein was shown to be a disulfide-linked dimer, where the unique PSI had been processed out of the sequence (White et al., 1999). It has been reported that the PSI-deletion mutant of cyprosin is not processed by itself, indicating that in the absence of PSI, plant aspartic peptidases fail to autolyze from the proform to the mature form. Nothing is known of the enzyme(s) responsible in plants, but the excision of each insert is imprecise, resulting in the generation of a complex mixture of heterogenous, mature aspartic peptidase within the tissues of each of the plants that has been studied, for example, from seeds of barley (Sarkinnen et al., 1992), pumpkin (Hiraiwa et al., 1997), and *Arabidopsis thaliana* (Mutlu et al., 1998), from rice (Asakura et al., 1995), and from flowers of the cardoon, *Cynara cardunculus* (Heimgartner et al., 1990; Verissimo et al., 1996; Ramalho-Santos et al., 1998). Commonly, the enzymes thus generated are heterodimers with molecular weights in the region of 40,000 to 45,000. These complexity of natural isoforms is compounded even further by the expression of several genes in the plant tissues, each encoding closely related enzymes so that physicochemical and enzymatic characterization of naturally occurring aspartic peptidases isolated directly from plants has been made rather difficult.

F. Membrane Bound Aspartic Peptidases

β-secretases are membrane bound aspartic peptidases when compared with other mammalian aspartic proteinases such as Cathepsin D and renin, which are soluble. β-secretase is involved in the production of the amyloid plaques formed by the accumulation of the 4-kDa amyloid (Aβ) peptide, which is a characteristic feature of Alzheimer's Disease (Glenner and Wong, 1984; Masters et al., 1985). β-secretase has been identified to be a transmembrane aspartic proteinase based on the different experimental approaches such as expression cloning, searching a database of *Candida elegans* proteins and the human homologs as well as using an inhibitor to purify the protein (Hussain et al., 1999; Vassar et al., 1999; Lin et al., 2000). The β-secretase, also referred to as Asp2, BACE (Beta site APP Cleaving Enzyme) or memapsin2, is reported to contain the two active site motifs, DTGS at residues 93–96 and DSGT at residues 289–292, which are characteristic of aspartic proteinases (Howlett et al., 2000). A second closely related aspartic proteinase, referred to as Asp1 or BACE 2 or Down's region aspartic proteinase or memapsin1, was reported and shown to have 55% homology to Asp2 (Hussain et al., 2000; Howlett et al., 2000). Asp1 and Asp2 show 25 to 30% homology at the amino acid level to other human aspartic proteinases (Hussain et al., 1999; Vassar et al., 1999; Lin et al., 2000). A unique feature of Asp1 and Asp2, which distinguishes them from the other human aspartic proteinases, is the presence of a C-terminal extension that includes a transmembrane domain and a signal peptide. Asp2 has been reported to have four predicted glycosylation sites in the protein sequence. A distinguishing feature of the protein is the pro-domain (amino acids 22–45), which is shorter than that of other human aspartic proteinases. A mutation of either aspartic acid residues present in the active site results in reduced production of the Aβ peptide (Hussain et al., 1999; Vassar et al., 1999).

A second enzyme is also reported to be involved in the pathway leading to the production of the amyloidogenic Aβ peptide, which is referred to as γ-secretase and was suggested to be an aspartic proteinase similar to Cathepsin D. However, the nature of the γ-secretase has not been established.

III. PUTATIVE ASPARTIC PEPTIDASES

Families of peptidases (U22, U33, U34, U23, U24, U25, U11, and U4) are strictly categorized as of unknown catalytic type, but they exhibit certain indications of aspartic type of peptidases.

A. Putative Transposition Endopeptidases

The putative endopeptidases of family U22 are encoded by open reading frames that correspond to the *pol* gene of retroviruses (Inouye et al., 1986), whereas that of the *copia* transposon (U23) is encoded by the open reading frame corresponding to the Rous sarcoma virus *gag* gene (Yoshika et al., 1990). The *gypsy* transposon from *Drosophila* is homologous to *Drosophila* transposons in family U22 and has been suggested to contain a peptidase (Garfinkel et al., 1991). For family U23, deletion of part of the 5' end of yeast *TyB* (including the Asp-Ser-Gly tripeptide) prevents processing, and similar results were obtained by another group subsequently (Garfinkel et al., 1991). For the product of *copia* transposon in *Drosophila*, autocatalytic processing has been shown to be necessary for the release of the protein VPL from the polyprotein precursor, and mutation of the putative catalytic Asp prevents processing (Yoshika et al., 1990). The autocatalytic processing of the *copia* transposon of *Drosophila*, thought to be mediated by the transposon endopeptidase, was not significantly inhibited by pepstatin. Family U24 comprises only the putative endopeptidase of maize transposon *bs1* (Johns et al., 1989).

B. Thermopsin Family (U16)

Among the few peptidases that are known from archaebacteria, there is a subtilisin (family S8) and a multicatalytic endopeptidase complex (S25). However, thermopsin from the thermophilic *Sulfolobus acidocaldarius* shows no relationship to any other protein. The enzyme has a pH optimum of 2, maximally active at 70°C, and it is covalently attached to the cell membrane. Thermopsin is apparently synthesized as a precursor with a 41-residue prepropeptide. Sensitivity to inhibition by pepstatin suggests a possible distant relationship to the pepsin clan; however, the typical Asp-Xaa-Gly motif is not present, and there is no evidence of an internal duplication.

C. Scytalidopepsin B Family (U18)

Pepstatin-insensitive peptidases active at low pH values are known from a variety of fungi (*Aspergillus, Scytalidum*) and bacteria (*Xanthomonas, Pseudomonas, Bacillus*). The bacterial enzymes are inhibited by the carboxyl-specific carbodiimides and by peptide aldehyde tyrostatin. Unlike enzymes from the pepsin family, these endopeptidases are thermostable. Scytalidopepsin B from *Scytalidium* and aspergillopepsin II (*Aspergillus* peptidase A) have been sequenced and are found to be homologous. Aspergillopepsin II is a secreted enzyme, synthesized as a precursor. Activation involves not only removal of the 59-residue prepropeptide, but also excision of an internal 11-residue peptide to produce a two-chain molecule. The gene for the peptidase does not include introns (Inoue et al., 1991).

D. Leader Peptidase II Family (U11)

Bacterial cell walls contain large quantities of murein lipoprotein. This is a small protein that has N-terminal cysteine substituted on sulfur with the $CH_2(OOCR_1)CH(OOCR_2)CH_2$- group, and the C-terminal lysine is bound to the membrane peptidoglycan (murein) through the ε-amino group. Secretion of the lipoprotein from the cytoplasm is mediated by a leader peptide, which is cleaved by a specialized peptidase of the inner membrane known as leader peptidase II. Leader peptidase II is strongly inhibited by the antibiotic globomycin, but it is also inhibited by pepstatin, which suggests that the enzyme may be an aspartic endopeptidase.

E. Pseudomonapepsin Family (U25)

Pseudomonapepsin, an acid endopeptidase from *Pseudomonas* species, is not inhibited by the standard inhibitors for the pepsin family, pepstatin, DANLME, or EPNP, but it is inhibited by tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal). Pseudomonapepsin is a secreted enzyme, synthesized as a precursor with a signal peptide and a large propeptide, which is autocatalytically activated by cleavage at a Leu-Ala bond (Oda et al., 1994).

F. Sporulation Sigma Factor Processing Peptidase Family (U4)

Bacilli produce spores under the direction of a protein known as σ (sigma)^E, which switches on the genes necessary for sporulation. The σ factor is produced as a precursor, and the peptidase believed to be responsible for processing it is the product of the *spoIIGA* gene. Because of the presence of an Asp-Ser-Gly motif in the processing peptidase, the enzyme has been assumed to be an aspartic peptidase.

The peptidase is located in the inner membrane and possesses five membrane-spanning domains and large cytoplasmic domain that contain the putative catalytic Asp.

G. Families of Peptidases of Unknown Catalytic Mechanism

There are a number of incompletely characterized peptidases that cannot be assigned to any catalytic type and show no homology to peptidases of known family. These are listed in Table 1.

IV. PEPTIDASE INHIBITORS

Peptidases are responsible either directly or indirectly for all bodily functions, including cell growth, differentiation, and death (apoptosis), cell nutrition, intra- and extracellular protein turnover (house-keeping and repair), cell migration and invasion, and fertilization and implantation. These functions extend from the cellular level to the organ and organism level to produce cascade systems such as homeostasis and inflammation, and complex processes at all levels of physiology and pathophysiology. Any system that encompasses normal and abnormal bodily functions must have effective regulatory counterparts, that is, peptidase inhibitors. Hence, the research interest in peptidase inhibitors has evoked tremendous attention in many disciplines. Multicellular organisms possess endogenous protein peptidase inhibitors to control proteolytic activity. Most of these inhibitory proteins are directed against serine peptidases, although some are known to target cysteine, aspartyl, or metallopeptidases. Indeed, inhibitors of serine, cysteine, and metallopeptidases are distributed ubiquitously throughout the biological world. In sharp contrast, however, naturally occurring inhibitors of aspartic peptidases are relatively uncommon and are found in only certain specialized locations.

Traditionally, peptidase inhibitors have been developed by natural product screening for lead compounds with subsequent optimization or by empirical substrate-based methods (West and Fairlie, 1995). The optimization involves replacement of the hydrolysable amide bonds by a nonhydrolyzable isostere and optimizing inhibitor potency through trial-and-error structural modifications that progressively reduce the peptide nature of the molecule. This substrate-based drug design has been substantially improved in recent years with the availability of three-dimensional structure information for peptidases, permitting receptor-based design. The structural information about the active site of the receptor (or peptidase) and selection of designed molecules with the aid of computers has helped to design receptor-based inhibitors. Combinatorial chemistry also presents opportunities both to discover new molecular entities for assaying and to optimize lead structures for development of peptidase inhibitors.

A. Inhibitors of Aspartic Peptidase

As an enzyme family, aspartic peptidases are a relatively small group. Nevertheless, they have received enormous attention because of their significant roles in human diseases. The best-known examples are the involvement of renin in hypertension, cathepsin D in metastasis of breast cancer, and the peptidase of human immunodeficiency virus (HIV) in acquired immune deficiency syndrome (AIDS). Therefore, the new understanding of the structure and function relationships of these enzymes has a direct impact on the design of inhibitor drugs. Moreover, as structure and function are closely related among the aspartic peptidases, model enzymes have been particularly informative. A comprehensive list of the potent aspartic protease inhibitors is given in Table 2.

Aspartic peptidases are uniquely susceptible to inhibition by pepstatin and by the active site-directed affinity labels, diazoacetyl norleucine methyl ester and EPNP [epoxy-(*p*-nitrophenoxy)propane]. Each of the latter reacts specifically with the side-chain carboxyl of a distinct aspartic acid residue to inactivate the enzyme. Together, these residues contribute to the catalytic mechanism and provide the basis for nomenclature for this class of enzyme. Aspartic peptidase-inhibitor crystal structures are currently available on the PDB database for viral peptidases (HIV-1, HIV-2, SIV, FIV), Cathepsin D, renin, renin/chymosin, penicillopepsin, secreted aspartic peptidase, pepsin, mucoropepsin, retropepsin, saccharopepsin, rhizopuspepsin, and plasmapepsin II.

Aspartic peptidases generally bind 6 to 10 amino acid regions of their polypeptide substrates, which are typically processed, with the aid of two catalytic aspartic acid residues in the active site (James and Sielecki, 1997). Thus, there is usually considerable scope for building inhibitor specificity for a particular aspartic peptidase by taking advantage of the collective interactions between a putative inhibitor, on both

TABLE 1
Members of Families Aspartic Proteases[a]

Family	EC	Database Code
Family U7: Endopeptidase IV		
Endopeptidase IV (*Escherichia coli*)	-	SPA_ECOLI, LICA_HAEIN
Minor capsid protein presursor C (bacteriophage λ)	-	VCAC_LAMBD
soh B gene product (*E. coli*)	-	(M73320)
Family U2: Aminopeptidase iap		
Alkaline phosphatase isozyme conversion protein (*E. coli*)	-	IAP_ECOLI
Family U5: Tail-specific protease		
Tail-specific protease (*E. coli*)	-	(M75634)
OrfX (*Agmenellum*)	-	(X63049)
Family U5: Murein endopeptidase		
Penicillin-insensitive murein endopeptidase (*E. coli*)	-	MEPA_ECOLI
Family U8: Bacterophage murein endopeptidase		
Murein endopeptidase (bacterophage)	-	ENPP_
Family U9: Prohead endopeptidase		
Prohead endopeptidase (bacteriophage T4)	-	PCCP_BPT4
Family U3: Spore endopeptidase		
Spore endopeptidase (*Bacillus*) (9124)	-	(M55262),
Family U20: γ-D-Glutamyl-L-diamino acid opeptidase II		
γ-D-Glutamyl-L-diamino acid opeptidase II (*Bacillus spaericus*)	-	(X64809)
Family U26: *Enterococcus* D-Ala-D-Ala carboxypeptidase		
D-Ala-D-Ala carboxypeptidase (*Enterococcus*)	-	(M90647)
Family U29: Encephalomyelitis virus proteinase 2A		
Proteiase 2A (Theiler's muriene encephalomyletis virus)	-	POLG_TMEVD
Proteiase 2A (Encephalomyocarditis virus)	-	POLG_EMCV
Family U27: *Lactococcus* ATP-dependent proteinase		
ATP-dependent proteinase (*Lactococcus*)	-	(X67821)
Family U28: Aspartyl dipeptidase		
Aspartyl dipeptidase (*Salmonella*)	-	*b*

[a] EC is the enzyme nomenclature number (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, "Enzyme Nomenclature 1992," Academic Press, Orlando, Florida, 1992, and supplement); - indicate that no EC number has been assigned. Literature references to the individual proteins are generally to be found in the database entries for which the codes are given.

[b] Conlin, C. A., Hakensson, K., Lijas, A., and Miller C. G. (1994) *J. Bacteriol.* **176**, 166.

TABLE 2
Potent Aspartic Protease Inhibitors

Inhibitor	Source	Enzyme	Disease	IC_{50} or K_i Values (μg/ml or nM)	References
17-kDa Inhibitor	Ascaris lumbricoid es	Pepsin Cathepsin E	Acidity Cancer	0.5 3	Kageyama, 1998.
Pluoripotent inhibitor	Sea Anemone	Cathepsin D	Cancer	NA	Lenarcic and Turk, 1999.
8-kDa inhibitor	Yeast	Protease A or Saccharopeps in	Yeast infections	1.1	Phylip et al., 2001.
Pepstatin and derivatives	Streptomy ces species	Pepsin	Acidity	1.5-10	Umezawa et al., 1970.
Unsymmetrical peptidyl ureas	Synthetic	Pepsin	Acidity	0.3-30*	Dales et al., 2001.
Saquinavir	Synthetic	HIV-1 Protease	AIDS	0.12	Roberts et al., 1990.
Ritonavir	Synthetic	HIV-1 Protease	AIDS	NA	Lea and Faulds., 1996.
Indinavir	Synthetic	HIV-1 Protease	AIDS	0.52	Lacy et al., 1996.
Nelfinavir	Synthetic	HIV-1 Protease	AIDS	2	Shetty et al., 1996
Amprenavir	Synthetic	HIV-1 Protease	AIDS	0.6	Kim et al., 1995.
Lopinavir	Synthetic	HIV-1 Protease	AIDS	1.3**	Carrillo et al., 1998
CGP-73547	Synthetic	HIV-1 Protease	AIDS	0.004-0.1*	Bold et al., 1998.
Palinavir	Synthetic	HIV-1	AIDS	31**	Lamerre et

TABLE 2 (continued)

		Protease			al., 1997.
Mozenavir	Synthetic	HIV-1 Protease	AIDS	0.3	Lam et al., 1994.
Tiprinavir	Synthetic	HIV-1 Protease	AIDS	5**	Turner et al., 1998.
ATBI	Bacterial	HIV-1 Protease	AIDS	18	Dash et al., 2001
CGP 38 560	Synthetic	Renin	Hypertension	0.7	Buhlmayer et al., 1988.
Zankiren	Synthetic	Renin	Hypertension	1.1	Kleinert et al., 1992.
BILA 2157 BS	Synthetic	Renin	Hypertension	1.4	Simoneau et al., 1999.
The substituted piperidines	Synthetic	Renin	Hypertension	5*	Oefner et al., 1999.
SC-5003	Synthetic	Plasmepsin	Malaria	500	Francis et al., 1994.
Allophenylnorstatine derivatives	Synthetic	Plasmepsin	Malaria	70	Nezami et al., 2002.
A-70450	Synthetic	SAPS	Candidal infections	1.4	Abad-Zapatero et al., 1996.
Diamonidiol-based peptidomimetics	Synthetic	SAPS	Candidal infections	3.2-90*	Skrbec and Romeo., 2002.
Lycernuic acid C	Plant	SAPS	Candidal infections	20	Zhang et al., 2002
Apigenin-4'-O-(2",6"-di-O-p-coumaryl-)beta-D-glucoside	Plant	SAPS	Candidal infections	8.5	Zhang et al., 2002.
OM99-2	Synthetic	β-Secretase	Alzheimer's Disease	1	Lin et al., 2000.
OM00-3	Synthetic	β-Secretase	Alzheimer's Disease	0.3	Hong et al., 2002.

*: Values are expressed in μM.

**: Values are expressed in pM.

sides of its scissile amide bond, and a substantial portion of the substrate-binding groove of the enzyme. Some aspartic peptidase also have one or more flaps that close down on top of the inhibitor, further adding to inhibitor peptidase interactions and increasing the basis for selectivity. The scissile amide bond undergoes nucleophilic attack by a water molecule, which is itself partially activated by deprotonated catalytic aspartic acid residue. The protonated aspartic acid donates a proton to the amide bond nitrogen, generating a zwitterionic intermediate, which collapses to the cleaved products. The water molecule that binds between the enzyme and inhibitor is thought to position a peptide substrate, stretching the peptide bond out of planarity toward a tetrahedral transition state that is stabilized by a second water molecule (Chatfield and Brooks, 1995).

Aspartic peptidase inhibitors can be grouped under two categories by their molecular nature, (1) proteinaceous inhibitors, and (2) low-molecular-weight inhibitors.

1. Proteinaecous Inhibitors

In a sharp contrast to the ubiquitous presence of multiple forms of proteinaceous inhibitors of other classes of peptidases from different sources of plants, animals, and microorganisms, there is a paucity of proteinaceous inhibitors of aspartic peptidases. With the exception of macroglobulins, which inhibit peptidases of all classes, individual protein inhibitors inhibit only peptidases belonging to a single mechanistic class. Protein inhibitors of aspartic peptidases are relatively uncommon and are found in only a few specialized locations (Bennet et al., 2000). Few of the examples include renin-binding protein in mammalian kidney, which, intriguingly, has now been identified to be the enzyme, *N*-acetyl-D-glucosamine-2-epimerase (Kay et al., 1983; Phylip et al., 2001), a 17-kDa inhibitor of pepsin and cathepsin E from the parasite *Ascaris lumbicoides* (Kageyama, 1998; Ng et al., 2000), proteins from plants such as potato, tomato, and squash (Kreft et al., 1997; Christeller et al., 1998), and a pluoripotent inhibitor from sea anemone of cysteine peptidase as well as cathepsin D (Lenarcic and Turk, 1999). There is a report of an 8-kDa polypeptide inhibitor from yeast, which inhibits the vacuolar aspartic peptidase (peptidase A or saccharopepsin).

2. Low-Molecular-Weight Inhibitors

In contrast to the proteinaceous nature of the peptidase inhibitors from plants and animals, the inhibitors produced by microorganisms are of smaller molecular nature. The presence of peptidase inhibitors in microorganisms came into existence from the studies on antibiotics because they act as inhibitors of enzymes that are involved in growth and multiplication. Extracellular proteolytic enzymes hydrolyze organic nitrogen compounds in the medium and are thought to be harmful to cells. The production of inhibitors of the proteolytic enzymes by microorganisms has probably evolved as a mechanism to provide cell protection. Specific inhibitors of microbial origin have been used as useful tools in biochemical analysis of biological functions and diseases. Polysaccharide sulfates have been reported to be pepsin inhibitors; however, their antipepsin activity is weak, and the effect of such polyanionic compounds is not specific.

Pepstatin, a low-molecular-weight aspartic peptidase inhibitor, isolated from various species of *Streptomyces,* is a specific inhibitor of pepsin (Umezawa et al., 1970) (Figure 1). *Streptomyces testacus* was reported to produce various pepstatins that differed from one another in the fatty acid moiety (C_2-C_{10}). A pepstatin containing an isovaleryl group has been most widely used for biological and biochemical studies. Moreover, as minor components, pepstanone containing (S)-3-amino-5-methylhexane-2-one instead of the C-terminal (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA), and hydroxypepstatin containing L-serine instead of L-alanine, have also been isolated. Pepstatin containing an acetyl group and propanoyl or isobutyryl groups were isolated from *Streptomyces naniwaensis* and *Streptomyces* no. 2907.

Pepstatins, pepstanones, and hydroxypepstatins have almost identical activity against pepsin and cathepsin D. However, pepstatin is more effective against renin than are pepstanone or hydroxypepstatin, and its potency against renin increases with the increasing numbers of carbon atoms in the fatty acid moiety. Esters of pepstatin, pepstatinal and pepstatinol possess anti-pepsin activity similar to pepstatins. Several pepstatin analogs have also been synthesized to date. AHMHA and its *N*-acyl derivative exhibit no potency toward pepsin; however, *N*-acetyl-valyl-AHMHA is active, and the addition of another valine between the acetyl and valyl groups does not increase their activity. The addition of L-alanine to the C-terminal group

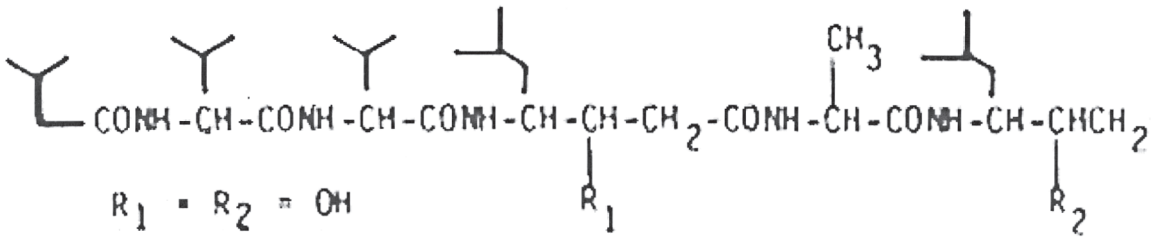


FIGURE 1. Chemical structure of pepstatin, a pepsin inhibitor.

increases the activity about 100 times. This suggests that the acetyl-valyl-AHMHA-L-alanine is the smallest molecular structure that exhibits inhibition against pepsin and cathepsin D similar to pepstatin. Acetyl-L-valyl-L-valyl-[(3S, 4R)-4-amino-3-hydroxy-6-methyl] heptanoic acid prepared by chemical synthesis shows absence of activity. This suggests that the 4S-configuration of AHMHA is essential for activity.

The bacterial enzyme that hydrolyzes the isovaleryl bond in pepstatin has been identified, and from the residual peptide benzoyl-L-valyl-AHMHA-L-alanyl-AHMHA and L-lactyl-L-valyl-AHMHA-L-alanyl-AHMHA have been synthesized. These analogs are more water-soluble than pepstatin and have almost identical activity against pepsin and cathepsin D. However, these water-soluble analogs have much weaker activity against renin when compared with pepstatin. The addition of aspartic acid or arginine to the C-terminus of pepstatin increases its water solubility. Such water-soluble analogs have same activity against renin as does pepstatin and also have a hypotensive action (Rich, 1985). Pepstatin also inhibits carageenin-induced edema and suppresses the generation of Shay rat ulcer. Therapeutic effects on stomach ulcers in man have also been observed. Pepstatin has been reported to be effective against experimental muscle dystrophy and enhances the effect of leupeptin. Pepstatin also inhibits leukokinin formation and ascites accumulation in ascites carcinoma of mice. Pepstatin inhibits the growth of *Plasmodium beghei* and inhibits focus formation in murine sarcoma virus (Yuasa et al., 1975).

Recently, a new class of peptidomimetics, the unsymmetrical peptidyl ureas, have emerged as powerful inhibitors of aspartic peptidases (Dales et al., 2001). These were developed using mechanism-based and substrate-based design techniques and using the computational method GrowMol (Ripka et al., 2001). These newly synthesized inhibitors possess a distinct advantage over the natural inhibitors. The natural inhibitors such as antipain, elastinal, etc. contain urea bonds in place of the amide bonds between the P3 and P4 residues and not between the P1 and P2 residues. Ureas have the ability to form stronger hydrogen bonds than the amide groups. The synthesis of ureas as inhibitors was therefore a natural choice. These synthetic inhibitors of porcine pepsin were generated through computational programs that analyze the target enzyme structure, predict inhibitor structures, and analyze the enzyme-inhibitor complex formed. These structures would help to chemically build up molecules of medicinal value and would give rise to newer classes of drugs.

B. HIV-1 Peptidase Inhibitors

The peptidase of the human immunodeficiency virus (HIV-1 PR) has proven to be an attractive drug target due to its essential role in the replicative cycle of HIV. Several low-molecular-weight inhibitors of HIV-1 PR are now used in humans, including saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir. These are the first successful examples of receptors/structure-based designer drugs and were developed using structures of compounds bound in the active site of HIV-1 PR and with the knowledge of inhibitors of other aspartic peptidases (e.g., renin) (Kempf and Sham, 1996). All HIV-1 PR inhibitors developed so far target the active site substrate-binding groove of the homodimeric enzyme, a long cylindrical cavity that binds 6 to 7 amino acids via ionic, van der Waals, or hydrogen bonding interactions (Kempf and Sham, 1996). Two catalytic aspartates in the center of this cavity promote amide bond hydrolysis. Figure 9 depicts the crystal structure of the HIV peptidase complexed with a representative inhibitor.

Saquinavir became the first peptidase inhibitor designed from a three-dimensional structure of a pep-

tidase (structure-based design) to be approved for human use in 1996 (Roberts et al., 1990; Pakyz et al., 1997; Patick et al., 1998), despite its low oral bioavailability due to poor absorption and extensive first-pass degradation by cytochrome P450 (Wacher et al., 1998). It is active in cell culture against both HIV-1 and HIV-2 viruses and in combinations with ritonavir, an inhibitor of cytochrome P450 (Kempf et al., 1997), lead to greatly increased plasma concentrations. Ritonavir itself is a potent inhibitor of HIV-1 PR with high oral bioavailability (Lea and Faulds, 1996). Indinavir or Crixivan is another potent inhibitor of HIV-1 and HIV-2 peptidase that halts the spread of HIV infection in MT4 lymphoid cells and is orally bioavailable. In humans, Indinavir is rapidly absorbed in fasting state, there is significant binding to plasma proteins, and the main degradation pathway is via cytochrome P450 (Lacy et al., 1996). The mesylate salt of nelfinavir, approved for human use in 1997, is a lipophilic peptidase inhibitor with good oral bioavailability in rats and monkeys (Shetty et al., 1996; Kaldor et al., 1997). Amprenavir, is a water-soluble, orally bioavailable inhibitor with long half life, allows less frequent administration of drug, thereby having the potential for less side effects with respect to other marketed HIV peptidase inhibitors described above (Kim et al., 1995; Adkins and Faulds, 1998; Fung et al., 2000; Noble et al., 2000).

Viral resistance to "monotherapy" with any of these drugs is a significant problem (Erickson, 1995). Serial passages of HIV-1 *in vitro* in the presence of increasing concentrations of a peptidase inhibitor cause a rapid emergence of drug-resistant viral strains of HIV-1. Thus, new HIV peptidase inhibitors with different resistance profiles are still being actively pursued. A number of second-generation inhibitors have been developed. Lopinavir or ABT-378 was designed to inhibit mutant peptidases produced in response to ritonavir (Carrillo et al., 1998; Sham et al., 1998; Hurst et al., 2000). It is 10-fold more potent against ritonavir-resistant strains and displays lower binding to serum proteins. Although the oral bioavailability of lopinavir is very poor, when administered with ritonavir the bioavailability was enhanced (Sham et al., 1998). Kaletra, the HIV peptidase inhibitor was developed as a mixture of lopinavir and ritonavir by Abbott. It was the first second-generation inhibitor of HIV peptidase to be used successfully as a recommended drug, when the primary therapy with other peptidase inhibitors failed (Wlodawer, 2002). CGP-73547 inhibits indinavir and saquinavir-resistant strains of HIV-1, is orally bioavailable (Bold et al., 1998). One of the most promising preclinical candidates for HIV peptidase inhibition is palinavir. This compound is a very potent, orally active inhibitor of HIV-1 and HIV-2 peptidases with high antiviral activity (Lamerre et al., 1997). An orally bioavailable inhibitor called as Mozenavir was developed by DuPont Merck and the University of Uppsala. Mozenavir has a seven-membered cyclic urea ring as the starting molecule (Lam et al., 1994). Mozenavir has a Ki of 0.3 n*M* and is highly potent in cell cultures. A very different inhibitor, tiprinavir, was developed by Pharmacia and Boehringer-Ingelheim. It is a nonpeptidic molecule with a Ki of 5 pM (Turner et al., 1998). Tiprinavir is soluble and highly bioavailable with significant activity against multidrug resistant HIV-1 strains (Thaisrivongs et al., 1999; Rusconi et al., 2000). L-735, 524, a hydroxyaminopentane amide class peptidomimetic is a highly potent and orally bioavailable HIV peptidase inhibitor (Dorsey et al., 1994; Vacca et al., 1994). The Ki values of L-735, 524 for HIV-1 and HIV-2 are 0.38 n*M* and 2.45 n*M*, respectively. This seven-fold difference combined with its enhanced oral bioavailibility offer chances of using this molecule or close structural analogues as effective agents.

A report of the inhibitors of HIV-1 peptidase dimerization has been published recently. These compounds indicate the first studies of nonpeptidic inhibitors of HIV-1 peptidase dimerization (Song M et al., 2001). Compound 1 contained 4-(2-aminoethyl)-6-dibenzofuranpropionic acid as a conformationally restricted linker. Compound 2 was a nonpeptidic beta strand mimetic, 2-[3-([2-[9-fluorenylmethoxy) carbonyl] hydrazine] carbonyl)-4-methoxyanilino]-2-oxoacetic acid. The Ki values of these compounds were 5.4 and 9.1 μ*M*, respectively. A peptide inhibitor has been isolated from an extremophilic *Bacillus* species. It is reported to be a tight binding, noncompetitive inhibitor with a Ki value of 17.8 n*M* against HIV peptidase (Dash et al., 2001). The recent approaches in the designing of peptidase inhibitors have focused on the dimer interface involving the residues 1–5 and 95–99 of the HIV peptidase. The sequence of the cleavage sites changes with that of the peptidase (Goodenow et al., 2002). New reports of the HIV peptidase inhibitors have suggested that resistance-evading inhibitors can be designed (Louis et al., 1998; Rosin et al., 1999; Dash et al., 2001). Even though a multitude of peptidase inhibitors have been reported,

only six, viz., saquinavir, nelfinavir, ritonavir, indinavir, lopinavir, and amprenavir, have been approved as drugs by USFDA (United States Food and Drug Administration) in the treatment of AIDS (Figure 2). Newly emerging resistant strains of HIV enhance the need for newer and more potent drugs. Hence, the research in this field needs to be intense.

C. Renin Inhibitors

The aspartic peptidase renin is involved in the rate-limiting step of the renin-angiotensin (RAS) system, by hydrolyzing the α_2-globulin angiotensinogen to release the 10-residue peptide angiotensin I. Because of its specificity, renin inhibitors are antihypertensive agents similar in action to ACE inhibitors, and AII antagonists, but are free of some side effects associated with ACE inhibitor administration. For example, Zankiren (A-72517), a potent inhibitor of human plasma renin, is the peptidic inhibitor with significant oral absorption (Kleinert et al., 1992). Renin inhibitors have been developed mainly by modifying substrate fragments from the angiotensinogen cleavage site (Rosenberg, 1995), but their clinical progress has been hampered by their peptidic character, which confers low stability and poor oral bioavailability in humans. Another hurdle in the development of renin inhibitors has been the high cost of production, compared with current antihypertensives such as ACE inhibitors and AII receptor antagonists. Generally, renin inhibitors need to interact with five subsites (S4-S1') of the enzyme to bind tightly and selectively

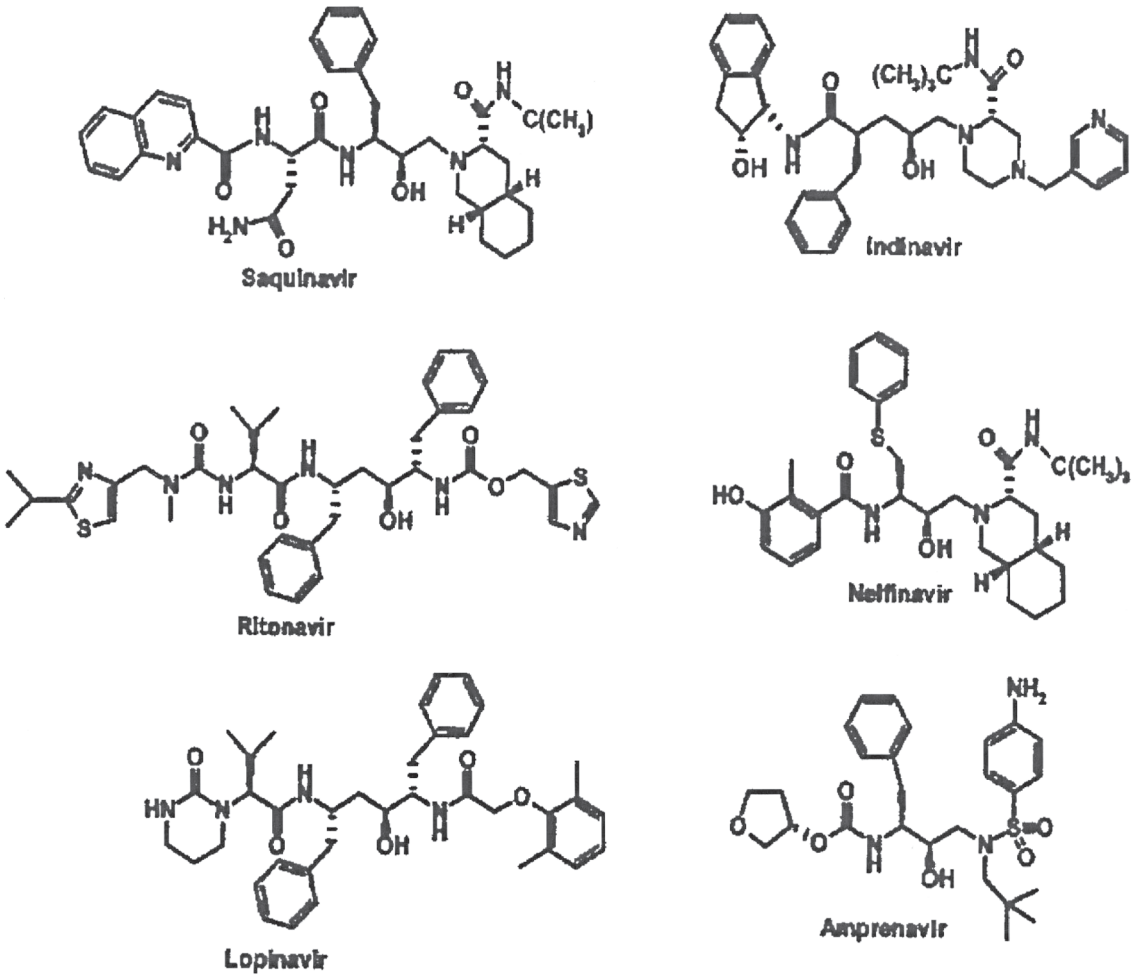


Figure 2. Chemical formulae of the FDA approved inhibitors of HIV-1 protease.

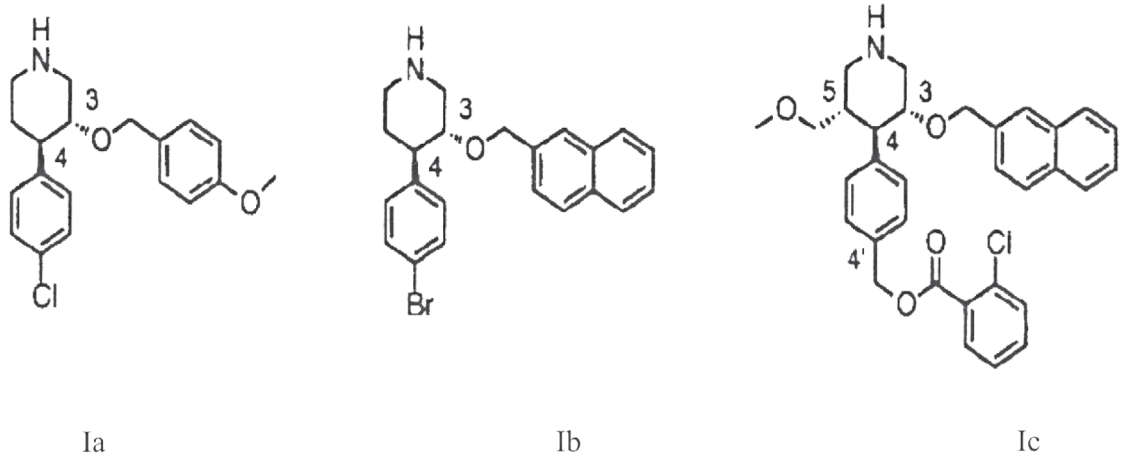
compared with only three for ACE inhibitors. Consequently, renin inhibitors tend to have higher molecular weight, have more stereo centers, and thus are more expensive to manufacture.

Several renin inhibitors with low molecular weight, less peptidic character, and improved oral bioavailabilty have emerged recently. CP-108 671 was designed from the cleavage site of angiotensinogen and the structure of the general aspartic peptidase inhibitor pepstatin (Hoover et al., 1995). It uses a cyclohexylnorstatine transition-state analogue, a(R)-benzylsuccinate, at P3 for chymotrypsin stability and is a potent inhibitor of human plasma renin. It is highly selective over most aspartic peptidases but does weakly inhibit cathepsin D. BILA 2157 BS is another potent renin inhibitor with some selectivity toward cathepsin D and oral activity (Simoneau et al., 1999). A combination of the X-ray crystal structure of CGP38560 bound renin (Buhlmayer et al., 1988), and previous information (Goschke et al., 1997) that the S3 subsite can be accessed by extending the P1 residue has helped in developing several other nonpeptidic inhibitors with good activity and specificity. These nonpeptidic, low-molecular-weight compounds represent excellent progress toward the necessary features (oral bioavailability and economic production) for renin-binding drugs but may require improved selectivity. Recently, a new class of renin inhibitors, the substituted piperidines, have emerged (Oefner et al., 1999), which proposes a novel paradigm for the inhibition of the monomeric aspartic peptidases. The inhibitors have been developed from a simple 3,4-disubstituted piperidine lead compound. Two representative compounds from this class were tested against recombinant human renin. The first compound, trans (3R, 4R)-2-naphthyl-methoxy-4-(4-bromophenyl)-piperidine, inhibited renin with a IC_{50} of 5 μ*M*. The bromophenyl substituent allowed the nitrogen of piperidine to close in on the two catalytic carboxylates. Structural variation gave rise to the 3,4,5-trisubstituted-piperidine derivative. This derivative had an IC_{50} of 2 n*M* and formed a better complex with renin. Major induced fit adaptations were found around the active site of the enzyme with the modified derivative. The structural analysis of the complex resulted in more optimization of the inhibitors (Figure 3). Piperidine moiety can now be used as a central template in the synthesis of a class of nonpeptidomimetic inhibitors against aspartic peptidases.

D. Plasmepsins Inhibitors

Plasmepsins I and II, found in the malarial parasite *Plasmodium falciparum*, are aspartic peptidases that are essential for the degradation of its major food source, human hemoglobin (Dame et al., 1994). The inhibition of these enzymes, which have 73% and 35% sequence homology with human cathepsin D, therefore is considered a viable therapeutic strategy for the treatment of malaria (Coombs et al., 2001). Both plasmepepsin I and II are believed to initially cleave the Phe33-Leu34 peptide bond of the α-chain of hemoglobin, followed by cleavage of the polypeptides into smaller fragments that are subsequently processed by the cysteine peptidase falcipain (Francis et al., 1997). SC-5003, the first peptidomimetic inhibitor reported to selectively inhibit plasmepsin I and II, was active *in vitro* against the live parasite, preventing hemoglobin degradation (Francis et al., 1994). X-ray crystallographic structure of plasmepepsin II complexed to pepstatin A have been used to develop peptidic inhibitors, which starves the live parasite *in vitro* as well as inhibits human cathepsin D (Silva et al., 1996).

Currently, combinatorial synthesis is being used to generate inhibitor libraries for these enzymes. Another class of inhibitors, the allophenylnorstatine-based inhibitors, have been shown to be effective and potent inhibitors of plasmempsin II (Nezami et al., 2002). The allophenylnorstatine scaffold contains four different positions where separate functional groups can be introduced (Figure 4). This improves the binding affinity and selectivity toward the selected target. The KNI series of inhibitors that incorporates the allophenylnorstatine moiety with a hydroxymethylcarboxamide isostere is the most effective inhibitor series. The exception is KNI-529, which has the antidiastereoisomer phenylnorstatine replacing the allophenylnorstatine. The best compound of this series, KNI-727, shows a Ki of 70 n*M*. The reaction is favored both enthalpically and entropically. Good oral bioavailability and low toxicity indicates that allophenylnorstatine is a very good scaffold for the development of inhibitors with clinical applications. All compounds developed to date are potent inhibitors of human cathepsin D, so better selectivity needs to be attained in orally active inhibitors of these enzymes. Interestingly, a combination of cysteine and aspartic peptidase inhibitors was found recently to be more effective than either compound alone in inhibiting *Plasmodium-*



Ia Ib Ic

FIGURE 3. Chemical structures of renin inhibitors, 4-halophenyl piperidine compound 1a, trans (3R, 4R)-2-naphthyl-methoxy-4-(4-bromophenyl)-piperidine 1b and 3,4,5-trisubstituted piperidine derivative 1c.

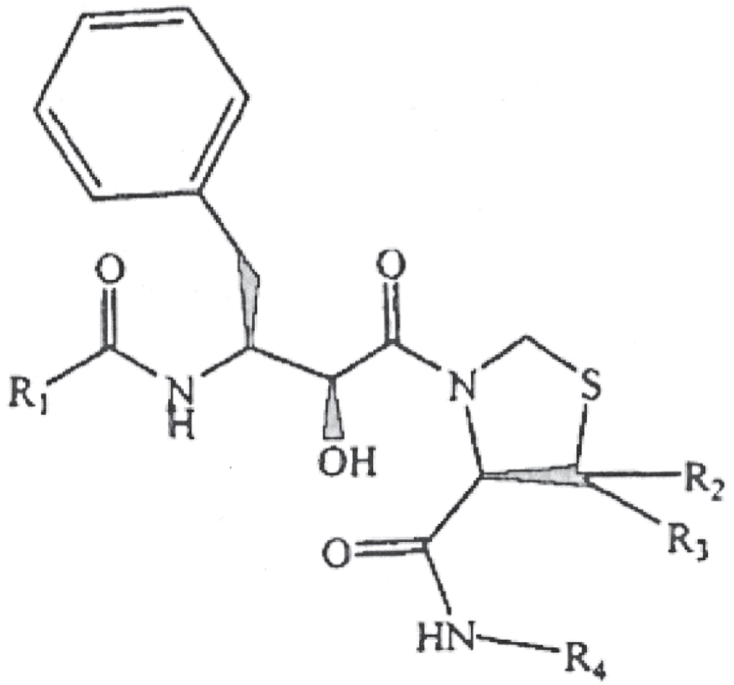


FIGURE 4. Chemical structure of the allophenylnorstatine scaffold. R_1, R_2, R_3, R_4 indicate the places where different chemical functional groups can be introduced to generate a series of inhibitors.

mediated hemoglobin degradation in both culture and a murine malaria model (Semenov et al., 1998). This synergy suggests that combination therapy may be a viable strategy for antimalarial treatment regimes of the future.

E. Cathepsin D Inhibitors

Human cathepsin D is an intracellular aspartic peptidase found mainly in lysosomes. It has a number of "housekeeping" functions, including the degradation of cellular and phagocytosed proteins. The enzymes may be involved in a variety of disease states, including cancer and Alzheimer's disease. Clinical studies have shown that cathepsin D is overexpressed in breast cancer cells, and this seems to be associated with an increased risk of metastasis due to enhanced cell growth (Rochefort and Liaudet-Coopman, 1999). Cathepsin D or a similar aspartic peptidase is also thought to be involved in formation of the β-amyloid peptide in Alzheimer's disease (Papassotiropoulos et al., 1999; Wolfe et al., 1999). The availability of selective and potent inhibitors will help to further define the role of cathepsin D in disease and possibly lead to therapeutic agents.

Relatively few inhibitors of cathepsin D have been reported, partly because of its uncertain role as a viable target for therapeutic intervention. Human cathepsin D was co-crystallized with pepstatin A, and its structure (Baldwin et al., 1993) has promoted some inhibitor studies. One study suggests that the entropy and solvation effects are key determinants of high affinity for pepstatin-cathepsin D binding (Majer et al., 1997). Although a general inhibitor of aspartic peptidases, pepstatin A remains the most potent inhibitor known. There have been reports of cyclic inhibitors designed from the X-ray structures using the fact that the enzyme-bound conformation of the P2 and P3' residues of pepstatin are in close proximity to each other (Silva et al., 1996). This allows cyclization of the inhibitor, thereby increasing the proteolytic stability of the three-amide bonds in the cycle. Combinatorial approaches have been carried out for the development of inhibitors to prove the methodology for the optimized specificity against other aspartic peptidases.

F. Secreted Aspartic Peptidase Inhibitors

The *Candida* yeast strains *C. albicans, C. tropicalis*, and *C. parapsilosis* exist in small quantities in a healthy intestinal tract, but they become a health problem when the immune system is compromised. Such opportunistic infections arise in AIDS patients where *C. albicans* is a serious pathogen of the mucous membranes (Gruber et al., 1999). It is also the major cause of vaginitis (De Bernardis et al., 1999) and has been implicated in liver toxicity and in the development of multiple chemical allergies. *C. tropicalis* is the predominant cause of fungal infections in neutropenic cancer patients. These organisms have the ability to secrete into the host (Naglik et al., 1999) several aspartic peptidases (SAP, secreted aspartic peptidase) of broad specificity. These peptidases are thought to be linked to the virulent effects of *Candida* strains in humans as peptidase-deficient mutants reduce the virulence (Hube et al., 1997; Sanglard et al., 1997). Therefore, these enzymes are becoming attractive targets for therapeutic attack. Nine SAPs have been identified in the genome of *C. albicans* to date (SAP1-9) (Monod et al., 1998). From mutation experiments, SAP2 seems to be the dominant isoenzyme for the normal progression of systemic infection, while SAP1 and 3 are also important for overall virulence of *C. albicans* (Naglik et al., 1999). SAP4-6 appears to play a role in the process of induction of SAP2 (Sanglard, D. et al., 1997). X-ray crystal structures have been determined for SAP2 complexed to pepstatin (Cutfield et al., 1995), a close homologue SAP2X bound to the same inhibitor (Abad-Zapetero et al., 1998), and a SAP enzyme of *C. tropicalis* (Symerski et al., 1997).

Very little inhibitor design has been reported for SAP2. A-70450 was originally designed to inhibit renin and later found to be nonselective inhibitor of the SAP of *C. albicans*. This inhibitor incorporates the (*S*)-hydroxylethylene isostere with the hydroxyl group positioned in the crystal structure between two catalytic aspartate residues. Interestingly, the terminal methylpiperazine ring of A-70450 is found in a boat conformation that occupies the S3 subsite of the enzyme together with the benzyl group of the ketopiperazine ring. The large S3 subsite is not found in other aspartic peptidases, and this difference could be exploited to develop selective inhibitors for SAP2 (Abad-Zapetero et al., 1996). SAPs have been reported to be inhibited by indinavir and ritonavir in the range of 1 to 10 μ*M* (Cassone et al., 1999; Gruber et al., 1999). Reduced amide monohydroxyethylene and diamonidiol-based transition state peptidomimetics were also reported to inhibit SAPs effectively (Skrbec and Romeo, 2002). A few natural products were iso-

lated from *Lycopodium cernuum* out of which lycernuic acid C and apigenin-4'-*O*- (2", 6"-di-*O*-*p*-coumaryl)-beta-D-glucoside showed inhibition of SAP with IC_{50} values of 20 and 8.5 µg/ml (Zhang et al., 2002) (Figure 5). Further research efforts are needed to screen natural sources for the identification of new inhibitors.

G. β-Secretase Inhibitors

The feasibility of an inhibitor drug against β-secretase for the treatment of Alzheimer's Disease was substantiated by the success in the design of inhibitor drugs against HIV peptidase and the apparent tolerance of the β-secretase gene deletion in mice (Cai et al., 2001; Luo et al., 2001). A first generation inhibitor OM99-2, an 8-residue transition analog with Ki near 1 n*M* was designed based on the substrate specificity (Lin et al., 2000., Ghosh et al., 2000). The crystal structure of the β-secretase-OM99-2 complex resolved at 1.9 Å indicated that S_3' and S_4' subsites were not well formed in β-secretase (Hong et al., 2002). A further detailed subsite preference of β-secretase (Turner et al., 2001) and preferred binding residues led to the designing of a second generation inhibitor, OM00-3 with a Ki of 0.3 n*M* from a combinatorial inhibitor library (Hong et al., 2002). The crystal structure of β-secretase and OM00-3 at 2.1 Å resolution was determined using the molecular replacement method. The interactions of the P_1/P_1' region of OM00-3 with the substrate binding cleft of β-secretase and the conformation of the inhibitor from P_3 to P_2' are the same as those for the OM99-2-β-secretase complex (Hong et al., 2002). The S_4 pocket deduced from the crystallographic studies of β-secretase involves Glu^{11}, Gln^{73}, Thr^{232}, and Arg^{307}. The latter was shown to form several ionic bonds with the carboxylate oxygen atoms of inhibitor P4 Glu. These multiple interactions with the peptidase contribute to the inhibitor binding. In OM00-3, the leucines at P_3 and P_1 show contacts with the S_3 site of the enzyme as well as with each other and stabilize the conformation of the inhibitor. The locations and the nature of the S_3' and S_4' binding sites have been defined for the first time. It has been demonstrated that valine at P_2' provides better binding with the enzyme. Other binding modes provided for the P_2 and P_4 side-chains could help in the future designing of inhibitors. The current findings suggest that designed inhibitors should have a strong binding side chain such as Val at P_2 (Figure 6). The new binding mechanisms proposed at P2 and P4 suggest scope for further inhibitor design. The recent reports of β-secretase inhibitors have raised the possibilities of inhibitors as therapeutic agents in Alzheimer's Disease.

V. SEQUENCE HOMOLOGY

In the present review article, we have analyzed the amino acid sequences of aspartic peptidase inhibitors in an attempt to study the homologies between them that will shed light on their evolutionary

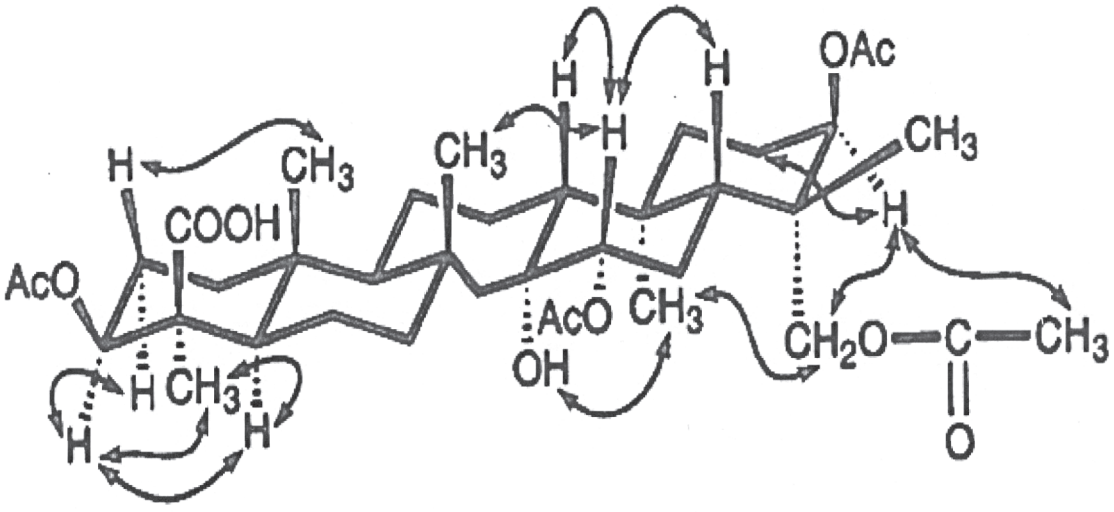


FIGURE 5. Lycernuic acid C: Inhibitor of SAP isolated from *Lycopodium cernuum*.

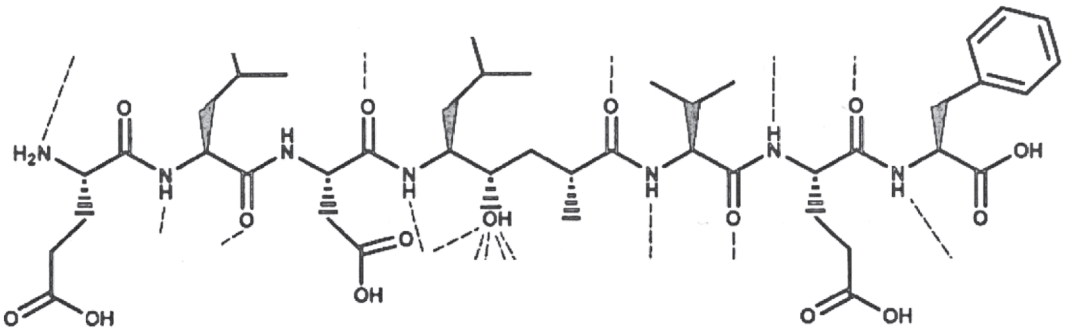


FIGURE 6. Schematic representation of β-secretase inhibitor; OM00-3.

characteristics and functional correlation. Protein sequence homology has become routine in computational biology serving as a starting point in the phylogenetic analysis of proteins and in the prediction of the secondary structure of proteins. The comparison of the amino acid sequences assists in the study of their evolution. However, no reports of sequence homology studies of aspartic peptidase inhibitors are available. Aspartic peptidase inhibitors from diverse origins such as plants, animals, and microbes have been selected from the Swiss-Prot entries. Sequence data from eight inhibitors from potato, five from nematodes, and five synthetic peptides were available and have been aligned using the CLUSTAL W online software for multiple alignments (Thompson et al., 1994). The amino acid sequence analysis indicated that considerable homology existed between the members of the same group, whereas no homology was observed between members of different groups. The dendrogram drawn suggested that the inhibitors could be classified into three distinct groups (Figure 7). The inhibitors from potato, which are reported to be cathepsin D and pepsin inhibitors, were classified as Group 1. The inhibitors classified under group 2 showed inhibition against pepsin, whereas the group 3 inhibitors were reported to be renin inhibitors. The further analysis of the results indicated that the eight aspartic peptidase inhibitors in group 1 showed variations that might have arisen out of mutations or genetic shuffling in a single gene locus giving rise to single amino acid variations interspersed among highly conserved regions (Figure 8A). The analyses of group 2 indicated that a stretch of 21 amino acids was conserved with variations at amino acid positions 2, 5, 7, 15, and 16. The most predominant amino acids at these positions were aspartic acid, methionine, glutamine, glutamine, and lysine, respectively. The aspartic acid at position 2 was substituted by valine in two of the sequences. Methionine at position 5 was replaced by serine occassionally. The two glutamines at positions 7 and 15 were substituted by leucine, glycine, or arginine, while lysine at position 16 was replaced by alanine, serine, or glutamine (Figure 8B). The analysis of the synthetic peptides constituting group 3 showed that two histidines and phenylalanine were conserved (Figure 8C). The variations in the amino acids, however, did not affect the inhibitory activity of the inhibitors.

From the survey of the genome databank of NCBI, it was noted that the DNA sequences for the aspartic peptidase inhibitors were not available. This could be attributed to the fact that very few natural protein inhibitors have been investigated for their gene sequence. The peptide sequences were derived as cleavage products, and as a result the nature of their DNA sequence may not have been deduced.

Girdwood et al. (1998) have determined the functional relationship between the protein sequences of aspartic peptidase inhibitors derived from nematodes belonging to the family Filaridae. Their studies are aimed at correlating similar gene sequences from *Ascaris, Onchocerca,* and other related nematodes to assess their function as aspartic peptidase inhibitors.

VI. INHIBITOR DESIGN AND FUTURE PROSPECTS

Most aspartic peptidase inhibitors that have been developed to date bind to their target enzyme through

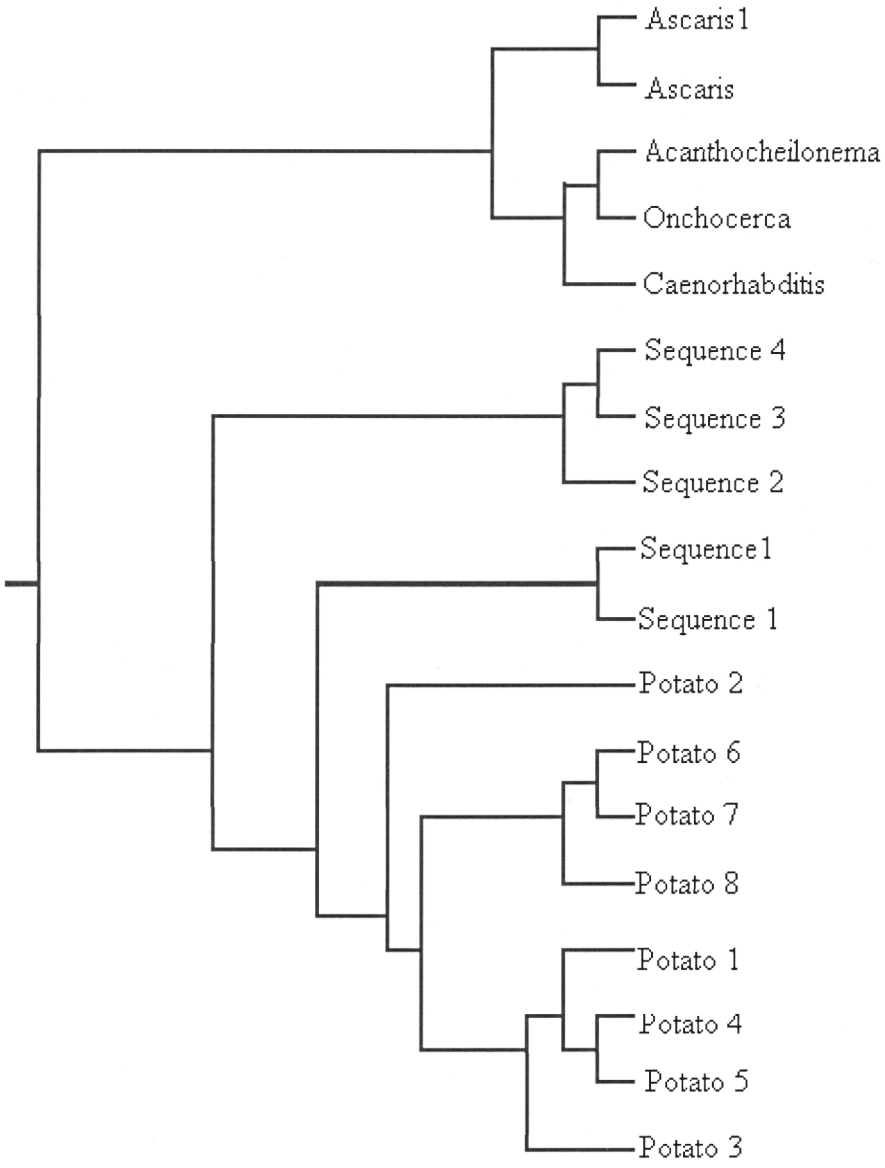


FIGURE 7. Dendrogram showing the relationship among the aspartic protease inhibitors, The dendrogram was created by the Genomenet ClustalW server (Kyoto Center) (Thompson et al., 1994). The differences between the sequences are proportional to the length along the horizontal axis.

A:

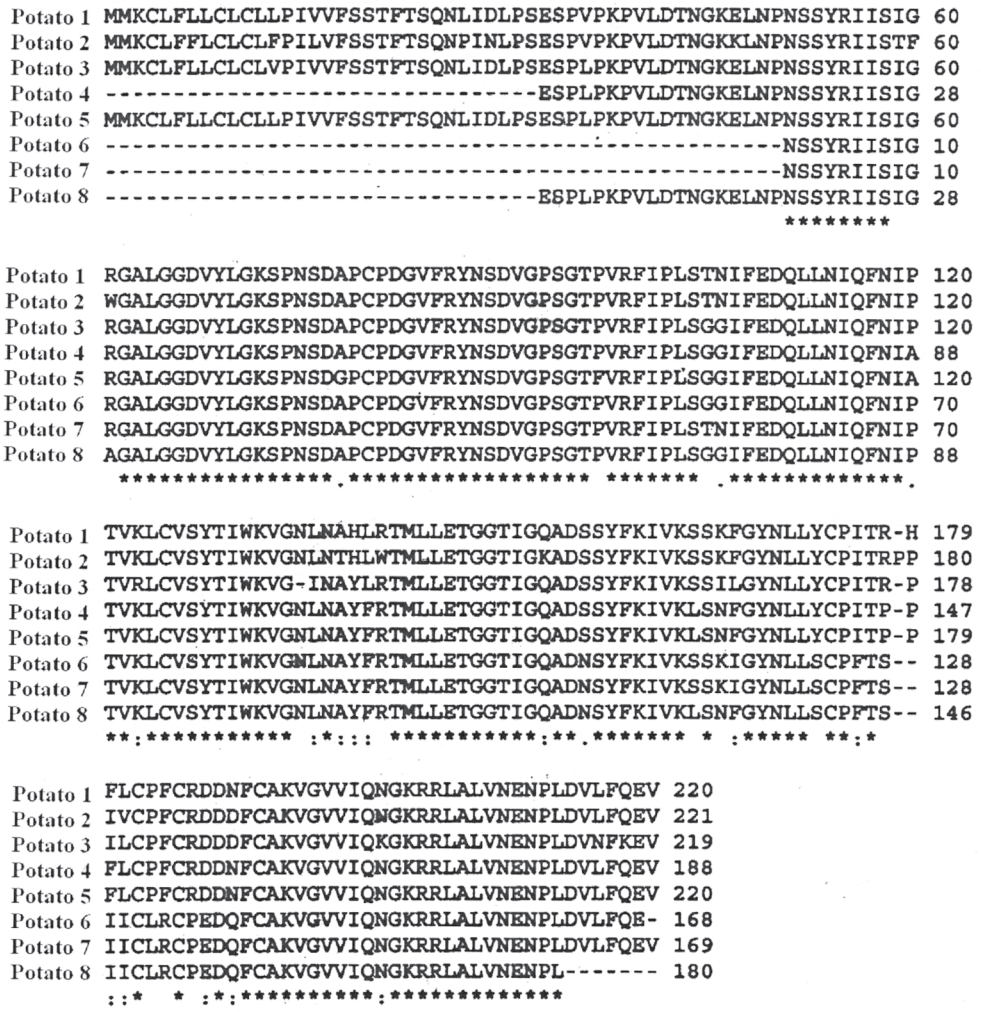


FIGURE 8. Homology alignments of the aspartic protese inhibitor sequences. The inhibitor sequences have been selected from the SWISS-PROT entries and aligned using the CLUSTALW software for multiple alignment (Thompson et al., 1994). Identical amino acid residues are marked as * and the conserved amino acid residues are marked as :. Inhibitors: A: From potato (Group 1) B: From nematodes (Group 2) C: Synthetic inhibitors (Group 3).

B:

```
Acanthocheilonema ----MKILSCLLLCTITVLEGNVMNRHNKRFAGFNVAGIGGTAGCVVVDNKLFANGFFLR 56
Onchocerca        RTTTMKILFCLLLLAITALEAGVVKRYNKRFAGFNVAGIGGNAGCVVVDNKLFANSFFLR 60
Caenorhabditis    ----MKLLALVALCAVGVASH--RDKRQLSIGTISVSGAGGSTGCVVTGNVLYANGIRLR 54
Ascaris 1         ------------MHVWLILSLASLWTSSIAYSQFLFSMSTGPFICTVKDNQVFVANLPWT 48
Ascaris           --------------------------------QFLFSMSTGPFICTVKDNQVFVANLPWT 28
                                                  : .:   *   *.* .* ::. .:

Acanthocheilonema ELTAEEQREFAQYVEESNKYKEELKVSLEERRKGWQ-----IARQSEKGAKILSTITEKN 111
Onchocerca        ELTTEEQRELAQYIEDSNRYKEEVKESLEERRKGWQ-----LARDGKEDSKVLSALAEKK 115
Caenorhabditis    NLTSSEQSELATYQTEVEQYKTQLRNILSQRRENLRNRLMSQGRNQQQQSNDVSSQGGND 114
Ascaris 1         MLEGDDIQVGKEFAARVEDCTNVKHDMA------------------------------- 76
Ascaris           MLEGDDIQVGKEFAARVEDCTNVKHDMA------------------------------- 56
                   *  .:      :    : .   :

Acanthocheilonema ---LPKPPKKPSFCTAADTTQYYFDGCMVQNNKIFVGQSYVRDLTADEAKELKSFDVKMT 168
Onchocerca        ---LPKPPKKPSFCSAGDTTQYYFDGCMVQNDKIYVGRAYVRDLTPDEVTQLKTFDAKMT 172
Caenorhabditis    DGSIPKAPEKPSFCTAEDTTQYYFDGCMVQGNKVYVGGQYARDLSSDEISELQTFDTQQT 174
Ascaris 1         ----PTCTKPPPFCGPQDMKMFNFVGCSVLGNKLFIDQKYVRDLTAKDHAEVQTFREKIA 132
Ascaris           ----PTCTKPPPFCGPQDMKMFNFVGCSVLGNKLFIDQKYVRDLTAKDHAEVQTFREKIA 112
                      *. .: *.** . * . : * ** * .:*:::.  *.***:..:  ::::*  : :

Acanthocheilonema AYQKYLSSSIQQQMNSLFGDKTNLLNLFTN---THLESTSQASEATTIPTTTQTPVEAPE 225
Onchocerca        AYQKYLSS-------------------------------------------------- 180
Caenorhabditis    AYQNAVQSQMQSQVQGLFGGSDFLSALFGGDRFNQQQQRQQPSSTTPASTSSTTLPPKPT 234
Ascaris 1         AFEEQQEN-----------------------------QPPSSGMPHGAVPAGGLSPPP 161
Ascaris           AFEEQQEN-----------------------------QPPSSGMPHGAVPAGGLSPPP 141
                  *:::  ..

Acanthocheilonema TPSFCVPIY 234
Onchocerca        --------- 
Caenorhabditis    VPQFCTAIF 243
Ascaris 1         PPSFCTVQ- 169
Ascaris           PPSFCTV-- 148
```

C:

Synthetic renin inhibitors

```
Sequence 4    ----PHPFHFFVY-X-
Sequence 3    ----PHPFHLVIHK--
Sequence 2    DRVYIHPFHL------
Sequence 1    DRVYIHKFHLLVYS--
Sequence 1    DRVYIHPFHLLVY--S
                    *  **
```

FIGURE 8. (continued)

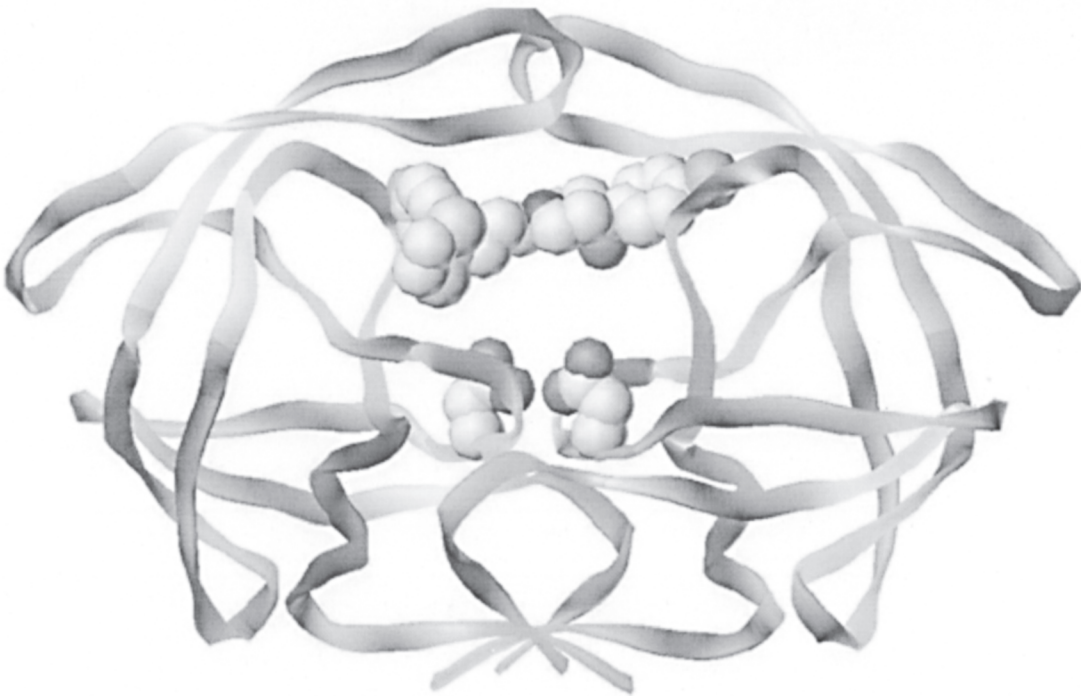


FIGURE 9. Crystal Structure of HIV Protease with a representative inhibitor. The computer generated image of the HIV-1 Protease bound with a representative inhibitor is shown. The HIV-1 protease and other retroviral proteases have the structural feature called "the flap region". The flap remains flexible and allows substrate and ligand access to the active site by opening and folding the tips into hydrophobic pockets thus exerting a central role in the protease activity. In the protease each monomer contributes one Asp-Thr-Gly triad, aa: 25(25') – 26(26') –27(27'). The inhibitor interacts with the triad in the active site of the protease and induces inward movement of the flaps. The structure of the HIV-1 protease is as described in PDB ID. 1AID (Rutenber et al., 1993).

noncovalent interactions (i.e., hydrogen bonds, ionic, or van der Waal's contacts). Therefore, these compounds are reversible inhibitors of peptidases, and effective inhibition relies on the enzyme having greater affinity for the inhibitor than its natural substrate. High affinity for any particular aspartic peptidase has been achieved by trying to maximize the number of noncovalent interactions that the inhibitor makes with the enzyme. One approach that has proven very successful in this regard is the incorporation of a transition-state isostere into designed inhibitors. A transition-state isostere is defined as a functional group that can mimic the tetrahedral transition state of amide bond hydrolysis but cannot itself be hydrolyzed by the enzyme. It has been hypothesized that stable structures, which can resemble the transition state of an enzyme reaction, will be bound more tightly than the substrate for the enzyme-catalyzed reaction. Studies on the general aspartic peptidase inhibitor pepstatin (which incorporates the statine transition-state isostere) suggest that this increased affinity, which can be as great as 10^4-fold, is not only due to mimicry of the transition state of amide bond hydrolysis but also due to the displacement of the catalytic water molecule hydrogen bonded to the catalytic aspartates.

The susceptibility of the individual aspartic peptidases to inhibition by these compounds varies considerably. The *Ascaris* proteins for example are effective inhibitors of human, pig, and chicken pepsins, pig gastricin, and (rabbit) cathepsin E with a weaker influence on human gastricin and little or no effect of the other aspartic peptidases tested. This selectivity of inhibition is also observed with the pepsin inhibitor peptide obtained after activation of pepsinogen. It has been known that one of the peptides released on activation of (pig) pepsinogen binds to pepsin above pH 4.5 to stabilize the enzyme at higher pH values and to inhibit it. This inhibitor has been identified as the peptide released in the first step in the sequential activation of pig (and cow) pepsinogen(s) and is de-

rived from the first 16/17 residues in the zymogens (Dunn et al., 1983). This differential susceptibility to inhibition by naturally occurring compounds persists into what is perhaps the best-known category, the pepstatins. Members of this peptidase family, for example, renin, pepsin, cathepsin D, and human immunodeficiency virus-peptidase, are generally typecast on the basis of their susceptibility to inhibition by acetylated pentapeptides, isovaleryl-, and acetyl-pepstatin. However, the two most recently identified human aspartic peptidases, β-site Alzheimer's precursor protein cleavage enzyme and β-site Alzheimer's precursor protein cleavage enzyme 2 (Vassar et al., 1999), are not inhibited by this classic type of inhibitor of this family of enzymes. On the basis of these observations with naturally occurring inhibitors, it would seem to be possible to design synthetic counterparts that should be specific for individual enzymes. The rationale behind such inhibitors has been to synthesize a peptide of an appropriate length and containing the amino acid residues known to be present in the naturally occurring substrate for the enzyme but with the introduction of statine in place of the two residues contributing to the scissile bond of the substrate. Such an approach has led to the synthesis of highly potent inhibitors of human renin and calf chymosin (Powell et al., 1984).

An analogous yet different strategy where a chemical modification of the scissile peptide bond is used to introduce a nonhydrolyzable analogue of the tetrahedral transition state formed during hydrolysis was also reported (Szelke et al., 1982). Instead of using the naturally occurring statine residues as the centerpiece around which the inhibitor is constructed, the complete amino acid sequence of the substrate is retained in the inhibitor except that the scissile peptide bond between P_1- P_1' of the substrate is replaced by the nonhydrolyzable analogue of the transition state. Using this approach, a tight-binding inhibitor of human renin has been developed that is a synthetic analogue based on the sequence of residues known to occur on either side of the scissile peptide bond of the angiotensinogen but with a reduced -CH_2-NH- isostere in place of the -CONH- of the substrate.

It is also possible, of course, to device such inhibitors not on the sequence of residues found in naturally occurring protein substrates but based on synthetic peptides that are known to be good substrates for certain enzymes. Thus, it would appear that although naturally occurring inhibitors of aspartic peptidases may have little physiological significance in regulating their target enzymes *in vivo*, nevertheless such compounds and their synthetic counterparts have proved of inestimable value in facilitating distinction among the different types of aspartic peptidases. Such inhibitors can be utilized for diagnostic purposes to establish the nature of a "newly isolated" peptidase. As an illustration of this, consider the inhibition of human pepsin and gastricin by isovaleryl and lactyl-pepstatin (Reid, 1984). Although considerable progress has been made with the structure, activity, and importance (biological and commercial) of the aspartic peptidases, much remains to be learned about the distinctions of the various architecture and how these are reflected in the functions of the enzymes. Inhibitors (naturally occurring and synthetic) have permitted detailed biochemical and crystallographic investigations to be made, but an understanding of the selectivity of such inhibitors may be of just as much importance for the design and synthesis of specific inhibitors for use therapeutically in controlling individual aspartic peptidases.

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