Design of Substrate-Based Inhibitors of Human β-Secretase

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Abstract: By use of the effectively cleaved β-secretase (BACE) substrate (**1**), incorporation of a statine in P_1 resulted in a weak inhibitor **13** of the enzyme. Further substitution of P_1'-Asp by P_1'-Val in **13** results in a potent inhibitor **22** of BACE. Removal of the $P_{10}-P_5$ residues on the N-terminal part of inhibitor **22** resulted in no loss of potency (**23**). C-terminal truncations of inhibitor **22** generally led to significant loss of potency.

Introduction. The proteolysis of the membrane-anchored amyloid precursor protein (APP) results in the generation of the amyloid β (Aβ) peptide that is thought to be causal for the pathology and subsequent cognitive decline in Alzheimer's disease (AD).[1,2] The amyloid approach postulates that agents that decrease Aβ levels in vivo will have therapeutic benefit in AD. Evaluation of such agents for sustained reduction of brain Aβ levels can be done in animal models for Aβ and plaque deposition.[3,4] The two specific proteases involved in the production of the Aβ peptide are the β- and γ-secretases. The mechanistic class of γ-secretase, which liberates the carboxy terminus of the peptide, is not fully established but is thought to be an unusual aspartyl protease.[5] We have recently disclosed compounds that inhibit γ-secretase in cells and demonstrate reduction of brain Aβ levels in PDAPP transgenic mice.[6]

A number of groups, including our laboratory, have published on the isolation and cloning of β-secretase (BACE), the enzyme involved in the proteolytic formation of the amino terminus of the Aβ peptide and shown it to be a membrane-bound aspartyl protease.[7−9] The cleavage of APP by BACE occurs on its luminal side and is considered to be the rate-limiting step in the processing of APP to Aβ.[2] BACE is thus an attractive therapeutic target for the design of inhibitors of Aβ production. Purification of the enzyme activity from the human brain was achieved in our laboratory by a sequential four-step affinity−purification procedure using an immobilized peptide inhibitor (**22**). The development of this inhibitor and its congeners is described in this communication.

Design. Our initial foray into the design of inhibitors of BACE began with the definition of the P_1 and P_1' specificity for the enzyme. The substrate $P_{13}-P_5'$ (**1**) is effectively cleaved by the enzyme and was used as the starting point for this design process. Substitutions at the P_1 and P_1' sites in **1** were made to explore substrate specificity and provide a potential structural basis for the initial inhibitor design. The enzyme shows high substrate preference in P_1 for leucine **(1)** and phenylalanine (**5**), while all other P_1 substitutions resulted in peptides that were not cleaved by the enzyme under the conditions of the study. In contrast, the P_1' specificity of the enzyme appears to be less stringent with P_1'-Ala (**16**) showing maximal cleavage while other residues in P_1' afforded substrates that were cleaved to a lesser extent. Details of the substrate preference study for BACE are to be published in a separate manuscript.

In addition to the substrate cleavage analysis, these analogues were also evaluated as inhibitors of the enzyme activity (Table 1). Inhibition of the enzyme was determined using the MBPC125Swe (maltose-binding-protein C-125 Swedish) substrate. Measurement of the cleavage products was done by ELISA as previously reported.[5] None of the substrates with P_1 variations were inhibitors of the enzyme activity. On the other hand, several of the substrates with P_1' variations show significant inhibition of the enzyme, with the valine analogue (**13)** being the most potent ($IC_{50} \approx 3\ \mu M$) of the entire set. Interestingly, the alanine analogue (**16**), which was identified as the best substrate in the cleavage analysis, was not an inhibitor of the enzyme. Product inhibition by the cleavage products of **13** does not appear to be involved because both the N- and C-terminal cleavage products did not inhibit the enzyme.

Having developed an understanding of the P_1 and P_1' preferences for the enzyme, we next introduced a noncleavable residue in the substrate analogues that exhibited affinity for the BACE active site as determined by the substrate cleavage analysis (Table 2). Incorporation of statine for P_1-leucine of the $P_{10}-P_5'$ substrate **1** resulted in a weak inhibitor of the enzyme (**17**, $IC_{50} \approx 60\ \mu M$). Removal of the C-terminal arginine (P_5') from **17** had no effect on the potency of the inhibitor (**18**, $IC_{50} \approx 40\ \mu M$). Hence, all other analogues were prepared in the 14-residue, des-Arg peptide series. Replacement of the aspartyl group of **18** with alanine (**21**) and valine (**22**) resulted in significant enhancement in potency, with the valine analogue (**22**) being the most potent ($IC_{50} \approx 0.03\ \mu M$, Table 2). In our laboratory inhibitor **22** was further used in the affinity purification of the crude human brain preparation to yield purified β-secretase that was subsequently sequenced and cloned.[4] The preference for the *S*-hydroxyl isomer of statine (**18** vs **19**) and the lack of inhibition by the O-acetylated derivative (**20**) demonstrate that BACE, a membrane-bound protease, has an inhibitor profile similar to those of other known aspartyl proteases.[10] The acetyl derivative (**20**) was prepared from acetylstatine (**49**), whose synthesis is shown in Scheme 1. The *O*-acetyl-boc-statine was incorporated into the peptide as an *R*,*S* mixture, and the isomers then were separated by HPLC. Identification of the absolute stereochemistry of the isomers was done by analytical HPLC of a sample after base hydrolysis of the actetate group and comparison with the retention time of analogue **18**. Synthesis of the peptidic inhibitors was done using standard Boc-resin-based chemistry from Boc-amino acids and Boc-statine.

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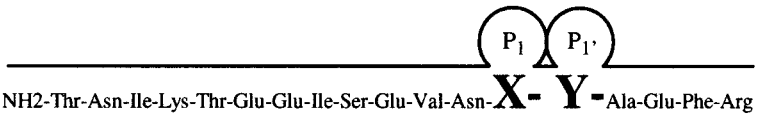
Table 1. β-Secretase Substrates−Analogue Inhibitors



compd	X	Y	IC_{50} (μM)[a]
1	Leu	Asp	>200
2	Ala	Asp	>200
3	Gly	Asp	>200
4	Met	Asp	>200
5	Phe	Asp	>200
6	Arg	Asp	>200
7	Glu	Asp	>200
8	Val	Asp	>200
9	Thr	Asp	>200
10	His	Asp	>200
11	Leu	Gly	119
12	Leu	Leu	45
13	Leu	Val	3
14	Leu	Lys	40
15	Leu	Phe	40
16	Leu	Ala	>200

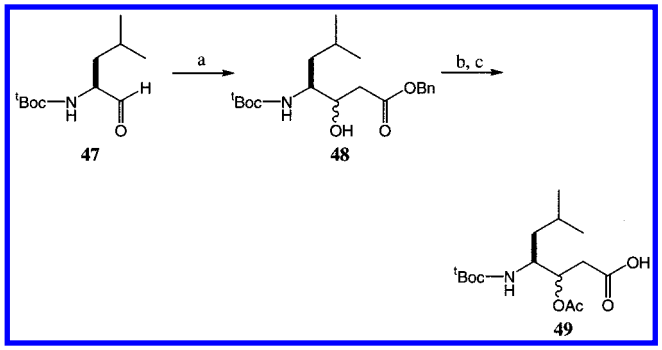
[a] Concentration necessary to inhibit 50% of enzyme activity in MBPC125Swe assay, average of two runs.

Table 2. β-Secretase Substrate Derived Inhibitors

	compound	IC_{50} (μM)[a]
17	NH2-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta(*S*-OH)-D-A-E-F-R	60
18	NH2-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta(*S*-OH)-D-A-E-F	40
19	NH2-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta(*R*-OH)-D-A-E-F	>200
20	NH2-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta(*S*-OAc)-D-A-E-F	>200
21	NH2-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta(*S*-OH)-A-A-E-F	0.5
22	NH2-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta(*S*-OH)-V-A-E-F	0.03

[a] Concentration necessary to inhibit 50% of enzyme activity in MBPC125Swe assay, average of two runs.

Scheme 1. Synthesis of *R,S*-Acetoxystatine[a]



[a] (a) LDA, benzyl acetae, THF; (b) Ac_2O, pyr; (c) H_2Pd/C, EtOH.

Truncation of the N- and the C-terminal portions of inhibitor **22** was done to determine the effects of key terminal residues on activity and the minimal size requirement for the inhibitor peptide. On the N-terminal side, residues P_{10}−P_5 could be removed without loss in potency (**22** vs **23**, Table 3). However, further removal of the N-terminal residues between P_5 and P_1 resulted in substantial loss of potency (Table 3). On the C-terminal side an Ala scan through the tetrapeptide sequence reveals that while there was a significant loss in potency on replacing any of the residues in this sequence, no single residue contributed to all of the inhibitory activity. However, truncation of the C terminus by removal of the C-terminal phenylalanine resulted in some loss of potency (**28** vs **37**) while removal of both C-terminal phenylalanine and glutamic acid resulted in significant to complete loss of potency (**28** vs **38**, Table 4).

Table 3. N-Terminal Inhibitor Modifications

	X-Sta-Val-Ala-Glu-Phe-COOH	
entry	X	IC_{50} (μM)[a]
22	NH2-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val0Asn-	0.03
23	NH2-Glu-Val-Asn-	0.04
24	NH2-Glu-Ala-Asn	2.5
25	NH2-Val-Asn-	9.0
26	NH2-Asn-	>150
27	*N*-acetyl-Val-Asn-	0.5
28	*N*-acetyl-Val-Met-	0.3

[a] Concentration necessary to inhibit 50% of enzyme activity in MBPC125Swe assay, average of two runs.

Table 4. C-Terminal Inhibitor Modifications

	Ac-Val-Met-Sta-X	
compd	X	IC_{50} (μM)[a]
28	Val-Ala-Glu-Phe-COOH	0.3
29	Ala-Ala-Glu-Phe-COOH	5.0
30	Val-Ala-Ala-Phe-COOH	2.0
31	Val-Ala-Glu-Ala-COOH	1.2
32	Asp-Ala-Glu-Ala-COOH	>100
33[b]	tleu-Ala-Glu-Ala-COOH	20
34	Phe-Ala-Glu-Phe-COOH	15
35	Thr-Ala-Glu-Phe-COOH	100
36	Pro-Ala-Glu-Phe-COOH	>200
37	Val-Ala-Glu-COOH	1.0
38	Val-Ala-COOH	>100
39	Val-COOH	>100

[a] Concentration necessary to inhibit 50% of enzyme activity in MBPC125Swe assay, average of two runs. [b] tleu = *tert*-leucine.

Finally, effects of modification of the noncleavable residue were determined using analogues **40**−**42** (Table 5). The "statine" analogue **28** and "AHPPA"[4(*S*)-amino-3-hydroxy-5-phenylpentanoic acid] **40** were equipotent,

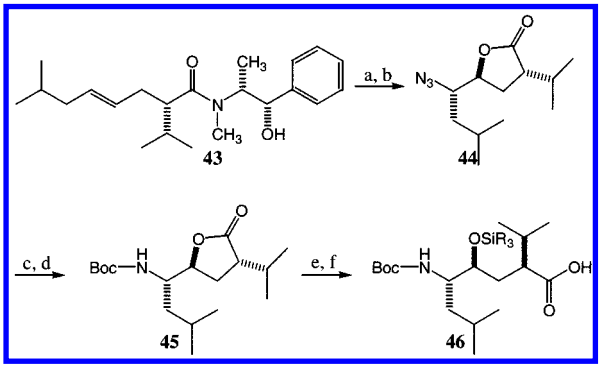
Table 5. Statine Modifications of Inhibitors

Ac-Val-Met-**X**-Ala-Glu-Phe-COOH

entry	X	IC50 (μM)[a]
28	'Statine'	0.3
40	'AHPPA'	0.5
41	'ACHPA'	5.0
42	'Hydroxyethylene'	0.02

[a] Concentration necessary to inhibit 50% of enzyme activity in MBPC125Swe assay, average of two runs.

Scheme 2. Synthesis of Hydroxyethylene Moiety[a]



[a] (a) NBS, HOAc, THF, H_2O; (b) NaN_3, DMF; (c) H_2/Pd/C, EtOH; (d) Boc_2O, Hunigs base, dioxane; (e) NaOH, dioxane/H_2O; (f) imidazole, TBDMSiCl, DMF; or TBDMSiOTf, Hunigs base, CH_2Cl_2.

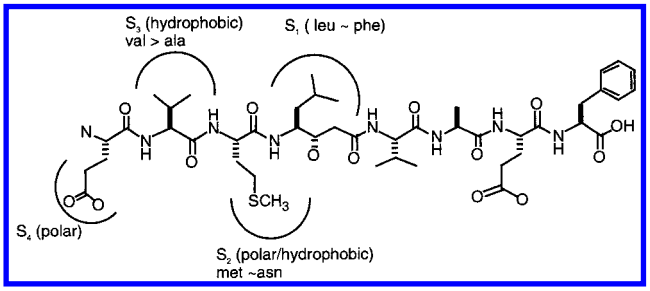


Figure 1. Proposed inhibitor-binding requirements for S_1−S_4 subsites.

while the "ACHPA"[4(*S*)-amino-5-cyclohexylpentanoic acid] **41** was 10-fold less potent. The hydroxyethylene derivative **42** showed significant enhancement in potency, suggesting that the hydroxyethylene replacement of the "stat-val" central core could result in very potent inhibitors. The hydroxyethylene analogue is similar to the BACE inhibitors reported by Tang et al.[11−13] The inhibitor **42** was constructed using standard solid-phase synthesis and protected hydroxyethylene **46.** The synthesis of **46** is outlined in Scheme 2.[14]

On the basis of the SAR from these peptidic inhibitors, a model for the S_1−S_4 inhibitor-binding subsites of BACE can be developed (Figure 1). The S_1 site can

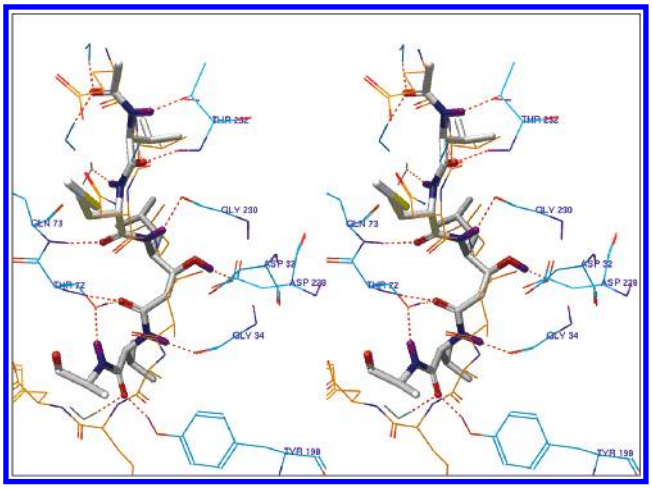


Figure 2. Modeled structure of analogue **28** (white) bound to BACE. Red dashes indicate hydrogen bonding to the inhibitor. The model was constructed using the crystal structure of BACE bound to inhibitor OM99-2 (orange), reported by Tang et al.[11] The C-terminal residues of the inhibitor showed no clearly preferred conformation and are omitted from the figure.

accommodate a Leu or Phe side chain, while S_2 can accommodate both polar and hydrophobic residues. Similarly S_3 prefers branched hydrophobic side chains and S_4 accommodates polar acidic side chains. A model (Figure 2) of inhibitor **28** constructed using the crystal struture of OMP-99 as described by Tang et al.[11] shows key H-bond interactions made by the inhibitor to the enzyme in the S_1−S_3 subsites. The C-terminal portion of the inhibitor showed no preferred conformation.

Conclusion. We have developed a series of substrate-based potent inhibitors of the human brain β-secretase and have generally defined the requirements for the S_1−S_4 subsites of the enzyme.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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