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Inhibitors of β-Amyloid Formation Based on the β-Secretase Cleavage Site

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A series of inhibitors of β-amyloid formation have been developed based on the β-secretase cleavage site (VNL-DA) of the Swedish mutant Amyloid Precursor Protein. A simple tripeptide aldehyde was found to be the most potent (IC_{50} = 700 nM) in the series displaying an inhibitory profile which is different from reported inhibitors of β-amyloid formation. © 2000 Academic Press

Alzheimer's Disease (AD) is a neurodegenerative disease that is characterized by amyloid plaques, extracellular cerebrovascular deposits of insoluble aggregates of beta amyloid peptide (Aβ) in the brain [1]. The disease leads to progressive dementia and neuronal cell death, but its pathogenesis remains obscure. Aβ peptides ranging from 39 to 43 amino acids in length are found in normal cells, the two major forms being $Aβ_{1–40}$ and $Aβ_{1–42}$ found in the ratio of ~9:1, respectively. In amyloid plaques the major form is $Aβ_{1–42}$ which appears to seed precipitation of other Aβ peptides [2]. It is currently thought that Aβ amyloid deposition leads to, rather than results from, the complex pathology that characterizes AD [3].

Aβ peptides are derived from proteolysis of larger integral membrane-spanning proteins known as β-amyloid precursor proteins (APPs). APP is processed by at least three as yet unidentified proteases termed the β, γ and α secretases although a novel aspartic protease has recently been identified which displays characteristics consistent with β-secretase [4–7]. The α-secretase cleavage site is near the middle of the Aβ sequence and therefore gives rise to non-amyloidogenic APP fragments. The unknown proteases responsible for cleaving APP at the N- and C-termini of Aβ have been termed β- and γ-secretases, respectively. There is experimental evidence suggesting that β-secretase first cleaves the soluble extracellular domain of APP releasing a membrane-associated 12 kDa C-terminal fragment, which is then processed by γ-secretase to release $Aβ_{1–42}$ [8]. Since this peptide aggregates to insoluble amyloid deposits in the brain parenchyma and vasculature which are thought to be toxic to the surrounding neuropil, preventing the formation of Aβ by inhibiting β and/or γ secretases may be a viable therapeutic strategy [9].

To date, only a few inhibitors of Aβ formation have been reported, with potencies in the low μM range. Calpain inhibitors with aldehyde termini (e.g., MDL28170 Cbz-Val-Phe-H[9], IC_{50} ~ 200 μM; calpain inhibitor 1 Ac-Leu-Leu-Nle-H, 50% inhibition at 50 μM; calpeptin Cbz-Leu-Nle-H, 57% inhibition at 5 μM; MG132 Cbz-Leu-Leu-Leu-H, 55% inhibition at 5 μM) are believed to inhibit proteolytic processing at the γ-secretase site either directly or indirectly. Quantifying the levels of $Aβ_{1–40}$ and $Aβ_{1–42}$ produced in HEK or COS-1 cells in the presence of these inhibitors has shown that while levels of $Aβ_{1–40}$ decreases, levels of $Aβ_{1–42}$ either remain the same or increase. This observation has led to the proposal for existence of two distinct γ-secretases [10, 11], one responsible for $Aβ_{1–40}$ generation and the other $Aβ_{1–42}$. Recent evidence however seems to cast some doubt on this hypothesis [12]. A few patents also describe the synthesis and activity of inhibitors of aspartyl proteases (e.g., Cathepsin D) [13] and calpain as weak (μM) inhibitors of Aβ formation via blockade of γ-secretase [14]. Only one report has dealt with a substrate-based approach to the design of inhibitors of γ-secretase (1–42) [15].

Compounds described that block Aβ formation by inhibiting processing at the β-secretase cleavage site are even fewer in number and include the irreversible serine protease inhibitor AEBSF (IC_{50} ~ 1 mM), a pyrrolidine (IC_{50} ~ 40 μM) and Cbz-Leu-Leu-Lene-H (IC_{50} = 800 nM) [16]. We now describe some substrate-based inhibitors of Aβ formation which are designed from the β-secretase cleavage site (VNL-DA) of APP.

β-Secretase cleaves APP at the N-terminus of Aβ by recognizing the region Val594 to Ala598 (VKM-DA) of APP695 and it is highly sequence specific [17]. With the exception of the Swedish mutation (VNL-DA) which increases (5- to 8-fold) Aβ production, amino acid substitutions of residues 594–598 dramatically reduce levels of βA4 production. We therefore decided to use the substrate sequence of the Swedish mutation, and

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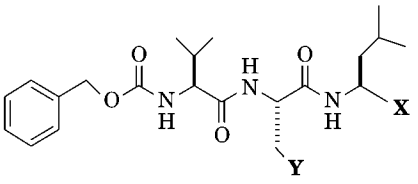
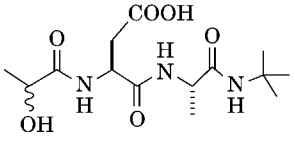
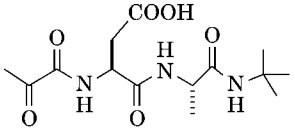
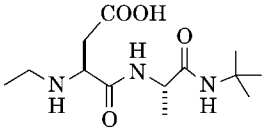
TABLE 1

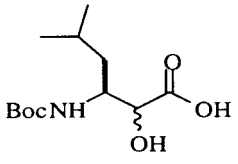
Structure–Activity Data for Designed Compounds



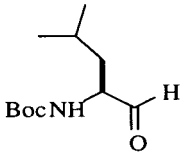
	Y	X	% Inhibition Total Aβ	% Inhibition Total $A\beta_{1-42}$
1.	$-CONH_2$	—CHO	73[a]	45[a]
2.	$-CONH_2$	$-CH_2OH$	0[b]	0[b]
3.	$-CONH_2$	—CN	0[b]	0[b]
4.	$-CONH_2$	—C(O)NHOH	0[b]	0[b]
5.	$-CONH_2$		0[b]	0[b]
6.	$-CONH_2$		0[b]	0[b]
7.	$-CONH_2$		34[c]	39[c]
8.	$-CH(CH_3)_2$	—CHO	IC_{50} = 700 nM	IC_{50} = 2.5 μM

Note. The inhibitor data are the average of three independent experiments where a = % inhibition at 25 μM, b = % inhibition at 100 μM, c = % inhibition at 50 μM.

replace the cleavable amide bond with various transition state isosteres that are effective in other inhibitors of proteases. Aldehydes are effective electrophilic isosteres in inhibitors of serine and cysteine proteases, nitriles form a reversible complex with cysteine proteases, hydroxamates inhibit metalloproteases, norstatine and reduced amide isosteres in **6** and **8** have also been used for aspartic protease inhibition, while α-keto amides have been used extensively in inhibitors of cysteine and serine proteases. These compounds were synthesized by standard procedures.



9



10

Briefly, **1** was synthesized from Boc-Leu-OMe, deprotected with TFA and sequentially coupled to BocAsn-(Trt)-OH then Cbz-Val-OH with BOP/DIPEA in DMF. The resulting tripeptide, Cbz-Val-Asn-Leu-OMe, was reduced to the alcohol **2** with $LiBH_4$ followed by oxidation with the Dess-Martin reagent to give **1.** The nitrile **3** was synthesized from Boc-Leu-NH_2 which was dehydrated with $POCl_3$ and the resultant nitrile intermediate coupled to BocAsn(Trt)-OH followed by Cbz-Val-OH to give **3.** The reaction of basic hydroxylamine solution with Cbz-Val-Asn-Leu-OMe gave the desired hydroxamic acid **4** [18]. The aldehyde **8** was made by a similar procedure as described for **1.** Compounds **5, 6,** and **7** were synthesized in solution by a stepwise assembly starting from H-Asp(Obzl)-Ala-NtBu and sequentially coupling to the peptide chain, the α-hydroxy acid **9** with BOP reagent or the aldehyde **10** by reductive amination with $NaCNBH_3$ (see scheme). Both intermediates were further elaborated by coupling Boc-Asn-OH and Cbz-Val-OH and a final deprotection with LiOH to give compounds **6** and **8.** Oxidation of the secondary alcohol of **6** with Dess-Martin reagent gave the α keto amide **7.**

Compounds were assayed using CHO cells, stably transfected with wild-type APP751 [19], grown to confluence in 6-well plates, and treated with inhibitors in 1.5 mL complete media for 24 h (Table 1).

The medium was collected, centrifuged to remove suspended cells, and secreted APP cleavage products in the conditioned media were immunoprecipitated with specific antibodies. The immunoprecipitates were analyzed by SDS–PAGE and fluorography via a previously published method [20]. The inhibitor data are the average of three independent experiments.

The aldehyde **1** showed inhibition of Aβ formation at low μM concentrations. The alcohol precursor **2,** nitrile **3** or hydroxamic acid **4** were not active when incubated with CHO cells at a concentration of 100 μM. As nitrile **3** is expected to be specific for cysteine proteases over serine, this result suggests that the aldehyde **1** might be inhibiting a serine protease. Of the longer peptides **5–7,** only the compound incorporating the reduced amide isostere (**7**) was active, albeit weakly. As all these inhibitors need to cross cell membranes for enzyme inhibition, the asparagine residue of **1** was substituted for the more hydrophobic isosteric replacement leucine to give compound **8** (logP = 3.9). This simple tripeptide aldehyde was the most active compound of the series (IC_{50} = 700 nM for inhibition of total Aβ and IC_{50} = 2.5 μM for Aβ_{1-42}). The most active compounds above (**1, 7,** and **8**) all inhibited the formation of both total Aβ *and* Aβ_{1-42} in a dose-dependent manner. They thus display a different inhibitory profile compared to the structurally similar peptide aldehydes MDL28170 Cbz-Val-Phe-H, Calpain inhibitor 1 Ac-Leu-Leu-Nle-H, calpeptin Cbz-Leu-Nle-H, and MG132 Cbz-Leu-Leu-Leu-H. This observation is consistent with either preferential inhibition of β-secretase or inhibition of a discrete γ-secretase which processes APP at the sites which lead to Aβ_{1-40} and Aβ_{1-42} formation respectively.

Although **8** (z-Val-Leu-Leu-H, IC_{50} = 700 nM) is sevenfold more potent than the peptide aldehyde inhibitor, z-Leu-Leu-Leu-H (MG 132, $IC_{50} \sim 5$ μM), the two compounds are structurally similar and thus potentially could target the same secretase. Were this true, the greater potency of **8** could be explained by greater cell permeability (CLog P = 3.4 vs 3.9), the former being 5 times less hydrophobic. However if compound **8** does inhibit γ-secretase rather than β-secretase, as for MG 132, it should block formation of Aβ_{1-40} preferentially over Aβ_{1-42}. Instead we find that **8** inhibits secretion of both Aβ_{1-42} *and* total Aβ in a dose-dependent manner. Since both tripeptide **1** and pentapeptide **7** contain residues that span the β-secretase cleavage site, they are also expected to be specific inhibitors of β-secretase or an upstream event leading to inhibition of β-secretase activity. Indeed they inhibit formation of total Aβ *and* Aβ_{1-42}, just like **8,** and this is consistent with the conclusion that they inhibit the action of β- rather than γ-secretase. As compound **7** contains the reduced amide isostere it would be expected to inhibit an aspartyl protease activity and could be acting on the β-secretase enzyme that has been recently reported.

In conclusion we have described a series of inhibitors of Aβ formation designed from the β-secretase cleavage site of APP. The most potent were the tripeptide aldehydes **1** and **8** which bear a striking similarity to previously described γ-secretase inhibitors but display a different inhibitory profile. We have inferred that **1** and **8** inhibit β-secretase, but the possibility remains that they also act on the γ-secretase cleavage sites of APP. Compound **8** is one of the most potent inhibitors of Aβ formation reported thus far, and we are attempting to design more potent analogues as well as determine of the specific site of action of these compounds using antibodies specific to $APP_s\beta$, P3 and $APP_s\alpha$ in a cell-based assay.

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REFERENCES

1. Glenner, G. G., and Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* **120,** 885–890; Jarrett, J. T., Berger, E. P., and Lansbury, P. T., Jr. (1993) *Biochemistry* **42,** 4693–4697.
2. Hardy, J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2095–2097.
3. Schenk, D. B., Rydel, R. E., May, P., Little, S., Panetta, J., Lieberburg, I., and Sinha, S. (1995) *J. Med. Chem.* **38,** 4141–4154.
4. Vassar, R., *et al.* (1999) *Science* **286,** 735–741.
5. Yan, R., *et al.* (1999) *Nature* **402,** 533–537.
6. Sinha, S., *et al.* (1999) *Nature* **402,** 537–540.
7. Hussain, I., *et al.* (1999) *Mol. Cell Neurosci.* **14,** 419–427.
8. Paganetti, P. A., Lis, M., Klafki, H. W., and Staufenbiel, M. (1996) *J. Neurosci. Res.* **46,** 283–293.
9. Higaki, J., Quon, D., Zhong, Z., and Cordell, B. (1995) *Neuron* **14,** 651–659.
10. Citron, M., Diehl, T. S., Gordon, G., Biere, A. L., Seubert, P., and Selkoe, D. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13170–13175.
11. Klafki, H., Abramowski, D., Swoboda, R., Paganetti, P. A., and Staufenbiel, M. (1996) *J. Biol. Chem.* **271,** 28655–28659.
12. Zhang, L., Song, L., and Parker, E. M. (1999) *J. Biol. Chem.* **274,** 8966–72.
13. (a) Dovey, H. F., *et al.* (1995) Canadian Patent Application, *Chem. Abstr.* **129,** 689; (b) Felsenstein, K., Smith, D. W., Poss, M. A., and Charturvedula, P. (1997) US Patent 5,703, 129.
14. McDonald, I. A., Albrecht, E., and Munoz, B. (1998) US Patent 5,703,129.
15. Wolfe, M. S., Citron, M., Diehl, T. S., Xia, W., Donkor, I. O., and Selkoe, D. J. (1998) *J. Med. Chem.* **41,** 6–9.
16. Varghese, J., Latimer, L. H., Tung, J. S., and Dappen, M. (1997) Annual Reports in Medicinal Chemistry, Vol. 32, pp. 12–20. Academic Press, San Diego.
17. Citron, M., Teplow, D. B., and Selkoe, D. J. (1995) *Neuron* **14,** 661–670.
18. Fieser, L. F., and Fieser, M. (1967) Reagents for Organic Synthesis, Vol. 1, John Wiley and Sons, New York.
19. Koo, E. H., and Squazzo, S. L. (1994) *J. Biol. Chem.* **269,** 17386–17389.
20. Citron, M., *et al.* (1997) *Nat. Med.* **3,** 67–72.