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Review

Design of Potent Aspartic Protease Inhibitors to Treat Various Diseases

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In this retrospective, personal review covering our research from the late 1980s until 2007, we outline nearly two-decade worth of our own work on several aspartic protease inhibitors including those affecting renin, HIV-1 protease, plasmepsins, β-secretase, and HTLV-I protease and we report on aspartic protease inhibitors as potential drugs to treat hypertension, AIDS, malaria, Alzheimer's disease and adult T-cell leukemia, HTLV-I associated myelopathy / tropical spastic paraparesis, and various, respectively, associated diseases. Herein, we describe our methods for rational substrate-based drug design of peptidomimetics that potently inhibit the activity of renin, HIV-1 protease, plasmepsins, β-secretase, and HTLV-I protease accordingly, using an appropriately selected inhibitory residue that contained a hydroxymethylcarbonyl isostere. Although this non-hydrolyzable isostere mimics the transition state that is formed during protein cleavage of a substrate, the isostere-containing inhibitor is not cleaved. We highlight our optimization studies in which we used various techniques and tools such as truncation studies, natural and non-natural amino acid substitution studies, various moieties to promote chemical and pharmacological stability, X-ray crystallography, computer-assisted docking and dynamic simulations, quantitative structure-activity relationship studies, and various other methods that this review can barely mention.

Keywords: Beta-secretase inhibitor / HIV Protease inhibitor / HTLV Protease inhibitor / Plasmepsin inhibitor / Renin inhibitor

Received: December 19, 2007; accepted: February 26, 2008

DOI 10.1002/ardp.200700267

Introduction

For decades, peptides have become an increasingly important class of molecules in biochemistry, physiology, and medicinal chemistry, to name a few. People hold special interests in peptides because many peptides possess potent pharmacological properties. Peptides are complex molecules in which amino acids are covalently linked together by amide bonds, and each peptide sequence is unique with regard to its chemical and physical properties. Many peptide sequences, termed substrates, are specifically recognized by proteases and digested. Our work deals with the aspartic protease class of enzymes, in which two catalytic aspartic acid residues of the protease cleave a substrate. For nearly two decades, we have focused on synthesizing aspartic protease inhibitors based on the sequence of the substrate. Hence, our peptidic inhibitors are designed to compete for the interactions of substrate and protease.

The main design of our inhibitors is based on the mimicry of a transition state formed during amide hydrolysis by an aspartic protease (Fig. 1). In general, the most widely accepted mechanism is an acid-based system involving two active aspartic acid residues in the active site and a water molecule that resides between them. These two aspartic acid residues act as a proton donor and acceptor, respectively, to catalyze the hydrolysis of

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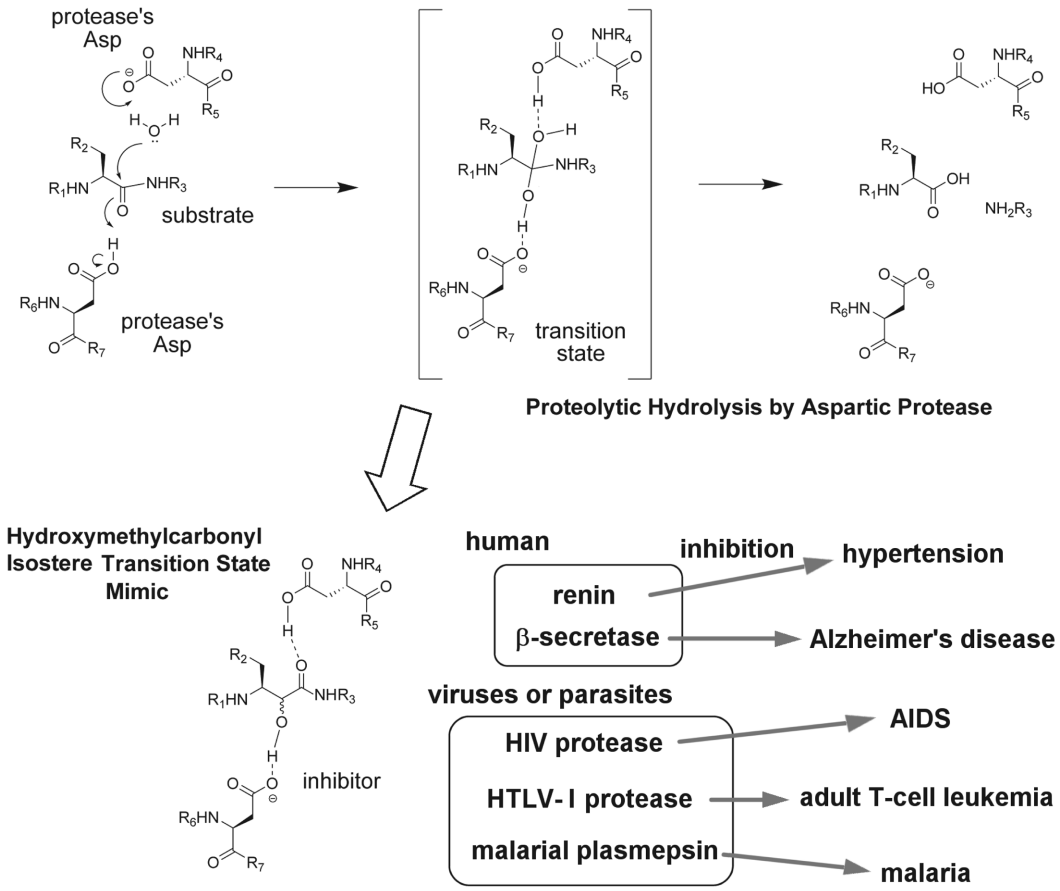

Figure 1. Design of the aspartic protease inhibitors possessing a hydroxymethylcarbonyl isostere as a substrate transition state mimic, and the overview of our strategy for developing anti-incurable disease drugs using these inhibitors.

peptide bonds in substrates. The water molecule is partly activated by an aspartate and makes a nucleophilic attack at a specific carbonyl carbon in the substrate. The carbonyl oxygen, in turn, captures a proton from another aspartic acid in the active site, resulting in a tetrahedral intermediate. This intermediate is the crucial transition state. Re-stabilizing from the transition state, the amino moiety from the substrate becomes a better leaving group, and the substrate is cleaved into two peptide fragments. In designing aspartic protease inhibitors, we envisioned the synthesis of a peptidic substrate analogue that contains a non-hydrolyzable transition state isostere in place of the normal hydrolyzable P1–P1′ amide bond. When a hydroxymethylcarbonyl isostere, a functional group that can mimic the tetrahedral transition state, formed during amide bond hydrolysis but cannot be hydrolyzed itself by the protease, is introduced in a substrate inhibitor, the protease's biochemical actions are suspended. The critical hydroxyl group, as a transition state mimic, forms hydrogen bond interactions with the carboxylic acid moieties of the two catalytic aspartic acid residues in the protease. The carbonyl oxygen in the inhibitor is also involved with one of the aspartic acids to form a hydrogen bond network. Several X-ray diffraction crystallographic data [1–5], NMR data [6], and docking simulation studies [7–8] of our inhibitors in complex with their respective proteases, *i.e.* HIV-1 protease and plasmepsin IV [9], provide evidence for the optimized interactions between our inhibitors and their protease's active sites. Especially, for the first time, our NMR studies of an HIV-1 protease-inhibitor complex revealed a catalytic mechanism of aspartic protease involving such hydrogen network [6].

On the design of substrate-based peptidic inhibitors, we often concern ourselves with reducing the size of the inhibitor and introducing special functional groups so as to improve dissolution, cellular penetration, membrane permeability, and intestinal absorption. We also focus on

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replacing optimized natural amino acid residues with structurally similar non-natural amino acid residues, thereby reducing the peptidic nature of the inhibitor, so as to avoid premature digestion of the inhibitor by other proteases. Three-dimensional data derived from X-ray diffraction crystallography and NMR spectroscopy serve as the foundations of our computer-assisted docking and dynamic studies. Most importantly of all, biochemical activity data obtained from enzyme assays and pharmacological results guide our structure-activity relationship studies to further design more potent and pharmacologically favoured inhibitors.

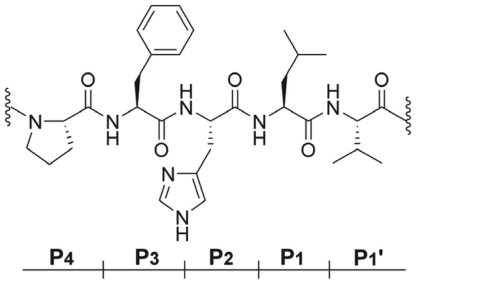
In the following sections, we hope to comprehensively present our rational substrate-based drug design method for elucidating the pharmacophore and developing inhibitors against renin, HIV-1 protease, plasmepsins, β-secretase, and HTLV-I protease.

Results and discussion

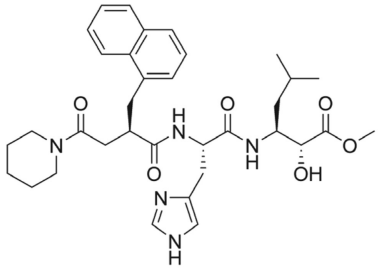
Renin inhibitors

The renin-angiotensin-aldosterone system is a complex system that regulates blood pressure and electrolyte balance. The system involves many organs and has an assortment of feedback mechanisms. Renin is a highly specific aspartic protease that selectively cleaves angiotensinogen **1** to generate a decapeptide, angiotensin I, which is subsequently cleaved by a non-specific dipeptidyl carboxypeptidase, angiotensin-converting enzyme, to form a potent octapeptidic vasopressor, angiotensin II. Many antihypertensive agents, which are involved in the renin-angiotensin-aldosterone system, inhibit the angiotensin-converting enzyme or antagonize the angiotensin II receptor.

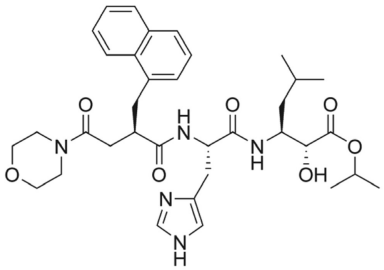
Our main purpose for this project was to design and synthesize potent and orally bioactive renin inhibitors. A portion of the sequence for angiotensinogen **1** is Pro-Phe-His-Leu-Val, within which the scissile bond is between P1 Leu and P1' Val (Fig. 2). At first, we designed and synthesized renin inhibitor **2** that looked fairly similar to the P4-P1 angiotensinogen segment **1** in which the P4 Pro residue was enlarged to a piperidinyl moiety to better occupy the S4 renin binding site [7]. A succinic acid residue, having a P3-P4 retro-inverso amide bond, was introduced to reduce the likelihood of premature degradation by other proteases. The P3 Phe side-chain was replaced by a 1-methylnaphthyl moiety to avoid recognition and proteolytic degradation by chymotrypsin, especially at the P3-P2 Phe-His amide bond, and, at the same time, also to promote a tighter fit in the S3 subsite. The P2 His residue was kept as in angiontensinogen **1** to maintain hydrogen bond interactions with the protease's Ser233, while the P1 residue was simply capped with a methyl ester. As for the P1 inhibitory residue, we selected allonorstatine, (2*S*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acid, and norstatine, (2*R*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acid, as hydroxymethylcarbonyl containing residues



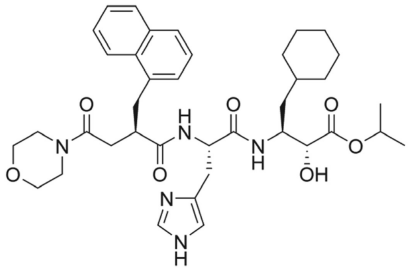
1, a segment of angiotensinogen



2, IC_{50} = 41 nM



3, IC_{50} = 6.7 nM



4, IC_{50} = 2.4 nM

Figure 2. A segment of angiotensinogen **1** and peptidic renin inhibitors **2**–**4**. The IC_{50} values represent competitive inhibition against angiotensinogen in human renin.

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because of their structural similarity to the P1 Leu in angiontensinogen **1**. The difference between allonorstatine and norstatine is the chirality of the carbon next to the hydroxyl group. Comparatively, a norstatine moiety, as the critical P1 hydroxymethylcarbonyl residue, provided an inhibitor **2** that was considerably more potent than one containing an allonorstatine residue.

The P4 residue was then further optimized to a morpholinyl moiety and the P1-cap moiety was replaced by an isopropyl ester to resemble angiontensinogen's P1' Val residue **1**, resulting in a more potent renin inhibitor **3** [10].

Interestingly, when we modified the P1 norstatine to a cyclohexylnorstatine moiety, (2*R*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid, thereby becoming more structurally different from angiotensinogen's P1 Leu **1** residue, we observed an increased inhibitory potency against renin in our most potent compound **4** (KRI-1314) [11]. However, a P1 phenylnorstatine moiety, (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid, would result in a reduction in inhibitory potency.

In the end, we developed the low molecular weight compound **4** that exhibited highly selective renin inhibitory activity *in vitro* while being stable against monkey-liver homogenates, human plasma, and chymotrypsin [11]. Oral administration of 10 mg/kg to salt-depleted monkeys resulted in a fall of 10–20 mmHg of mean blood pressure and reduced plasma-renin activity for a 5-hour period. In other words, renin inhibitor **4** was an orally bioavailable, effective, and long-lasting antihypertensive drug.

HIV protease inhibitors

Acquired immunodeficiency syndrome (AIDS) is a collection of symptoms and infections caused by the human immunodeficiency virus (HIV). In the later stage of the disease, patients are susceptible to opportunistic infections and cancers, and eventually succumb to death. As a part of the virus' cell cycle, HIV protease cleaves precursor polyproteins into polyproteins [12–14]. These polyproteins are then further processed, assembled, and developed into a mature virion leading to the pathogenesis of AIDS. Inhibition of the virus-specific aspartic protease would result in the production of immature and non-infectious virions [15–18].

A feature, which is a basis for rational design of selective inhibition against non-human proteases, is, that proteases of HIV type 1 (HIV-1) and other retroviruses recognize the Xaa-Pro sequence as the cleavage site, whereas mammalian aspartic proteases do not [19]. The p17/p24 (also known as MA/CA, matrix / capsid, **5**) and TF/PR, transframe / protease **6**, cleavage site regions of HIV-1 polyproteins share a similar P1-P1′ Xaa-Pro sequence (Fig. 3). In consideration that sequence **11**'s P3 Gln and P1 Tyr residues did not seem to have any apparent benefit on binding to HIV-1 protease, we designed an inhibitor where the P4-P1 residues would have a similar sequence as that of sequence **6**, while the P1′-P3′ residues would be identical to sequence **5**. For the critical P1 hydroxymethylcarbonyl isostere residue, an allophenylnorstatine moiety provided more potent inhibition than a phenylnorstatine moiety as seen in compound **7** [20]. Similarly, a compound with a P1 allocyclohexylnorstatine residue, (2*S*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid, was more potent than one containing a cyclohexylnorstatine residue, although the corresponding allophenylnorstatine and phenylnorstatine residues provided more potent inhibitory activity against HIV-1 protease [21].

In order to obtain smaller inhibitors, the P4-P3 and P2′-P3′ residues were excluded from the scaffold. A benzyloxycarbonyl protection group was optimized as a P2-cap isostere of the former P3 Phe residue, while a P1′-cap *tert*-butylamine was optimized to replace the bulky P2′ Ile residue [21, 22]. The resulting tripeptidic inhibitor **8** was a bit less potent than the former heptapeptidic inhibitor **7**.

To increase fit in the large hydrophobic S3 subsite, the P2-cap moiety was optimized to a 1-napthyloxyacetyl moiety [22]. The P2 natural amino acid, Asn, was replaced by a non-natural amino acid L-methanesulfonylalanine. Indeed, the designed tripeptidic inhibitor **9** exhibited similar HIV-1 protease inhibitory activity as heptapeptidic inhibitor **7**.

Considering the subtle balance of lipophilicity-hydrophilicity and molecular size with regard to penetration across the cell membrane and non-specific adsorption in blood, the P2-cap moiety was replaced by a 5-isoquinolinyloxyacetyl moiety (cf. P3 residue of orally bioavailable renin inhibitor **4**) and the P1′ Pro moiety was exchanged for a non-natural amino acid residue L-thiazolidine-4-carboxylic acid [22]. The ensuing compound, kynostatin-272 (also known as KNI-272, **10**), exhibited equipotency to compound **9**. Kynostatin-227 (also known as KNI-227, **11**) with a P1′ L-5,5-dimethylthiazolidine-4-carboxylic acid moiety seemed to exhibit a slight improvement in inhibitory activity against HIV-1 protease, possibly due to the conformational constraint and hydrophobicity of the bulky dimethyl group. Both inhibitors KNI-272 and KNI-227 were superpotent and highly selective HIV-1 protease inhibitors (IC_{50}, K_i) with excellent antiviral activity in cells (EC_{50}) [22, 23]. Particularly, KNI-272 also had high antiviral activity against a wide spectrum of HIV strains and a low cytotoxicity profile against non-infected cells (LC_{50} > 20 μM). The crystal structure of HIV-1 protease-inhibitor (KNI-272) complex (Protein Data Bank ID:

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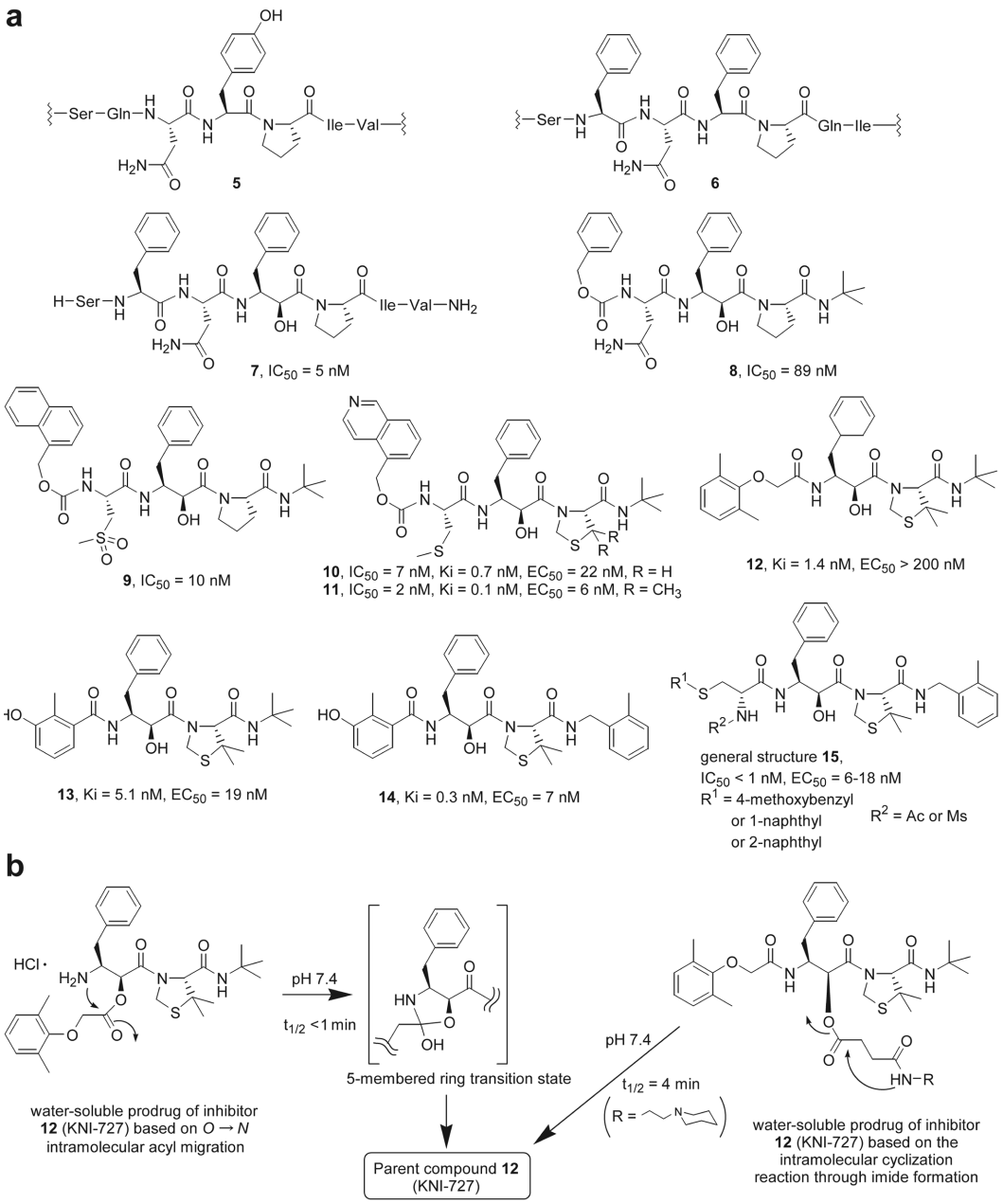

Figure 3. a) Cleaving regions of HIV-1's p17/p24 **5** and TF/PR **6** sequences, and peptidic HIV protease inhibitors **7**–**15**. The IC_{50} and K_i values represent HIV protease inhibitory activity in non-cellular assays, while the EC_{50} values represent HIV antiviral activity in cellular assays. b) The examples of two novel classes of water-soluble prodrugs derived from HIV-1 protease inhibitors. These prodrugs can be converted non-enzymatically and rapidly to the corresponding parent drugs under physiological conditions.

1HPX), and a view of the hydrogen network between KNI-272 and the two catalytic aspartic residues of enzyme, which was revealed by our NMR studies [6], are depicted in Fig. 4. Although KNI-272 showed good oral bioavailability in a human clinical trial, the plasma half-life was very short ($t_{1/2\beta}$ = 23 min, intravenous), which suggested that the inhibitor must be further optimized for a more desirable pharmacokinetic profile [24].

An overlay of solution, crystalline, and complex structure of KNI-272 in computer-assisted experiments revealed that except for the P2-cap moiety, all coordinates were similar [25]. This observation suggested that the P2-P2′ residues form the pharmacophore of the inhibitor. As a result, we opted to discard the P2-cap moiety and altered the design of the P2 residue to a P1-cap moiety [24]. According to an extensive literature search, a

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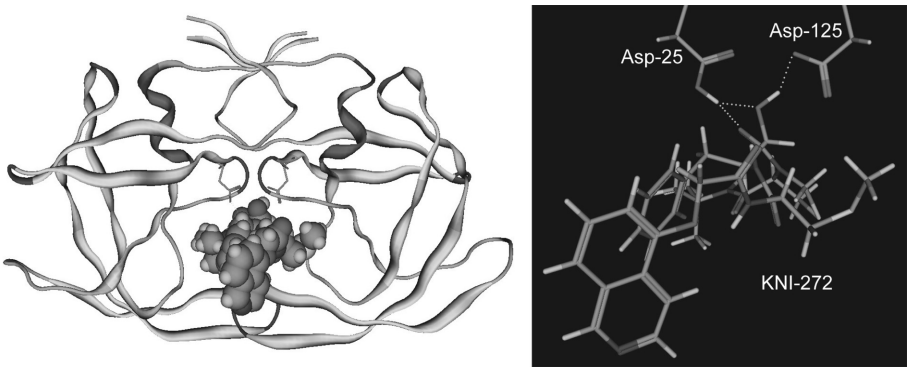

Figure 4. HIV protease-inhibitor **10** (KNI-272) complex and the interaction between KNI-272 and protease's active site.

variety of ligands optimized for binding to the S2 subsite of HIV protease had been reported for hydroxyethylamine inhibitors, namely 2,6-dimethylphenoxyacetyl and 3-hydroxy-2-methylbenzoyl groups. For example, a 3-hydroxy-2-methylbenzoyl group is present in clinically-used drug nelfinavir. Consequently, we synthesized the potent HIV protease inhibitors JE-1482 (also known as KNI-727, **12**) and JE-533 (also known as KNI-577, **13**), respectively. However, although KNI-727 exhibited very high HIV-1 protease inhibitory activity, its cellular antiviral activity was less than desirable. On the other hand, KNI-577 exhibited potent cellular antiviral activity. KNI-577 was further optimized at the P2′ moiety to take in consideration the symmetrical nature of the homodimeric protease, *i.e*. the aromatic P1-cap moiety would reside in the S2 subsite while the aromatic P1′-cap moiety would be accommodated by the S2′ pocket. The resulting dipeptidic inhibitor **14** (also known as KNI-764, JE-2147, SM-319777, or AG-1776) was a highly potent inhibitor of HIV-1 protease that exerted potent cellular antiviral activity with a relatively longer plasma half-life ($t_{1/2\beta}$ = 93 min) than tripeptidic inhibitor **10** (KNI-272) ($t_{1/2\beta}$ = 23 min). Moreover, KNI-764 had moderate oral bioavailability and duodenal absorption as well as a low cytotoxicity profile (LC_{50} > 20 μM). Additionally, KNI-764 was also effective against all HIV-1 and HIV-2 strains along with clinical HIV-1 resistant variants due to its flexibility and adaptability to the mutated HIV proteases [2, 26, 27]. Several attempts at designing pseudo-symmetric hydroxymethylcarbonyl-hydrazide HIV-1 protease inhibitors could not best KNI-764's cellular antiviral activity [28].

According to the literature, D-amino acid residues had been used as alternatives for the P1-cap moiety in the design of HIV-1 protease inhibitors. While maintaining the P1 to P1′-cap moieties as in KNI-764, we designed and synthesized inhibitors with a P2 D-amino acid residue so as to offer more design possibilities. Surprisingly, we discovered that superpotent HIV-1 protease inhibition and high cellular antiviral activity could be maintained in our general tripeptidic inhibitors **15**, despite some significant structural changes to certain moieties, R^1 and R^2 [29]. In particular, the IC_{50} value for KNI-1966 (R^1 = 1-naphthyl, R^2 = mesyl in general structures **15**, shown in Fig. 3) is 6.4 nM. However, changing the sulfide atom to a sulfoxide group reduced cellular antiviral activity. This group of inhibitors **15**, possessed favourable selective cytotoxicity profiles and admirable cellular antiviral activity against different HIV strains, including strains that were resistant against one or both clinically used drugs nelfinavir and ritonavir. Our current research deals with the further development of general inhibitors **15**.

Low water-solubility of anti-AIDS drugs is a serious problem [30–33] causing undesirable pharmaceutical properties such as erratic oral absorption and poor oral bioavailability. To overcome this low water-solubility setback, one effective strategy is to convert the water-insoluble parent drugs into hydrophilic prodrugs by covalently attaching appropriate solubilizing moieties such as phosphates [34, 35], sugars [36, 37], and amino acids [38, 39] that can enzymatically release the parent drugs. Phosphate-type water-soluble prodrugs of HIV-1 PR inhibitors were reported by Thaisrivongs and co-workers [32]. In order to develop practical anti-AIDS drugs, we developed two novel classes of water-soluble prodrugs derived from HIV-1 protease inhibitors that could be converted non-enzymatically to the corresponding parent drugs under physiological conditions. The prodrugs based on $O \rightarrow N$ intramolecular acyl migration, which possessed an *O*-acyl-β-hydroxy-α-amino acid structure, could spontaneously release these parent drugs via a 5-membered ring transition-state [40–45]. The concept of this prodrug's class was applied to other fields, *e.g*., paclitaxel prodrugs [45–50], novel synthetic strategies for the peptides containing difficult sequences [51–53], and "click peptides"

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as useful tools for molecular-pathological studies of Alzheimer's disease using chemical biology technology [54–56]. Another class of our prodrugs possessing self-cleavable linkers, the succinyl group, could be converted to the corresponding parent drugs via a spontaneous imide formation by intramolecular nucleophilic reaction [57, 58]. The concept of the latter prodrug's class was also applied to the double-drugs that could release two different-type anti-AIDS drugs [59].

Plasmepsin inhibitors

Malaria is a disease caused by a plasmodium parasite that feeds on the hemoglobin of an infected victim. The parasite produces a family of proteases, called plasmepsins, that degrades the host's hemoglobin, consequently leading to the symptoms of malaria and death of the host. Inhibition of plasmepsins would essentially starve the parasite. Four plasmepsins, plasmepsin I, II, and IV, as well as histo-aspartyl protease, have been implicated with hemoglobin degradation. Although plasmepsin I, II, and IV are classical aspartic proteases, histo-aspartyl protease, as the name suggests, involves an aspartic acid and a histidine. Among the four proteases, plasmepsin I and II are known to readily cleave hemoglobin between Phe33 and Leu34 of the a-globin subunit. Between these two plasmepsins, the structure and active site specificity of plasmepsin II is better known.

Because phenylnorstatine and allophenylnorstatine are structurally similar to Phe, several potent HIV-1 protease inhibitors including compounds **10** and **12**–**14** (KNI-272, -727, -577, and -764, respectively) were tested, out of curiosity, against plasmepsin II [60]. Surprisingly, the compounds could inhibit plasmepsin II with Ki values of 1 μM or less. Selectivity over a homologous aspartic protease, cathepsin D, was also determined. Cathepsin D is mainly associated with intracellular catabolism in mammalian lysosomal compartments, and involved in hormone and antigen processing. Inhibitor **12** (KNI-727) exhibited the highest selectivity for plasmepsin II among the tested compounds. At the significant P1 hydroxymethylcarbonyl residue near the catalytic site of the enzyme, an allophenylnorstatine residue is preferred over phenylnorstatine, allonorstatine, and allocyclohexylnorstatine residues when it pertains to inhibitory activity against plasmepsin II [60, 61]. Moreover, at the P1′ position, similar to the cases with HIV-1 protease, the increased bulkiness due to the methyl groups of dimethylthiazolidine evidently contributed to an increased inhibitory activity when compared with a thiazolidine moiety. Among several P1′-cap moieties, 1-amino-2-indanol was best accommodated by the active site of plasmepsin II [61, 62]. The optimization of the P1′-cap moiety

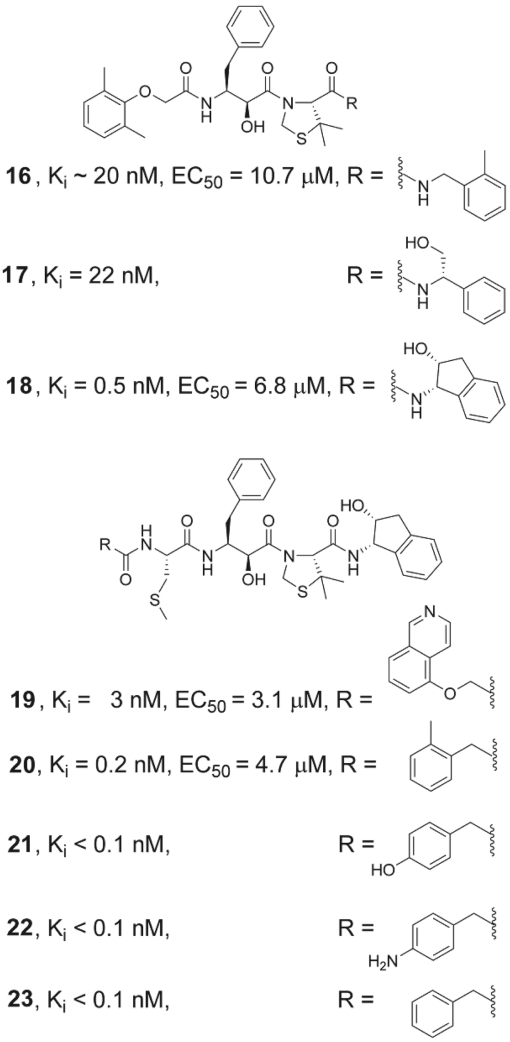

Figure 5. Plasmepsin inhibitors **16**–**23** against malaria. The Ki values represent plasmepsin II protease inhibitory activity, while the EC50 values represent plasmepsin antiparasitic activity in cellular assays.

began with compound **16** in which the P1′-cap moiety was borrowed from HIV-1 protease inhibitor **14** (Fig. 5) [25, 26]. Computer-assisted docking simulations suggested a possible hydrogen bond interaction within the S2′ pocket and an equipotent inhibitor **17** was derived [61, 62]. Restricting the flexibility of the P1′-cap moiety with an (1*S*,2*R*)-1-amino-2-indanol brought us to our most potent plasmepsin II inhibitor **18** (KNI-10006). Indeed, the effects of both the hydrophobic indane structure and its spatially arranged hydroxyl group which allowed for proper hydrogen bond interactions with plasmepsin II greatly contributed to the high potency of inhibitor **18** (KNI-10006).

Simultaneous inhibition of all four plasmepsins would lead to a faster starvation of the parasite. By such action,

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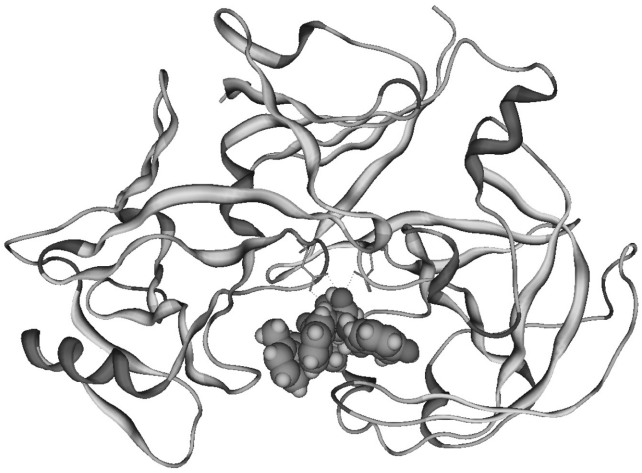

Figure 6. Crystal structure of plasmepsin IV-inhibitor **14** (KNI-764) complex.

the appearance of drug resistant mutants would also be impeded because the parasite would have to mutate several proteins in order to overcome the inhibition. Such an adaptive drug with the ability to inhibit several members of a protein family would be more advantageous than a combination regimen of multiple medications, due to cost-effectiveness, dosage, adverse effects, and compliance. Inhibitors **12** (KNI-727) and **18** (KNI-10006) were both assayed for inhibitory activity against all four plasmepsins [61]. Although KNI-727 was effective against plasmepsin I, II, and IV, the compound exhibited low inhibitory activity against histo-aspartyl protease. Plasmepsin IV is a critical enzyme in the design of plasmepsin inhibitors as antimalarial disease drugs, from the aspect of amino acid sequence homology among the four plasmepsins [4]. Optimization for plasmepsin IV might lead to the inhibition of all plasmepsins. Hence, we revealed the crystal structure of plasmepsin IV (derived from *Plasmodium malariae*) inhibitor **14** (KNI-764) complex as shown in Fig. 6 [4, Protein Data Bank ID: 2ANL]. However, this result showed that the inhibitor bound to the enzyme in an unexpected orientation, *i.e.* the allophenylnorstatine occupied the S1' pocket, and the P2 group was found outside the S2 pocket [4]. On the other hand, KNI-10006 was clearly more potent than KNI-727 and could effectively inhibit all four proteases. Of course, because histo-aspartyl protease is not a true aspartic protease, inhibition to the histo-aspartyl protease by KNI-10006 was less efficient than inhibition against the other three plasmepsins. Moreover, KNI-10006 was selective for plasmepsin II over human pepsin [61]. However, KNI-10006 could also potently inhibit cathepsin D, thereby reducing its selectivity for plasmepsin II [62].

In our design of other plasmepsin inhibitors, we backtracked to tripeptidic HIV-1 protease inhibitor **11** (KNI-227) and derived a P1′-cap aminoindanol analogue **19** that exhibited potent plasmepsin II inhibition with slightly improved cellular antiparasitic activity over KNI-10006 [63]. The P2-cap moiety was elaborated to benzylcarboxylate derivatives **20**–**23**, based on inhibitory potency against plasmepsin II and computer-assisted docking experiments. We observed that within the context of compounds **20**–**23**, substitution to the phenylacetyl group seemed to be detrimental for plasmepsin II inhibition, except when the *para*-position was substituted by a hydrogen-bond donor group such as an amino or hydroxyl moiety. Further modifications are currently being investigated.

β-Secretase inhibitors

Alzheimer's disease is essentially an age-related disease that deprives memory, insight, judgement, abstraction, and language from its elderly victims. Over time, the degenerative disease degrades the nervous and muscular systems, ultimately leading to death of the patient. The etiology of Alzheimer's disease begins with a type I single transmembrane glycoprotein β-amyloid precursor protein (APP), which has multifunctional roles associated with growth, development, and aging of the central nervous system [64, 65]. Alzheimer's disease is a result of a disruption of APP's normal processing. In a non-disease causing pathway, APP is cleaved by α-secretase. In a pathological pathway, however, APP is cleaved at a different position by β-secretase, an aspartic protease, to form β-amyloid [66–69].

According to the amyloid hypothesis [70–74], β-secretase [BACE1: β-site APP (amyloid precursor protein) cleaving enzyme] appears promising as a molecular target for therapeutic intervention in Alzheimer's disease [75–79]. The incidence of developing Alzheimer's disease is higher in patients with a Swedish mutant than wild type APP sequence. Using a substrate of a Swedish mutant **24** in which the P2-P1 Lys-Met residues of the wild type is mutated to Asn-Leu (Fig. 7), we investigated the modification of the P1 Leu residue to an inhibitory hydroxymethylcarbonyl isosteric unit of either norstatine, allophenylnorstatine ((2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid), phenylnorstatine or phenylthionorstatine ((2*R*,3*R*)-3-amino-2-hydroxy-4-(phenylthio)butyric acid) [79–82]. The difference between allophenylnorstatine and phenylnorstatine is the chirality of the carbon next to the hydroxyl group (cf. allonorstatine and norstatine). The P1 phenylnorstatine and phenylthionorstatine moieties were equally favored for β-secretase inhibition. Considering that phenylnorstatine was smaller than phenylthio-

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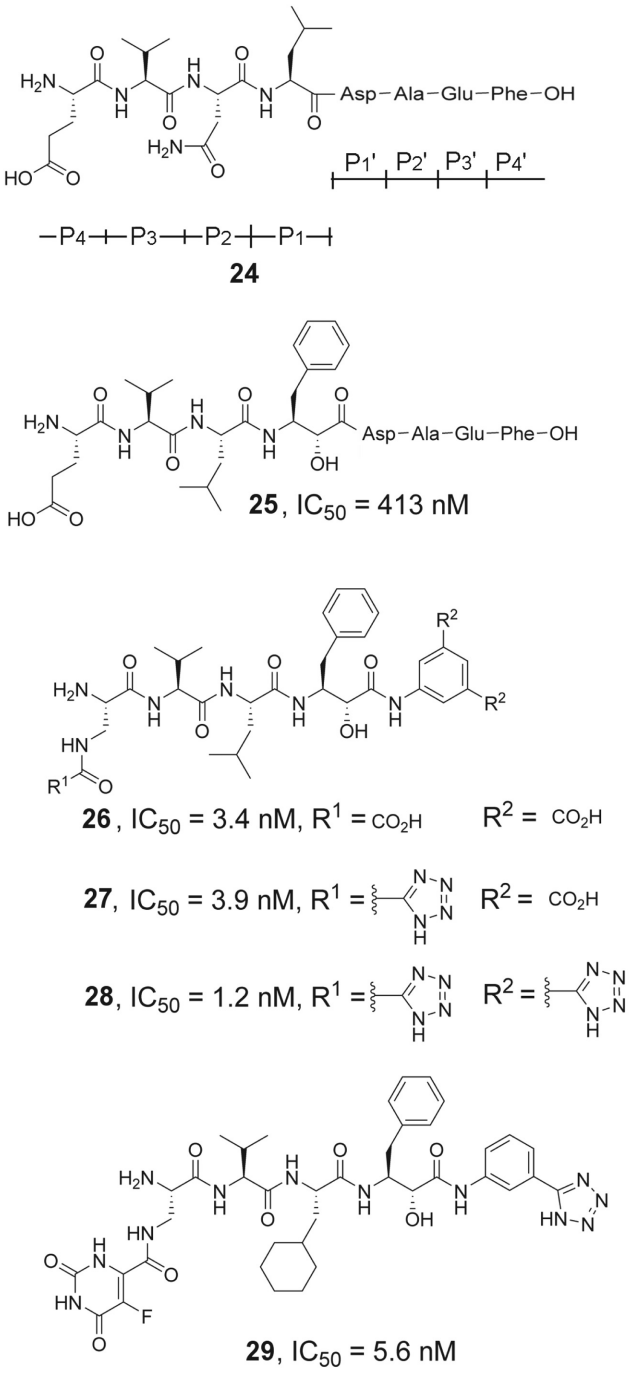

Figure 7. A substrate **24** of β-secretase and peptidic β-secretase inhibitors **25**–**29**. The IC_{50} values represent β-secretase inhibitory activity in non-cellular assays.

norstatine and small-sized compounds would most likely have higher membrane permeability, the ensuing compounds were designed around a P1 phenylnorstatine moiety. Several natural amino acid residues were substituted at the P3, P2, P1′, P2′, and P3′ positions to optimize the inhibitor [79]. The more potent compound **25** (KMI-008) differed from the Swedish substrate by a P2 Leu and P1 phenylnorstatine. KMI-008 exhibited moderate inhibitory activity against recombinant β-secretase and decreased sAPPβ secretion, a soluble form of APP generated by the action of β-secretase, from COS-7 cells co-transfected with both APP and β-secretase.

Several compounds were synthesized by step-wise Gly substitution at each residue of KMI-008 [83]. The result from the Gly-scan study in conjunction with a truncation study on KMI-008 suggested that residues P4′ to P2′ could be removed without abolishing β-secretase inhibitory activity. Subsequently, we discarded the P2′-P4′ residues. To increase the number and vigour of hydrogen-bond interactions, we optimized the P4 residue to an N^{β}-oxalyl-*L*-2,3-diaminopropinonic acid and the P1′ residue to an isophthalic moiety. The resulting pentapeptide **26** (KMI-370) was smaller and exhibited much more potent β-secretase inhibitory activity than octapeptide **25** (KMI-008). KMI-370 dose-dependently inhibited the secretion of sAPPβ from HEK293 cells transfected with β-secretase. However, the P4 side-chain of KMI-370 can isomerize under physiological conditions to its a-form, *i.e.* Nα-oxalyl-*L*-2,3-diaminopropinonic acid, and thereby become inactive [84]. Consequently, we designed in a tetrazole moiety, 1*H*-tetrazole-5-carbonyl, as a bioisostere of the P4-free carboxylic acid of the oxalyl group, and synthesized compound **27** (KMI-429) that exhibited improved stability while maintaining high inhibitory potency. While *in-vitro* HEK293 cell assays revealed that pentapeptide **27** (KMI-429) dose-dependently inhibited sAPPβ production, *in-vivo* injections of compound **27** into the hippocampus of APP wild type and transgenic Tg2576 mice, respectively, inhibited both amyloid-β production and release of sAPPβ in both models [85]. Our docking simulation study showed that KMI-429 was optimized to bind to the active site of BACE1, as shown in Fig. 8 [84]. When the two carboxylic acid groups from the P1' residue of KMI-429 were replaced by tetrazole rings, the resulting compound **28** (KMI-684) exhibited slightly more potent inhibitory activity against β-secretase [86]. Compounds with either a single carboxylic acid or tetrazole group at the *meta*-position of the phenyl P1' residue exhibited slightly lower inhibitory potency than their respective disubstituted compounds **27** (KMI-429) or **28** (KMI-684).

However, we suspected that KMI-684 was too polar to efficiently penetrate organic membranes because of the presence of three tetrazole moieties [87]. Consequently, optimization and substitution studies of the P4 tetrazole group with hydrogen-bond acceptor groups at the P4 diaminopropionic moiety led us to a 5-fluoroorotyl group.

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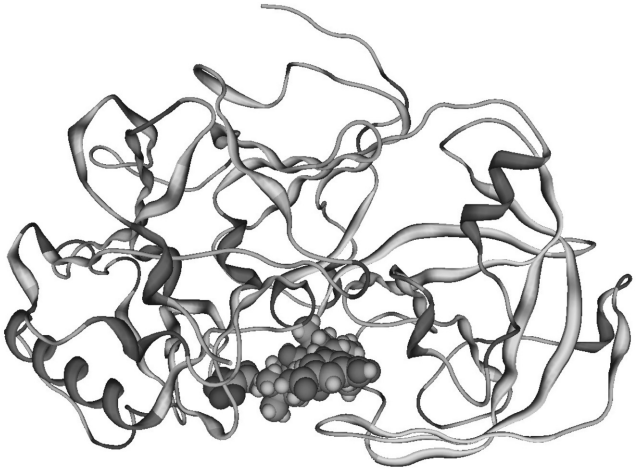

Figure 8. β-Secretase-inhibitor **27** (KMI-429) complex.

The P2 residue was optimized to cyclohexylalanine that would increase hydrophobicity. Moreover, being a non-natural amino acid, cyclohexylalanine could avoid recognition and premature metabolism by other proteases. We also determined that one tetrazole group, instead of two, at the P1′ residue was adequate to maintain potent β-secretase inhibitory activity without rendering the compound too polar. The resulting inhibitor **29** (KMI-574) exhibited high potency against β-secretase, and most importantly, higher inhibition of sAPPβ production in cultured HEK293 cells than a KMI-684 analogue with one P1′ tetrazole group. We derived a quantitative structure-activity relationship equation correlating cellular inhibition with β-secretase inhibition by enzymatic assay, calculated log P, and calculated molar refractivity. Log P represents hydrophobicity, whereas molar refractivity denotes bulkiness. We predicted that compounds with high β-secretase inhibition by enzymatic assay, calculated log P values of less than -1.006 and calculated molar refractivity values of around 21.080 would exhibit potent cellular inhibition against β-secretase expressing cells.

Using the knowledge that we have obtained from our peptidomimetic β-secretase and literature studies, we are currently focussing our effort on the smaller non-peptidomimetic β-secretase inhibitors.

HTLV-I Protease inhibitors

The human T-cell leukemia virus type 1 (HTLV-I) presents as adult T-cell leukemia (ATL) an aggressive and fatal lymphoproliferative disorder. Some viral carriers will develop a severe, progressive, demyelinating inflammatory disease called HTLV-I associated myelopathy / tropical spastic paraparesis (HAM/TSP) that results in symmetrical paralysis of the lower limbs [88–89]. Several inflammatory diseases are also implicated. Currently available treatments for HTLV-I-associated diseases alleviate symptoms and are not curative, and patients would eventually die due to opportunistic infections or HTLV-I associated disease. Much like its distant HIV cousin, HTLV-I is a retrovirus that processes its unique precursor polyproteins via an aspartic protease. Unfortunately, potent HIV-1 protease inhibitors such as compounds **10**, **12**–**14** (KNI-272, -727, -577 and -764, respectively), and ritonavir were ineffective against HTLV-I protease [90].

Similar to the development of our HIV-1 protease inhibitors, the design of our HTLV-I protease inhibitors began with substrate **30** of HTLV-I's Gag precursor polyprotein that is processed at the MA/CA cleavage site (Fig. 9). For the inhibitory P1 residue, among allonorstatine, phenylnorstatine, and allophenylnorstatine residues, we observed that an allophenylnorstatine residue seemed to be best accommodated by the HTLV-I protease active site [90, 92]. Of interest, similar to the case presented in the design of β-secretase inhibitors (cf. **24** and **25**), although the norstatine and allonorstatine residues shared a more structural commonality with Leu than phenylstatine and allophenylnorstatine, respectively, the corresponding aspartic protease favoured the matching phenylstatine and allophenylnorstatine residues. Octapeptidic inhibitor **31**, containing a P1 allophenylnorstatine residue and the P4-P2 and P1′-P4′ sequences borrowed from nonapeptidic substrate **30**, exhibited moderately potent inhibitory activity against HTLV-I protease. In consideration of the excellent results we obtained with a P1′ dimethylthiazolidine residue in HTLV-I's cousin, HIV, an equipotent analogue **32** of inhibitor **31** with a non-natural amino acid P1′ dimethylthiazolidine residue was designed to avoid premature Pro recognition and degradation by other proteases. A truncation study on the octapeptidic analogue **32**, commencing from the *N*- and *C*-termini, led to an expected less potent hexapeptidic HTLV-I protease inhibitor **33** [90, 91]. Knowing that HTLV-I protease also cleaves different substrates at many cleavage sites of various precursor polyproteins, the natural amino acid residues of compound **33**, namely the P3-P2 and P2′-P3′ residues, were systematically substituted with residues from a small library of natural amino acids. The resulting compound **34** exhibited high potency against HTLV-I protease. We noted that inhibitor **34** was composed of a fair number of Ile residues, and decided to pursue our substitution study with non-natural amino acid residues, thereby removing peptidic features from the design to prevent premature proteolysis [92]. We selected L-*tert*-leucine and L-(+)-a-phenylglycine as possible replacements for Ile and methodically substituted each Ile residue of compound **34** to optimize for

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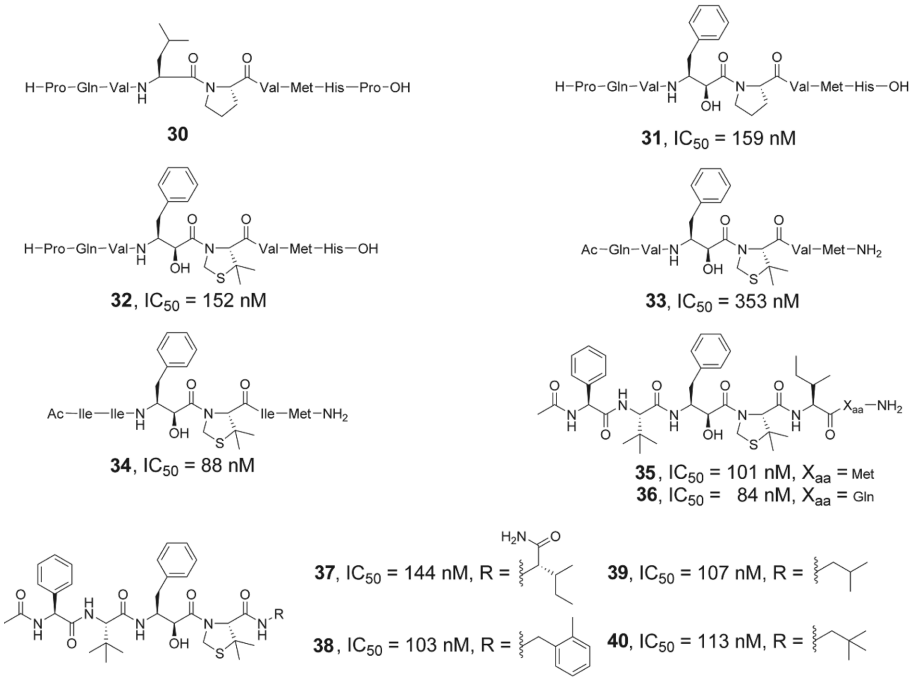

Figure 9. A MA/CA substrate **30** and several inhibitors **31–40** of HTLV-I protease. The IC_{50} values represent HTLV-I protease inhibitory activity.

inhibitory potency, and eventually derived highly potent inhibitor **35**. During the optimization process, it became apparent that the P3′ residue had come to be a lower determinant of activity, because a P3′ replacement of the non-polar Met residue in compound **35** by a polar amino acid, Gln, in compound **36** maintained high inhibitory activity against HTLV-I protease. We subsequently removed the P3′ residue to uncover a moderately potent inhibitor **37** of HTLV-I protease. In the case for HIV protease inhibitors **14** and **15**, the P2′ Ile residue could be successful substituted by an *ortho*-methylbenzylamine P1′-cap moiety without being a detriment to HIV-1 protease inhibition (cf. **7**). Accordingly, we replaced the P2′ Ile residue in HTLV-I protease inhibitor **37** by an *ortho*-methylbenzylamine P1′-cap moiety to discover a fairly potent inhibitor **38**. A preliminary optimization study suggested that increased bulk around the β-carbon of the P1′-cap moiety favoured the inhibitory activity against HTLV-I protease, as exemplified by inhibitors **39** and **40**. Interestingly, computer-assisted docking experiments suggested that, in the symmetrical homodimeric HTLV-I protease, the P1′ and P1′-cap moiety resided in the respective mirror pockets of the S1 and S2 where the corresponding P1 and P2 side-chain fit. In our present work, we are exploring the P2-cap moiety and other P1′-capping groups to maximize inhibitory activity against HTLV-I protease.

During our course of study, we came to realize that although HIV and HTLV-I are both from the *Retroviridae* family, HTLV-I protease had a higher specificity than HIV protease [90, 91]. In other words, potent HTLV-I protease inhibitors often also exhibited potent inhibition against HIV-1 protease. However, potent HIV-1 protease inhibitors did not necessarily possess potent inhibitory activity against HTLV-I protease. If such a need arises, HTLV-I protease inhibitors can be further developed as HIV-1 protease inhibitors.

Closing remarks

Designing aspartic protease inhibitors often begins with the shortest substrate that can be processed by the protease. The first few inhibitors often look similar to the substrate except for the scissile P1 position. The critical P1 cleavage point in the substrate is replaced by a non-hydrolyzable residue to mimic the transition state of the substrate during peptide hydrolysis. All of our work revolves around inhibiting residues that contain a hydroxymethylcarbonyl isostere. From these initial inhibitors, several truncation and natural amino acid substitution studies are performed to minimize the size of the inhibitors and optimize the efficiency of inhibition, especially when one considers that most proteases can process several different substrates. Natural amino acid residues are then methodically replaced by non-natural amino acid residues so as to avoid premature recognition and digestion of the inhibitors by other proteases. From this point on, most of the work relies on quantita-

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tive structure-activity relationship equations, computer-assisted docking, and dynamic simulations that require three dimensional coordinates obtained from various sources such as X-ray diffraction crystallography or NMR studies, and various other techniques that help identify structural preferences by the protease. The target of the research also shifts from optimizing enzyme inhibition to improving membrane permeability, reducing drug-resistance, minimizing adverse drug effects, enhancing solvent miscibility, increasing oral bioavailability, and considering various other pharmaceutics, pharmacokinetic, and pharmacodynamic factors. When it comes to rational drug design, although a fair amount of tedious trials-and-errors are still involved, the reward of finding a cure to an incurable or difficult to treat disease is certainly worth the struggle.

This research was supported in parts by The Frontier Research Program; The 21st Century COE Program from The Ministry of Education, Culture, Sports, Science and Technology, Japan; The Japan Health Sciences Foundation; and Japan Society for the Promotion of Science's Post-Doctoral Fellowship for Foreign Researchers.

The authors have declared no conflict of interest.

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