Microbial Aspartic protease inhibitors

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Abstract

Aspartic proteases are relatively a small group of proteolytic enzymes. Over the last decade, they have received tremendous research interest as potential targets for pharmaceutical intervention as many have been shown to play significant roles in physiological and pathological processes. Despite numerous efforts, however, the only inhibitors for aspartic proteases currently in the market are directed against the HIV protease of viral origin. Nevertheless, several inhibitors including those targeting renin-angiotensin system and β-secretase are in clinical or preclinical developments and few other aspartic proteases are discussed as potential drug targets. Currently the research strategies are focusing on the need for improved comprehension of protease-regulated cascades, along with precise selection of targets and improved inhibitor specificity. There is plethora of synthetic inhibitory compounds targeting aspartic proteases; however there are few reports documented in literature on biologic inhibitors from microorganisms. The present chapter is a comprehensive state-of-the-art review describing the aspartic protease inhibitors from microbial origin. In addition, the chapter highlights the detailed kinetic interactions of inhibitors with clinically relevant aspartic proteases. The chapter also illustrates the bifunctional role of aspartic protease inhibitors.

Keywords: - Aspartic proteases, Peptidic inhibitors, HIV protease inhibitor, Kinetic interactions, Bifunctional inhibitors.
Introduction

The diversity and specificity of proteases constitutes the basis for their serendipitous nature and multifaceted physiological activities. Proteases participate in most aspects of cell nutrition, physiology, signaling cascades and microbial pathogenesis (Ward et al., 2009). Their activity, if uncontrolled, would be destructive to the cell or organisms and therefore must be precisely regulated. The most significant aspect of protease action is the control of protease activity to limit cleavage to intended substrates without destruction of functional proteins. Many proteases are also essential for propagation of diseases, and hence inhibition of different proteases is emerging as a promising approach in medicinal application for cancer, obesity, hepatitis, herpes, cardiovascular, inflammatory, neurodegenerative diseases, and various infectious and parasitic diseases (Rao et al., 1998). Aspartic proteases are relatively a small group of proteolytic enzyme 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 (Dash et al., 2003). Cathepsin D inhibitors have broadened the knowledge of structure, mechanism and contribution of cathepsin D in therapy of diseases (Gacko et al., 2007). These inhibitors are mainly synthetic molecules; however there is paucity on biologic inhibitors from microbes. The application of biologic inhibitors will stimulate renewed interest in the therapeutic targeting of aspartic proteases. The mechanism by which these inhibitors modulate the proteases anticipates the development of biologic inhibitors has lead molecules for clinical approval in the near future. The present chapter is a comprehensive state-of-the-art review describing the aspartic protease inhibitors from microbial origin.

Classification and mechanism of proteases

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins. Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as hemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms (Rao et al., 1998)). In general the active sites of protease are flanked on one or both sides by one or more subsites capable of accommodate the side chain of specific single amino acid residue from the substrate. The degree of amino acid specificity delineated by the active site and the subsites ultimately defines the general biocatalytic properties and especially the specificity of the proteolytic reaction.
Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure. Depending on their site of action, proteases are categorized into two major groups, i.e., exoproteases and endoproteases. Exoproteases act only near the ends of the N or C termini of the polypeptide chains and are classified accordingly as amino- and carboxyproteases, respectively. Endoproteases attack peptide bonds in more central locations of the polypeptide chain more remote from the N and C termini and indeed free amino or carboxyl groups are known to inhibit or retard enzyme action (Ward et al., 2009). Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases and two newly established families, i.e., glutamic acid proteases and threonine proteases. There are a few miscellaneous proteases which do not precisely fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Dash et al., 2003).

**Serine protease**

As the name implies, serine proteases contain a serine group in their active site, which is essential for substrate binding and cleavage. Generally, serine proteases are characterized by their broad substrate specificity, and their activity extends beyond purely peptidase to include esterase and amidase activities. Serine proteases exist among exoprotease, endoprotease, oligoprotease, and omega protease groups. Important representative enzyme groups include the chymotrypsins (SA), subtilisins (SB), carboxyprotease C (SC), and Escherichia D-Ala-D-Ala protease A (SE), and these have primary structures that are totally unrelated. Serine proteases are characterized by having a conserved glycine-containing peptide, Gly-Xaa-Ser-Yaa-Gly, associated with the catalytic serine. A common reaction mechanism in the form of a catalytic center containing serine as a nucleophile, aspartate as an electrophile, and histidine as a base, is exhibited by groups SA, SB, and SC, respectively. Interestingly, distinctive protein folding strategies among these groups accomplish similar geometric orientations of these residues, suggesting a convergent evolutionary background. Some groups may be differentiated from the latter groups in that they lack the serine-aspartate-histidine catalytic center.

Serine proteases generally exhibit pH optima in the range 7–11 and manifest isoelectric pH values in the range 4–6. Serine alkaline proteases, produced by certain bacteria including *Arthrobacter*, *Streptomyces*, and *Flavobacterium* species, filamentous fungi, including *Conidiobolus*, *Aspergillus*, and *Neurospora* species, and some yeasts represent the largest subgroup of serine proteases, and are active at the higher end of the above pH range (pH optimum ~10) with an unusually high isoelectric pH around 9. These enzymes are inhibited by diisopropylfluorophosphate (DFP) or a potato protease inhibitor, but not by tosyl-L-phenylalanine-chloromethyl ketone (TPCK) or tosyl-L-lysine-chloromethylketone (TLCK), which inhibit other serine proteases. These enzymes are characterized as having substrate specificities similar to but broader than that of chymotrypsin, where the carboxyl side of the peptide bond being attacked contains a tyrosine, phenylalanine, or leucine residue. The second largest family of serine proteases contains the subtilisins, which are best represented by subtilisin Carlsberg and subtilisin
BPN, produced by *Bacillus licheniformis* and *B. amyloliquefaciens*, respectively. The active site conformations of both the enzymes are similar to trypsin and chymotrypsin despite their contrasting molecular structures. While the subtilisin from *Conidiobolus coronatus* exhibits catalytic similarities with subtilisin Carlsberg, its protein structure is distinct.

**Thiol / Cysteine proteases**

Cysteine proteases generally may be assigned to one of the following four groups according to their side chain specificities: (1) papain-like (includes clostripain and streptopain), (2) trypsin-like with preference for cleavage at the arginine residue, (3) specific to glutamic acid, and (4) others. Most have neutral pH optima. All cysteine proteases have cysteine/histidine catalytic dyad, although the order of these residues, Cys-His or His-Cys, may vary. They generally need reducing agents such as sodium bisulphite, hydrogen cyanide, or cysteine for activity retention. Sulphydryl agents such as p-chloromercuribenzoate are inhibits or denaturants, whereas DFP and metal-chelating agents are not. Clostripain (*Clostridium histolyticum*), which shows high specificity for arginyl residues contributing the carboxyl group to the peptide bond contrasts with papain in that it requires calcium for activity. Streptopain (*Streptococcus* sp.) manifests broad specificity toward synthetic substrates and oxidized insulin B chain. Biocatalysis is mediated by a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. These enzymes have broad specificity and also attack amide ester, thiol ester, and thiono ester bonds. The enzyme initially binds noncovalently to the substrate, after which acylation of the enzyme occurs together with release of the first product. Water reacts with the acyl enzyme releasing the second product through deacylation.

**Metalloproteases**

The divalent metal-requiring metalloproteases are a very diverse group of proteases, which include both endoproteases and exoproteases. They are inhibited by chelating agents such as ethylenediaminetetraacetic acid, but not by sulphydryl agents or DFP. Thermolysin, a neutral zinc protease produced by *B. stearothermophilus*, is one of the most thoroughly characterized metalloproteases where a histidine and glutamine participate in the active site, providing a ligand for zinc and a catalytic function, respectively. As its name suggests, thermolysin exhibits high thermostability and has a half-life of 1h at 80 °C. Four calcium atoms enhance the thermostability of the protein. The metalloprotease collagenase is produced by a variety of microorganisms, including *Achromobacter iophagus* and *Clostridium histolyticum*. *Pseudomonas aeruginosa* produces the neutral metalloprotease elastase as well as alkaline metalloproteases. The alkaline protease I from *Myxobacter* lyses the cell walls of *Arthrobacter* crystallites, AU1 whereas *Myxobacter* protease II cannot lyse the bacterial cells. Generally, the metal-binding site motif includes the motif His-Glu-Xaa-Xaa-His. Biocatalysis requires bound divalent cations, and removal of the metal through dialysis or with chelating agents causes inactivation. In thermolysin, Glu143 participates in the nucleophilic attack of a
water molecule on the carbonyl carbon of peptide bond being cleaved, which has been polarized by the Zn ion.

**Glutamic acid and Threonine proteases**

These new class of proteases, glutamic acid protease was derived from a former pepstatin-insensitive carboxyl protease and threonine proteases was discovered in 1995 as part of proteasome complex. Glutamic acid proteases have been discovered from *Stylidium lignicola* and *Aspergillus niger var. macrosporus*. The catalytic mechanism is based on the two enzymes from the aspergilloglutamic and scytalidoglutamic proteases. The active site diad glutamic acid and glutamine play a critical role in substrate binding and catalysis. These amino acids along with their associated water molecules act as nucleophiles to exhibit an acid-base mechanism distinct from that of the aspartic proteases. The glutamic acid acts as a general acid in the first phase of catalysis, donating a proton to the carbonyl oxygen of the scissile peptide bond of the substrate. Simultaneously, an OH– is donated by water associated with the active site of the enzyme to the carbonyl oxygen of the peptide bond of the substrate. Sometimes, two water molecules are involved in the reaction. The transition state of the substrate is thought to be stabilized by hydrogen bonding with the two catalytic residues. Then, glutamic acid donates a proton to the amide nitrogen atom of the scissile peptide bond triggering the breakdown of the tetrahedral intermediate and thus effecting peptide bond cleavage. The glutamine residue is then responsible for recovering the original state of the glutamic acid residue. Theronine proteases are part of a multicomponent proteasome complex in microbial cells. The archaebacterial proteasome has 14 active sites in the inner channel, one on each β subunit. The hydrolytic sites are spatially separated from the intracellular components. Recent reports have indicated that the active site nucleophile is the hydroxyl group on the threonine at the N-terminus of the β subunit. The replacements of the terminal threonine by serine in archaebacterial proteasomes allows complete proteolytic activity. Therefore, the conservation of the threonines in the active sites of all threonine proteases from bacteria to eukaryotes is unclear. Looking at the diverse functions of the threonine proteases in bacteria and mammals, it is evident that the phylogenetically ancient proteasome has undergone adaptations that favor different functions in different physiological situations.

**Aspartic proteases**

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (13), and have been placed in clan AA. The members of families A1 and A2 are known to be related to each other, while those of family A3 show some relatedness to A1 and A2. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. The members of the pepsin family have a bilobal structure with the active-site cleft located between the lobes. The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr.
Retropepsins are monomeric, that is, they carry only one catalytic aspartate, and thus dimerization is required to form an active enzyme and the motif Xaa is Ala. Penicillopepsin and endothiapepsin biocatalysis is mediated by a general base catalytic mechanism with a lytic water molecule participating in the reaction. The specificity of the catalysis has been explained on the basis of available crystal structures. The structural and kinetic studies also have suggested that the mechanism involves general acid-base catalysis with lytic water molecule that directly participates in the reaction (Figure 1). This is supported by the crystal structures of various aspartic protease-inhibitor complexes and by the thiol inhibitors mimicking a tetrahedral intermediate formed after the attack by the lytic water molecule (Rao et al., 1998).

The aspartic proteases are inhibited by pepstatin. They are also sensitive to diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP) in the presence of copper ions. In general pepsins and other aspartic proteases exhibit broad based specificity towards cleavage in peptides consisting of at least six hydrophobic amino acids at specific substrate positions (Dash et al., 2003). Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Microbial aspartic proteases can be broadly divided into two groups, (i) pepsin-like enzymes produced by Aspergillus, Penicillium, Rhizopus, and Neurospora and (ii) rennin-like enzymes produced by Endothia and Mucor sp (Rao et al., 1998).

In contrast to serine and cysteine proteases, catalysis by aspartic proteases 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 (Northrop, 2001; Dunn, 2002).

Aspartic protease inhibitors

Proteases 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, protease inhibitors. Hence, the research interest in protease inhibitors has evoked tremendous attention in many disciplines. Multicellular organisms possess endogenous protein protease inhibitors to control proteolytic activity. Most of these inhibitory proteins are directed against serine proteases, although some are known to target cysteine, aspartyl, or metalloproteases. Indeed, inhibitors of serine, cysteine, and metalloproteases are distributed ubiquitously throughout the biological world. In sharp
contrast, however, naturally occurring inhibitors of aspartic proteases are relatively uncommon and are found in only certain specialized locations.

Protease inhibitors can be generally classified into 2 large groups based on their structural dichotomy: low molecular weight peptidomimetic inhibitors and protein protease inhibitors composed of one or more peptide chains (Dash et al., 2003). Protease inhibitors can be further classified into 5 groups (serine, threonine, cysteine, aspartyl and metalloprotease inhibitors) according to the mechanism employed at the active site of proteases they inhibit. Some protease inhibitors interfere with more than one type of protease. For example, the serine family of protease inhibitors (serpins) is generally thought of as active against serine proteases, yet contains several important inhibitors of cysteine proteases as well. Proteolytic inhibition by protease inhibitors can occur via 2 mechanisms: irreversible trapping reactions and reversible tight binding reactions (Rawlings et al., 2004). Inhibitors which bind through a trapping mechanism change conformation after cleaving an internal peptide bond and “trap” the enzyme molecule covalently; neither the inhibitor nor protease can participate in further reactions. In tight-binding reactions, the inhibitor binds directly to the active site of the protease; these reactions are reversible and the inhibitor can dissociate from the enzyme in either the virgin state, or after modification by the protease (Fear et al., 2007).

Traditionally, protease 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 non hydrolyzable isostere and optimizing inhibitor potency through trial and- error structural modifications that progressively reduce the peptide nature of the molecule. This substrate/receptor- based drug design has been substantially improved in recent years with the availability of three dimensional structures. The structural information about the active site of the receptor (or protease) 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 protease inhibitors.

As an enzyme family, aspartic proteases 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 protease 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 proteases, model enzymes have been particularly informative.

Aspartic proteases 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 protease-inhibitor crystal structures are currently available on the PDB database for viral proteases (HIV-1, HIV-2, SIV, FIV), Cathepsin D, renin, renin/chymosin, penicillopepsin, secreted aspartic protease, pepsin, mucoropepsin, retropepsin, saccharopepsin, rhizopuspepsin, and plasmapepsin II.

Aspartic proteases 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 protease by taking advantage of the collective interactions between a putative inhibitor, on both sides of its scissile amide bond, and a substantial portion of the substrate-binding groove of the enzyme. Some aspartic protease also have one or more flaps that close down on top of the inhibitor, further adding to inhibitor protease 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 protease inhibitors can be grouped under two categories by their molecular nature, (1) proteinaceous inhibitors, and (2) low-molecular-weight inhibitors.

**Proteinaceous Inhibitor**

In a sharp contrast to the ubiquitous presence of multiple forms of proteinaceous inhibitors of other classes of proteases from different sources of plants, animals, and microorganisms, there is a paucity of proteinaceous inhibitors of aspartic proteases. With the exception of macroglobulins, which inhibit proteases of all classes, individual protein inhibitors inhibit only proteases belonging to a single mechanistic class. Protein inhibitors of aspartic proteases 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 lumbricoides* (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 pluripotent inhibitor from sea anemone of cysteine proteases 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 proteases (proteases A or saccharopepsin).
Low-Molecular-Weight Inhibitors

In contrast to the proteinaceous nature of the proteases inhibitors from plants and animals, the inhibitors produced by microorganisms are of smaller molecular nature. The presence of proteases 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 proteases inhibitor, isolated from various species of \textit{Streptomyces}, is a specific inhibitor of pepsin (Umezawa et al., 1970). Pepstatin also inhibits the activities of cathepsin D, cathepsin E, renin, pseudorenin, aspartyl proteases produced by microorganisms.

\textit{Streptomyces testacus} was reported to produce various pepstatins that differed from one another in the fatty acid moiety (C2-C10). 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 \textit{Streptomyces naniwaensis} and \textit{Streptomyces} no. 2907 (Dash et al., 2003).

Pepstatins, pepstanones, and hydroxypepstatins have almost identical activity against pepsin and cathepsin D. Pepstatin also binds to procathepsin D and inhibits its autoactivation. 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. The inhibition of aspartyl proteases by pepstatin depends to a large extent on the presence of acid residue in their structure. The inhibitory effect of pepstatin on cathepsin D does not depend on the carbon chain length of acid radicals (Gacko et al., 2007). Esters of pepstatin, pepstatinal and pepstatinol possess antipepsin 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 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-AHMHAL- alanyl-AHMHA and L-lactyl-L-valyl-AHMHA-Lalanyl- 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 proteases (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 (Dash et al., 2003).

**Inhibitors of pepsin**

Pepsin is a well studied enzyme whose activity has been extensively studied since Northrop crystallized it in 1929 (Northrop, 1930). It belongs to the family of the aspartic proteases, together with cathepsin D, quinosine, renin and the HIV-protease. The recognition of the HIV-protease as a member of this family (Pearl and Taylor, 1987) has renewed the interest in this type of enzymes and in their inhibition (Velazquez-Campoy et al., 2000).

Although in many cases the etiologic agent of the ulcer is unknown, it is accepted that ulcerative processes occur as results of a balance between aggressive endogenous factors such as acid, pepsin and the maintenance of the integrity of the gastric mucosa, and environmental factors like the use of non-steroidal anti-inflammatory drugs (NSAID), the presence in the gastro-intestinal mucosa of *Helicobacter pylori*, smoking habit, environmental stress, and population feeding habits (Lanza, F. L. 1984). Pepsin is a major aggressive factor in the GERD (Gastro- Easophgeal Reflux Diseases) (Tobey et al., 2001). Recently, in accompany with a remarkable advance in the pharmacological studies with
regard to ulceration of digestive organs, many attempts are directed to the aspect of enzymatic action of pepsin in order to solve the causes of ulceration as well as developments of ulcers. Because there is no drug that produces 100% remission of gastroduodenal ulcers (Friedman and Peterson 1998), and because there are thousands of cases involving this kind of illness, which constitutes a huge health problem, to study substances with potential anti-ulcerogenic activity is not only important, but vital. It is necessary to seek anti-ulcerogenic agents that are more effective, less toxic and cheaper.

There is a wide range of specific inhibitors that can bind to the active site and electively remove the activity of pepsin. One of the best known ones is pepstatin (Umezawa, 1970) that, at acidic pH, tightly binds to the catalytic site of both pepsin and its precursor: pepsinogen. The pepstatin—pepsinogen complex, however, cannot be formed above pH 3 because the active site is blocked by a propeptide sequence. Below pH 5, pepsinogen is self-cleaved to produce active pepsin (Dash et al., 2003). A simplified analogs of pepstatin A representing ‘tripeptides’ with two valine residues which are C-terminated by an amino alcohol moiety was found to exhibit inhibition against pepsin (Kratzel et al., 2000). 1,2-Epoxy-3-(p-nitrophenoxy)propane (EPNP) is known to inhibit pepsin and other aspartic proteinases by reacting with the active site aspartic acid residue. However, the reaction is considerably slow in general, and therefore, it is desirable to develop similar reagents that are capable of inhibiting these enzymes more rapidly. Hishashi et al., 2008 have reported the synthesis of a novel inhibitors which have a reactive epoxide group linked with peptide by a hydrazide bond, with a general structure: Iva-L-Val-L-Val-(L-AA)n-N2H2-ES-OEt (Iva, isovaleryl; AA, bulky hydrophobic or aromatic amino acid residue; ES, epoxysuccinyl). These inhibitors were shown to inhibit porcine pepsin remarkably faster than EPNP.

Alkalo Thermophilic Bacillus Inhibitor (ATBI) is a hydrophilic peptidic aspartic protease inhibitor extracellularly produced by an alkalothermophilic Bacillus sp isolated from the soil sample of a hot spring at Vajreswari, Maharashtra, India (Dey et al., 1991). ATBI was found to inhibit pepsin with a two-step inhibition mechanism. Kinetic analysis showed that pepsin is competitively inhibited by ATBI. The progress curves are time-dependent and consistent with slow-tight binding inhibition. The $K_i$ values for the first reversible complex (EI) of ATBI with pepsin was $(17 \pm 0.5) \times 10^{-9}$ M, whereas the overall inhibition constant, $K_i^*$ was $(55 \pm 0.5) \times 10^{-12}$ M. Comparative analysis of the kinetic parameters with pepstatin, the known inhibitor of pepsin, revealed a higher value of $k_5/k_6$ for ATBI (Dash et al., 2001).

A low molecular weight aspartic protease inhibitor (API) was isolated from a thermotolerant Bacillus licheniformis and exhibited a slow tight binding mechanism of the aspartic protease (AP) pepsin. The inhibitor was found to be specific for pepsin, showed very weak inhibitory activity against other aspartic proteases and did not show any inhibitory activity against other classes of proteases. The amino acid analysis and CD-spectra analysis of API suggest the peptidic nature. The kinetic studies of pepsin–API interactions reveal that API is a slow-tight binding competitive inhibitor with the IC$_{50}$ and $K_i$ values of $4.0\text{nM}$ and $(3.83\text{nM}$–$5.31\text{nM})$ respectively (Kumar and Rao., 2006).
Inhibitors of HIV protease

HIV-1 protease (PR) has been classified as an aspartic protease that functions as a homodimer, based on its primary amino acid sequence, its inhibition by pepstatin, and its crystal structure (Ratner et al., 1985; Richards et al., 1989). The retroviral protease is encoded in the viral pro gene for all retroviruses, including HIV-1 (Oroszlan et al., 1990; Pettit et al., 1993). During the replication cycle of HIV, gag and gag-pol gene products are translated as polyproteins. These proteins are subsequently processed by the virally encoded protease to yield structural proteins of the virus core, together with essential viral enzymes including the protease itself. The active site of HIV-1 PR is composed of the carboxylate side chains of two Asp residues, one from each subunit of the dimer (Navia et al., 1989; Lappatto et al., 1989). The aspartic proteases are a large family of enzymes with diverse functional roles that also share a number of structural features (Davies, 1990; Tang et al., 1978). One such feature is called the flap, which lies above the active site cleft. By sequence alignment, the conserved sequence domain of the flap region begins at position 47 of the HIV-1 PR sequence and extends through the Gly at position 52. These residues form a short stretch of b-sheet followed by a turn that ends with the conserved Gly at position 52. The crystallographic structures of HIV-1 PR-inhibitor complexes demonstrated that the binding of a peptide analogue inhibitor or a peptide substrate involves numerous hydrogen-bonding interactions with the highly mobile flaps (Miller et al., 1989). Movement of these flaps apparently accompanies the binding of the peptide analogue or substrate, which binds in an extended β-sheet, such that hydrogen bonds are established between the complementary carbonyl oxygens and the amide protons of the peptide within the flaps. The presumed function of the flap-peptide interactions is to entrap and align the scissile peptide sequence in the HIV-1 PR active site (Rodriguez et al., 1993). HIV-1 PR has been an attractive target for the development of drugs against AIDS (Blundell et al., 1989). The rational design of HIV-1 PR inhibitors may be considered under two broad categories based on (a) the substrate specificity and (b) the structural homology of HIV-1 PR dimer (Darke et al., 1988). Plethoras of synthetic inhibitory compounds targeting the active site of the HIV-1 PR have been reported (Lang et al., 1993; Wlodawer and Erickson, 1993). Saquinavir became the first protease inhibitor designed from a three-dimensional structure of a protease (structure-based design) to be approved for human use in 1996, despite its low oral bioavailability due to poor absorption and extensive first-pass degradation by cytochrome P_{450}. It is active in cell culture against both HIV-1 and HIV-2 viruses. 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. Newly emerging resistant strains of HIV enhance the need for newer and more potent drugs (Dash et al., 2003). However, there is a lacuna of literature on biological inhibitors from microorganisms.

HIV-1 PR was inhibited by a peptidic inhibitor, ATBI isolated from an extremophilic Bacillus sp. and the kinetic parameters revealed ATBI as a noncompetitive and tight binding inhibitor with the IC_{50} and K_{i} values 18.0 and 17.8 nm respectively. The
A hydrophilic peptide was found to be a potent inhibitor with a Mr of 1147, and an amino acid sequence of Ala-Gly-Lys-Lys-Asp-Asp-Asp-Pro-Pro-Glu. Fluorescence spectroscopic studies revealed that ATBI binds in the active site of the HIV-1 PR and is the first report of a noncompetitive inhibitor from an extremophilic microorganism. It is well established that Trp-42 is present adjacent to the flaps, and the flap regions of HIV-1 PR are the only dynamically flexible portions of the enzyme. The enzyme inactivation is caused by the loss of the flexibility of the flaps restricting the entry and exit of the polypeptide substrate and products (Dash and Rao., 2001).

Although bioactive peptides can be produced chemically by a variety of synthetic strategies, recombinant peptides production of 5–50 amino acid size range offers the potential for large-scale production. Expression of very short polypeptide chains can sometimes be problematic in microbial systems such as E. coli, however some peptides have been expressed as a part of fusion proteins (Rutenber et al., 1993; Brandwijk et al., 2005; Gavit and Better, 2000; Li et al., 2000). There is a paucity of reports on cloning of low molecular weight peptidic inhibitors of aspartic proteases from microbial sources. Vathipadiekal et al., 2010 have described the cloning of a synthetic gene coding for ATBI through a gateway cloning approach. The peptide sequence data of ATBI was exploited to synthesize the complementary oligonucleotides, which were annealed and subsequently cloned in frame with the gene for GST in E. coli. When setting up a process for production of a recombinant protein, the normal approach is to first try to express the protein of interest in E. coli. Alternative expression systems are used only if the product is biologically inactive after production due to lack of essential post-translational modifications, incorrect folding or when the recovery of the native protein is too low (Georgiou, 1996).

The recombinant peptide ATBI was found to be functionally active in vitro against HIV-1 protease, pepsin, and fungal aspartic protease. A reduction in the activity of recombinant peptide as compared to natural ATBI was observed, which could be explained by several reasons. One of the reasons could be the heterologous host system used for expression. The appropriate cellular microenvironment present in the natural host may be crucial factor for the optimal activity of ATBI. This could be circumvented by producing the recombinant ATBI in Bacillus sp. in the native system. The difference in the mode of production of ATBI in E. coli versus Bacillus can also be one of the factors contributing for the reduced activity observed for recombinant ATBI. Bacillus expression systems have significant advantages over E. coli expression systems in that protein of interest are secreted due to an active secretory system (Wong, 1995). However the present results validate the use of synthetic gene of ATBI in further developing the inhibitor as a useful clinical agent as well as for sub cloning the gene to various other host systems including Bacillus for retaining the optimal activity. Also the protocol described in this study may be used to clone pharmaceutically important peptide molecules (Vathipadiekal et al., 2010).

An almost two-decade research effort by academic and pharmaceutical institutions resulted in the successful commercialization of seven drugs that are potent inhibitors of HIV-1 protease activity and which, if used correctly, are highly effective in managing
viral load. However, identification of clinical viral isolates that are resistant to these drugs indicates that this is a significant problem and that new classes of inhibitors are continually needed. Screening of microbial extracts followed by bioassay-guided isolation led to the discovery of a natural hinnuliquinone, a C2-symmetric bis-indolyl quinone natural product that inhibited the wild-type and a clinically resistant (A44) strain of HIV-1 protease with $K_i$ values of 0.97 and 1.25 µM, respectively. Crystallographic analysis of the inhibitor-bound HIV-1 protease helped explain the importance of the C2-symmetry of hinnuliquinone for activity (Singha et al., 2004).

**Kinetic studies on enzyme-inhibitor interactions**

A number of enzymatic reactions do not respond to the presence of competitive inhibitors instantly but rather display a slow onset of the inhibition. In some cases the inhibitor interacts slowly with the enzyme, and in others the formation of the enzyme-inhibitor complex takes place in a very short time. Such inhibition is called slow-binding inhibition, and the inhibitor is referred to as the slow-binding inhibitor (Szedlacek and Duggleby, 1995; Sculley et al., 1996). From the kinetic point of view, the possible mechanisms for the slow-binding inhibition phenomena are described in Scheme 1. Scheme 1a assumes that the formation of an EI complex is a single slow step and the magnitude of $k_3I$ is quite small relative to the rate constants for the conversion of substrate to product. However, Scheme 1b demonstrates the two-step slow-binding inhibition, where the first step involves the rapid formation of a reversible EI complex, which undergoes slow isomerization to a stable, tightly bound enzyme-inhibitor complex, EI*, in the second step. Inhibitors which inhibit the enzyme-catalyzed reactions at concentrations comparable to that of the enzyme and under conditions where the equilibria are set up rapidly are referred as tight-binding inhibitors. The establishment of the equilibria between enzyme, inhibitor, and enzyme-inhibitor complexes in slow-binding inhibition occurs slowly on the steady-state time scale (Morrison, 1982), which has been thoroughly reviewed (Kati et al., 1998; Yiallouros et al., 1998; Ploux et al., 1999). Understanding the basis of the isomerization of the EI complex to the EI* complex could lead to the design of inhibitors that allow titration of the lifetime of the EI* complex. Early investigations of pepsin and the fungal aspartic proteases gave rise to a variety of mechanistic proposals, which were resolved in favor of the mechanisms shown in Scheme 1 (James et al., 1992). The future development of slow-tight binding inhibitors will undoubtedly depend on application of kinetic techniques that yield quantitative information about the properties of the inhibitors.

**Inhibition kinetics of ATBI with aspartic proteases**

Considering the physiological importance of the aspartic proteases and their role in various diseases, there is a lacuna in the studies of the mechanism of inhibition by slow-binding inhibitors. To our knowledge, the best-known slow-binding inhibitors of pepsin are pepstatin (Umezawa et al., 1978) and its analogues (Rich and Sun, 1980; Gelb et al., 1985). However, the hydrophobic nature of pepstatin holds a disadvantage for its poor oral bioavailability. The evaluation studies of the kinetic parameters of ATBI, a slow-
tight binding inhibitor of the aspartic proteases (AP), pepsin and aspartic protease from *Aspergillus saitoi* (F-prot) showed competitive inhibition.

Examination of the progress curves suggested that, in the absence of ATBI, the steady-state rate of proteolytic activities was reached rapidly, whereas in its presence, the rate decreased in a time dependent manner. The progress curves also revealed a time range where the conversion of EI to EI* was minimal. For a low concentration of ATBI, the range was 0-3 and 0-4 min for pepsin and F-prot, respectively, within which classical competitive inhibition experiments can be used to determine the $K_i$ values. From such experiments, $K_i$ for ATBI was determined to be $(17 \pm 0.8) \times 10^{-9}$ M for pepsin and $(3.2 \pm 0.5) \times 10^{-6}$ M for F-prot. The $K_i$ associated with the formation of the reversible enzyme-inhibitor complex (AP-ATBI) determined by the Dixon method supported the above values. The apparent rate of reaction $k$ from the progress curves when plotted versus the inhibitor concentration followed a hyperbolic function, indicating a twostep inhibition mechanism. The overall inhibition constant, $K_i^*$ were $(55 \pm 0.5) \times 10^{-12}$ M for pepsin and $(5.2 \pm 0.6) \times 10^{-8}$ M for F-prot respectively (Dash et al., 2001).

ATBI was found to inhibit the purified recombinant HIV-1 PR with an IC50 value (50% inhibitory concentration) of 18 nM. The inhibition of the HIV-1 PR followed a sigmoidal pattern with increasing concentrations of the inhibitor. However, the secondary plot (the slope of inhibition graph versus inhibitor concentration) was not linear, suggesting that the application of Michaelis-Menten inhibition kinetics was not appropriate in this study. The inhibition constant $K_i$, determined by the classical double reciprocal plot and also by Dixon plot was 17.8 nM, which is almost equal to the IC50 value of the inhibitor. The Lineweaver-Burk’s reciprocal plot showed that ATBI was a noncompetitive inhibitor of the HIV-1 PR. For the inhibition kinetic studies, the HIV-1 PR activity was monitored in the presence of various concentrations of inhibitor and substrate as a function of time. A very rapid inhibition of the HIV-1 PR was observed, which necessitated measuring all of the kinetic parameters at second order association conditions. The $\alpha$-values obtained for ATBI were reasonably constant, and the average calculated value of $\alpha$ in the presence of substrate is $8.25 \pm 0.50 \times 10^{-4} \text{ s}^{-1}$. The association rate constants $\beta$ in the presence and absence of substrate were calculated from the plot of the HIV-1 PR inhibition versus time. The values of $\beta$ were not affected by the presence of the substrate, indicating that the presence of substrate had no implication on the interaction of the inhibitor and enzyme. The reciprocal of the values of $\beta$ were plotted as a function of the substrate concentration. The plotted line did not fit a linear plot but was a good fit for a rectangular hyperbola, revealing noncompetitive inhibition. The mechanism of inhibition of the HIV-1 PR was further deciphered by the plot of $\Delta \beta$ versus substrate concentration. Noncompetitive inhibition was represented by the straight line (Dash et al., 2001).

**Fluorometric analysis of enzyme-inhibitor interactions**

To delineate the conformational changes induced in the aspartic proteases (pepsin and F-prot) due to the binding of ATBI, the fluorescence spectra of the enzyme-inhibitor complexes were monitored. The tryptophanyl fluorescence spectra of pepsin and F-prot
exhibited an emission maxima ($\lambda_{\text{max}}$) at ~342 nm, as a result of the radiative decay of the $\pi-\pi^*$ transition from the Trp residues. The binding of ATBI resulted in a concentration-dependent progressive quenching of the emission spectra of the enzymes. However, $\lambda_{\text{max}}$ of proteases indicated the absence of the blue or red shift in the intrinsic fluorescence, negating any drastic gross conformational changes in the three-dimensional structure of the enzymes. To monitor the isomerization of AP-ATBI to AP-ATBI*, the intrinsic tryptophanyl fluorescence of the complexes as a function of time was measured. Upon the addition of ATBI, a rapid decrease in the quantum yield of fluorescence was observed, followed by a slow decline to a final stable value over a period of 60 and 25 s for pepsin and F-prot, respectively, indicating an exponential decay of the fluorescence intensity. The magnitude of the rapid fluorescence decrease as a function of time was found to be similar to the total fluorescence quenching observed at a specific ATBI concentration. Both AP-ATBI and AP-ATBI* complexes have the same intrinsic fluorescence. Further, titration was performed in which an increased concentration of ATBI was added to the enzymes. The magnitude of the initial rapid fluorescence loss ($F_0 - F$) increased hyperbolically, corroborating the two-step, slow-tight binding inhibition of the aspartic proteases by ATBI. The estimated value of $K_i$ determined by fitting the data for the magnitude of the rapid fluorescence decrease ($F_0 - F$) was $(18.9 \pm 0.5) \times 10^{-9} \text{M}$, and the $k_5$ value determined from the data derived from the slow decrease in fluorescence was $(8.3 \pm 0.5) \times 10^{-4} \text{s}^{-1}$ for pepsin. These rate constants are in good agreement with that obtained from the kinetic analysis of pepsin; therefore, the initial rapid fluorescence decrease can be correlated to the formation of the reversible complex AP-ATBI, while the slow, time dependent decrease reflected the accumulation of the tight-bound slow dissociating complex AP-ATBI* (Dash et al., 2001).

The localized conformational changes induced in the HIV-1 PR due to the interaction with ATBI were investigated by fluorescence spectroscopic studies. The sequence data indicated the presence of four Trp residues A-6, A-42, B-6, and B-42, two on each monomer of the HIV-1 PR (19). The visualization and accessibility calculations of these Trp residues revealed that they are present on the surface of the enzyme and thus are excellent probes to monitor the changes in the tertiary structure due to ligand binding. Therefore, the conformational changes induced in the HIV-1 PR upon binding of ATBI were monitored by exploiting the intrinsic fluorescence by excitation of the $\pi-\pi^*$ transition in the Trp residues. The fluorescence emission spectra of the HIV-1 PR exhibited an emission maxima ($\lambda_{\text{max}}$) at 342 nm as a result of the radiative decay of the $\pi-\pi^*$ transition from the Trp residues, confirming the hydrophilic nature of the Trp environment. The titration of the native enzyme with increasing concentrations of ATBI resulted in a concentration dependent quenching of the tryptophanyl fluorescence. However, the $\lambda_{\text{max}}$ of the fluorescence profile indicated no blue or red shift, revealing that the ligand binding caused reduction in the intrinsic protein fluorescence. A progressive quenching in the fluorescence of the HIV-1 PR at 342 nm was observed concomitant to the binding of substrate (Lys-Ala-Arg-Val-Nle-nitro-Phe-Glu-Ala-Nle-amide). Further, to throw light upon the mechanism of inactivation of the HIV-1 PR by ATBI, the interaction of two representative competitive inhibitors, N-acetyl-Leu-Val-Phe-Al (where Al is aldehyde) (Sarubbi et al., 1993) and pepstatin were analyzed by steady-state intrinsic fluorescence measurements. The binding of the competitive inhibitors led to the
decrease in the quantum yield of the tryptophanyl fluorescence as indicated by the quenching of the emission spectra of the HIV-1 PR. The comparative analysis of the intensity changes in the fluorescence spectra of the HIV-1 PR upon binding of the substrate or the known active site-based inhibitors was found to be similar to that of ATBI, suggesting that ATBI binds in the active site of the enzyme (Dash et al., 2001). Figure 2 represents the proposed mechanism of HIV-1 PR inhibition by ATBI.

**Chemical modification of ATBI and assessment of its antiproteolytic activity**

The functional groups involved in the inhibitory activity of ATBI were elucidated by employing chemical modifiers with specific reactivity. The amino acid sequence of ATBI revealed the presence of Lys, Asp, and Glu residues with ionizable side chains. The involvement of these groups in the mechanistic pathway was investigated using WRK (Woodward’s reagent K), a carboxyl group modifier, and TNBS (2, 4, 6-trinitrobenzenesulphonic acid), an amine group modifier of lysine. Semi logarithmic plots of residual inhibitory activity against the aspartic proteases as a function of time were linear. The modification of the carboxyl groups of ATBI by WRK was monitored by the differential absorption at 210/340 nm. Analysis of the order of reaction for pepsin and F-prot yielded a slope of 1.67 and 1.64, respectively, and thus suggested the involvement of two carboxyl groups in enzyme inactivation. TNBS caused time- and concentration-dependent loss of the inhibitory activity of ATBI. A reaction order of 0.75 and 0.79 for pepsin and F-prot, respectively, determined from the slope of the double logarithmic plot, indicated the involvement of a single amine group of ATBI in the enzyme inactivation (Dash et al., 2001).

**Inhibition kinetics of API from *Bacillus licheniformis* with pepsin**

During the initial kinetic analysis, the inhibitor showed competitive inhibition against pepsin in vitro. The 1:1 molar ratio of the interaction of inhibitor with pepsin classified it under the “tight-binding inhibitor” group (William and Morrison, 1979; Wolfenden, 1976). Inhibitor was found to inhibit pepsin with an IC\textsubscript{50} value (50% inhibitory concentration) of 4.2nM and inhibition constant K\textsubscript{i}, determined by the different methods was found between 3.83nM to 5.31nM, which are almost equal to the IC\textsubscript{50} value of the inhibitor. The Lineweaver–Burk reciprocal plot shows that inhibitor was a competitive inhibitor of pepsin. For the inhibition kinetic studies, the pepsin activity was monitored in the presence of various concentrations of inhibitor and substrate as a function of time. In the region of K\textsubscript{i}, both k\textsubscript{4}I and k\textsubscript{5} values would be low. These low rates of association and dissociation would lead to slow-binding inhibition. Alternatively, binding may also involve two steps where there is a rapid formation of an initial collisional complex, E\textsubscript{I} that subsequently undergoes slow isomerization to form the final tight complex E\textsubscript{I*}. The nature of these changes has been discussed (Lenarcic and Turk, 1999; Jencks, 1975). The extent of E\textsubscript{I*} formation depends on the affinity of the E\textsubscript{I} complex and the relative rates of formation of E\textsubscript{I*} and its relaxation to E\textsubscript{I}. Slow binding inhibitor can also arise due to an initial slow interconversion of the enzyme E into another form E\textsuperscript{*} which binds the inhibitor by a fast step. As found in most of the ground-state inhibitors, formation of the
first reversible complex AP–API was too rapid to be measured at steady-state kinetics and was likely to be near diffusion control. The rate of formation of the second enzyme inhibitor complex, AP–API*, was slow and relatively independent of the stability of the AP–API complex or of the ability of the inhibitor to stabilize the AP–API* complex. Thus the major variable for slow-binding inhibition is \( k_7 \), the first-order rate at which AP–API* relaxes to AP–API. An equivalent statement is that the apparent inhibitor constant, \( K_i^* \), depends on the ability of the inhibitor to stabilize the AP–API* complex. It is interesting to comment on the kinetic data of inhibitor in the light of the extensive kinetic analysis of pepstatin, a known tight-binding inhibitor of pepsin. The values of \( K_i \) and \( K_i^* \) for inhibitor were observed higher than those of pepstatin. The slow-binding inhibitors combine at the active site and induce conformational changes that cause the enzyme to clamp down in the inhibitor, resulting in the formation of a stable enzyme–inhibitor complex. The time-dependent inhibition kinetics of pepsin by inhibitor followed a two-step mechanism, which was also reflected in the quenching pattern of the fluorescence (Kumar and Rao., 2006).

**Fluorometric studies of pepsin - API binding**

The kinetic analysis revealed a two-step inhibition mechanism, where the EI complex isomerizes to a tightly bound, slow dissociating EI* complex. This isomerization is a consequence of the conformational changes induced in pepsin due to the binding of API. The tryptophanyl fluorescence of pepsin exhibited an emission maxima (\( \lambda_{\text{max}} \)) at 342nm, as a result of the “radioactive” decay of the \( \pi-\pi^* \) transition from the Trp residues. The binding of inhibitor resulted in a concentration-dependent quenching of the fluorescence with saturation reaching at or above 10nM inhibitor. The absence of blue or red shift in \( \lambda_{\text{max}} \) negated any drastic gross conformational changes in the three-dimension structure of the enzyme due to API binding. Binding of API resulted an exponential decay of the fluorescence intensity as indicated by a sharp decrease in the quantum yield of fluorescence followed by a slower decline to a stable value. Furthermore, titration of inhibitor against pepsin revealed that the magnitude of the initial rapid fluorescence loss \( (F_0-F) \) increased in a saturation-type manner, which corroborated the two-step slow-tight binding inhibition of pepsin by inhibitor. The value of \( K_i \) determined by fitting the data for the magnitude of the rapid fluorescence decrease \( (F_0-F) \) was 4.74±0.19nM. The subtle conformational changes induced during the isomerization of EI to EI* was monitored by analyzing the tryptophanyl fluorescence of the complexes as a function of time. The \( k_6 \) and \( K_i \) values were determined from the data derived from the slow decrease in fluorescence were 2.42±0.2s\(^{-1}\) and 5.31±0.5 respectively. These rate constants are in good agreement with that obtained from the kinetic analysis. Therefore, the initial rapid fluorescence decrease can be correlated to the formation of the reversible complex EI, whereas the slow, time dependent decrease reflected the accumulation of the tight bound slow dissociating complex EI* (Kumar and Rao., 2006)

**Bifunctional aspartic protease inhibitors**

In recent years, considerable efforts have been expended in the design and synthesis of glycosidase inhibitors, not only to understand about the active site structures and
mechanisms of these interesting enzymes but also in generating new therapeutic agents. Specific inhibitors of glycosidases have proved valuable in a number of applications ranging from mechanistic studies to possible therapeutic uses such as control of blood glucose levels, viral infectivity through interference with normal glycosylation of coat proteins, against cancer, bacterial infections, and as insecticides. A number of naturally occurring reversible glycosidase inhibitors such as nojirimycin, castanospermine, swainsonine, and acarbose have been reported. Another class of inhibitors is the synthetic analogues of sugars containing reactive groups such as epoxides, isothiocyanates, and α-halocarbons. There are reports of mechanism-based inhibitors such as conduritol epoxides, the quinone methide-generating glycosides, and the glycosylmethyl triazenes (Dash et al., 2002).

In some cases, especially from cereals, bifunctional protease/amylase inhibitors are reported. These inhibitors have specific action against mammalian and insect amylase and trypsin enzyme. Cereal inhibitors have generally low molecular weight (10,000–50,000). These inhibitors have natural role in the control of endogenous amylase activity or in the defense against pathogen and pests. Amylase/trypsin inhibitors are reported to be anti-nutritional factor and have therapeutic application. Apart from this defense mechanism these bifuncional inhibitors are potentially valuable ‘two-in-one’ affinity ligands for the purification of proteases and amylases (Saxena et al., 2010).

**Xylanase / aspartic protease inhibitor**

Xylanases (1,4-β-D-xylan xylanohydrolase) are glycosidases that catalyze the hydrolytic cleavage of β-1,4-linked polymers of D-xylose. They have raised enormous interest in the past decade in view of their application in clarification of juices and wines, conversion of renewable biomass into liquid fuels and in development of environmentally sound pre-bleaching processes in the paper and pulp industry (Kulkarni et al., 1999). Although extensive studies have been carried out on the industrial applications of xylanases, there is a paucity of reports on their molecular enzymology and clinical implications. Recently, glycosidases have been studied with a clinical perspective of locating enzyme-allergens (Tarvainen and Keskinen, 1991). Some of these enzymes, including xylanases and cellulases have been found to cause occupational and non-occupational allergies, such as respiratory and irritant contact dermatitis. Therefore, from the biomedical point of view, inhibitors of this class of enzymes will have tremendous importance in the near future. In addition, inhibition of cellulolytic and hemicellulolytic enzymes have potential applications to prevent the degradation of wood and cloth by the action of the hydrolytic enzymes present in the gut of termites.

The three-dimensional structures of family 10 xylanases have revealed the extended substrate binding cleft in which the surface residues are linked by an extensive hydrogen bonding network. The clefts form deep grooves consistent with their endo-mode action and comprise a series of subsites, each one capable of binding a xylose moiety. The active site of xylanase contains two essential catalytic groups, one playing the role of acid/base and the other functioning as a nucleophile. These two groups have been identified as carboxyl groups and a covalent intermediate is formed, which undergoes
hydrolysis to afford hemiacetal with net retention of anomeric stereochemistry. The transition states leading to and from the covalent intermediate have substantial oxacarbonium ion character, as indicated by kinetic isotope effects and by the effects of electron-withdrawing substituents on the sugar ring upon reaction rate (Kulkarni et al., 1999). Analysis of active site amino acids that play an important role in substrate binding and in catalysis has been greatly facilitated by solving the crystal structure of family 10 xylanases covalently linked to mechanism-based cellobiosyl and syllobiosyl inhibitors. To gain further insight into the details of the hydrolytic mechanism of glycosidases, specific inhibitors are necessary, which can act as mechanistic and structural probes. A diverse array of extremely potent, basic, nitrogen-containing inhibitors has been developed over the years, and they have been found to be of great utility in the study of the glycosidase mechanism (Stutz, 1999). However, there have been very few reports of naturally occurring inhibitors of xylanases and to our knowledge no reports of peptidic inhibitors of this class of enzyme from extremophilic organisms.

The bifunctional nature of ATBI was established by its potency toward Xyl I, the xylanase purified from the Thermomonospora sp. The steady-state kinetics revealed time-dependent competitive inhibition of Xyl I by ATBI, consistent with two-step inhibition mechanism. The inhibition followed a rapid equilibrium step to form a reversible enzyme-inhibitor complex (EI), which isomerizes to the second enzyme-inhibitor complex (EI*), which dissociated at a very slow rate. The rate constants determined for the isomerization of EI to EI*, and the dissociation of EI* were $13 \pm 1 \times 10^{-6} \text{ s}^{-1}$ and $5 \pm 0.5 \times 10^{-8} \text{ s}^{-1}$, respectively. The $K_i$ value for the formation of EI complex was $2.5 \pm 0.5 \mu\text{M}$, whereas the overall inhibition constant $K_i^*$ was $7 \pm 1 \text{nM}$. The conformational changes induced in Xyl I by ATBI were monitored by fluorescence spectroscopy and the rate constants derived were in agreement with the kinetic data. Thus, the conformational alterations were correlated to the isomerization of EI to EI*. ATBI binds to the active site of the enzyme and disturbs the native interaction between the histidine and lysine, as demonstrated by the abolished isoindole fluorescence of o-phthalaldehyde (OPTA)-labeled Xyl I. The inactivation of Xyl I is due to the disruption of the hydrogen-bonding network between the essential histidine and other residues involved in catalysis. Figure 3 depicts a model for the probable interaction between ATBI or OPTA with Xyl I (Dash et al., 2002).

**Chitinase / aspartic protease inhibitor**

Chitinases (EC 3.2.1.14) catalyze the hydrolytic cleavage of β-1,4-glucosidic linkage of chitin and belong to the family 18 of glycoside hydrolases (Henrissat and Romeu, 1995). These enzymes are reported to play an important role in many diseases like arteriosclerosis (Boot et al., 1999), colorectal cancer (Cintin et al., 1999) and its level is now considered as a diagnostic hallmark in Gaucher disease (Hollak et al., 1994). Chitinase from Aspergillus fumigatus was related with causing pneumonia and other fatal invasive infections in immuno-compromised hosts (Cohen, 1991). Chitinases of *T. vaginalis* involved in pathogenicity are an interesting target for drugs (Loiseau et al., 2002).
Chitinase inhibitors have chemotherapeutic potential against fungi (Hollis et al., 2000), insects (Sakuda et al., 1987) and malaria transmission (Vinetz et al., 2000). Recently, chitinase inhibitors were also suggested to have anti-inflammatory potential against asthma and allergic diseases, including atopic dermatitis and allergic rhinitis (Zhu et al., 2005). Although a few synthetic chitinase inhibitors for family 18 exist, the majority of potent inhibitors are natural products. These vary widely in structure and include cyclic peptides e.g. argifin and argadin (Rao et al., 2005; Andersen et al., 2008); psammaplin A, allosamidin, styloguanidines and xanthine derivatives such as theophylline, caffeine and pentoxifylline (Andersen et al., 2005; Rao et al., 2005). Anderson et al., 2005 and Sunazuka et al., 2009 have also reported linear fragments of argifin (mono-, di-, tri- and tetra peptide) which inhibited chitinase competitively. A phage display library with disulfide-cyclized peptides inhibiting ChiA and ChiB from *Serratia marcescens* in nanomolar range was screened and reported by Petter et al., 2008. The detailed knowledge of the binding modes of these molecules with their biological targets is now being studied and provides a framework within which inhibitor efficiency and selectivity may be optimized in a rational manner. Inhibition of *Plasmodium falciparum* midgut chitinase by allosamidin results in complete disruption of the oocyst development (Shahabuddin et al., 1993). Inhibitors like allosamizoline (Shohei et al., 1996), argifin (Omura et al., 2000) and argadin (Arai et al., 2000) show a good chitinase inhibitory activity that may be useful probes for the histological study and application as an optical biosensor system (Sakuda et al., 1998). Chitinase inhibitors like psammaplinA, diketopiperazine (Izumida et al., 1996) and styloguanidines (Houston et al., 2002) display anti-fungal activity. C2-Dicaffeine shows low micro molar affinity for a model chitinase and has desirable drug-like properties (Schuettelkopf et al., 2006).

A bifunctional low molecular weight, linear, peptidic inhibitor API, from thermo-tolerant *Bacillus licheniformis* is reported to exhibit a slow tight binding inhibition against ChiA. The bifunctionality of the inhibitor can be defined as it was previously reported to inhibit an aspartic protease, Pepsin (Kumar and Rao, 2006). The ChiA–API kinetic interactions reveal noncompetitive, irreversible and tight binding nature of API with $I_{50}=600 \text{ nM}$ and $K_i=510 \text{ nM}$. The inhibition progress curves show a two-step slow tight binding inhibition mechanism with the rate constant $k_5=8.7\pm1\times10^{-3} \text{ s}^{-1}$ and $k_6=7.3\pm0.6\times10^{-5} \text{ s}^{-1}$. CD-spectra and tryptophanyl fluorescence analysis of ChiA incubated with increasing API concentrations confirms conformational changes in enzyme structure which may be due to irreversible denaturation of enzyme upon binding of API. Chemical modifications by WRK abolished the anti-chitinase activity of API and revealed the involvement of carboxyl groups in the enzyme inactivation. Abolished isoindole fluorescence of OPTA-labeled ChiA demonstrates the irreversible denaturation of ChiA upon incubation with API for prolonged time and distortion of active site of the enzyme (Kumar and Rao, 2010). The mechanism of peptide binding to ChiA is illustrated in Figure 4.

**Inhibitor design and future prospects**

The family of aspartic proteases, although rather small, contains a number of validated and potential drug targets making drug discovery efforts in this area very fruitful and exciting. There have been substantive advances in our understanding of the use of
protease inhibitors as therapeutic agents. Several synthetic protease inhibitors have been approved by the FDA for therapy of HIV and hypertension. These drugs represent prime examples of structure based drug design. Moreover, the inhibitory principles and compounds, which have been established and discovered, now enable mechanism-based drug discovery across the whole family. The sequencing of the human genome and the resulting knowledge on all human aspartic proteases allows an exhaustive profiling of inhibitors for specificity towards all family members thereby reducing the risk of unwanted side effects.

A number of natural and peptidomimetic inhibitors performed well in different phases of clinical testing to treat other human disorders, including cancer, inflammation, cardiovascular, neurodegenerative, and various infectious diseases. Despite this impressive progress, there is much to learn about the cross talk between signal transduction pathways and protease activation cascades. Additionally, development of successful protease inhibitors for clinical use is reliant on maximizing bioavailability, specificity, and potency of inhibition of the target enzyme. Ideally, localizing protease inhibitors to a single target area of the body may also help minimize the potential for complications and detrimental side effects. There is the further issue of the development of drug resistance to protease inhibitors in the face of a build up of substrate pressure, and selection of catalytically active mutant or other salvage proteases that do not have complementarity for carefully designed inhibitors of wild type proteases. The future appears to still hold considerable promise for protease inhibitors. We can anticipate new, overexpressed proteases from genomic/biochemical comparisons made between normal/diseased cells, host/ pathogen, healthy/unhealthy subjects leading to more effective and efficient validation of proteases as drug targets. New advances in protein chemistry will lead to faster production and greater quantities of pure recombinant proteases and advances in structural biology (crystallography, NMR spectroscopy) will produce faster and more accurate inhibitor-protease structures. 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 proteases.

Discovery of novel selective inhibitors can proceed only through combination of screening of chemical libraries, rational design, computational technology, and exploration of natural compounds. The exploitation of vast microbial diversity will also generate large amount of biologic aspartic protease inhibitors. Furthermore, future research into the synergistic capabilities of inhibitors will help elucidate the most effective combination therapies. These advances, together with more careful attention to inhibitor conformation, mechanism of action, and drug-like composition are expected to result in more potent, more selective, more bioavailable inhibitors with a higher probability of success in the clinic.
References

Scheme 1 E stands for the free enzyme, I is the free inhibitor, EI is a rapidly forming pre-equilibrium complex, and EI* is the final enzyme–inhibitor complex. Binding between the enzyme and inhibitor may either involve a single step, having slow association and dissociation rates (a), or have an initial fast binding step, followed by a slow reversible transformation of EI to another entity, EI* (b), or have an initial slow interconversion of the enzyme E into another form, E*, which binds to the inhibitor by a fast step (c). $k_{cf}$ and $k_{cf}^{-1}$ stand for the rate constants for forward and backward reaction, respectively, for the conversion of the enzyme.
Figure 2 Schematic representation of the proposed mechanism of inhibition of the HIV-1 PR by ATBI. Secondary structure of the HIV-1 PR is shown by the stereoview ribbon diagram. The Trp residues A-42 and B-42 are adjacent to the flap region, whereas the Trp residues A-6 and B-6 are far from the flap region. The HIV-1 PR and other retroviral proteases have the structural feature called the “flap region” (shown above the arrows), which is important for the substrate binding and catalysis. The binding of the inhibitor (as indicated by the solid block) in the active site induces inward movement of the flaps (as indicated by the arrows). The noncompetitive nature of ATBI, along with its multiple nonbonded interactions with the flaps, is responsible for the loss of the dynamic flexibility of the flaps, resulting in the inactivation of the HIV-1 PR. The structure of the HIV-1 PR is as described in PDB ID.1AID. (Dash et al., 2001).
Figure 3 Schematic representation of the stereo view model depicting the probable mechanism of OPTA and ATBI binding to the active site of the Xyl I. The active site of the Xyl I has been modeled based on the x-ray crystallographic structure of a similar thermostable family 10 xylanase from *Thermoaosanus aurantiacus* (22) (PDB ID 1TUX) using the software uanta/SYBYL, MSI. The active site of Xyl I includes the essential Glu, His, and Lys residues. A, the chemoaffinity label OPTA (shown in purple color) contains two aldehyde groups, one of which binds to the primary amine of the Lys and the other group reacts with the secondary amine of the imidazole ring of His of Xyl I resulting in the release of two water molecule (not shown). These chemical reactions result in the formation a fluorescent isoindole derivative. B, preincubation of ATBI with Xyl I resulted in the binding of the inhibitor in the active site of the enzyme. Based on our results, we propose that the Asp residues in the ATBI (shown in purple color) form hydrogen bonds (solid dashed lines) with the free amine group of the Lys and the secondary amine of the His of Xyl I. The other charged residues (not shown) can form many non-bonded interactions with the active site residues of Xyl I. These interactions in conjunction with the tight binding nature of ATBI probably prevent the binding of OPTA to the His and Lys residues, thus an isoindole derivative failed to be formed with the ATBI-preincubated Xyl I. (Dash et al., 2002).
Figure 4 Hypothetical schematic representation of the model depicting the probable mechanism of API binding to ChiA. The active site and proximal residues of the ChiA have been modeled based on the x-ray crystallographic structure of ChiA from Serratia marcescens (PDB code 1EDQ) using the software Hyperchem 7.5 (evaluation version). The active site of ChiA includes the essential Asp313, Glu315, Tyr163-390 and Trp539 residues. (A) The chemoaffinity label OPTA (shown in red color) contains two aldehyde groups, one of which binds to the primary amine of the Lys 142, 267, 37 residues and the other group reacts with the secondary amine of the imidazole ring of His 263, 186, 137 residues of ChiA resulting in the release of water molecules (not shown). These chemical reactions result in the formation a fluorescent isoindole derivative. (B) Pre-incubation of ChiA with API resulted in the binding of free -COOH group of Asp-1 residue of API form covalent bond with the amine group of the Lys-142, 267, 37 residues. The free NH2 group of Aps-1 of API form covalent bond with secondary amine of the imidazole ring of His 263, 186, 137 residues of ChiA (dashed lines). These interactions in conjunction with the tight binding nature of API probably prevent the binding of OPTA to the Lys and His residues, thus an isoindole derivative failed to be formed with the ChiA pre-incubated with API. (Kumar and Rao., 2010).