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BACE1 Inhibitor Peptides: Can an Infinitely Small k_{cat} Value Turn the Substrate of an Enzyme into Its Inhibitor?

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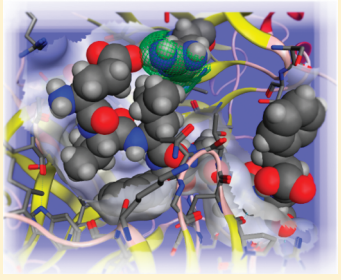
ABSTRACT: Recently, we reported substrate-based pentapeptidic β-secretase (BACE1) inhibitors with a hydroxymethylcarbonyl isostere as a substrate transition-state mimic. These inhibitors showed potent BACE1 inhibitory activity in enzyme and cell assays, with KMI-429 showing *in vivo* inhibition of Aβ production. We also designed and synthesized nonpeptidic and small-sized BACE1 inhibitors using "*in-silico* conformational structure-based design". By studying the structure−activity relationship of these inhibitors, we found that the $\sigma-\pi$ interaction of an inhibitor with the BACE1-Arg235 side chain played a key role in the inhibition of BACE1. We speculated that a peptide capable of binding to the BACE1-Arg235 side chain via the $\sigma-\pi$ interaction might exhibit BACE1 inhibitory activity. Hence, we designed and synthesized a series of peptides that were modified at the P_2 position and found that some of these peptides exhibited a potent BACE1 inhibitory activity despite their structural similarity to the BACE1 substrate.



KEYWORDS: *Alzheimer's disease, β-secretase, BACE1, BACE1 inhibitor, substrate, inhibitor peptide*

β-Secretase, also known as β-site amyloid precursor protein cleaving enzyme 1 (BACE1), is a promising molecular target for developing anti-Alzheimer's disease drugs,[1−7] mainly because it triggers amyloid β (Aβ) peptide formation by cleaving the amyloid precursor protein (APP) at the *N*-terminus of the Aβ domain.[8−13] Recently, we reported potent pentapeptidic BACE1 inhibitors[5,14−19] (KMI-420, KMI-429, KMI-684, and KMI-574) possessing phenylnorstatine [Pns: (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] to be substrate transition-state mimics as shown in Figure 1. Among them, KMI-429[15,16] exhibited strong inhibition of BACE1 activity in cultured cells, and on direct administration into the hippocampi of APP transgenic and wild-type mice, it caused significant reduction in Aβ production.[16] We also designed and reported nonpeptidic and small-sized BACE1 inhibitors, KMI-1027 (IC_{50} = 50 nM)[20] and KMI-1303 (IC_{50} = 9 nM),[21] possessing a heterocyclic scaffold at the P_2 position as shown in Figure 2. KMI-1027 was designed from KMI-429 as a lead compound using the *in-silico* conformational structure-based design, which focused on a conformer of docked inhibitor in BACE1. On establishing that a slightly attractive force such as stacking or $\sigma-\pi$ interaction between the guanidino-plane of BACE1-Arg235 and the P_2 region of the inhibitor plays a key role in the mechanism of BACE1 inhibition,[20] we designed a potent BACE1 inhibitor, KMI-1303, which possesses an electron-rich halogen atom that seemed to interact with the electron-poor guanidino-π orbital of BACE1-Arg235 by Coulomb's force.[20]

Until now, most of the peptidic BACE inhibitors possessing a substrate transition-state analogue, including the ones reported by us, have been designed from the Swedish mutant APP sequence (EVNL*D), which is cleaved faster than the wild-type APP sequence (EVKM*D) by BACE1.[6] The K_m values of the Swedish mutant APP type substrate (9 μM) and wild-type APP type substrate (7 μM) are similar, suggesting that the affinities

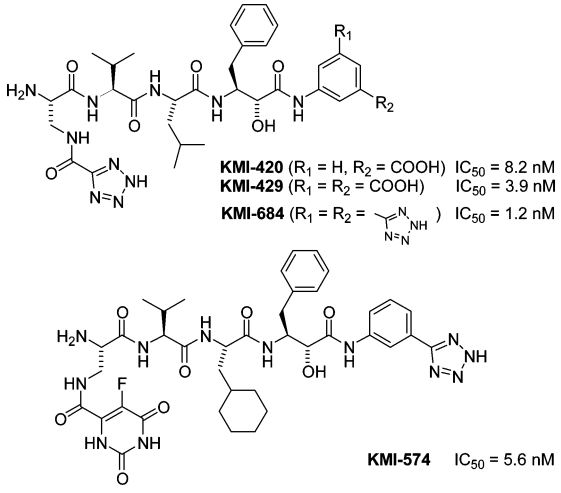


Figure 1. Peptidic BACE1 inhibitor with a substrate transition-state analogue.

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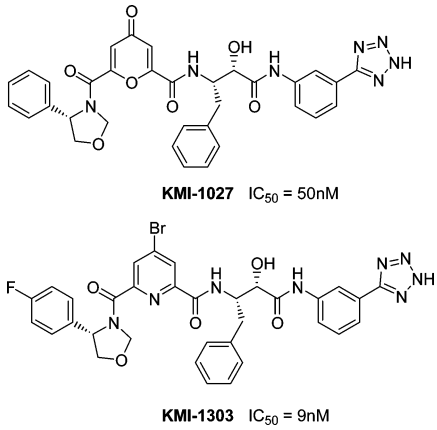


Figure 2. Nonpeptidic BACE1 inhibitors.

for both types of substrates against BACE1 are comparable. However, the Swedish mutant type substrate that has very high k_{cat} value (0.02 s^{-1}) elicited higher catalytic efficiency by BACE1 than did the wild-type APP type substrate (0.002 s^{-1}).[22] We speculated that the hydrogen-bonding interaction between BACE1-Arg235 and the P_2-Asn side chain of the Swedish mutant substrates activates the turnover required for enzymatic catalysis, thereby improving the k_{cat} value. However, strong attractive forces, such as a hydrogen bonding, seem to be unfavorable for enzyme inhibition. In fact, our KMI compounds possessing a hydrogen-bond acceptor or donor, such as the Asn residue, at the P_2 position showed the lowest BACE1 inhibitory activity.[5,21] As described above, we found the interaction between BACE1-Arg235 and its inhibitors to be significant; the guanidino-plane of BACE1-Arg235 in most publicly available X-ray crystal structures of BACE1−inhibitor complexes showed similar figures flopping over the P_2 region of the inhibitors. Moreover, the nearest distances between the guanidino-plane and the P_2 regions showed similar values of about 3 Å. We hypothesized that the guanidino-plane of Arg235 pushed down on the P_2 region of the inhibitors, causing them to be affixed in the active site of BACE1. In addition, a slightly attractive force, such as stacking or $\sigma-\pi$ interaction, might pack down the inhibitors effectively in the active site. Hence, we speculated that such an interaction at the S_2 site reduces the turnover of an enzyme, and a substrate possessing a vastly reduced k_{cat} value might turn into an inhibitor (Figure 3). Herein, we designed a series of peptides that were modified at the P_2 position.

Peptides **1**−**22** were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis methods; the synthesis of peptide **15** is outlined in Scheme 1. In brief, the Fmoc-protected amino acid was attached to 2-chlorotrityl chloride resin using diisopropylethylamine (DIPEA) in 1,2-dichloroethane (DCE). The Fmoc group was removed with 20% piperidine in DMF, and peptide bonds were formed using diisopropylcarbodiimide (DIPCDI) as a coupling reagent in the presence of 1-hydroxybenzotriazole (HOBt). After the peptide chain was elongated, cleavage from resin was achieved using trifluoroacetic acid (TFA) in the presence of *m*-cresol, thioanisole, and water. All peptides were purified by preparative RP-HPLC. BACE1 inhibitory activity was determined by an enzyme assay using a recombinant human BACE1 and a fluorescence resonance energy transfer (FRET) substrate as previously reported.[5,14−21] The enzymatic reaction was performed by incubating BACE1 and the FRET substrate (7-methoxycoumarin-4-yl)acetyl-Ser-Glu-Val-Asn-Leu*Asp-Ala-Glu-Phe-Arg-Lys(2,4-dinitrophenyl)-Arg-Arg-NH_2) together with 2.0 or 0.2 μM KMI compounds, in incubation buffer, and the *N*-terminal cleavage fragment of the substrate was analyzed by RP-HPLC with fluorescence detection.

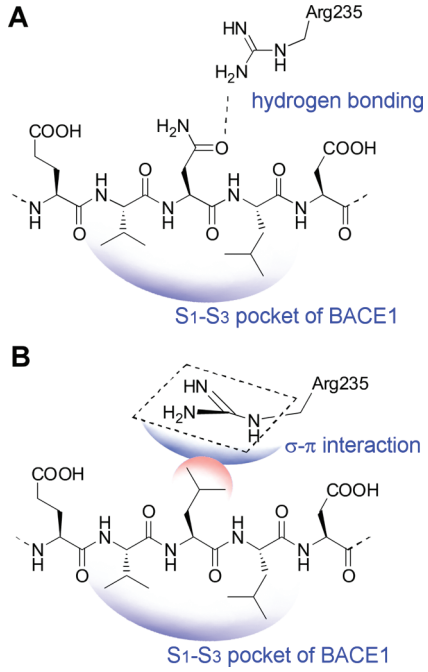
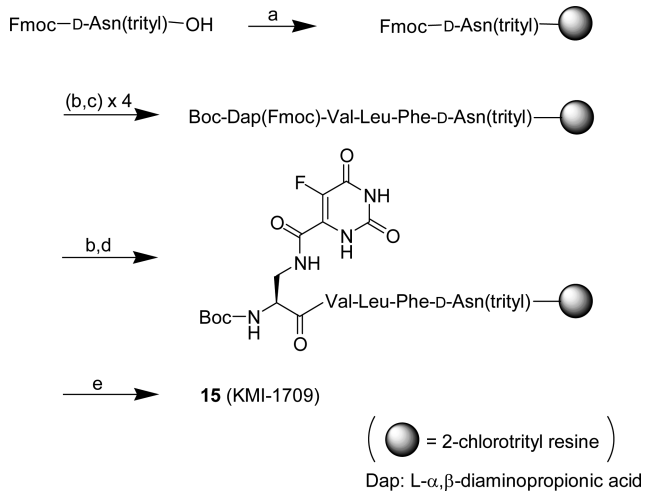


Figure 3. Interaction of substrates with the BACE1-Arg235. (A) Swedish mutant type substrate. (B) BACE1 inhibitor peptide with a P_2 Leu residue. The P_2 Leu side chain seemed to interact with the guanidino-plane of BACE1-Arg235 by a weak $\sigma-\pi$ interaction.

Scheme 1. Synthesis of Inhibitor 15[a]



[a]Reagents and conditions: (a) 2-Chlorotrityl chloride resin, DIPEA, DCE; (b) 20% piperidine/DMF; (c) protected amino acid, DIPCDI, HOBt/DMF; (d) 5-fluoroorotic acid, DIPCDI, HOBt/DMF; (e) TFA, *m*-cresol, thioanisole, H_2O.

Because our KMI compounds with a P_2 Leu residue and P_1 substrate transition-state analogue exhibited potent BACE1 inhibitory activity and the side chain of P_2 Leu seemed to interact with the guanidino-plane of BACE1-Arg235 by a weak $\sigma-\pi$ interaction, we first synthesized some peptides possessing a Leu residue at the P_2 position as shown in Table1. The octapeptides **1**−**6** exhibited weak inhibitory activity. Because

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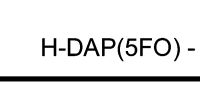
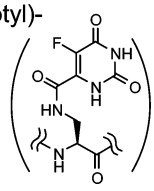
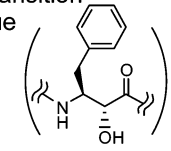
Table 1. Peptides That Showed BACE1 Inhibitory Activities

Compound	P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	BACE1 inhibition % at 2 μM
1 (KMI-1634)	H-Glu - Val - Leu - Phe - Ser - Ala - Glu - Phe-OH	6
2 (KMI-1638)	H-Glu - Val - Leu - Phe- D-Ser -Ala - Glu - Phe-OH	28
3 (KMI-1635)	H-Glu - Val - Leu - Phe - Cys - Ala - Glu - Phe-OH	<5
4 (KMI-1639)	H-Glu - Val - Leu - Phe- D-Cys -Ala - Glu - Phe-OH	28
5 (KMI-1632)	H-Glu - Val - Leu - Phe - Dap - Ala - Glu - Phe-OH	7
6 (KMI-1633)	H-Glu - Val - Leu - Phe- D-Dap-Ala - Glu - Phe-OH	15
7 (KMI-1705)	H-Glu - Val - Leu - Phe-D-Ser-OH	23
8 (KMI-1708)	H-Glu - Val - Leu - Phe-D-Asn-OH	29
9 (KMI-1006)	H-Glu - Val - Leu - Phe-OH	8
10 (KMI-1504)	H-Glu - Val - Lys - Phe-OH	10
11 (KMI-1855)	H-Glu - Val - Asn - Phe-OH	<5

Table 2. Peptides with a 5-Fluoroorotyl Moiety at the P4 Position

Compound	P_4 P_3 P_2 P_1 P_1'	BACE1 inhibition % at 2 μM	BACE1 inhibition % at 0.2 μM	IC_{50} (nM)
12 (KMI-1791)	H-DAP(5FO)[*1]- Val - Leu - Phe - Ser -OH	85	48	263
13 (KMI-1706)	H-DAP(5FO) - Val - Leu - Phe-D-Ser -OH	94	57	138
14 (KMI-1792)	H-DAP(5FO) - Val - Leu - Phe - Asn -OH	89	56	173
15 (KMI-1709)	H-DAP(5FO) - Val - Leu - Phe-D-Asn -OH	95	65	86
16 (KMI-1858)	H-DAP(5FO) - Val - Asn - Phe - Asn -OH	<5	—	—
17 (KMI-1793)	H-DAP(5FO) - Val - Leu - Phe-D-Ala -OH	93	53	169
18 (KMI-1794)	H-DAP(5FO) - Val - Leu - Phe-D-Val -OH	94	69	82
19 (KMI-1707)	H-DAP(5FO) - Val - Cha[*3]- Phe-D-Ser -OH	92	54	163
20 (KMI-1710)	H-DAP(5FO) - Val - Cha - Phe-D-Asn -OH	92	58	141
21 (KMI-1795)	H-DAP(5FO) - Val - Leu - Phe - OH	90	54	184
22 (KMI-1857)	H-DAP(5FO) - Val - Asn - Phe - OH	<5	—	—
KMI-446	H-DAP(5FO) - Val - Leu - Pns[*2]– 	99	82	26

[*1]DAP(5FO): N^{β}-(5-Fluoroorotyl)-L-2,3-diaminopropionic acid 

[*2]Pns: a substrate transition -state analogue 

[*3]Cha:L-cyclohexylalanine

peptides **2**, **4**, and **6** containing a D-amino acid residue showed slightly higher inhibitory activity, pentapeptides **7** and **8** possessing a D-amino acid were synthesized, and they replicated the inhibitory activity of the octapeptides. Peptide **9**, which lacked the P_1' residue, also showed slightly weak inhibitory activity; on the other hand, it is noteworthy that peptide **11**, with the P_2—Asn residue corresponding to the Swedish mutant APP sequence, showed no inhibitory activity. Peptide **10**, however, with the P_2-Lys residue corresponding to the wild-type APP sequence, showed slightly higher inhibitory activity than did **9** and **11**. This supports the fact that the K_m value of the wild-type APP type substrate is slightly lower than that of the Swedish mutant APP type substrate, and thereby, the wild-type APP sequence, rather than the Swedish mutant APP sequence, possesses higher affinity against BACE1.

Previously, we reported that replacing the carboxylic acid of the P_4-Glu residue in peptidic KMI-compounds with its bioisostere, such as a tetrazole ring or a 5-fluoroorotyl group, improved their BACE1 inhibitory activities.[17,18] Hence, we designed a series of BACE1 inhibitors containing a 5-

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fluoroorotyl group in the P_4-amino acid residue as shown in Table 2. Peptides **12**−**15** and **17**−**20** showed potent BACE1 inhibitory activity. In particular, peptides **13**, **15**, and **17**−**20**, possessing a D-amino acid residue at the P_4 position, exhibited potent inhibitory activity comparable to that of KMI-446[18] (99% inhibition at 2 μM, IC_{50} = 26 nM) with a substrate transition-state analogue. In contrast, peptide **16**, with the P_2−Asn residue corresponding to the Swedish mutant APP sequence, showed no inhibitory activity, as predicted. Although we reported that KMI compounds possessing a cyclohexylalanine (Cha) residue at the P_2 position showed improved BACE1 inhibitory activity,[18] peptides **19** and **20** with a Cha residue showed inhibitory activity similar to that of the corresponding peptides **13** and **15**. Because peptide **21** lacking the P_1' residue also showed potent BACE1 inhibitory activity, peptide **21** that is released from peptides **12**−**15**, **17**, and **18** by cleavage at their P_1−P_1' amide bonds might inhibit BACE1. Hence, we performed the incubation experiment using the same concentrations of BACE1 and BACE1 inhibitors used in our BACE1 inhibitory assay. However, peptides **12**−**15** were not cleaved by incubation for 1 or 24 h (1 h corresponds to the BACE1 inhibition assay incubation time), and therefore, peptide **21** was not detected by HPLC analysis of the incubated buffer solution.

How can a common peptide without a substrate transition-state analogue inhibit enzyme activity? One possible mechanism of inhibition is that the transition-state of these peptides might stably bind to BACE1 because of their vastly reduced k_{cat} value, which is a result of the σ−π interaction between the P_2 region of peptides and BACE1-Arg235. Although this hypothesis seems reasonably explained, it is difficult to prove. However, examples of inhibitors with the trifluoromethylketone moiety as a transition-state analogue that can bind to an enzyme, such as cysteine or serine protease, and form complexes with the tetrahedral structure of trifluoromethylketone's carbonyl carbon at the C-terminus of inhibitors, have already been reported.[23] Another possibility is that inhibitor peptides might bind to an active site of BACE1 without change in the peptide bond. To investigate this, we performed the docking simulation study under the MMFF94x force field using MOE software (Chemical Computing Group Inc., Canada). Octapeptide **2** bound in BACE1 (PDB ID: 1FKN) was shown in Figure 4A. The reason that octapeptides **2**, **4**, and **6**, with a D-amino acid, showed higher inhibitory activity than peptides **1**, **3**, and **5**, with a corresponding L-amino acid, seems to be the small space corresponding to a water molecule between the peptides and the two catalytic side chains of BACE1. This space might be important for the cleavage mechanism of an aspartic protease. The hydrophilic P_1'-side chains of octapeptides **2**, **4**, and **6** might occupy this space, allowing them to bind stably to BACE1. On the other hand, pentapeptides **7**, **8**, **13**, **15**, and **17**−**20** with a D-amino acid also showed higher inhibitory activity than did pentapeptides with a corresponding L-amino acid. We considered that the same phenomenon occurred in octapeptides and pentapeptides but for different reasons. It is possible that the α-carboxylic group and the side chain at the P_1' position of these pentapeptides counterchange their positions, permitting effective binding to the active site of BACE1 as shown in Figure 4B. This is supported by the fact that pentapeptide **18**, with a bulky Val residue at the same position, also showed potent inhibitory activity and is unable to fit into the small space. Additionally, the α-carboxylic group of the P_1' residue might interact with the hydrophilic S_1'-pocket of BACE1.

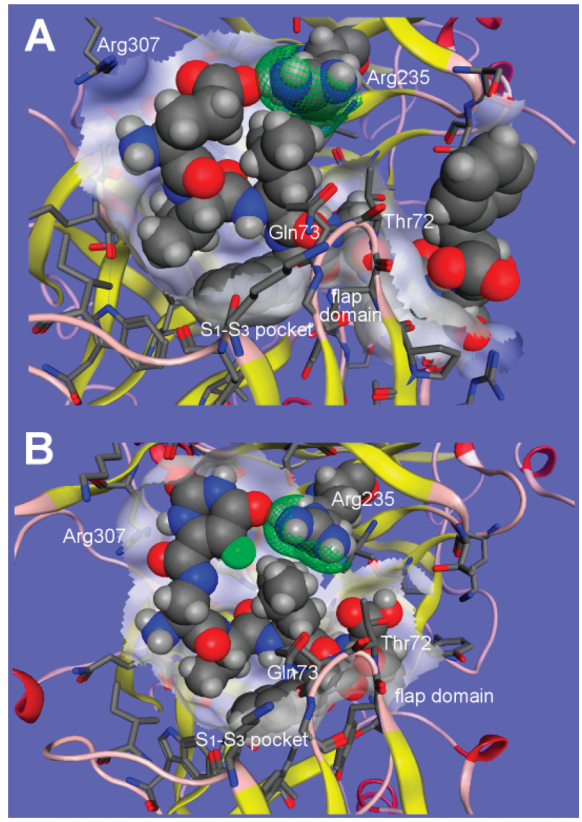

Figure 4. Octapeptide **2** (A) and pentapeptide **13** (B) bound in BACE1 (PDB ID: 1FKN). Inhibitor peptides and Arg235 were depicted by space-filling modeling. The cartoon and stick models indicate BACE1 enzyme. The P_1' regions of the inhibitors are behind the terms "Thr72" situated in the right upper parts of flap domains in both models A and B.

In conclusion, we designed a series of peptides possessing a P_2-Leu residue that seemed to reduce the turnover in the BACE1 inhibitory mechanism, and we found some peptides with weak inhibitory activity. We also synthesized a series of peptides with a 5-fluoroorotyl moiety in the P_4-amino acid residue. Peptides KMI-1706, KMI-1709, KMI-1793, and KMI-1794 showed potent BACE1 inhibitory activity, comparable to that of KMI-446 with a substrate transition-state analogue. KMI-1858, possessing the same P_2-Asn residue as the Swedish mutant APP that seemed to have higher affinity for the active site of BACE1, showed no inhibitory activity, suggesting that the interaction of inhibitors and substrates with BACE1-Arg235 plays a critical role in the inhibition and catalytic action of BACE1, respectively. These findings, describing the interaction of BACE1-Arg235 with the P_2 position of inhibitors/substrates, may be important for developing the next generation of BACE1 inhibitors. To the best of our knowledge, this is the first study to report that the concepts for designing substrates and inhibitors are fundamentally different. Thus, the finding that a common peptide without a substrate transition-state analogue can inhibit BACE1 activity can spur future research toward developing "a gene-based therapy using a DNA that codes for peptide sequence with BACE1 inhibitory activity".

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Notes

The authors declare no competing financial interest.

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