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β-Secretase inhibitors: Modification at the P_4 position and improvement of inhibitory activity in cultured cells

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Abstract—Recently, we reported potent and small-sized β-secretase (BACE1) inhibitors KMI-570 and KMI-684 in which we replaced carboxylic acid groups at the P_1' position of KMI-420 and KMI-429, respectively, with tetrazole derivatives as carboxylic acid bioisosteres. These modifications improved significantly BACE1 inhibitory activity and chemical stability. In this study, the acidic tetrazole ring of the P_4 position of KMI-420 and KMI-570, respectively, was replaced with various hydrogen bond acceptor groups. We found BACE1 inhibitor KMI-574 that exhibited potent inhibitory activity in cultured cells as well as *in vitro* enzymatic assay.

According to the amyloid hypothesis,[1] β-secretase [BACE1: β-site APP (amyloid precursor protein) cleaving enzyme] is a molecular target for therapeutic intervention in Alzheimer's disease (AD),[2–6] because BACE1 triggers amyloid β (Aβ) peptide formation by cleaving APP at the N-terminus of the Aβ domain.[7–12] Recently, we reported small-sized BACE1 inhibitors KMI-420 (**1**) and KMI-429 (**2**),[13] that contained phenylnorstatine [Pns: (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] as a substrate transition-state mimic.[14] KMI-429 exhibited effective inhibition of BACE1 activity in cultured cells, and significant reduction of Aβ production *in vivo* (APP transgenic and wild-type mice).[13b] Furthermore, KMI-570 (**3**) and KMI-684 (**4**), in which the carboxylic acids at the P_1' position of KMI-420 and KMI-429, respectively, were replaced with tetrazole rings, showed more potent BACE1 inhibitory activity[15] (Fig. 1). According to structure–activity relationship

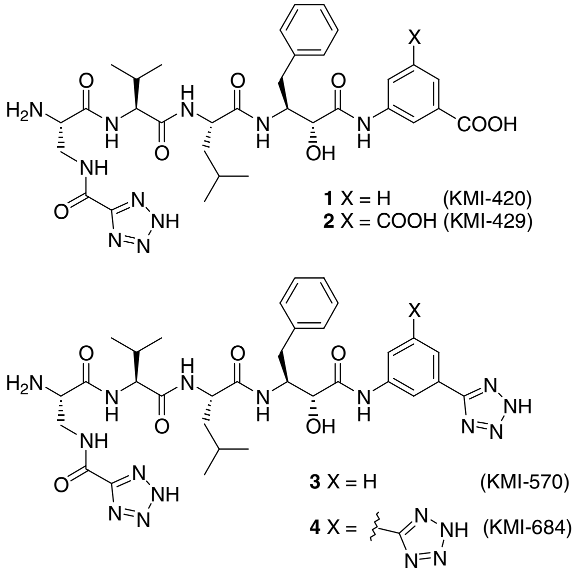

Figure 1. Structures of BACE1 inhibitors containing a tetrazole ring at the P_4 position.

Keywords: Alzheimer's disease; BACE1 inhibitor; Cultured cells.

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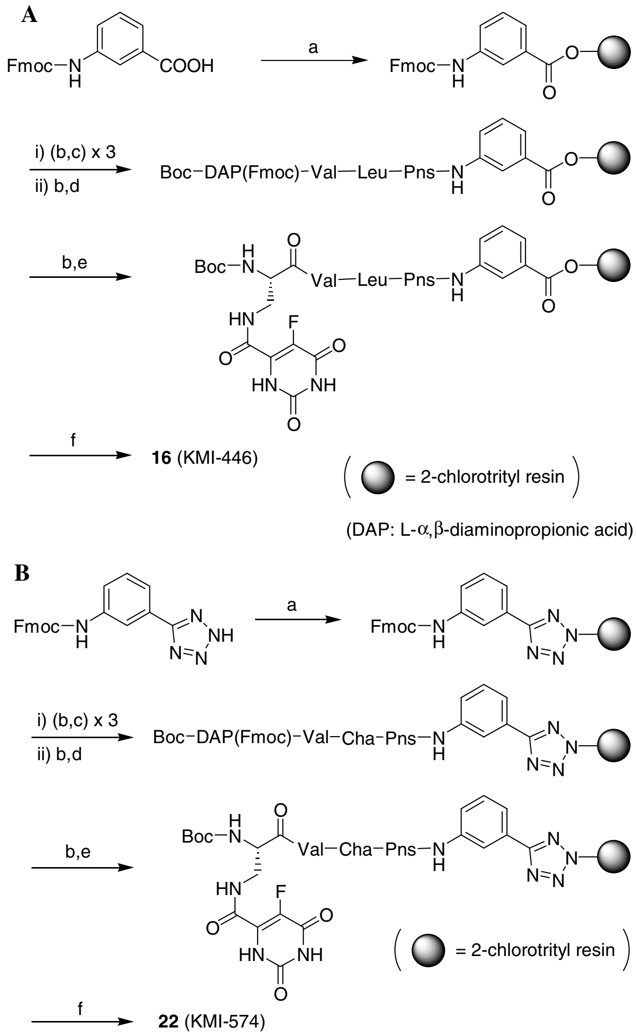
studies of KMI-compounds, an acidic moiety at the P_4 position is required for improving BACE1 inhibitory activity. Moreover, according to substrate specificity[16] and crystal structure[17] studies of BACE2, an acidic moiety at the P_4 position is thought to be important for selectivity against BACE2. Inhibition of BACE2, a homologue enzyme of BACE1,[18,19] by nonselective BACE1 inhibitors may compromise the desired decrease of Aβ, because BACE2 has been reported to regulate Aβ formation as well as α-secretase as an indirect antagonist of BACE1.[20,21] Hence, the presence of an acidic moiety or hydrogen bond acceptor at the P_4 position of BACE1 inhibitors would improve selectivity over BACE2, because of favored interactions with Arg307 in the S_4 pocket of BACE1 over the corresponding Gln in BACE2.[17] However, acidic moieties often possess low membrane permeability across the blood–brain barrier. In this paper, we replaced the respective acidic tetrazole ring at the P_4 position of KMI-420 and KMI-570 with other hydrogen bond acceptor groups and evaluated BACE1 inhibitory activity in cultured cells (BACE1-transfected HEK293 cells) in order to develop practical anti-Alzheimer's disease drugs. By replacing with a 5-fluoroorotyl group at the P_4 position and L-cyclohexylalanine (Cha) residue at the P_2 position likewise, we found BACE1 inhibitor KMI-574 that showed potent BACE1 inhibitory activity in cultured cells as well as *in vitro* enszymatic assay.

BACE1 inhibitors **5**–**22** were synthesized by Fmoc-based (9-fluorenylmethoxycarbonyl) solid-phase peptide synthesis methods according to previously reported procedures.[13a] As examples, the syntheses of inhibitors **16** and **22** are outlined in Scheme 1. Briefly, *N*-Fmoc-3-aminobenzoic acid or *N*-Fmoc-5-(3-aminophenyl)tetrazole was attached to 2-chlorotrityl chloride resin using diisopropylethylamine (DIPEA) in dichloromethane (DCM). The Fmoc group was removed with 20% piperidine in DMF and peptide bonds were formed using diisopropylcarbodiimide (DIPCDI) as coupling reagent in the presence of 1-hydroxybenzotriazole (HOBt). After elongating the peptide chain, cleavage from the resin was achieved using trifluoroacetic acid (TFA) in the presence of *m*-cresol and thioanisole. The crude peptide was purified by preparative RP-HPLC.

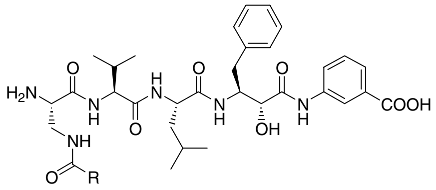


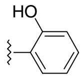
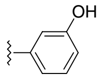
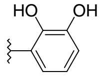
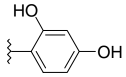
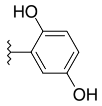
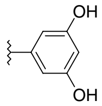
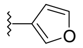
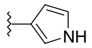
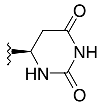
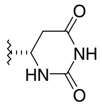
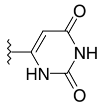
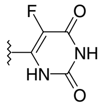
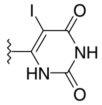
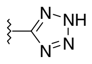
Scheme 1. Reagents: (a) 2-Chlorotrityl chloride resin, DIPEA, DCM; (b) 20% piperidine/DMF; (c) Fmoc-AA-OH, DIPCDI, HOBt, DMF; (d) Boc-DAP(Fmoc)-OH, DIPCDI, HOBt, DMF; (e) 5-fluoroorotic acid, DIPCDI, HOBt, DMF; (f) TFA, *m*-cresol, thioanisole.

BACE1 inhibitory activity of the inhibitors was determined by enzymatic assay using a recombinant human BACE1 and FRET (fluorescence resonance energy transfer) substrate as previously reported.[5] After the enzymatic reaction with BACE1 and 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, in incubation buffer with 2 or 0.2 μM KMI-compounds, the N-terminal cleavage fragment of the substrate was analyzed by RP-HPLC with fluorescence detection. BACE1 inhibitory activity in cultured cells was determined in the manner reported by Asai.[13b] HEK293 cells that stably expressed human BACE1 enzyme (BACE1-HEK293) were cultured in 60 mm dishes until 80–100% confluent (37 °C, 5% CO_2 incubator). After replacement by new serum-free medium with or without KMI-compounds (100 μM), BACE1-HEK293 cells were further incubated for 6 h. After precipitating the protein fraction by treatment with trichloroacetic acid, the fraction was mixed with sample buffer containing 2-mercaptoethanol and subjected to 10% SDS–PAGE. To detect sAPPβ (soluble APPβ: N-terminus domain that is released from APP by cleaving at the β-site by BACE1), Western blotting using anti-sAPPβ polyclonal antibody was performed. According to the fluorescence imaging of the bands on the blotting membranes, the amount of sAPPβ peptide, assumed to be the index of BACE1 activity, was measured. BACE1 inhibitory activity in cultured cells was determined by calculating the decrease rate of sAPPβ levels against the control using DMSO instead of KMI-compounds solution.

We selected inhibitor **1** (KMI-420), possessing a carboxylic acid at the P_1' benzene ring, as a parent compound and replaced its tetrazole ring at the P_4 position with various functional groups. The BACE1 inhibitory activity of compounds **5**–**17** is summarized in Table 1. Inhibitors **6**–**10** and **15**–**17**, in particular **9** and **16**, that contain

Table 1. Effects of P_4 modification on BACE1 inhibitory activity of KMI-420



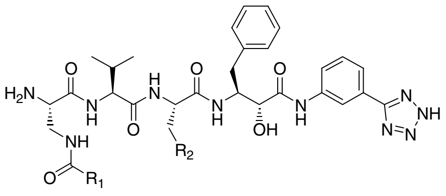
Compound (KMI No.)	R	BACE1 inhibition (%)		BACE1 inhibition (%) in cultured cells at 100 μM
		at 2 μM	at 0.2 μM	
5 (KMI-471)		75	—	29
6 (KMI-479)		91	49	57
7 (KMI-520)		89	75	~0
8 (KMI-521)		86	59	20
9 (KMI-522)		98	84	58
10 (KMI-473)		94	60	69
11 (KMI-523)		82	38	4
12 (KMI-468)		69	—	—
13 (KMI-508)		40	—	—
14 (KMI-509)		13	—	—
15 (KMI-467)		90	54	46
16 (KMI-446)		99	82	76
17 (KMI-510)		99	72	73
1 (KMI-420)		99	87	—

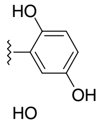
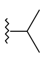
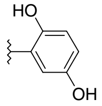
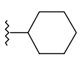
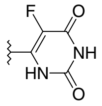
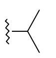
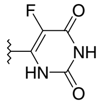
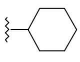
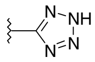
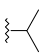
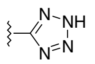
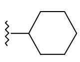
aromatic hydrogen bond acceptors at the P_4 position showed potent BACE1 inhibitory activity by *in vitro* enzymatic assay. In cultured cells, inhibitors **16** and **17** possessing ureide derivatives at the P_4 position exhibited higher BACE1 inhibitory activities in cultured cells than inhibitors **5–11** and **15**.

Replacing the Leu residue at the P_2 position with the unnatural amino acid Cha slightly enhanced BACE1 inhibitory activity (**18** cf. **3**, Table 2). Peptides containing unnatural amino acids are expected to possess improved stability *in vivo*. Using 2,5-dihydroxybenzoyl or 5-fluoroorotyl groups, corresponding inhibitors **9** and **16** at the P_4 position and Leu or Cha residues at the P_2 position, we synthesized inhibitors **19–22** that contain a tetrazole ring on the P_1' benzene ring. As shown in Table 2, inhibitors **19–22** exhibited potent BACE1 inhibitory activity by *in vitro* enzymatic assay that is similar to reference compounds **3** (KMI-570) and **18** (KMI-571). In cultured cells, compound **22** containing a P_4 5-fluoroorotyl group and P_2 Cha residue showed potent BACE1 activity, while compound **21** containing a P_2 Val residue and compounds **19** and **20** containing P_4 phenol derivatives showed moderate inhibitory activity. The difference in BACE1 inhibitory activity between *in vitro* enzymatic and cultured cell-based assay is thought to be dependent on the cell membrane permeability of the inhibitor. Because endogenous BACE1 is localized predominantly in the later Golgi and *trans*-Golgi network,[22,23] and thought to cleave at the β-site of APP on the *trans*-Golgi network mainly,[23] the synergistic effect of inherent BACE1 inhibitory activity by *in vitro* enzymatic assay and its cell permeability is believed to reflect BACE1 inhibitory activity in cultured cells.

Inhibitors **21** and **22** with 5-fluoroorotyl group at the P_4 position exhibited potent BACE1 inhibitory activity by *in vitro* enzymatic assay that is almost similar to that of inhibitor **3** with a tetrazole ring at the same position. Orotyl derivatives, although not classified as bioisosteres for carboxylic acid, and tetrazole rings play a role in enhancing BACE1 inhibitory activity. In order to find structure-based explanations, computational docking simulations were performed using available coordinates for BACE1 (PDB ID: 1W51), using previously reported methods.[13a] The structure of inhibitor **22** docked in BACE1, and that of superimposed previously reported inhibitor **4**, which possesses tetrazole rings at the P_4 and P_1' positions, are shown in Figure 2. In docking experiments, 5-fluoroorotyl and tetrazole-5-carbonyl

Table 2. Effects of P_4 modification on BACE1 inhibitory activity of KMI-570 and KMI-571



Compound (KMI No.)	R_1	R_2	BACE1 inhibition (%)		IC_{50}[a] (nM)	BACE1 inhibition (%) in cultured cells at 100 μM
			at 2 μM	at 0.2 μM		
19 (KMI-573)			99	93	—	65
20 (KMI-575)			99	92	—	69
21 (KMI-572)			100	98	6.5	63
22 (KMI-574)			100	97	5.6	84
3 (KMI-570)			100	98	4.8	66
18 (KMI-571)			100	98	3.3	59

[a] IC_{50} values in *in vitro* enzymatic assay.

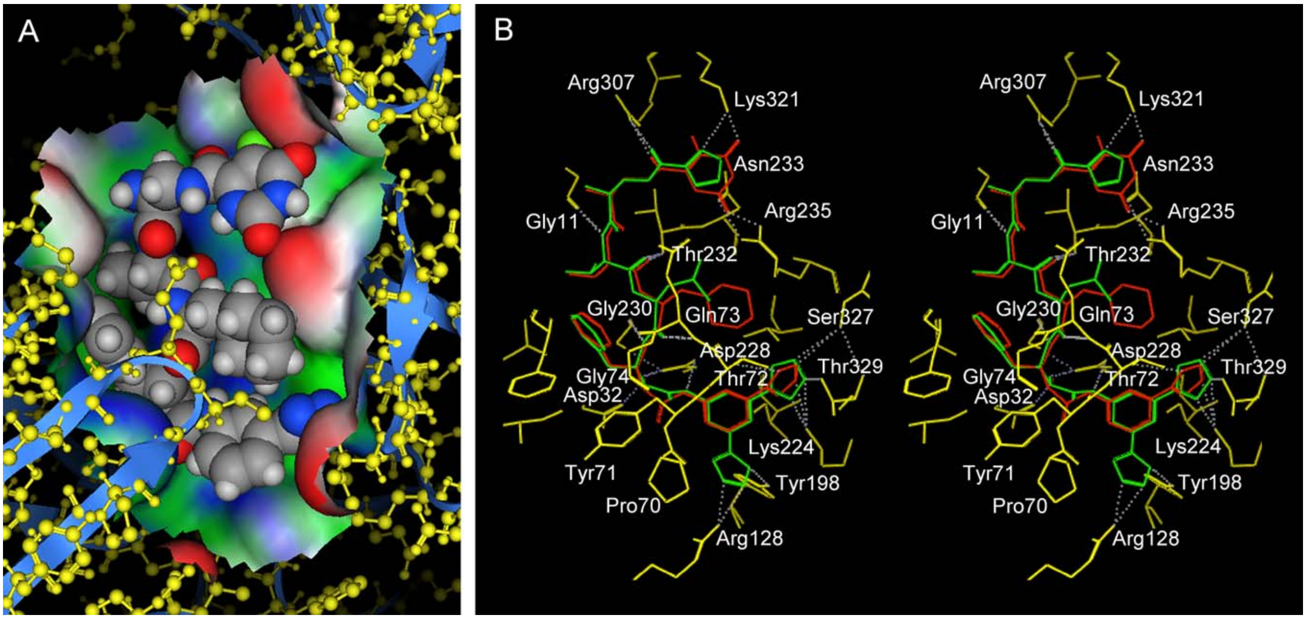


Figure 2. Docked inhibitor **22** (KMI-574) in BACE1 enzyme (PDB ID: 1W51). (A) Overview of docked inhibitor **22** (space-filling model) in the binding site of BACE1 enzyme. Yellow ball-and-stick and colored molecular-surface models indicate BACE1 and its binding site except for the flap domain from Pro70 to Gly74, respectively. (B) Stereoscopic view of superimposed inhibitors **22** (red lines) and **4** (KMI-684, green lines). Yellow and white dashed lines indicate BACE1 enzyme and hydrogen bond interactions, respectively.

groups each assumed the same pose in the binding pocket of BACE1 that consists of Arg307, Lys321, and Arg235, whereas the former group is larger than the latter. On the other hand, inhibitor **16**, which contained a fluorine atom on the aromatic ring, showed higher BACE1 inhibitory activity than inhibitors **15** and **17**. The strong electron-withdrawing fluorine atom raises the electrophilicity of the aromatic ring, consequently enhancing the hydrogen bond acceptor character of the P_4 side chain. This effect is likely to improve BACE1 inhibitory activity.

High affinity for the BACE1 enzyme does not necessitate high BACE1 inhibition in cultured cells because of various factors affecting cellular penetration. Bhhatarai and Garg have used calculated $\log P$ (Clog$P$) and calculated molar refractivity (CMR) values to predict the inhibitory activity of HIV-1 protease inhibitors.[24] (k_1 Clog$P$–k_2 ClogP^2) can be seen as the inhibitor's lipophilicity value, while (k_3 CMR–k_4 CMR^2) represents the inhibitor's steric effect, that both would favor internalization of the inhibitor into the cell. The following QSAR equation, derived from compounds **3**, **8**, **9**, **11**, and **15**–**22**, was obtained with a high regression fit ($r^2 = 0.933$) and significance ($p < 0.001$) when inhibitors **6**, **7**, and **10** were excluded as outliers by cross-validation.

$$\begin{aligned}\log(\mathrm{Inh_{cell}}) = {} & 1.889\log(\mathrm{Inh_{enz}}) - (0.169\mathrm{Clog}P \\ & - 0.042\mathrm{k_1Clog}P^2) - (4.351\mathrm{CMR} \\ & - 0.104\mathrm{CMR}^2) - 45.666 \qquad (1)\end{aligned}$$

$$n = 12,\ r^2 = 0.933,\ F = 16.679,\ p < 0.001$$

$\mathrm{Inh_{cell}}$ denotes percent BACE1 inhibition with 100 μM in cultured cells, while $\mathrm{Inh_{enz}}$ denotes percent BACE1 inhibition with 0.2 μM inhibitor by enzymatic assay. Clog$P$ and CMR values were calculated with ChemDraw Ultra 6, while statistical evaluations were performed in Microsoft Excel 2003.

The QSAR equation predicts that an inhibitor with Clog$P$ of less than −1.006 and CMR of 21.080 would exhibit potent cellular activity. It should be noted that an inhibitor with a low Clog$P$ value may have a detrimental CMR value, because relationships exist between Clog$P$ and CMR. Calculations also reveal that the inhibitor's intrinsic activity ($\mathrm{Inh_{enz}}$) is the major determinant, while Clog$P$ is a lesser determinant for cellular inhibitory activity than CMR, suggesting that CMR is a more accurate predictor than Clog$P$. Although the results obtained from the equation are rough estimates (Fig. 3), the QSAR equation is invaluable to design BACE1 inhibitors with high cellular activity, especially when the QSAR equation

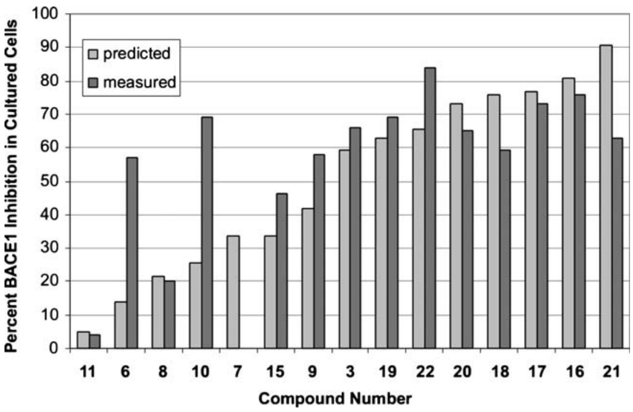


Figure 3. Predicted BACE1 inhibitory activities in cultured cells using multiple linear regression by Eq. 1 and their observed values for inhibitors **3**, **6**–**11**, and **15**–**22**.

is further refined as more cellular BACE1 inhibition data become available in future research.

In conclusion, BACE1 inhibitors were designed and synthesized using various hydrogen bond acceptor groups at the P_4 position and Cha residue replacement at the P_2 position. BACE1 inhibitor **22** (KMI-574) exhibited potent BACE1 inhibitory activity in cultured cells as well as *in vitro* enzymatic assay. These findings are a stepping stone forward to overcome a key issue in developing of anti-AD drugs, namely to improve cell and blood–brain barrier permeability.

Acknowledgments

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