Design and Synthesis of Statine-Based Cell-Permeable Peptidomimetic Inhibitors of Human β-Secretase

Roy K. Hom, Larry Y. Fang, Shumeye Mamo, Jay S. Tung, Ashley C. Guinn, Don E. Walker, David L. Davis, Andrea F. Gailunas, Eugene D. Thorsett, Sukanto Sinha, Jeroen E. Knops, Nancy E. Jewett, John P. Anderson, and Varghese John*

Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, California 94080

Received November 22, 2002

Abstract: We describe the development of statine-based peptidomimetic inhibitors of human β-secretase (BACE). The conversion of the peptide inhibitor **1** into cell-permeable peptidomimetic inhibitors of BACE was achieved through an iterative strategy of conceptually subdividing **1** into three regions: an N-terminal portion, a central statine-containing core, and a C-terminus. Replacement of the amino acid residues of **1** with moieties with less peptidic character was done with retention of BACE enzyme inhibitory activity. This approach led to the identification of the cell-permeable BACE inhibitor **38** that demonstrated BACE-mechanism-selective inhibition of Aβ secretion in human embryonic kidney cells.

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] Agents that inhibit Aβ production prevent plaque deposition in the brain and are postulated to be of therapeutic benefit in AD.[3] The two specific proteases involved in the production of the Aβ peptide are the β- and γ-secretases.

β-Secretase (BACE) has been shown by us and others to be a membrane-bound aspartyl protease.[4−6] 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. BACE cleavage results in the production of the β-C-terminal fragment (β-CTF), which is then further cleaved to Aβ by γ-secretase. BACE is highly enriched in the central nervous system. BACE knockout homozygote mice show complete absence of Aβ production and have no reported side effects.[7] BACE is thus an attractive therapeutic target for the design of inhibitors of Aβ production.

We have previously elaborated on the development of peptidic inhibitors of BACE.[8] Herein we describe the development of these compounds into statine-based peptidomimetics that were used in demonstrating selective inhibition of BACE in cells.

Design. Our goal was the conversion of the peptide inhibitors, such as **1**, into small-molecule BACE inhibitors that could potentially be used for Alzheimer's disease therapeutics (Figure 1). As a strategy, **1** was conceptually subdivided into three regions: an N-terminal portion, a central statine-containing core, and a C-terminus. These sections were individually targeted for modification to replace the amino acid residues with functionalities exhibiting less peptidic character, with retention of BACE enzyme activity. Inhibition of the enzyme was determined using the MBP-C125 (maltose-binding protein C-125) substrate assay as previously described.[4]

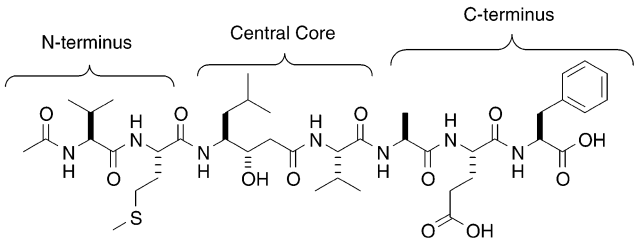
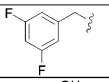
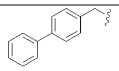
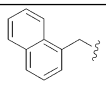
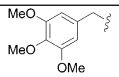
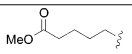
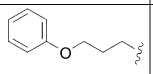
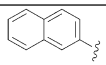
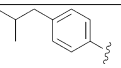
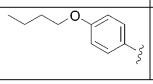


Figure 1. Division of peptide inhibitor **1** into strategic regions.

Table 1. N-Terminal Substitutions of Ac-VM

R-CO-[Sta]-Val-Ala-Glu-Phe		
Compound	R	IC_{50} (μM)[a]
2		130
3		140
4		17
5		56
6		42
7		94
8		50
9		25
10		48
11		28
12		27

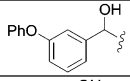
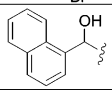
[a] Concentration necessary to inhibit 50% of enzyme activity in an MBP-C125 assay, with an average of two or more runs. The standard errors are as reported in the Supporting Information.

N-Terminal Modification. To probe the structure−activity requirements of the N-terminal binding sites, we synthesized a number of peptides with the common statine (Sta) core and C-terminus. Coupling of commercially available carboxylic acids to NH_2[Sta]VAEF-

* To whom correspondence should be addressed. Phone: 650-877-7623. Fax: 650-877-7486. E-mail: varghese.john@elan.com.

10.1021/jm025619l CCC: $25.00
Published on Web 04/11/2003

Table 2. Mandelate-like N-Termini

R-CO-[Sta]-Val-Ala-Glu-Phe		
Compound	R	IC_{50} (μM)[a]
13		28
14		41
15[b]		14
16[b]		6
17[b]		11
18[b]		3
19[b]		4

[a] Concentration necessary to inhibit 50% of enzyme activity in an MBP-C125 assay, with an average of two or more runs. The standard errors are as reported in the Supporting Information. [b] 1:1 diastereomeric ratio at the hydroxy-bearing methine carbon.

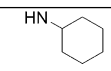
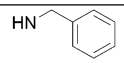
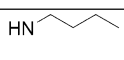
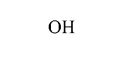
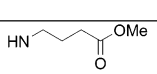
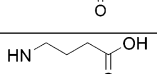
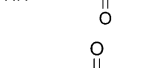
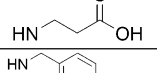
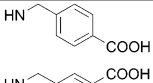
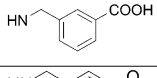
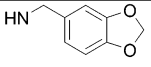
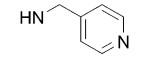
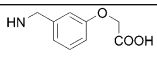
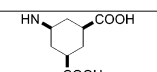
resin-bound peptide followed by standard peptide work-up quickly led to an N-terminal replacement to Ac-VM.

A variety of substituted phenylacetyl (compounds **2**−**7**), alkanoyl (**8** and **9**), and aroyl (**10**−**12**) N-termini were investigated (Table 1). The compounds all exhibited modest inhibitory activity toward BACE (IC_{50} = 17−140 μM), with significant attenuation relative to the activity of the base compound **1**. The most promising trend was the 10-fold increase in potency between the phenylacetyl (compound **2**) and the (*R*)-mandelyl N-cap (compound **4**). The corresponding (*S*)-mandelyl compound was essentially inactive (IC_{50} > 100 μM) in the same assay. Encouraged by this result, we further explored mandelate-like derivatives in the attempt to enhance BACE activity in this N-terminal series.

Table 2 illustrates the structure−activity data gathered around the mandelate N-terminal lead. Alkylation of the hydroxyl (compound **13**) and further functionalization of the hydroxyl-bearing carbon (**14**) both somewhat eroded enzyme inhibition. Substitution on the benzene ring with electron-donating groups led to increased activity (**15**−**19**), with 2-bromomandelyl (**18**) and (naphth-1-yl)hydroxyacetyl (**19**) yielding the most potent compounds in this series. While the corresponding carboxylic acids for compounds **13** and **14** were commercially available, the hydroxy acids involved in the synthesis of **15**−**19** were obtained in one step from the phenylacetic acids.[9]

C-Terminal Modification. Concurrently, we pursued the transformation of the C-terminal AEF region into more "druglike" groups (Table 3). Replacement of AEF with simple alkyl or aralkyl groups (**20**−**22**) completely eliminated any detectable inhibition. However, truncation of the AEF group to the valine free acid (**23**) afforded a compound with weak activity, which guided us toward the discovery of more potent carboxylic

Table 3. Initial Investigations into Replacements of AEF C-Terminus

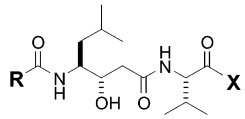
Boc-Val-Met-[Sta]-Val-X		
Compound	X	IC_{50} (μM)[a]
20		>200
21		>200
22		>200
23		94
24		47
25		17
26		30
27		4
28		4
29		>100
30		>100
31		5
32		0.3
33		10

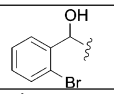
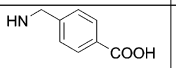
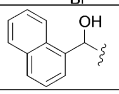
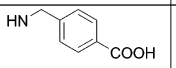
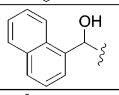
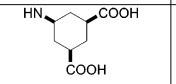
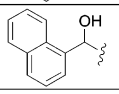
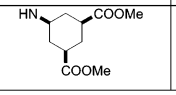
[a] Concentration necessary to inhibit 50% of enzyme activity in an MBP-C125 assay, with an average of two or more runs. The standard errors are as reported in the Supporting Information.

acid C-termini (**25**−**28**). There appeared to be a minimum spacer requirement because the three-carbon spacer (**25**) proved to be more inhibitory than one with only two carbons (**26**). In addition, rigid spacers were more favored, as evidenced by the improved potency of the benzoic acid derivatives **27** and **28**. The lack of activity change among compounds **27**, **28**, and **31** indicated low regiospecificity of the carboxylate interaction with the enzyme; however, attempts to replace the carboxylate with less polar groups (compounds **24**, **29**−**30**) all led to less potent analogues.

The relatively potent 4-methylaminobenzoic acid derivative (**27**) suggested that the carboxylate present was replacing one of the Glu or Phe carboxylates. This information led us to synthesize analogue **32**, which contained two carboxylates in a geometrically constrained format. Compound **32** exhibited a 10-fold increase in potency compared to the monocarboxylates (**27**−**28**, **31**); however, the diester (**33**)[10] still demonstrated lower potency. Nevertheless, it was notable that **32** was equipotent to the starting template **1**, making the aminocyclohexanedicarboxylic acid function an effective surrogate of Ala-Glu-Phe with regard to BACE inhibitory activity in this series.

Table 4. Combining N- and C-Terminal Modifications



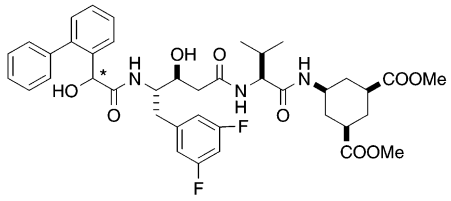
Compound[b]	R	X	IC_{50} (μM)[a]
34			40
35			50
36			3
37			140

[a] Concentration necessary to inhibit 50% of enzyme activity in an MBP-C125 assay, with an average of two or more runs. The standard errors are as reported in the Supporting Information. [b] 1:1 diastereomeric ratio at the hydroxy-bearing methine carbon.

Compounds with Both N- and C-Termini Modified. With reasonable N- and C-terminal replacements in hand, we focused on the synthesis of analogues containing both of these groups. The combination of the naphthyl N-terminus and the more potent carboxylate C-termini led to compounds with moderate to good inhibition of the BACE enzyme (Table 4). Consistent with the longer hexapeptide series, the 4-aminomethylbenzoic acid derivatives **34** and **35** were 10-fold less potent than the dicarboxylate derivative **36**, while the dimethyl ester **37** demonstrated 100-fold lower potency than its corresponding diacid.

BACE Inhibitors of Aβ Production in the Cellular Assay. Continuing in the combination of the active functional groups that we had discovered, we had realized in our earlier work that 4-amino-3-hydroxy-5-phenylpentanoic acid was comparable to Sta in terms of activity in the central portion of the molecule[8] and began to incorporate that moiety in our smaller inhibitors. Coupling this information with additional optimization of the N-terminus and central core led to the formation of **38** and **39** (Figure 2). The diastereomers were separable by chromatography.[11] The active diastereomer **38** (IC_{50} = 0.12 μM) also was effective in inhibiting Aβ secretion in transfected human embryonic kidney (HEK-293) cells (EC_{50} = 4.0 μM). This cell assay for screening inhibitors of Aβ production has been previously described. [3,13] The *R*-configuration can be assigned to the N-terminal hydroxyl of **38** based on the configuration of the active mandelate **4**; however, the absolute configuration has not been established. The corresponding diacid was more potent in the enzyme assay (IC_{50} = 0.02 μM) as a mixture of diastereomers but showed no cell activity (EC_{50} > 10 μM).

The two isomers **38** and **39** were then incubated with the HEK-293 cells to establish the mechanism of inhibition of Aβ. This was done through a previously described approach involving immunoprecipitation of APP fragments.[12] In these experiments (Figure 3), it was clear that at a dose of 10 times EC_{50} the active isomer (**38**) lowered β-secreted amyloid precursor protein (β-sAPP) levels in the cell without affecting α-secreted amyloid precursor protein (α-sAPP) levels, as would be expected with a BACE inhibitory mechanism. At 2 times EC_{50}, **38** had some effect on β-sAPP levels, as would be expected from a dose-dependent inhibitor, and still no effect on α-sAPP. In contrast, the essentially inactive **39** showed no difference on the levels of either α- or β-sAPP at either concentration shown.



38
isomer 1: IC_{50} = 0.12 μM[a]
EC_{50} = 4 μM[b]

39
isomer 2: IC_{50} = 10 μM[a]
EC_{50} > 10 μM[b]

Figure 2. Compounds used to demonstrate BACE selective lowering of Aβ in cells. IC_{50} is the concentration necessary to inhibit 50% of the enzyme activity in an MBP-C125 assay, with an average of two or more runs. EC_{50} is the concentration necessary to inhibit 50% of Aβ production in HEK-293 cells.

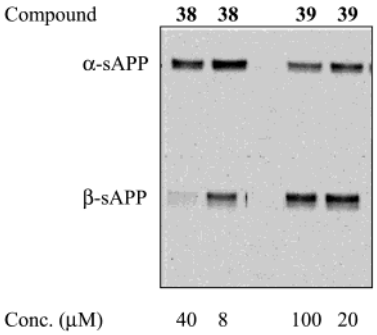


Figure 3. Western blot analysis of APP fragments in HEK-293 cells after treatment with compounds.

Conclusion. The heptapeptide BACE inhibitor **1** served as a convenient starting point from which smaller compounds, demonstrating BACE selective cellular inhibition, were developed. Through an iterative process of screening replacements to peptidic portions of the molecule at both the N- and C-terminal ends, we succeeded in identifying **38**, which demonstrated BACE selective inhibition of Aβ production in vitro. Further studies to evaluate **38** in vivo as well as to develop further refinements are ongoing and will be reported in due course.

Acknowledgment. The authors acknowledge Dr. Miguel Ondetti for all his advice in this work.

Supporting Information Available: Experimental details and characterization data for compounds **2**−**39** and BACE enzyme and Aβ cell assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Selkoe, D. J. Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **1999**, *399* (6738 Suppl), A23−A31.
(2) Sinha, S.; Lieberburg, I. Cellular mechanisms of β-amyloid production. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11049−11053.

(3) Dovey, H. F.; John, V.; Anderson, J. P.; Chen, L. Z.; De Saint Andrieu, P.; Fang, L. Y.; Freedman, S. B.; Folmer, B.; Goldbach, E.; Holsztynska, E. J.; Hu, K. L.; Johnson-Wood, K. L.; Kennedy, S. L.; Kholodenko, D.; Knops, J. E.; Latimer, L. H.; Lee, M.; Liao, Z.; Lieberburg, I. M.; Motter, R. N.; Mutter, L. C.; Nietz, J.; Quinn, K. P.; Sacchi, K. L.; Seubert, P. A.; Shopp, G. M.; Thorsett, E. D.; Tung, J. S.; Wu, J.; Yang, S.; Yin, C. T.; Schenk, D. B.; May, P. C.; Altstiel, L. D.; Bender, M. H.; Boggs, L. N.; Britton, T. C.; Clemens, J. C.; Czilli, D. L.; Dieckman-McGinty, D. K.; Droste, J. J.; Fuson, K. S.; Gitter, B. D.; Hyslop, P. A.; Johnstone, E. M.; Li, W.-Y.; Little, S. P.; Mabry, T. E.; Miller, F. D.; Ni, B.; Nissen, J. S.; Porter, W. J.; Potts, B. D.; Reel, J. K.; Stephenson, D.; Su, Y.; Shipley, L. A.; Whitesitt, C. A.; Yin, T.; Audia, J. E. Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in the brain. *J. Neurochem*. **2001**, *76*, 173−181.

(4) Sinha, S.; Anderson, J. P.; Barbour, R.; Basi, G. S.; Caccavello, R.; Davis, D.; Doan, M.; Dovey, H. F.; Frigon, N.; Hong, J.; Croak, K.; Jewett, N.; Keim, P.; Knops, J.; Lieberburg, I.; Power, M.; Tan, H.; Tatsuno, G.; Tung, J.; Schenk, D.; Seubert, P.; Suomensaari, S. M.; Wang, S.; Walker, D.; Zhao, J.; McConologue, L.; John, V. Purification and cloning of amyloid precursor protein β-secretase from human brain. *Nature* **1999**, *402*, 537−540.

(5) Yan, R.; Bienkowski, M. J.; Shuck, M. E.; Miao, H.; Tory, M. C.; Pauley, A. M.; Brashier, J. R.; Stratman, N. C.; Mathews, R. W.; Buhl, A. E.; Carter, D. B.; Tomasselli, A. G.; Parodi, L. A.; Heinrickson, R. L.; Gurney, M. Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* **1999**, *402*, 533−536.

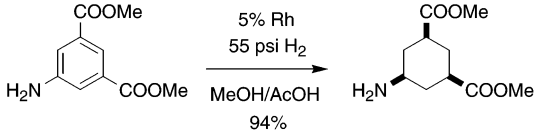
(6) Vassar, R.; Bennett, B. D.; Babu-Khan, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.; Amarante, P.; Loeloff, R.; Luo, Y.; Fisher, S.; Fuller, J.; Edenson, S.; Lile, J.; Jarosinski, M. A.; Biere, A. L.; Curran, E.; Burgess, T.; Louis, J. C.; Collins, F.; Treanor, J.; Rogers, G.; Citron, M. β-Secretase cleavage of Alzhemeier's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **1999**, *286*, 735−741.

(7) (a) Roberds, S. L.; Anderson, J.; Basi, G.; Bienkowski, M. J.; Branstetter, D. G.; Chen, K. S.; Freedman, S. B.; Frigon, N. L.; Games, D.; Hu, K.; Johnson-Wood, K.; Kappenman, K. E.; Kawabe, T. T.; Kola, I.; Kuehn, R.; Lee, M.; Liu, W.; Motter, R.; Nichols, N. F.; Power, M.; Robertson, D. W.; Schenk, D.; Schoor, M.; Shopp, G. M.; Shuck, M. E.; Sinha, S.; Svensson, K. A.; Tatsuno, G.; Tintrup, H.; Wijsman, J.; Wright, S.; McConlogue, L. BACE knockout mice are healthy despite lacking the primary β-secretase activity in brain: implications for Alzheimer's disease therapeutics. *Hum. Mol. Genet.* **2001**, *10*, 1317−1324. (b) Luo, Y.; Bolon, B.; Kahn, S.; Bennett, B. D.; Babu-Khan, S.; Denis, P.; Fan, W.; Kha, H.; Zhang, J.; Gong, Y.; Martin, L.; Louis, J.-C.; Yan, Q.; Richards, W. G.; Citron, M.; Vassar, R. Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. *Nat. Neurosci.* **2001**, *4*, 231−232. (c) Cai, H.; Wang, Y.; McCarthy, D.; Wen, H.; Borchelt, D. R.; Price, D. L.; Wong, P. C. BACE1 is the major β-secretase for generation of Aβ peptides by neurons. *Nat. Neurosci.* **2001**, *4*, 233−234.

(8) Tung, J. S.; Davis, D. L.; Anderson, J. P.; Walker, D. E.; Mamo, S.; Jewett, N.; Hom, R. K.; Sinha, S.; Thorsett, E. D.; John, V. Design of substrate-based inhibitors of human β-secretase. *J. Med. Chem*. **2002**, *45*, 259−262.

(9) Moersch, G. W.; Zwiesler, M. L. Synthesis of α-hydroxycarboxylic acids by aeration of lithiated carboxylic acids in tetrahydrofuran solution. *Synthesis* **1971**, *12*, 647−648.

(10) The ester required for the synthesis of **33** (1-amino-3,5-*cis*,*cis*-dimethyl cyclohexanedicarboxylate) was obtained in one step from commercial material:



To 10 g (47.85 mmol) of dimethyl 5-aminoisophthalate in 25 mL of AcOH and 50 mL of MeOH was added 5 g of 5% Rh on alumina in a high-pressure bottle, which was saturated with hydrogen at 55 psi and shaken for 1 week. The mixture was then filtered through a layer of diatomaceous earth and rinsed with methanol (3×). The filtrates were concentrated, and the crude solid was triturated with diethyl ether, affording 9.67 g (44.98 mmol, 94%) of the title compound as an off-white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.65 (s, 6H), 2.74 (tt, J = 11.5, 3.8 Hz, 1H), 2.39 (tt, J = 12.6, 3.6 Hz, 2H), 2.25−2.05 (m, 3H), 1.63 (br s, 2H), 1.46 (q, J = 12.8 Hz, 1H), 1.18 (q, J = 12.3 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.8, 51.7, 49.1, 41.2, 37.9, 30.1; MH^+ (CI) 216.1.

(11) (a) The 3,5-difluorophenylstatine portion of inhibitor **38** was synthesized from commercial 3,5-difluorophenylalanine (Synthetech) through the procedure outlined in the following. Wuts, P. G. M.; Putt, S. R. Synthesis of *N*-Boc statine and epi-statine. *Synthesis* **1989**, 951−953. (b) It could be surmised that the active isomer is the *R* form. However, X-ray crystallography of the isomer for definitive proof of this was not performed.

(12) The method for determination of in vitro Aβ reduction in human embryonic kidney cells and the immunoprecipitation protocol using these cells have been previously described in ref 3.

(13) Dovey, H. R.; Suomensaari-Chrysler, S.; Lieberburg, L.; Sinha, S.; Keim, P. S. Cells with a familial Alzheimer's disease mutation produce authentic beta-peptide. *NeuroReport* **1993**, *4*, 1039−1042.

JM025619L