

Review

New Directions for Protease Inhibitors Directed Drug Discovery

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ABSTRACT:

Proteases play crucial roles in various biological processes, and their activities are essential for all living organisms—from viruses to humans. Since their functions are closely associated with many pathogenic mechanisms, their inhibitors or activators are important molecular targets for developing treatments for various diseases. Here, we describe drugs/drug candidates that target proteases, such as malarial plasmepsins, β -secretase, virus proteases, and dipeptidyl peptidase-4. Previously, we reported inhibitors of aspartic proteases, such as renin, human immunodeficiency virus type 1 protease, human T-lymphotropic virus type I protease, plasmepsins, and β -secretase, as drug candidates for hypertension, adult T-cell leukaemia, human T-lymphotropic virus type I-associated myelopathy, malaria, and Alzheimer's disease. Our inhibitors are also described in this review article as examples of drugs that target proteases. © 2015 Wiley Periodicals, Inc. *Biopolymers (Pept Sci)* 106: 563–579, 2016.

Keywords: DPP-4; HCV NS3 protease; HIV-1 protease; plasmepsin; protease inhibitor; β -secretase

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INTRODUCTION

Enzymes play important roles in several biological processes including digestion, absorption, metabolism, and propagation. Their functions are essential for all living organisms and are closely associated with many pathogenic mechanisms. Enzyme inhibitors or activators therefore represent important molecular targets for the development of new disease treatments. Among enzymes, proteases are important molecular targets because their substrates are proteins, proteases, and peptides, which play crucial roles in all forms of life—from viruses to humans. Proteases are categorized into four major classes: aspartic proteases, serine proteases, cysteine proteases, and metalloproteases, which contain aspartic acid, serine, cysteine, and metallic ions, respectively, in their active sites. Renin was the first protease for which inhibitors were designed logically, using a computational approach (structure-based drug design; SBDD). Renin is an aspartic protease that is involved in the rate-limiting first step of the renin–angiotensin–aldosterone system (RAAS) and is a molecular target for antihypertensive agents.^{1–3} Renin cleaves angiotensinogen to release the decapeptide angiotensin I as shown in Figure 1A. Angiotensin I is subsequently cleaved by a nonspecific dipeptidyl carboxypeptidase, angiotensin-converting enzyme

This paper is dedicated to Emeritus Professor Haruaki Yajima on the occasion of his 90th birthday.

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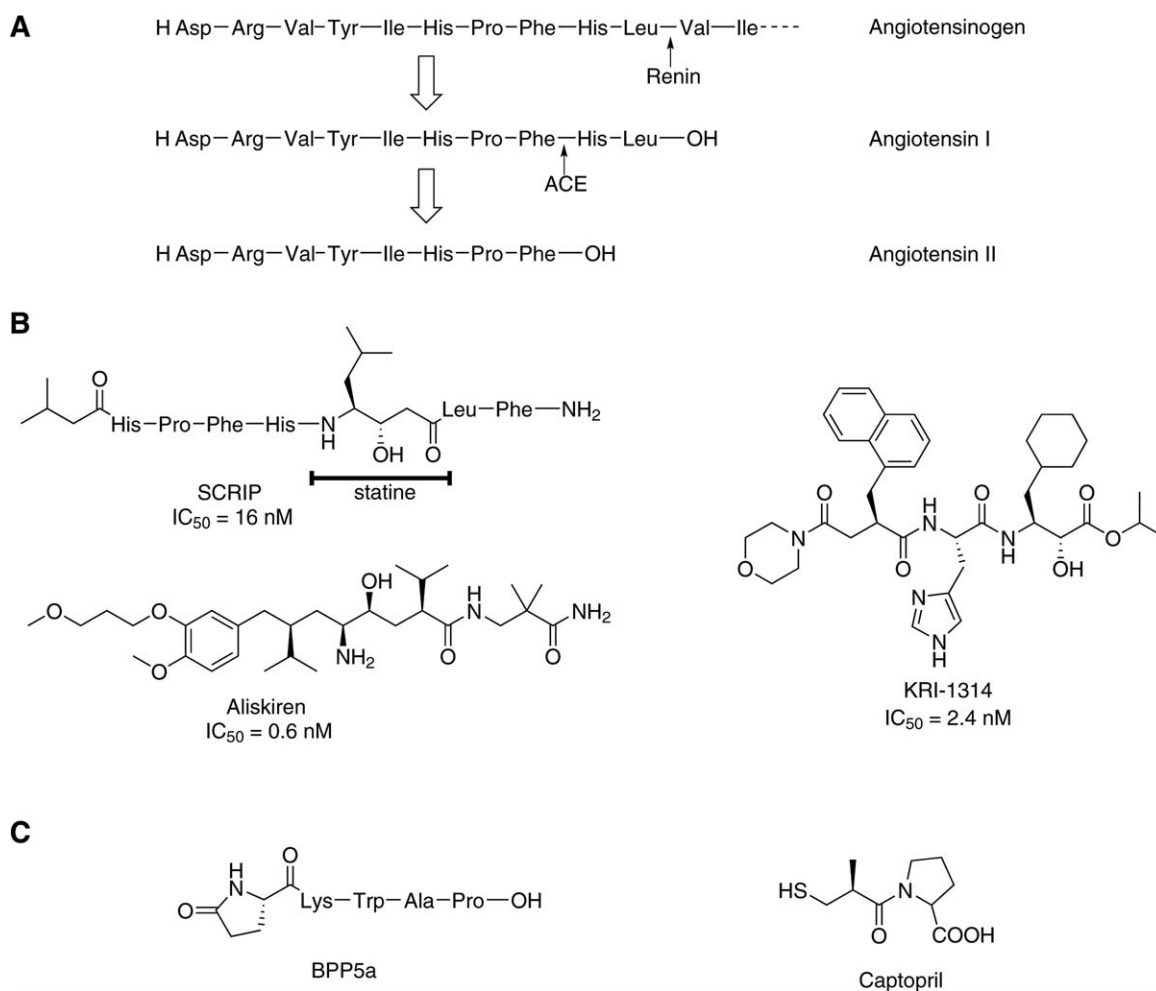


FIGURE 1 Renin and angiotensin-converting enzyme (ACE) inhibitors: (A) cleavage sites of renin and ACE; (B) renin inhibitors; (C) ACE inhibitors.

(ACE), to release the potent octapeptide vasopressor angiotensin II. The pepsin inhibitor pepstatin A, which features the γ -amino acid statine as the transition state analogue, was reported to inhibit the activity of renin, and since then, many renin inhibitors containing a transition state analogue have been developed.¹ Boger et al. designed a renin inhibitor, called statine-containing renin inhibitory peptide (SCRIP; half-maximal inhibitory concentration (IC₅₀) = 16 nM; Figure 1B), that had a statine residue at the P₁ position and contained a part of the angiotensinogen amino acid sequence.² We also designed and synthesized the potent, small inhibitor, KRI-1314 (IC₅₀ = 2.4 nM), that contains a norstatine-type residue as a transition state analogue.³ The first renin inhibitor drug, aliskiren (IC₅₀ = 0.6 nM; Novartis and Speedel), was approved by the US Food and Drug Administration (FDA) in 2007.

Aspartic proteases form a substrate transition state that has no covalent bonds between the substrate and the protease (as

discussed later), and as a result, inhibitors can be easily designed using a computational approach. The development of inhibitors for the metalloprotease ACE had to wait until the discovery of the bradykinin-potentiating peptide pyroglutamyl-Lys-Trp-Ala-Pro (BPP5a; Figure 1C). BPP5a was found in the venom of the snake *Bothrops jararaca* and inhibits ACE activity.⁴ The first ACE inhibitor drug, captopril, was approved by the FDA in 1981 and was designed to emulate the C-terminus Ala-Pro sequence of BPP5a.⁵ The proline residue of captopril docks in the S₂ pocket of ACE, and another residue docks in the S₁ pocket. The coordination bond that is formed between the sulfur atom of captopril and the zinc ion in the active site of ACE appears to contribute to the potent inhibition.

Subsequently, human immunodeficiency virus type 1 (HIV-1) protease inhibitors were designed using a computational approach to develop therapeutics for the treatment of acquired

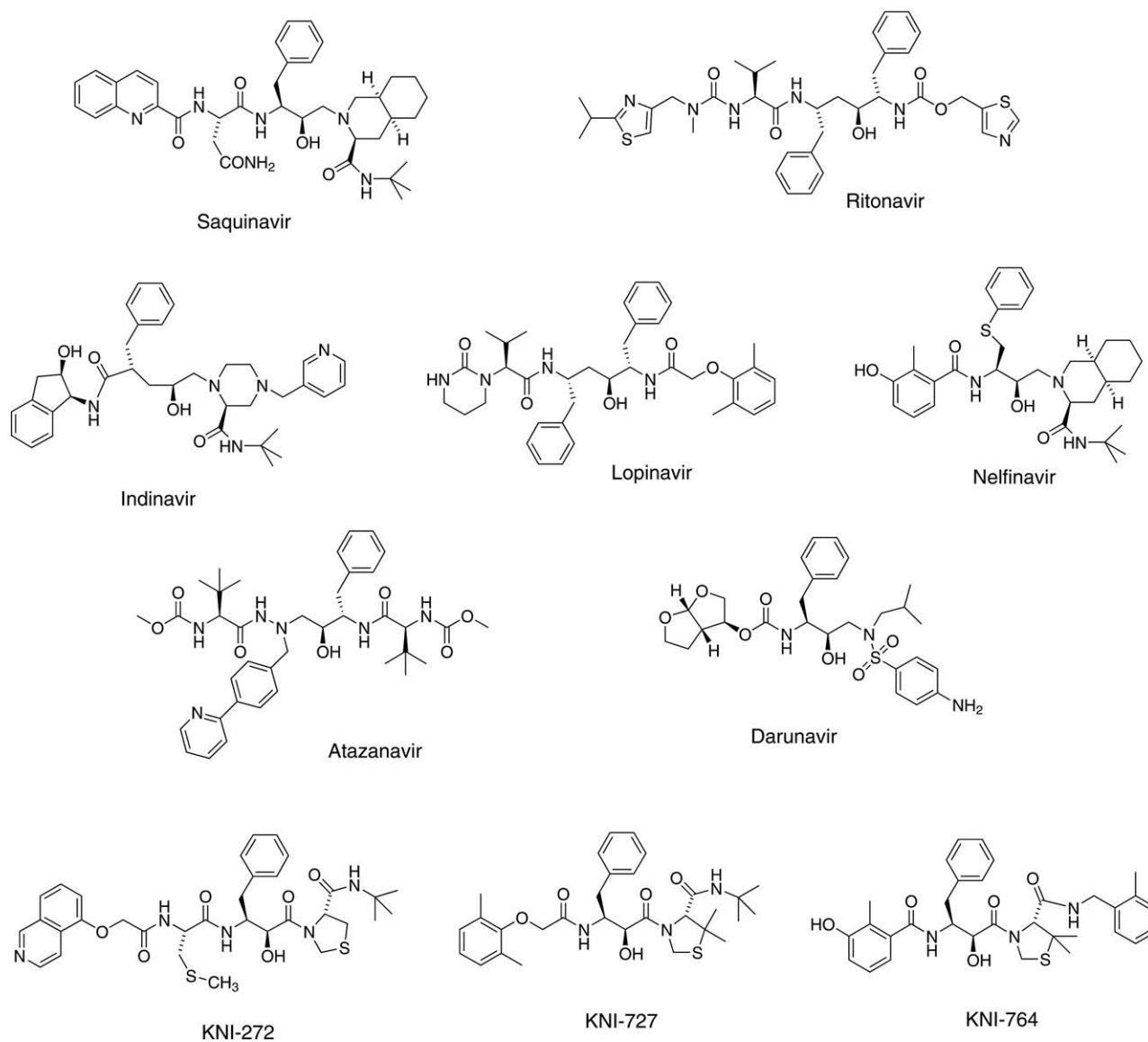


FIGURE 2 Human immunodeficiency virus type 1 (HIV-1) protease inhibitors.

immunodeficiency syndrome (AIDS). HIV-1 protease is also an aspartic protease, and its inhibitors can be designed using an approach similar to that used for the design of renin inhibitors. HIV-1 protease is responsible for processing the Gag and Gag-Pol polyproteins and enabling the proliferation of the retrovirus. The first HIV-1 protease inhibitor approved by the FDA, saquinavir (Figure 2), was developed using a computational approach. Many HIV-1 protease inhibitors, i.e., saquinavir, indinavir, nelfinavir, atazanavir, and darunavir, possess a hydroxyethylamine moiety that acts as a transition state analogue, whereas ritonavir and lopinavir possess a hydroxyethylene moiety as a transition state analogue. The use of these HIV-1 protease inhibitors in highly active anti-

retroviral therapy (HAART) has contributed considerably to overcoming AIDS.⁶ All HIV-1 protease inhibitors have a hydroxyl group in the transition state analogue residue and can strongly bind to the active site of the HIV-1 protease.

We have also reported a series of potent HIV-1 protease inhibitors such as KNI-272, KNI-727, and KNI-764 that contain a norstatine-type residue, allophenylnorstatine (Apns), as a transition state analogue (Figure 2).⁷ Furthermore, we have used the transition state concept to design inhibitors of several other aspartic proteases, such as malarial plasmepsins, β -secretase, and human T-lymphotropic virus type I (HTLV-1) protease.⁸⁻¹² Here, we describe the latest developments in protease inhibitor design, including our own work.

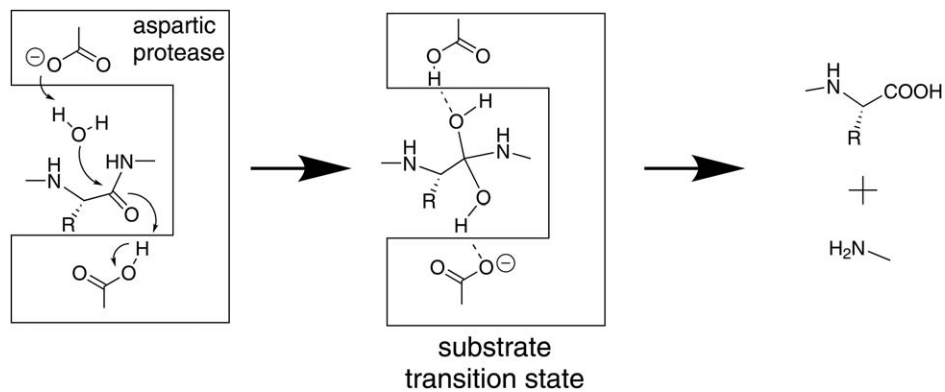


FIGURE 3 Mechanism of peptide bond cleavage by aspartic proteases *via* a substrate transition state.

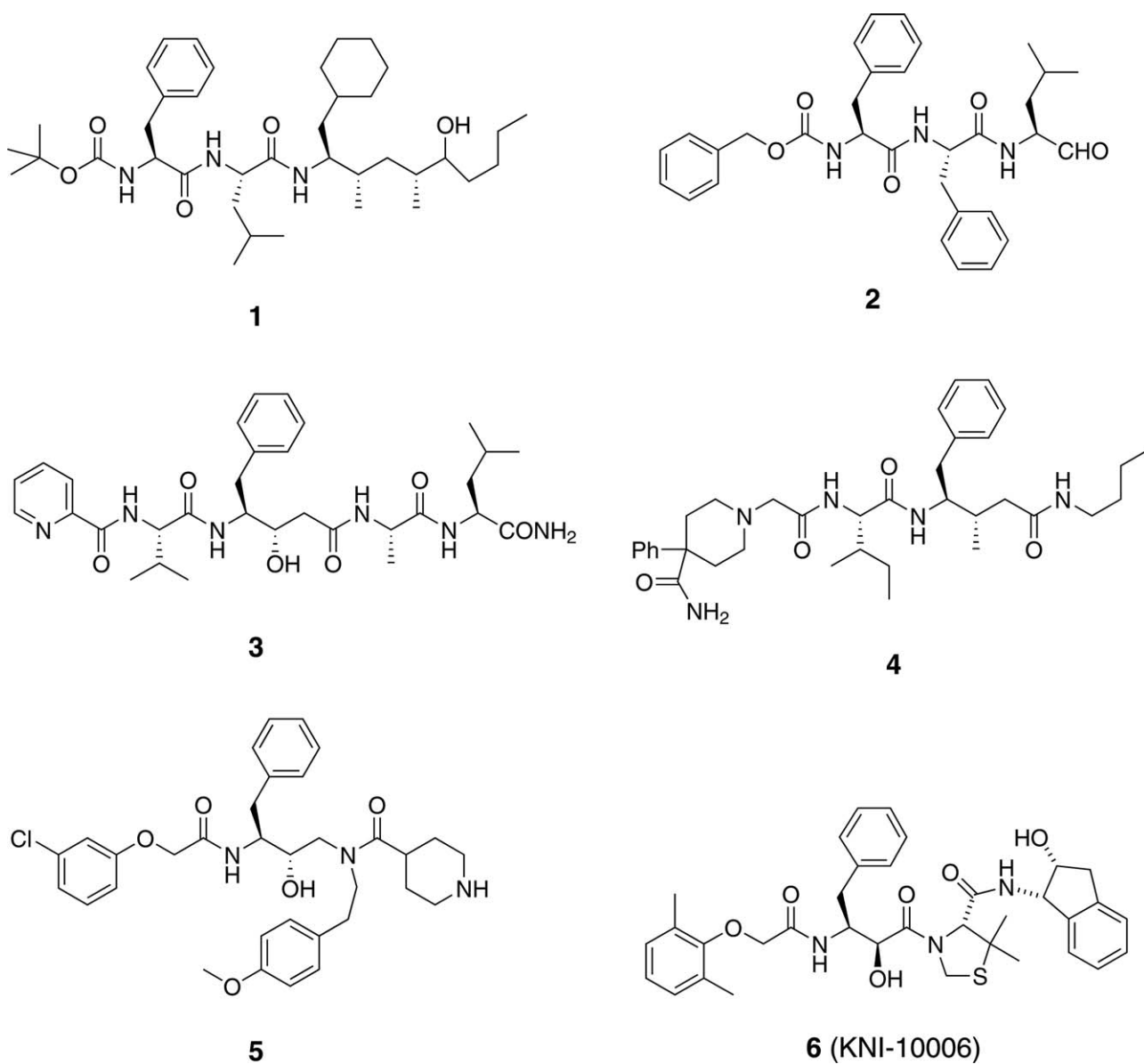


FIGURE 4 Plasmepsin inhibitors.

ASPARTIC PROTEASE INHIBITORS

Aspartic proteases have two catalytic aspartic acid residues at the active site. First, a water molecule is deprotonated by the carboxyl anion of one of the aspartic acid side chains; the resulting hydroxide ion then attacks the carbonyl carbon of the substrate at the cleavage site to form a tetrahedral transition state as shown in Figure 3. Rearrangement of this transition state causes the amide bond in the substrate to be broken. Although serine and cysteine proteases cleave peptide bonds *via* a transition state consisting of a covalent bond between the catalytic serine/cysteine and carbonyl carbon at the cleavage position of the substrate, aspartic proteases form a transition state based on noncovalent interactions between their carboxylic acid/carboxylate groups and the two hydroxyl groups of the substrate. The fact that the transition state in aspartic proteases involves no covalent bond formation makes the tetrahedral structures in the transition state relatively simple. This makes it possible to design aspartic protease inhibitors logically using a computational approach.

PLASMEPSIN INHIBITORS

Malaria is a disease caused by parasitic protozoa of the genus *Plasmodium* that feeds on the haemoglobin of an infected person. The protozoa produce a family of aspartic proteases, plasmepsins, which degrade the host's haemoglobin and eventually cause the symptoms of malaria and death of the host. Plasmepsins are currently being investigated as a molecular target for antimalarial drugs. Plasmepsins are known to consist of at least ten isoforms—Plm I, II, III, IV, V, VI, VII, IX, X, and histo-aspartyl protease (HAP)—that are encoded in the parasite's genome. Although most plasmepsins have two aspartic acid moieties in the catalytic site, one of the catalytic aspartic acid residues is replaced with histidine only in the case of HAP. Among the plasmepsin isoforms, Plm I and Plm II are known to readily cleave haemoglobin between the Phe33 and Leu34 residues of the α -globin subunit (Table I).

Francis et al. reported that **1** (SC-50083, Figure 4) selectively blocks the aspartic hemoglobinase, prevents haemoglobin degradation and kills the parasites. SC-50083 was screened from peptidomimetic aspartic protease inhibitors library.¹³ An aldehyde-type compound **2** (Ro 40-4388) was reported by Moon. Ro 40-4388 was identified from the inhibitors library and inhibited a chloroquine-resistant strain of *P. falciparum*.¹⁴ Silva et al. reported plasmepsin inhibitor **3**, which was screened from the inhibitors library of an aspartic protease, cathepsin D.¹⁵ Carroll et al. found **4** (PS 429694) from the statine-containing combinatorial library.¹⁶ Haque et al. reported **5** that was screened from hydroxyethylamine-type cathepsin D inhibitors library.¹⁷

Table I Cleavage Sites of Human Immunodeficiency Virus (HIV) Protease and Malarial Plasmepsins

A Cleavage sites of HIV-1 protease	
	P ₄ P ₃ P ₂ P ₁ P ₁ ' P ₂ ' P ₃ ' P ₄ '
p17/p24	-Gln-Arg-Gly-Tyr-Pro-Ile-Val-Gln-
p24/p1	-Ala-Arg-Val-Leu-Ala-Glu-Ala-Met-
p1/p9	-Ala-Thr-Ile-Met-Met-Gln-Arg-Gly-
p9/p6	-Pro-Gly-Asn-Phe-Leu-Gln-Ser-Arg-
TF/PR	-Ser-Phe-Asn-Phe-Pro-Gln-Ile-Thr-
PR/RT	-Thr-Leu-Asn-Phe-Pro-Ile-Ser-Pro-
RT/RN	-Ala-Glu-Thr-Phe-Tyr-Val-Asp-Gly-
RN/IN	-Arg-Lys-Ile-Leu-Phe-Leu-Asp-Gly-
B Cleavage sites of malarial plasmepsin I and II	
	P ₄ P ₃ P ₂ P ₁ P ₁ ' P ₂ ' P ₃ ' P ₄ '
Plm I α 33/34	-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro-
α 46/47	-Phe-Pro-His-Phe-Asp-Leu-His-Ser-
α 98/99	-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-
α 31/32	-Leu-Gly-Arg-Leu-Leu-Val-Tyr-
β 41/42	-Thr-Gln-Arg-Phe-Phe-Glu-Ser-Phe-
β 129/130	-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-
Plm II α 33/34	-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro-
α 108/109	-Leu-Leu-Val-Thr-Leu-Ala-Ala-His-
α 136/137	-Ser-Thr-Val-Leu-Thr-Ser-Lys-Tyr-
β 32/33	-Gly-Arg-Leu-Leu-Val-Val-Tyr-Pro-

TF: transframe protein, PR: HIV-1 protease, RT: reverse transcriptase, RN: ribonuclease, IN: integrase.

Because the cleavage sites of Plm I and Plm II are similar to that of HIV-1 protease (as shown in Table I), several potent HIV-1 protease inhibitors have been tested against plasmepsin II.¹⁸ Some HIV-1 protease inhibitors have been shown to inhibit plasmepsin II with an inhibitory constant (K_i) of 1 μ M or less. KNI-727 exhibited the highest selectivity for plasmepsin II among the tested compounds. Additionally, when designing inhibitor **6** (KNI-10006, Figure 4), we incorporated a hydroxyl-bearing indan derivative because the P₂' position of the α -globin subunit that is recognized by both Plm I and Plm II is a Ser residue and, as predicted, the hydroxyl group on the indan derivative appeared to mimic the hydroxyl group of the P₂'-Ser side chain. Inhibitors KNI-727 and KNI-10006 were assayed for inhibitory activity against the four main plasmepsins: Plm I, Plm II, Plm IV, and HAP.¹⁹ Although KNI-727 showed potent inhibitory activities against Plm I, Plm II, and Plm IV, it exhibited low inhibitory activity against HAP. A molecule that is able to inhibit the enzymatic activity of all the four main plasmepsins might lead to rapid starvation of the parasite and could be effective against a drug-resistant mutant

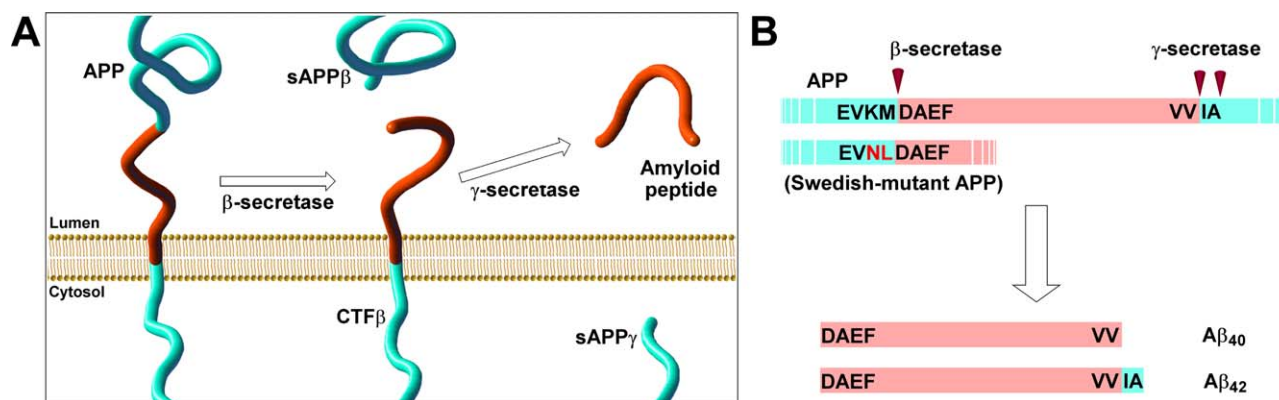


FIGURE 5 Processing pathway of amyloid precursor protein (APP); (A) formation of amyloid peptide from APP; (B) cleavage sites of APP.

of the parasite. KNI-10006 was found to be significantly more potent than KNI-727 and could effectively inhibit all four main plasmepsins.

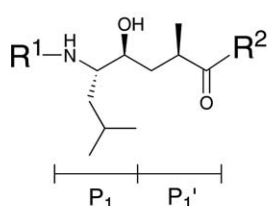
β-SECRETASE INHIBITORS

Amyloid β peptide ($A\beta$), the main component of plaques in the brains of AD patients, is formed by proteolysis of the amyloid precursor protein (APP).^{20–22} An aspartic protease β -secretase, also known as β -site APP-cleaving enzyme 1 (BACE1), triggers $A\beta$ formation by cleaving APP at the $A\beta$ domain N-terminus (Figure 5). Next, γ -secretase cleaves between either the Val and Ile, or the Ala and Thr residues in the C-terminus of the $A\beta$ domain to form either $A\beta_{40}$ or $A\beta_{42}$, respectively (Figure 5). $A\beta_{42}$ shows greater neurotoxicity and aggregability than $A\beta_{40}$ and appears to be a key biomolecular marker of AD pathogenesis. Based on the amyloid hypothesis, many inhibitors against β - or γ -secretases have been designed and synthesized as drug candidates for Alzheimer's disease.^{8–12} The fact that β -secretase-knockout transgenic mice live normally suggests that β -secretase is a promising molecular target for AD drug development.²³ Most β -secretase inhibitors possess a transition state analogue at the P_1 position and are designed based on the amino acid sequence of Swedish mutant APP, which has a double mutation around the β -site (at the K670N and M671L residues). Inhibition of the β -cleavage of the Swedish mutation of APP by β -secretase is important because this increases the levels of $A\beta_{42}$ and $A\beta_{40}$. Many APP mutations from AD patients have been reported, and these mutations accelerate $A\beta$ s levels or $A\beta$ s aggregabilities. Recently, a mutation on the β -site of APP—Ala(673)Thr—has been reported. This mutation was identified from a set of whole-genome sequence data of 1795 Icelandic people as a gene with

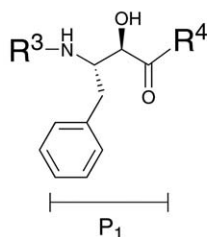
a low risk of AD and protects against AD and age-related decline. This finding appears to verify the validity of the amyloid hypothesis in the AD pathology.¹⁰

In 1999, Sinha et al. at Elan Pharmaceuticals succeeded in purifying β -secretase from the human brain by using an inhibitor with a statine residue as the substrate transition state analogue and then succeeded in cloning the enzyme.²⁴ Since then, many other research groups have reported β -secretase inhibitors. In 2000 and 2001, Ghosh et al. reported the potent inhibitors **7** (OM99-2, $K_i = 1.6$ nM) and **8** (OM00-3, $K_i = 0.3$ nM), which contained a hydroxyethylene unit as a substrate transition state analogue, and the first X-ray crystal structure of a complex between recombinant β -secretase and the inhibitor (Figure 6).^{25–29} We reported an octapeptidic β -secretase inhibitor, **9** (KMI-008, $IC_{50} = 413$ nM), which possessed a hydroxymethylcarbonyl (HMC) isostere as a substrate transition state analogue and a Leu residue at the P_2 position.³⁰ Moreover, a series of small and potent β -secretase inhibitors, such as **10** (KMI-429, $IC_{50} = 3.9$ nM), **11** (KMI-684, $IC_{50} = 1.2$ nM), and **12** (KMI-574, $IC_{50} = 5.6$ nM), were developed as lead compounds based on KMI-008 (Figure 6).^{30–39} In KMI-429 and KMI-684, the carboxylic group of KMI-008 was replaced with a carboxylic acid bioisostere, tetrazole ring, and these compounds were found to be more potent inhibitors.^{32–34} KMI-574, which possessed a 5-fluoroorotyl group at the P_4 position, showed improved inhibitory activity in cultured cells (HEK293 stably expressing human β -secretase).³⁵

In 2001, the first nonpeptidic β -secretase inhibitors, such as **13** (TAK-070; $IC_{50} = 2.93$ μ M; Figure 7),^{40,41} were reported by researchers at Takeda Chemical Industries. TAK-070 was found by screening a chemical library in the IMR32 human neuroblastoma cell line. An *in vitro* fluorescence resonance energy transfer (FRET) assay using recombinant human β -secretase



Compd	R ¹			R ²			K _i (nM)
	P ₄	P ₃	P ₂	P ₂ '	P ₃ '	P ₄ '	
7 (OM99-2)	Glu	Val	Asn	Ala	Glu	Phe	1.6
8 (OM00-3)	Glu	Leu	Asp	Val	Glu	Phe	0.3



Compd	R ³			R ⁴				IC ₅₀ (nM)
	P ₄	P ₃	P ₂	P ₁ '	P ₂ '	P ₃ '	P ₄ '	
9 (KMI-008)	Glu	Val	Leu	Asp	Ala	Glu	Phe	413
10 (KMI-429)	TCD	Val	Leu	R ³				3.9
11 (KMI-684)	TCD	Val	Leu	R ⁴				1.2
12 (KMI-574)	FOD	Val	Cha	R ⁵				5.6

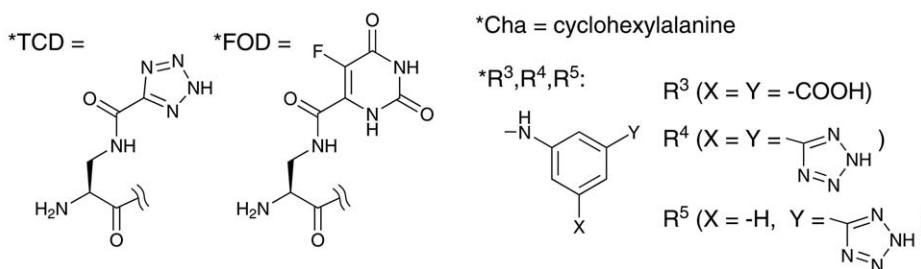


FIGURE 6 Early peptidomimetic β -secretase inhibitors.

and a competition study against a statine-containing, substrate-based inhibitor indicated that TAK-070 showed β -secretase inhibition in a dose-dependent and noncompetitive manner. Recently, Fukumoto and co-workers at Takeda Chemical Industries and the University of Tokyo carried out surface plasmon resonance (SPR) experiments that showed that TAK-070 binds to a specific region in the membrane-spanning portion of β -secretase by using C-terminally truncated β -secretase.⁴² This result suggests that TAK-070 does not have to target the catalytic site of β -secretase but only its membrane-spanning domain. However, most inhibitors that have subsequently been reported by other research groups target the active site of β -secretase.

Elan Pharmaceuticals reported a series of nonpeptidic β -secretase inhibitors, such as **14** (IC₅₀ = 20 nM; Figure 7) that possess a transition state analogue and were designed using an SBDD approach. Compound **14** has an isophthalic scaffold.^{43–49} The P₁ residues are preferentially arranged into planar aromatic rings because the S₁ site of β -secretase, which is formed by the cleft and flap domains, is narrow. Many inhibitors with an aromatic ring at the P₁ position were subsequently reported; for example, a research group at Merck Sharp and Dohme (MSD) reported the potent inhibitors^{50–60} **15**⁵² and

16⁶⁰ (IC₅₀ = 15 and 2 nM, respectively) with an isophthalic and a pyridine scaffold, respectively, and Ghosh et al. reported the potent inhibitor **17** (GRL-8234, IC₅₀ = 1.8 nM), which featured an isophthalic residue.^{61,62} Researchers at Novartis reported nonpeptidic β -secretase inhibitor **18** (IC₅₀ = 2 nM).^{63,64} Although **18** was designed based on peptidomimetic inhibitors by using the SBDD approach, it is notable because it forms a unique cyclic structure at the P₁ position where the hydroxyl and amino groups on the cyclic sulfone ring of **18** appear to interact with two Asp residues at the active site of β -secretase as well as acting as a transition state analogue.

Using a high throughput screening (HTS) approach, many inhibitors have been designed. Wyeth (merged with Pfizer in 2009) reported a series of β -secretase inhibitors based on some HTS hit compounds.^{65–71} The potent inhibitor **19** (IC₅₀ = 5 nM) was based on an HTS hit compound (IC₅₀ = 35 μ M). A research group at Johnson & Johnson and Astex Therapeutics identified an HTS compound (K_i = 900 nM) from their chemical library and designed the β -secretase inhibitor **20** (K_i = 20 nM).^{72,73} Recently, several clinical trials of β -secretase inhibitors have been carried out. In 2013, Eli Lilly announced that their β -secretase inhibitor (LY2886721) was terminated at the

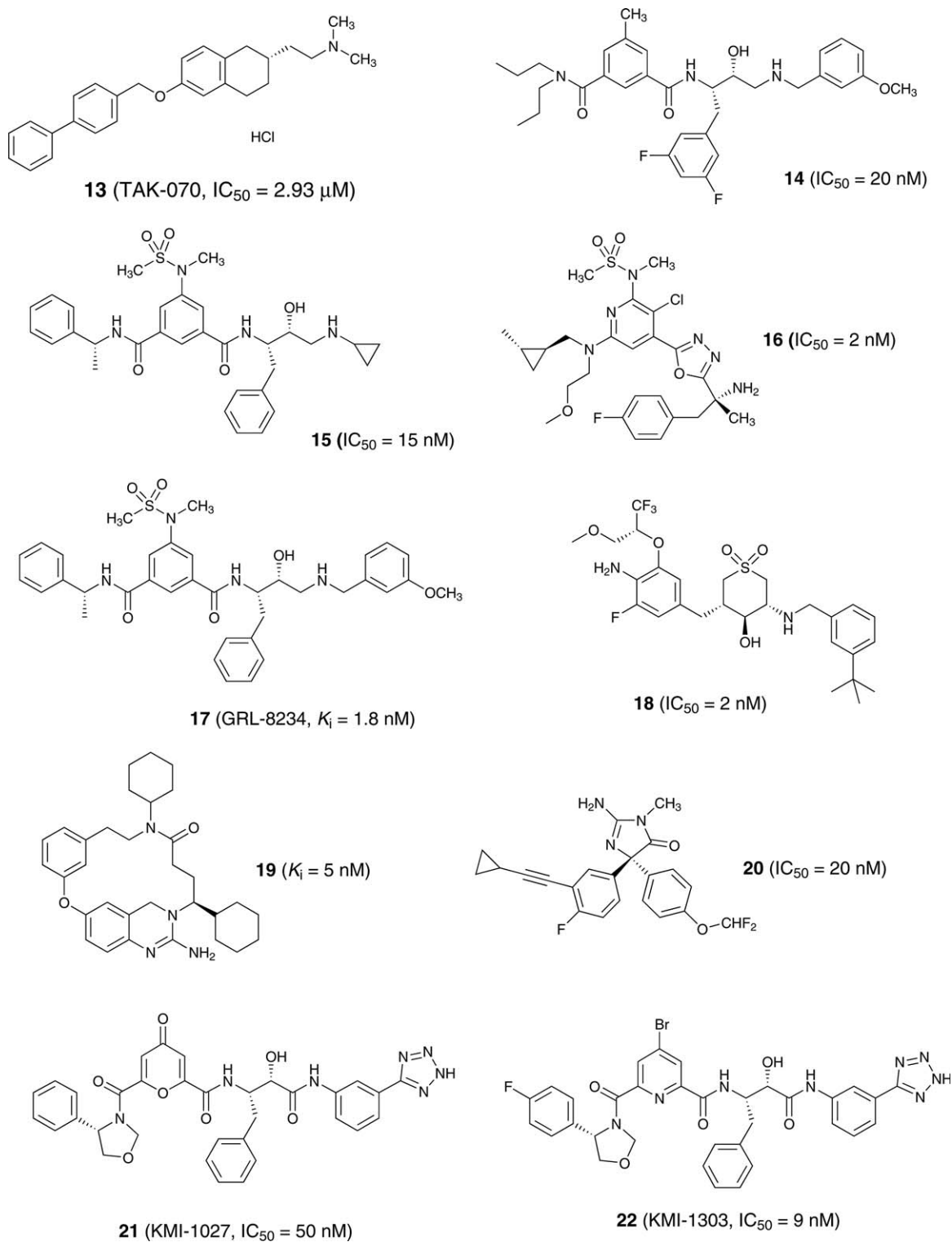


FIGURE 7 Nonpeptidic β -secretase inhibitors.

phase II clinical stage because of abnormal liver biochemical test results. However, such adverse events caused by other β -secretase inhibitors that are currently in clinical trials have not

been reported yet. Probably it appears to be a unique effect of LY2886721. Recently, Eli Lilly and AstraZeneca announced that they will collaborate with a 50:50 partnership on the Phase II/

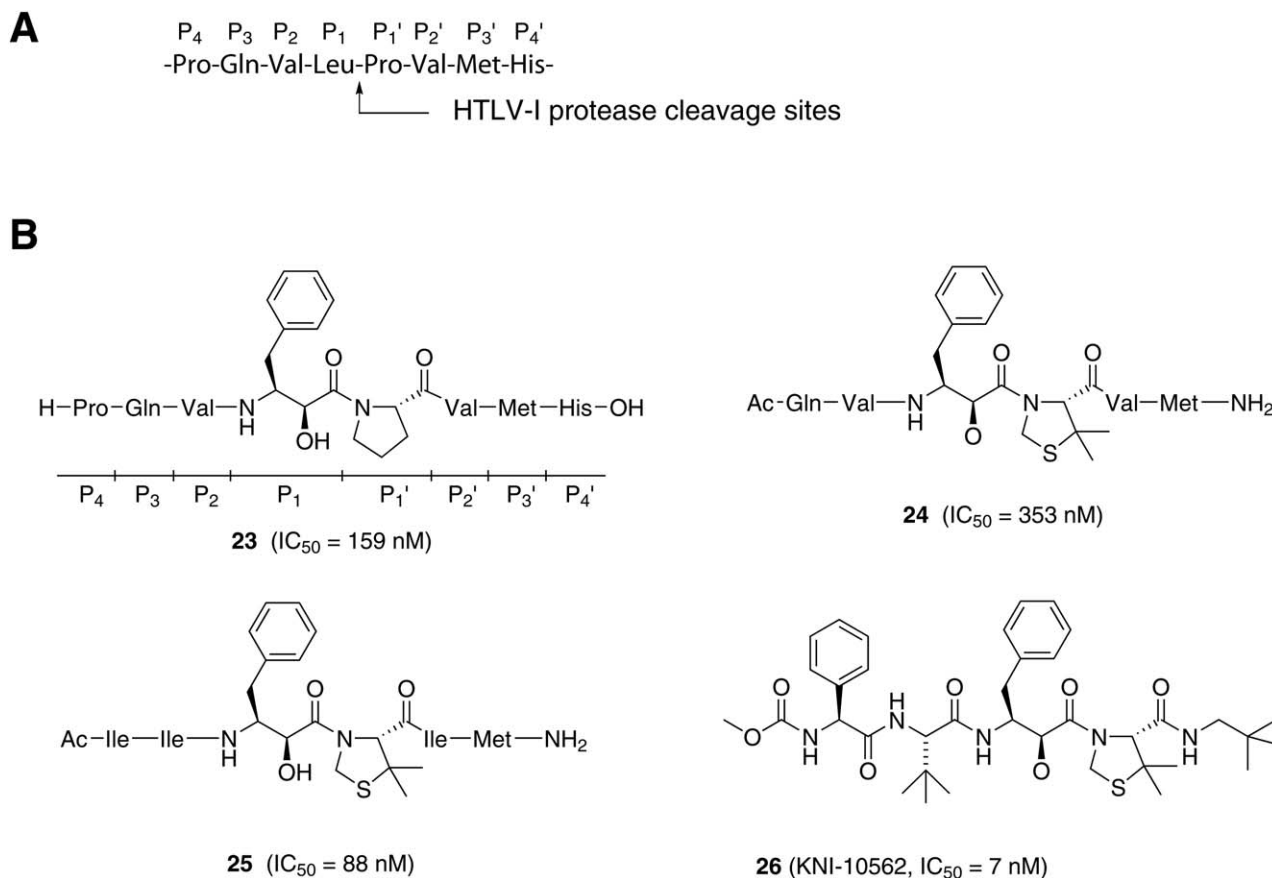


FIGURE 8 (A) Cleavage site of human T-lymphotropic virus type I (HTLV-I) protease; (B) HTLV-I protease inhibitors.

III trial of the β -secretase inhibitor (AZD3293) developed by AstraZeneca.⁷⁴ Although AD3839 was terminated at the phase I clinical stage, the clinical trial of AZD3293—the latest generation β -secretase inhibitor from AstraZeneca—was completed with encouraging results from both Alzheimer's patients and healthy participants. AD3293 effectively reduced the level of $A\beta$ in cerebrospinal fluid.

We also designed the nonpeptidic β -secretase inhibitors **21** and **22** that featured a transition state analogue, as shown in Figure 7.^{37,75–77} These inhibitors have a pyridinedicarboxylic or chelidonic scaffold at the P_2 position and were based on a virtual inhibitor with an isophthalamide moiety at the P_2 position that was designed using an *in silico* conformational SBDD approach.^{39,75} The oxazolidine ring fixes the turn structure between the phenyl ring at the P_3 position and the P_3 -aromatic ring, and the P_3 -phenyl ring can closely bind to the S_3 -subpocket of β -secretase. The quantum interaction of an inhibitor with the side chain of β -secretase-Arg235 plays an important role in the inhibition mechanism as previously mentioned.³⁷ We speculated that an electron-rich halogen atom could bind

to the electron-poor guanidine π -orbital by a quantum interaction and designed the potent inhibitor **22** with a halogen atom on the P_2 -heterocyclic ring.

HTLV-I PROTEASE INHIBITORS

Adult T-cell leukaemia (ATL) and HTLV-I associated myelopathy (HAM) are caused by infection with HTLV-I, a retrovirus that processes its precursor polyproteins via the unique aspartic protease HTLV-I protease. Since HTLV-I protease is similar to HIV-1 protease, potent HIV-1 protease inhibitors, such as our compounds KNI-727 and 764, and ritonavir were tested as HTLV-1 inhibitors. Unfortunately, these compounds were ineffective against HTLV-I protease.⁷⁸ Therefore, we designed some HTLV-I protease inhibitors using the substrate transition state concept as well as the development of renin and HIV-1 protease inhibitors as shown in Figure 8. Octapeptidic inhibitor **23** ($IC_{50} = 159$ nM) with a transition state analogue was designed based on a substrate of HTLV-I Gag precursor polyprotein that is processed at the MA/CA cleavage site as shown

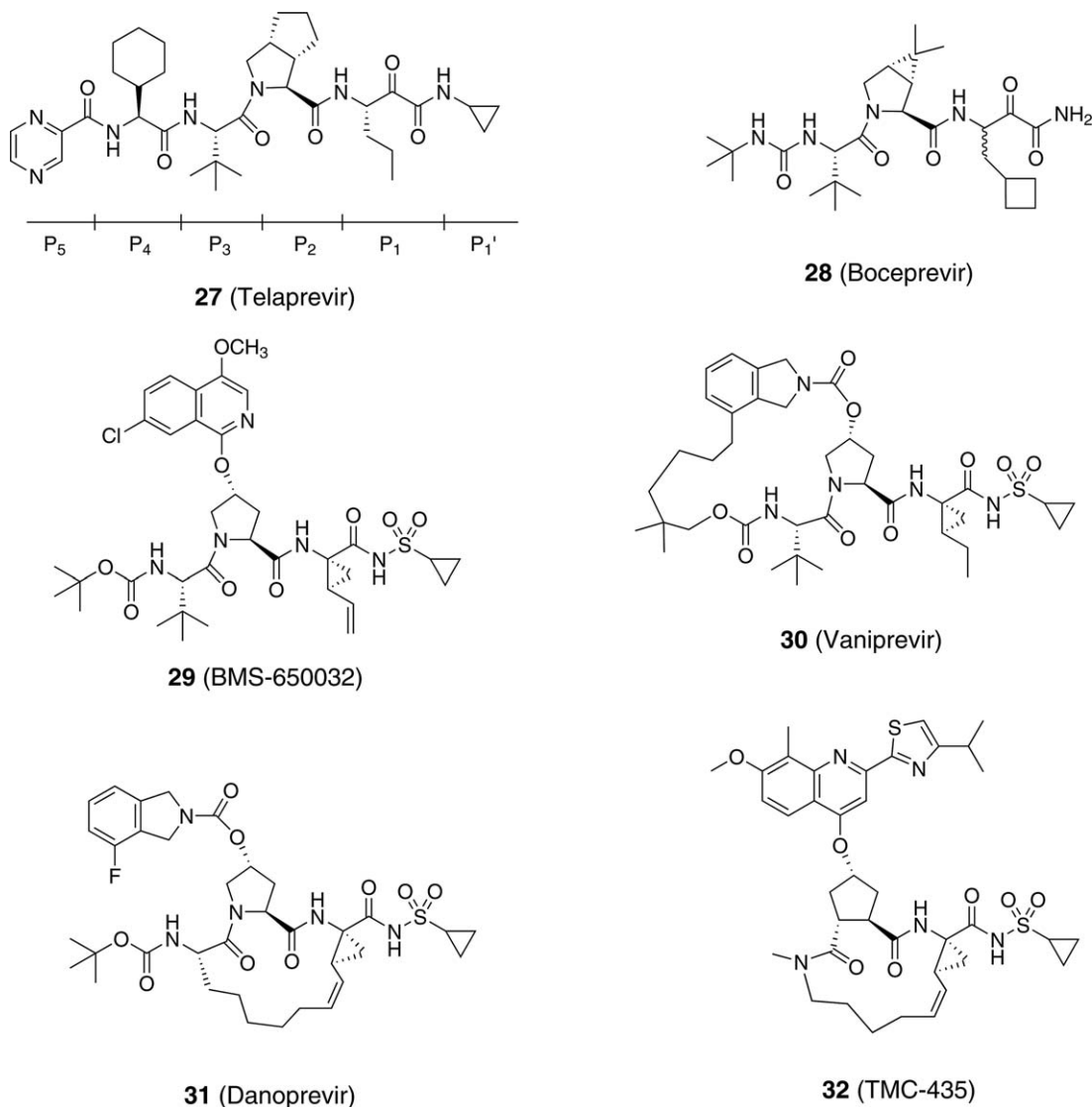


FIGURE 9 Hepatitis C virus NS3 protease inhibitors.

in Figure 8A. Compound **23** exhibited moderately potent inhibitory activity against HTLV-I protease. By inserting the non-natural amino acid dimethylthiazolidine (Dmt), which is a proline bioisostere, small hexapeptidic HTLV-I protease inhibitors **24** and **25** ($IC_{50} = 353$ and 88 nM, respectively) were obtained.⁷⁹ Subsequently, hydrophobic and branched amino acids were found to be preferred at the P₂–P₃ position, and the potent HTLV-I protease inhibitor **26** (KNI-10562, $IC_{50} = 7$ nM) possessing an *L*-*tert*-leucine and *L*-(+)- α -phenylglycine was designed.⁸⁰ Recently, we revealed the first X-ray crystal structures of inhibitor-HTLV-I protease complexes with KNI-10562 as a ligand.⁸¹ This crystal structure is very similar to that of the HIV-1 protease complex. This crystal structure is expected to be useful for designing more potent, next-generation HTLV-I protease inhibitor drugs.

SERINE PROTEASE INHIBITORS

Serine proteases generally have catalytic Ser, His, and Asp residues in the active site—the so-called “catalytic triad.” In this section, hepatitis C virus (HCV) NS3 protease, and dipeptidyl peptidase-4 (DPP-4) inhibitors are discussed as representative serine protease inhibitors.

Hepatitis C Virus NS3 Protease Inhibitors

Hepatitis C involves severe liver inflammation and is caused by an RNA virus, HCV. Interferon, HCV NS5b RNA polymerase inhibitor, cyclophilin inhibitor, and HCV NS3 protease inhibitor are well known as anti-HCV agents. Among them, NS3 protease is a serine protease that possesses a serine residue at the active site. The polyprotein that is encoded in the HCV

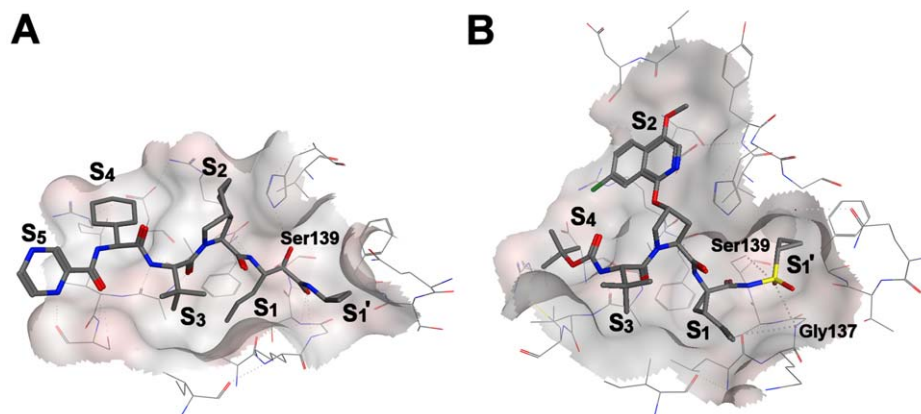


FIGURE 10 Hepatitis C virus (HCV) NS3 protease inhibitors docked in active site of enzyme. (A) Telaprevir, PDB ID: 3SV6. (B) BMS-650032, PDB ID: 4NWL.

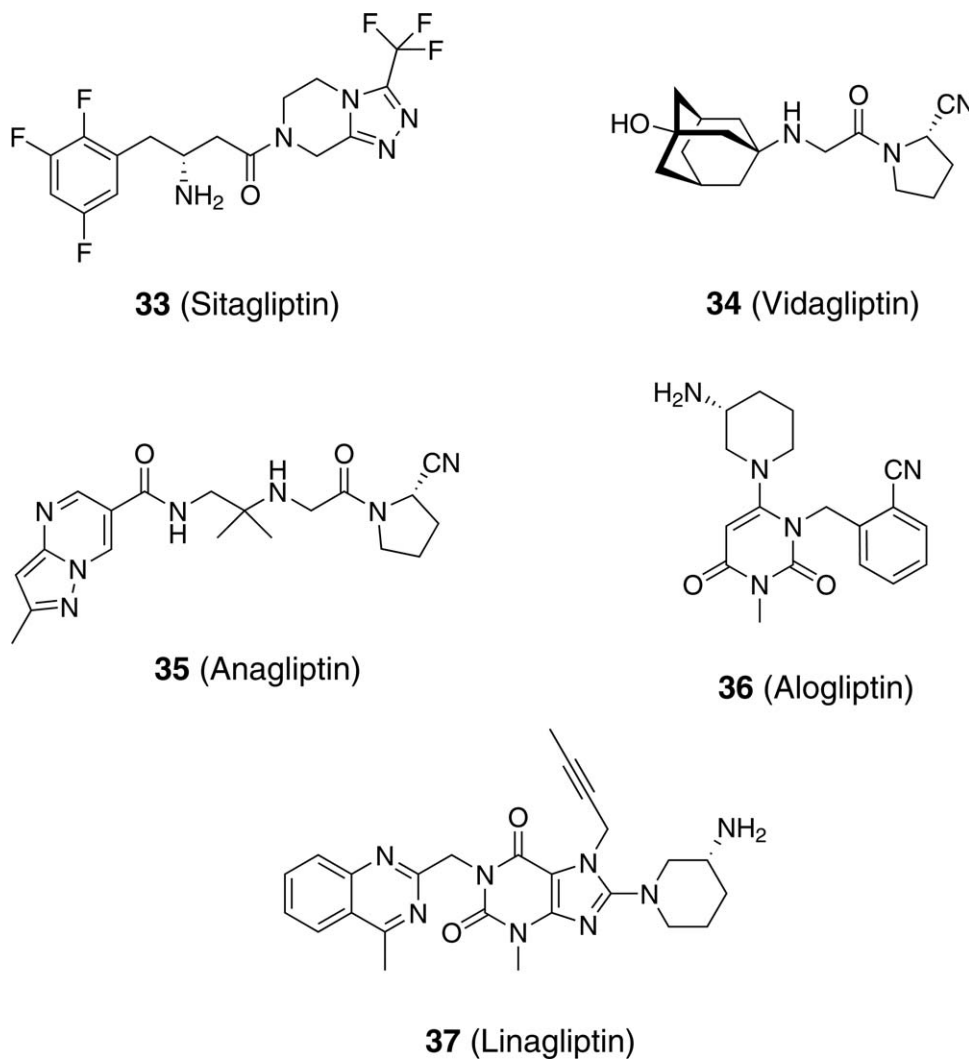


FIGURE 11 Dipeptidyl peptidase (DPP)-4 inhibitors.

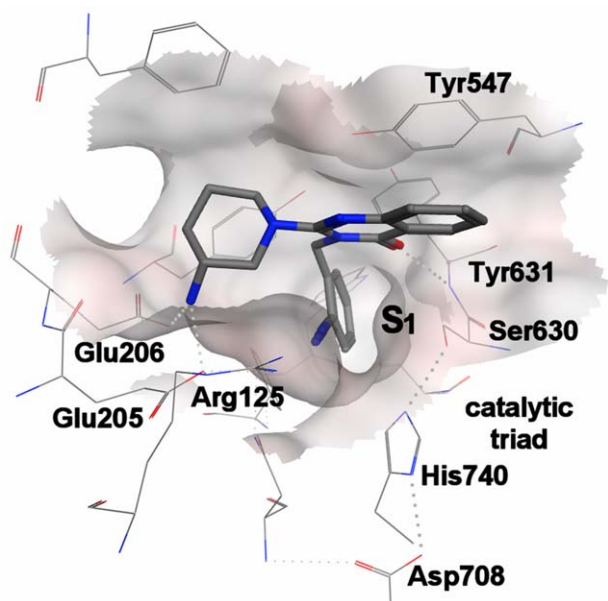


FIGURE 12 X-ray crystal structure of didipeptidyl peptidase (DPP)–4-alogliptin complex (PDB ID: 2ONC).

RNA is proteolytically cleaved into four structural proteins (C, E1, E2, and NS2-NS3-NS4A-NS4B-NS5A-NS5B) by the host's signal peptidase. Subsequently, six nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B are released by self-digestion.^{82–88} The NS3–NS4A complex is responsible for the release of four nonstructural proteins (NS4A, NS4B, NS5A, and NS5B) and is essential for HCV replication.

The first HCV NS3 protease inhibitor **27** (telaprevir, Figure 9)^{89–92} was codeveloped by Vertex Pharmaceuticals and Johnson & Johnson and approved for the treatment of HCV infection by the FDA in 2011. Telaprevir consists of six residues corresponding to the P_{1'} to P₅ positions and possesses an α -ketocarboxamide group at the P₁ position. Compound **28** (boceprevir)^{93,94} also possesses an α -ketocarboxamide group at the P₁ position and was developed by Schering–Plough (merged with MSD in 2009) and approved by the FDA in 2011. Inhibitors possessing an α -ketocarboxamide group would be designed to bind to the active site of the enzyme via covalent bonding between the carbonyl carbon of the α -ketocarboxamide group and the oxygen atom of the Ser139 side chain that is found at the active site. The X-ray crystal structure of the NS3–telaprevir complex (PDB ID: 3SV6) is shown in Figure 10A. As expected, it is shown that the inhibitor's carbonyl carbon binds to the Ser139 side chain via a covalent bond, and the carbonyl carbon forms a tetrahedral structure. It is most likely that the amide carbonyl group next to the carbonyl carbon stabilizes the tetrahedral structure. Subsequently, noncovalent inhibitors **29** (BMS-650032),⁹⁵ **30**

(vaniprevir),⁹⁶ **31** (danoprevir),⁹⁷ and **32** (simeprevir)⁹⁸ were reported by Bristol-Myers Squibb, MSD, Array BioPharma, and Medivir/Johnson & Johnson, respectively. Simeprevir was approved by the FDA in 2013 for use in combination with peginterferon- α and ribavirin. These inhibitors possess a cyclopropanesulfonylamide group at the N-terminus and appear to bind to the active site of the enzyme *via* hydrogen bonding. In the X-ray crystal structure of the NS3 protease–BMS-650032 complex (PDB ID: 4NWL), it is shown that one oxygen atom of the sulfonyl group interacts with the catalytic Ser139 side chain and the amino group of Gly137. The acidity of the sulfonyl group might allow the tight binding between the inhibitors and the hydrophilic active site that consist of Ser139 side chain and Gly137 amino group.

The biggest problem of these protease inhibitors is that drug-resistant variants can emerge rapidly by a single administration of these drugs. Hence, for the treatment of chronic hepatitis C, the NS3 protease inhibitors were commonly used in combination with PEGylated interferon (peginterferon) and a nucleoside inhibitor (ribavirin).⁹⁹ Recently, Bristol-Myers Squibb developed the first all-oral, interferon- and ribavirin-free, hepatitis C treatment—the NS3 protease inhibitor (asunaprevir) and NS5A replication inhibitor (daclatasvir) dual regimen.⁹⁸ Abbvie also developed an interferon- and ribavirin-free multiple dosing regimen consisting of an NS3 protease inhibitor (paritaprevir) with ritonavir in combination with an NS5A replication inhibitor (ombitasvir) and a non-nucleoside polymerase inhibitor (dasabuvir).¹⁰⁰

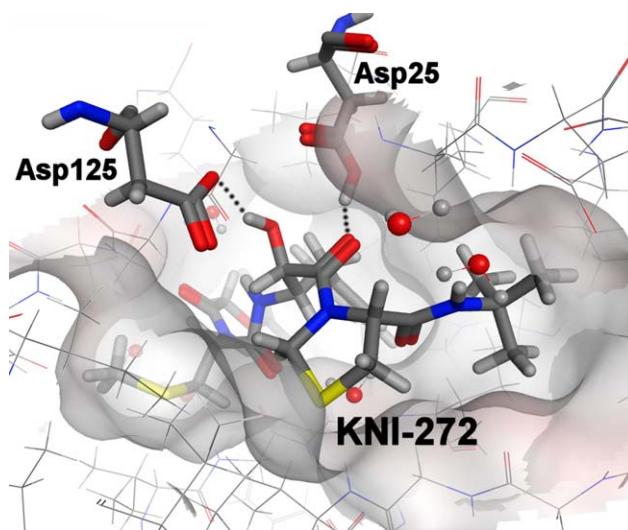


FIGURE 13 The crystal structure of human immunodeficiency virus (HIV)-protease-KNI-272 complex as determined by high-resolution X-ray and neutron diffraction. (PDB ID: 2ONC). The water molecules were depicted by ball and stick model.

DPP-4 INHIBITORS

Incretins are gastrointestinal hormones that increase insulin secretion in response to glucose levels. There are two main incretin hormones: glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP, also known as glucose-dependent insulinotropic peptide).^{101–105} Because these hormones are rapidly cleaved and inactivated by the serine protease DPP-4, its inhibitor is expected to be a drug candidate for type 2 diabetes treatment.^{106,107} DPP-4 recognizes the N-terminal Xaa-Pro residue of peptides, leading to the release of dipeptide Xaa-Pro. Since DPP-4 also recognizes the Xaa-Ala sequence, DPP-4 cleaves the N-terminus of GLP-1 and GIP—the N-terminal dipeptides being His-Ala and Tyr-Ala, respectively. Because Xaa-Ala residues were thought to exist at the N-terminus of many proteins/peptides, DPP-4 inhibitors were predicted to have many side effects. Additionally, it is known that the DPP family consists of nine enzymes. Among them, DPP-8 and DPP-9 have a similar structure and selectivity to DPP-4. Indeed, Lankas et al. reported some toxicities by the DPP-8/-9 inhibition of nonselective DPP-4 inhibitors—in rats, alopecia, thrombocytopenia, reticulocytopenia, enlarged spleen, multi-organ histopathological changes, and mortality, and in dogs, gastrointestinal toxicity.¹⁰⁸ A DPP-4 inhibitor for use as an antidiabetes drug therefore needs to be highly specific. Most DPP-4 inhibitors were designed based on the Phe-Pro sequence of the substrate. The first DPP-4 inhibitor **33** (sitagliptin, Figure 11) was developed by MSD and approved as an oral antidiabetes drug by the FDA in 2006. Sitagliptin has a β -amino acid unit and a unique cyclic amine corresponding to the Phe and Pro residues, respectively.¹⁰⁹ Inhibitors **34** (vidagliptin), **35** (anagliptin), and **36** (alogliptin) possess a cyanopyrrolidine group corresponding to the Pro residue and were developed by Novartis, Sanwa Kagaku Kenkyusho, and Takeda Pharmaceutical Company, respectively.^{110–112} Anagliptin was approved for use in Japan. The cyanopyrrolidine inhibitors are apparently designed based on the covalent bond formation between the cyano-carbon of the inhibitor and catalytic Ser630 side chain of the enzyme. However, alogliptin shows no covalent bonding with the Ser630 side chain in the X-ray crystal structure of the complex with the DPP-4 enzyme (PDB ID: 2ONC) as shown in Figure 11. The cyano group interacts with the Arg125 side chain of the enzyme, and the catalytic Ser635 side chain does not interact with alogliptin; instead, the binding relies on the docking of the inhibitor's phenyl ring into the hydrophobic S₁ pocket, the ionic bonding of inhibitor's amino group with Glu205 and Glu206 side chains, and the π - π stacking interaction between the inhibitor's quinazolinone ring and the Tyr547 side chain appear to allow a potent binding mode. Linagliptin developed by Boehringer Ingelheim and Lilly was

approved by the FDA in 2011. Linagliptin possesses no cyano group. The amino group, the unsaturated alkyl group, and the uracil ring of linagliptin interact with the Glu205 and Glu206 side chains, the S₁ pocket, and the Tyr547 side chain via ionic bonding, hydrophobic interaction, and π - π stacking, respectively, as well as that of alogliptin.

CONCLUSION

Many protease inhibitors have been developed as therapies for diseases such as hypertension, AIDS, adult T-cell leukaemia, malaria, Alzheimer's disease, hepatitis, and diabetes. Inhibitors of aspartic proteases such as renin, HIV-1 protease, malarial plasmepsin, β -secretase, and HTLV-I protease can be designed using the transition state analogue concept. There is no unified design concept in the case of metallo-, serine, and cysteine proteases. For the efficient development of inhibitors for these proteases, new design concepts or technology are required. Despite this difficulty, many drugs have been developed that are based on inhibition of the metalloprotease ACE, and the serine proteases HCV NS3 protease and DPP-4. Although severe acute respiratory syndrome (SARS) coronavirus main protease (M^{PRO}) was not discussed in this review article, M^{PRO} is categorized as a cysteine protease.¹¹³ As a potential treatment against future viral epidemics, the development of new design strategies for protease inhibitor design technologies is extremely important.

Aspartic protease inhibitors can be easily designed using a computational approach such as a docking simulation, *in silico* screening, or *in silico de novo* design because aspartic proteases form a tetrahedral substrate transition state that is relatively simple since it contains no covalent bonds between the enzyme and the substrate. On the other hand, cysteine/serine proteases form a covalent bond with the substrate in the transition state. It is therefore difficult to rationally design a transition state analogue inhibitor of cysteine/serine proteases using a docking simulation, because of the van der Waals repulsion that is generated between the inhibitor and the catalytic amino acid side chain. In the case of the metalloprotease ACE, inhibitor development had to wait until the discovery of a peptide in snake venom, and until then, the design of an inhibitor using a computational approach seemed impossible. In the case of the serine protease hepatitis C virus NS3 protease, α -ketocarboxamide inhibitors such as **27** and **28** were developed at an early stage. These inhibitors formed covalent bonds with the enzyme as shown in Figure 10A. Covalent inhibitors with a ketone or aldehyde group are generally unstable against nucleophilic agents or enzymes *in vivo*. Next, noncovalent inhibitors **29–32**, which feature a cyclopropanesulfonylamide group, were reported. Although the inhibitor's sulfonyl oxygen atom

ARTICLE HIGHLIGHTS

- Proteases are important molecular targets for curing various diseases, because they play crucial roles in various biological processes in all organisms – from viruses to humans.
- Since aspartic proteases form a substrate transition state that possesses no covalent bond between the active site of the enzyme and a substrate, inhibitors with a transition state analogue can be easily designed using a computational approach.
- The inhibitor development of metalloprotease ACE had to wait until the discovery of a peptide found in snake venom.
- Serine/cysteine proteases form a substrate transition state that contains a covalent bond between the catalytic Ser/Cys residue side chain and the substrate. As a result, their inhibitors were developed using various design concepts, not using a unified design concept as is the case for aspartic proteases.
- For the first time, the entire structure of HIV-1 protease in complex with the inhibitor (KNI-272) was determined using high-resolution X-ray and neutron crystallography, and its inhibitory mechanism could be revealed.

This box summarizes key points contained in the article.

interacts with the catalytic Ser side chain by hydrogen bonding (as shown in Figure 10B), the attractive force is weaker than that of a transition state inhibitor. These inhibitors showed potent inhibitory activities by elongation in the direction of S₂ sites. Compounds 30–32 were cyclized to fix their conformation when docked in the active site of the enzyme. Another group of serine proteases, the DPP-4 protease inhibitors 34–36, were designed in anticipation of the covalent bond formed between the inhibitor's cyano carbon and the catalytic Ser side chain. However, the X-ray crystal structure of the NS3 protease complex with 36 shows no covalent bond between the enzyme and the inhibitor as shown in Figure 12. As mentioned previously, it is difficult to rationally design metallo-, serine, and cysteine proteases based purely on the knowledge of the amino acid sequence of their substrate. Inhibitors such as ACE, HCV NS3 protease, and DPP-4 were developed using unique design concepts and bind to the catalytic side chain of the enzyme in a unique manner. In contrast, aspartic protease inhibitors can be designed logically based on the amino acid sequences of substrates. Many aspartic protease inhibitors have

a transition state analogue at the P₁ position, and their structures are simple. Recently, we determined the whole structure of the complex formed between the HIV-1 protease and KNI-272, which contains the location of protons, as shown in Figure 12.¹¹⁴ Although protons cannot be directly observed using conventional X-ray crystallography, the successful determination of all coordinates was achieved, for the first time, using high-resolution X-ray (at 1.4 Å) and neutron (at 1.9 Å) crystallography. As shown in Figure 3, in the cleavage mechanism of aspartic proteases, a carboxylic acid proton and a carboxyl anion in the active site of the enzyme interact with the substrate transition state *via* two hydrogen bonds. The transition state analogue of KNI-272 interacts with a carboxylic acid proton and a carboxyl anion in the same manner and accurately mimics the substrate transition state. Transition state analogue inhibitors allow potent inhibition of enzyme activity because the transition state analogue can bind strongly to the active site of the enzyme and is not cleaved enzymatically.

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