A Combinatorial Approach to the Identification of Dipeptide Aldehyde Inhibitors of β-Amyloid Production

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In an effort to rapidly identify potent inhibitors of Aβ production and to probe the amino acid sequence specificity of the protease(s) responsible for the production of this peptide, a large number of dipeptide aldehydes were combinatorially synthesized and manually evaluated for their inhibitory properties. The starting point for this study was the dipeptide aldehyde carbobenzoxyl-valinyl-phenylalanal previously shown to inhibit the production of Aβ in CHO cells stably transfected with the cDNA encoding βAPP695. Pools of related dipeptide aldehydes were combinatorially synthesized, and the most active pool was deconvoluted, resulting in the identification of the most active inhibitor of this pool. Systematic optimization of this inhibitor resulted in a series of dipeptide aldehydes with enhanced potencies relative to carbobenzoxyl-valinyl-phenylalanal. The most active dipeptide aldehydes were those that possessed hydrophobic amino acids at both the P1 and P2 positions. The most potent compound identified in this study was 3,5-dimethoxycinnamamide-isoleucinyl-leucinal with an IC_{50} of 9.6 μM, approximately 10-fold more active than carbobenzoxyl-valinyl-phenylalanal. In immunoprecipitation experiments using antibodies directed toward either Aβ1−40 or Aβ1−42, 3,5-dimethoxycinnamamide-isoleucinyl-leucinal, like carbobenzoxyl-valinyl-phenylalanal, preferentially inhibited the shorter 1−40 form of Aβ, whereas the longer 1−42 form was not as strongly inhibited. These results suggest that dipeptide aldehydes related to carbobenzoxyl-valinyl-phenylalanal inhibit Aβ through similar mechanisms and demonstrate the utility of a combinatorial synthesis approach to rapidly identify potent inhibitors of Aβ production.

Introduction

One of the pathological hallmarks of Alzheimer's disease is the presence of amyloid plaques in the brain tissue of afflicted individuals. A more detailed inspection of amyloid plaques revealed the presence of a 39−43-amino acid peptide called β-amyloid (Aβ) which is generated by the proteolytic processing of a 695−770-amino acid precursor called β-amyloid precursor protein (βAPP).[1]

βAPP is thought to be proteolytically processed via two major intracellular pathways. The first pathway involves the cleavage of βAPP within the Aβ domain[2,3] by a protease(s) called α-secretase. Despite numerous efforts to identify this protease, α-secretase currently remains unknown, although inhibitors of this enzyme have recently been described that provide clues regarding the class of protease to which α-secretase belongs.[4,5] The proteolytic cleavage of βAPP by α-secretase results in the constitutive secretion of the soluble extracellular portion of βAPP (α-sAPP)[6] and simultaneously produces a membrane-bound, 9−12-kDa carboxyl-terminal fragment (CTF) of βAPP.[7,8] This CTF is further cleaved at the carboxyl-terminus of Aβ by another unknown protease(s) called γ-secretase, resulting in the formation of a small 3-kDa secreted fragment of Aβ called p3.[9]

The second major βAPP-processing pathway involves the proteolytic cleavage of βAPP resulting in the formation of Aβ. In this pathway, a third unidentified protease(s) called β-secretase first cleaves βAPP at the Met^{-1}−Asp^{1} peptide bond releasing a carboxyl-terminally truncated, soluble form of βAPP (β-sAPP)[10] and generating a CTF containing the entire Aβ sequence.[8,11−13] Further processing of this CTF at the carboxyl-terminus of the Aβ domain by γ-secretase generates the 4-kDa Aβ peptide. The Aβ containing CTF of βAPP thus serves as the immediate amyloidogenic precursor of Aβ.[14]

Despite the efforts of a number of laboratories to elucidate the intracellular processing pathway for Aβ formation, the exact subcellular compartment in which the β- and γ-secretase activities reside remains unknown. Evidence has been reported for three different intracellular mechanisms leading to the generation of Aβ. The first, based on the observation that amyloidogenic CTFs are stabilized in cells treated with lysosomal inhibitors, is one that involves the pH-sensitive lysosomal−endosomal system where Aβ is thought to be formed in or enroute to the lysosome.[12,13,15−18] The formation of Aβ through clathrin-mediated endocytosis of surface βAPP is associated with this pathway.[12,19,20] A second mechanism of Aβ formation has been described based on the demonstration that lysosomal inhibitors have little or no effect on secreted Aβ levels suggesting that acidic nonlysosomal organelles such as the *trans*-Golgi network (TGN) or TGN-derived vesicles are the actual sites of Aβ formation.[9,10,14,17,21] Alternatively, it is possible that secreted, extracellular Aβ is formed within the lysosomal−endosomal system while intracellular Aβ is formed within the TGN.[22] A third possible

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mechanism for the production of Aβ has been described for neuronal cells and for the fibroblast cell line 293, where it appears that Aβ1−42 is formed in the endoplasmic reticulum/intermediate compartment which may be distinct from the site where Aβ1−40 is produced.[23−25] These findings imply that either Aβ1−42 and Aβ1−40 are produced by two distinct γ-secretase enzymes or a single protease differentially processes βAPP depending on its intracellular location.

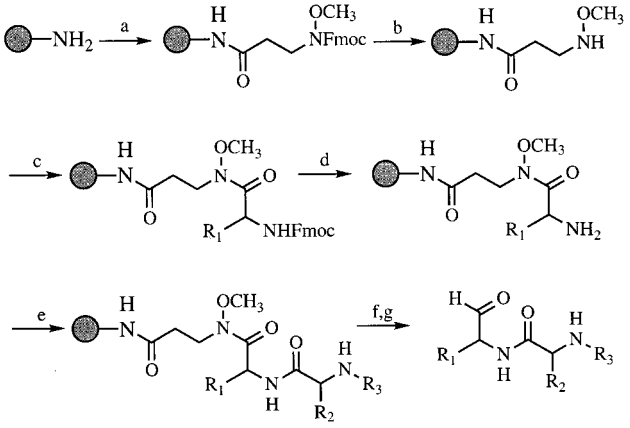
Since both β- and γ-secretase activities are required for the production of Aβ, these proteases are considered prime targets for therapeutic intervention; however, to date, these proteases remain unknown.[26] Protease inhibitors found to inhibit the production of Aβ in vitro provide clues regarding the type of protease(s) which may be involved. For instance, the ability of the general serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) to inhibit the production of Aβ but not p3 in a variety of cell types not only suggests that AEBSF is capable of inhibiting β-secretase but also suggests that β-secretase might belong to the serine protease class of proteolytic enzymes.[27]

Inhibitors of γ-secretase have also been reported. We recently showed that the peptide aldehyde carbobenzoxyl-valinyl-phenylalanal can inhibit the production of both Aβ and p3 in CHO cells expressing βAPP.[14] The concurrent accumulation of both amyloidogenic and nonamyloidogenic CTFs in treated cells suggests that carbobenzoxyl-valinyl-phenylalanal functions by way of inhibiting γ-secretase activity.[14] Three other peptide aldehyde inhibitors, *N*-acetyl-leucinyl-leucinyl-norleucinal (calpain inhibitor I), carbobenzoxyl-leucinyl-leucinyl-leucinal, and carbobenzoxyl-leucinyl-norleucinal (calpeptin), have since been shown to function in a similar fashion as carbobenzoxyl-valinyl-phenylalanal in cells expressing the Swedish double mutation (NL670/671) as well as in cells directly expressing the 100-amino acid amyloidogenic carboxyl-terminal fragment of APP.[28,29] These results provide evidence that related peptide aldehydes function similarly to inhibit γ-secretase.

A more detailed evaluation of the inhibitory activity of carbobenzoxyl-valinyl-phenylalanal demonstrates that this compound preferentially inhibits the production of Aβ1−40 versus Aβ1−42, providing strong evidence that the carboxyl-terminal heterogeneity of Aβ may be due to the presence of multiple γ-secretase enzymes, each with a distinct amino acid sequence specificity.[30] The preferential inhibition of Aβ1−40 has also been observed with other peptide aldehyde inhibitors[28,29] and has more recently been demonstrated for a peptide difluoro ketone inhibitor of γ-secretase.[31] Peptide inhibitors that inhibit primarily the longer and potentially more pathologically relevant 1−42 form of Aβ have yet to be reported. Since aldehydes and difluoro ketones are known to inhibit a variety of proteases including serine, cysteine, and aspartic acid proteases,[32−35] it is not known to which of these protease classes γ-secretase belongs.

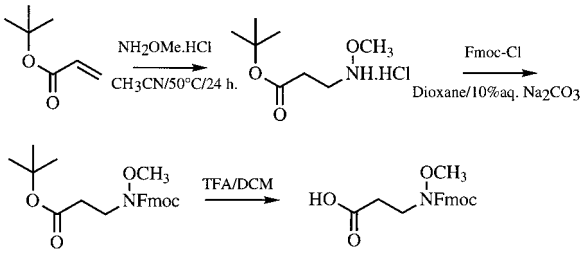
To find more potent peptide aldehyde inhibitors of Aβ production and to probe the amino acid specificity of γ-secretase, we used a combinatorial strategy to rapidly synthesize a large number of related dipeptide aldehydes. The strategy involved the synthesis of a combinatorial library of dipeptide aldehydes using the previously described (*N*-methoxyamino)propanoic acid linker.[36] Resulting aldehyde pools were evaluated for their ability to inhibit the production of Aβ in a whole cell assay using CHO cells stably transfected with βAPP. Taking carbobenzoxyl-valinyl-phenylalanal as our initial lead compound, the aldehydes synthesized in this study contained a variety of amino-terminal blocking groups and a number of different P1 and P2 amino acid side chains (P1 and P2 designation is according to the Berger and Schechter nomenclature for protease subsites[37]). Manual deconvolution of the resulting active pools using the whole cell assay identified individual aldehydes with marked improvements in Aβ inhibition. The relative inhibitory activities of these compounds provide a basis for the development of a structure−activity relationship for γ-secretase inhibitors that will be useful not only in the continued design of more potent and specific compounds of this class but also in the characterization of the protease(s) responsible for the γ-secretase cleavage of βAPP.

Scheme 1. Synthesis of Peptide Aldehydes Using 3-(*N*-Methoxyamino)propanoyl MBHA Resin[a]



[a] Reagents: (a) FMocNOMe-$(CH_2)_2CO_2H$/HOBt/DIPC/DIEA/DMF; (b) 50% piperidine/DMF; (c) Fmoc-amino acid/HOBt/DIPC/DIEA/DMF; (d) 50% piperidine/DMF; (e) solid-phase synthesis of desired molecule; (f) 50% TFA/DCM; (g) $LiAlH_4$/extract with EtOAc/concentrate, lyophilize to powder.

Scheme 2. Synthesis of 3-(Fmoc-*N*-methoxyamino)propanoic Acid



Results

The solid-supported synthesis of a mixture of peptide aldehydes was carried out using readily available reagents. The synthesis was carried out on an MBHA resin bearing an (*N*-methoxyamino)propanoyl linker as illustrated in Schemes 1 and 2. Following standard peptide synthesis using Fmoc amino acids, the orthogonally protected side chains were removed by treatment with trifluoroacetic acid in methylene chloride. This side chain-deprotected peptide was cleaved from the resin using a Weinreb reaction following treatment with

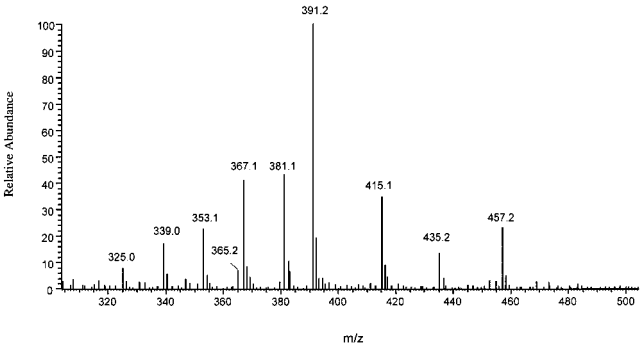
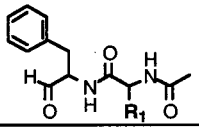
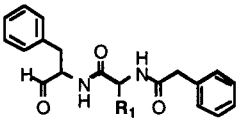
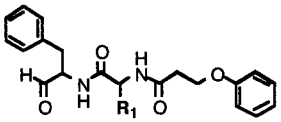
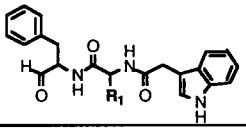
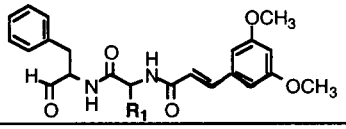
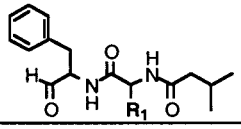
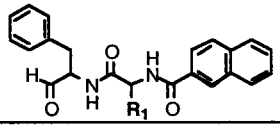
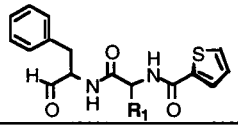
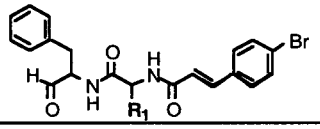
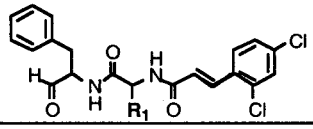


Figure 1. Electrospray ionization mass spectrum of aldehyde pool #3, containing a mixture of 10 peptide aldehydes. Molecular ion peaks indicate the presence of peptide aldehydes with the following R groups: Gly (325.0), Ala (339.0), 4-aminobutyric acid (353.1), D/L-Val (367.1), Leu, Ile (381.1), D/L-Phe (415.1). Molecular ion for the peptide aldehyde where the R group is proline (365.2) is very weak. Electron impact mass spectroscopy on this same pool shows the presence of this peptide aldehyde, MH^+ at 366 (data not shown). The peaks at 435.2, 457.2, and 391.2 possibly arise from fragmentation, sodium ion, or solvent molecule adduct formation as commonly seen during electrospray ionization and were not further characterized. The presence of the expected molecular ion peaks was used as the criterion to use a pool for biological assay.

lithium aluminum hydride in anyhdrous tetrahydrofuran (THF) at 0 °C. While the purities of each aldehyde pool were not quantitatively determined, pools of peptide aldehydes were routinely analyzed by reverse-phase HPLC (data not shown) and by electrospray ionization mass spectroscopy to ensure that the aldehydes that were intended to be synthesized were indeed present in the pool. An electrospray ionization mass spectrum of a typical library pool (pool #3) is shown in Figure 1. All major peaks present in this spectrum, with the exception of peaks 435, 457, and 391, correspond to individual aldehydes. The peaks at 435, 457, and 391 present in this pool are most likely sodium or solvent adducts or fragmentation ions formed during the ionization process. Following cleavage from the resin, the resulting pools of peptide aldehydes were in sufficiently pure form for testing directly in the whole cell assay. The major side product formed during the cleavage reaction was the corresponding alcohol, formed as a result of over-reduction. We tested representative alcohols in the Aβ EIA to make sure that they did not interfere with the assay. In all cases, the alcohols corresponding to active peptide aldehydes were found to be inactive in inhibiting Aβ production in N9 cells (data not shown).

To determine the stereochemical purity of the amino acid at the C-terminus of the peptide aldehydes generated using this synthetic method, the synthesis of single peptide aldehydes was carried out to ascertain the possibility of scrambling the stereochemistry of the α protons during the cleavage reaction. NMR analysis of the peptide aldehydes showed the presence of one aldehyde proton as a doublet. If there was substantial scrambling of the stereochemistry at that position, this single would appear as a doublet of doublets due to the splitting by two different Cα protons. A few of the compounds described in this study, including the most potent compound, was analyzed by NMR spectroscopy to rule out the possibility of significant scrambling of that stereocenter (data not shown).

Table 1. Structures of the Peptide Aldehyde Libraries Synthesized[a]

Pool #	Structure of the pool
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

[a] R_1 is a mixture containing glycine, alanine, D- or L-valine, D- or L-phenylalanine, proline, leucine, isoleucine, and 4-aminobutyric acid.

Based on the carbobenzoxyl-valinyl-phenylalanal lead described previously,[14] 10 pools of dipeptide aldehydes with Phe at position P1 were synthesized in order to systematically determine the optimum amino-terminal blocking group to incorporate into the dipeptide aldehyde. Each pool possessed a unique amino-terminal blocking group while the P1 residue was maintained as a Phe and the P2 residue was a collection of 10 different side chains (Table 1). Since the peptide inhibitors of Aβ production reported in the literature all tend to be fairly

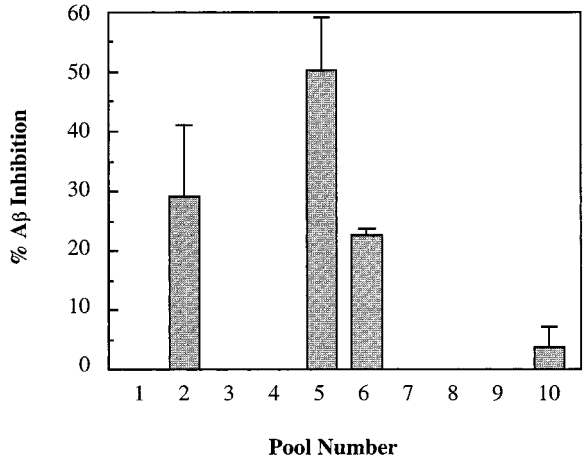


Figure 2. Inhibition of Aβtotal by pools of combinatorially synthesized aldehydes. Each pool of aldehydes was tested at a concentration of 25 μM in N9 cells. Percent Aβ inhibition values were based on the level of Aβ present in the conditioned medium of treated cells relative to a DMSO (no compound) control, as determined by EIA. Error bars represent the standard deviation of three assays.

hydrophobic as is the putative γ-secretase site of βAPP, the choice of the P2 residue was restricted to hydrophobic amino acids. This choice was also influenced by the desire to enhance the lipophilic nature of these peptide aldehydes in order to maximize cell permeability. Following synthesis, each pool was evaluated for its ability to inhibit the production of Aβ in N9 cells using the EIA (enzyme-linked immunosorbent assay) described in the Experimental Section. Three pools of aldehydes (pools #2, #5, and #6) were significantly active and showed greater than 20% inhibition of Aβ at a concentration of 25 μM (Figure 2). The remaining pools were relatively inactive with Aβ inhibition values below 10% at 25 μM. Of the three active pools, the most active pool was #5 containing 3,5-dimethoxycinnamamide as the amino-terminal blocking group. Studies with all subsequent aldehydes contained this amino-terminal group.

To determine the preferred P2 side chain within pool #5, the 10 individual dipeptide aldehydes comprising this pool were synthesized separately. Each of these individual aldehydes contained the 3,5-dimethoxycinnamamide amino-terminal blocking group and a Phe at the P1 position but varied at the P2 position. Figure 3 shows the relative abilities of these aldehydes to inhibit Aβ production in N9 cells at a concentration of 25 μM. All individual dipeptide aldehydes present in this pool were active and indicated that the observed activity of the pool was due to a sum of 10 partially active individual aldehydes and not due to the presence of any single compound with overwhelming activity. Of these, the aldehyde with an Ile at the P2 position was slightly more active than either the Val- or Leu-containing aldehyde. The deconvolution of another active pool (#6) gave similar results (Figure 3). Thus, the relative activities of dipeptide aldehydes varying at the P2 position were the same whether they possessed the 3,5′-dimethoxycinnamamide (pool #5) or the 3-methylbutanoyl (pool #6) amino-terminal blocking group.

Having determined the preferred amino-terminal blocking group and identifying Ile as a suitable P2 residue, a series of 16 individual aldehydes with varying P1 residues was synthesized to establish the optimum

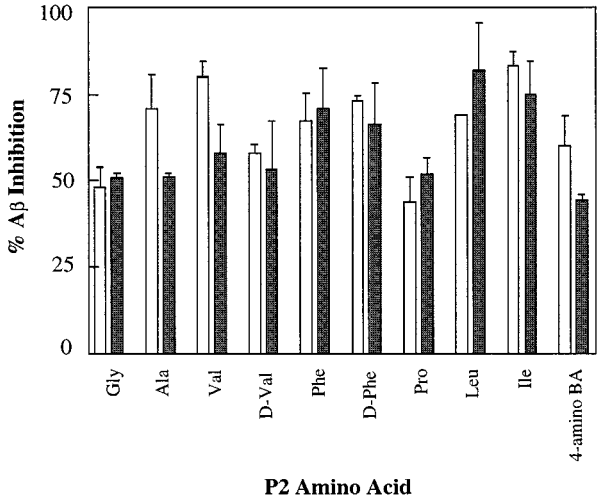


Figure 3. Aβtotal inhibition by dipeptide aldehydes differing at the P2 position. Individual dipeptide aldehydes were synthesized and assayed in the Aβ EIA at a concentration of 25 μM as described in Figure 2. 3,5-Dimethoxycinnamamide-(**P2**)-phenylalanal dipeptide aldehydes from pool #5 are represented by the white bars. 3-Methylbutanoyl-(**P2**)-phenylalanal dipeptide aldehydes from pool #6 are represented by the black bars. Error bars represent the standard deviation of three assays.

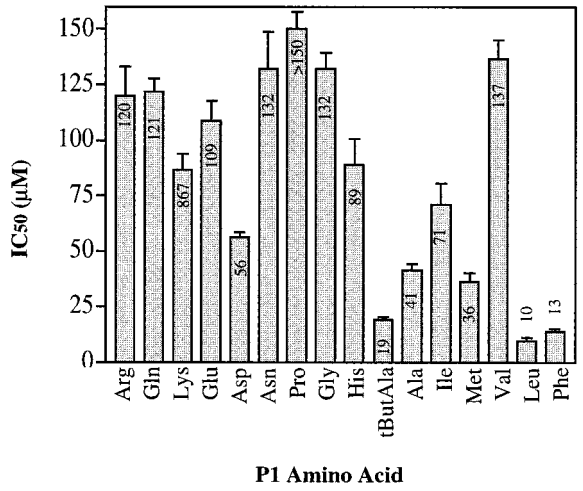
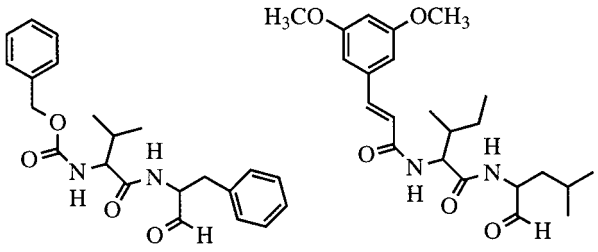


Figure 4. Inhibition of Aβtotal by dipeptide aldehydes differing at the P1 position. 3,5-Dimethoxycinnamamide-isoleucinyl-(**P1**)-aldehyde dipeptides were individually synthesized and assayed in the Aβ EIA as described in the Experimental Section. IC_{50} values are defined as the concentration of compound giving rise to 50% inhibition of Aβtotal relative to a DMSO (no compound) control. Error bars represent the standard deviation of three assays.

side chain at the P1 site. As established in the above experiments, each of these aldehydes contained the preferred 3,5-dimethoxycinnamamide amino-terminal blocking group and an Ile at the P2 position. These compounds were synthesized and assayed in the EIA, and their IC_{50} values were determined as described in the Experimental Section. Figure 4 illustrates the relative IC_{50} values for the 16 compounds synthesized. With the exception of Val at the P1 position, aldehydes with hydrophobic P1 residues tended to be the most active, while those with charged P1 amino acid side chains were relatively inactive. The most active aldehyde with an IC_{50} of 9.6 μM contained a Leu at the P1



carbobenoxyl-valinyl-phenylalanal I

Figure 5. Structures of carbobenzoxyl-valinyl-phenylalanal and 3,5-dimethoxycinnamamide-isoleucinyl-leucinal (compound **1**).

site, while the next most active aldehyde had a Phe at this position.

Having discovered a dipeptide aldehyde, 3,5-dimethoxycinnamamide-isoleucyl-leucinal (compound **1**), with an approximately 10-fold improvement in IC_{50} relative to the initial lead compound, carbobenzoxyl-valinyl-phenylalanal (Figure 5), it was of interest to determine whether the mechanism of action of this aldehyde was in any way similar to that of carbobenzoxyl-valinyl-phenylalanal. To determine this, N9 cells were metabolically labeled with [^{35}S]methionine/cysteine in the presence of 0−50 μM compound **1** for 5 h after which time the conditioned medium was harvested and immunoprecipitated with antibodies directed toward either Aβtotal (mAb 1101.1), Aβ1−40 (mAb 1702.1), or Aβ1−42 (mAb 108.1). The remaining cells were lysed and immunoprecipitated with antiserum BC-1, specific for the carboxyl-terminus of βAPP. The immunoprecipitated complexes were separated by polyacrylamide gel electrophoresis as described in the Experimental Section, and the resulting images were subjected to quantitative phosphorimage analysis. The results are shown in Figure 6. Panel A shows that, in the presence of increasing amounts of compound **1**, β- and α-CTFs accumulated to high levels during the 5-h treatment period. The accumulation of CTFs observed here mimics the effect observed with carbobenzoxyl-valinyl-phenylalanal[14] and suggests that compound **1** inhibits the γ-secretase step of βAPP processing. Panel B shows the extent of Aβtotal inhibition by compound **1** as determined by immunoprecipitation. The Aβtotal inhibition observed by immunoprecipitation was consistent with the IC_{50} determined previously by EIA (Figure 4). The approximately 3-kDa peptide that coprecipitated with Aβ contains the mAb 1101.1 epitope and is a variant of Aβ formed by cleavage between amino acids Tyr10 and Glu11 of the Aβ peptide.[14,38]

Having established that compound **1** was capable of inhibiting the production of Aβ in N9 cells, antibodies specific for either Aβ1−40 or Aβ1−42 were used to determine which form(s) of Aβ was inhibited. N9 cells were treated for 5 h with compound **1** at concentrations ranging from 0 to 50 μM. The conditioned media were harvested and immunoprecipitated with either mAb 1702.1 or mAb 108.1. The results are shown in Figure 7. Immunoprecipitating Aβ1−40 with mAb 1702.1 (Figure 7, panel A) showed that compound **1**, at concentrations greater than 12.5 μM, inhibited Aβ1−40 in a fashion similar to that observed for Aβtotal (Figure 6, panel B). However, at a concentration of 12.5 μM and

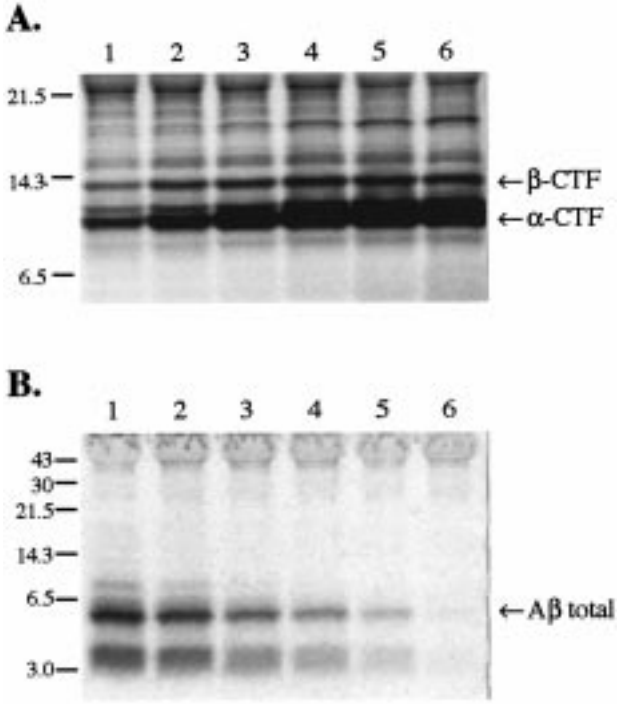


Figure 6. Immunoprecipitation of CTFs and Aβtotal from N9 cells treated with compound **1**. N9 cells were radiolabeled with [^{35}S]methionine/cysteine for 5 h in the presence of compound **1** at 0, 3.1, 6.2, 12.5, 25, and 50 μM (lanes 1−6, respectively). Immunoprecipitated samples were separated on a 16.5% Tris/tricine gel. Panel A: CTFs immunoprecipitated from the lysates of treated cells using antiserum BC-1. Panel B: Aβtotal immunoprecipitated from the conditioned medium of cells using mAb 1101.1.

below, compound **1** caused a slight increase in Aβ1−40 as detected by mAb 1702.1. Despite the fact that Aβ1−40 is the predominant species formed by N9 cells, this slight increase was not observed for Aβtotal immunoprecipitated with mAb 1101.1. It is likely that Aβ variants recognized by mAb 1101.1 but not by mAb 1702.1 could account for this difference.

In contrast to the effect on Aβ1−40, compound **1** did not inhibit Aβ1−42 as much over the same concentration range (Figure 7, panel B). Even at the highest concentration tested, compound **1** only inhibited approximately 15% of Aβ1−42 (Figure 7, panel B) versus >90% inhibition of Aβ1−40 (Figure 7, panel A). Thus, compound **1** preferentially inhibited the 1−40 from versus the 1−42 form of Aβ. Figure 8 shows the profiles for Aβ1−40, Aβ1−42, and Aβtotal inhibition by compound **1**, averaged over three independent immunoprecipitation experiments. The IC_{50} for Aβtotal inhibition as observed here by immunoprecipitation (∼15 μM) is consistent with the IC_{50} of ∼10 μM observed by EIA (Figure 4). The plots for Aβ1−40 and Aβ1−42 more clearly illustrate the preference of compound **1** to inhibit Aβ1−40 versus Aβ1−42. The IC_{50} value of compound **1** for inhibition of Aβ1−40 as determined by these immunoprecipitation experiments was 22 μM, slightly higher but consistent with the ∼15 μM observed for Aβtotal. In contrast, the IC_{50} value of compound **1** for inhibition of Aβ1−42 was above 50 μM and too high for accurate determination based on the concentration range of compound **1** used here.

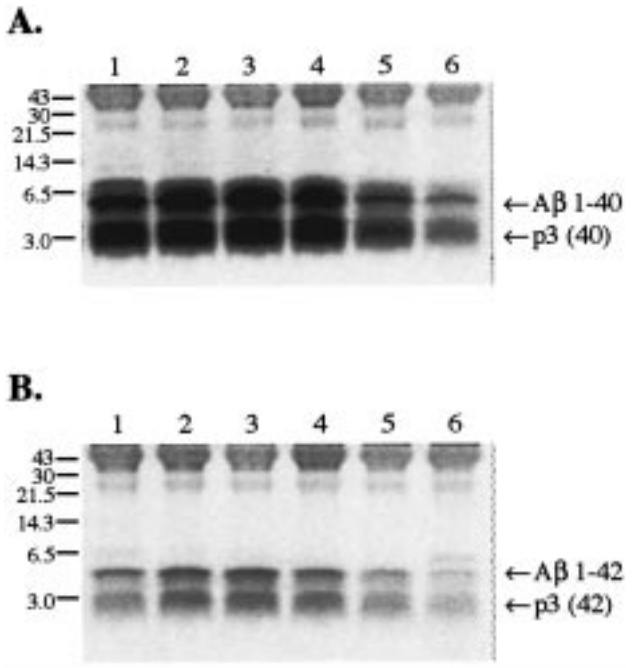


Figure 7. Effect of compound **1** on Aβ1−40 and Aβ1−42 production in N9 cells. N9 cells were radiolabeled with [^{35}S]-methionine/cysteine in the presence of compound **1** at 0, 3.1, 6.2, 12.5, 25, and 50 μM (lanes 1−6, respectively). Immunoprecipitated samples were separated on a Tris/bicine gel containing 8 M urea in order to resolve Aβ1−40 and Aβ1−42. Panel A: Aβ1−40 and p3 (40) immunoprecipitated with mAb 1702.1. Panel B: Aβ1−42 and p3 (42) immunoprecipitated with mAb 108.1.

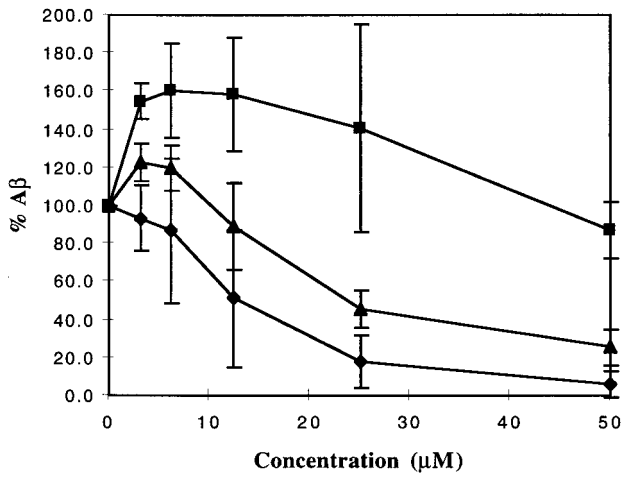


Figure 8. Inhibition of Aβ production in N9 cells by compound **1**. Aβtotal (◆), Aβ1−40 (▲), and Aβ1−42 (■) values are averages obtained from three independent immunoprecipitation experiments as described in Figures 6 and 7. Percent Aβ values were relative to a DMSO-treated control. Error bars represent the standard deviation of three independent experiments.

Discussion

The combinatorial synthetic method described in this report allows for the rapid generation of a large number of short dipeptide aldehydes with diverse amino-terminal functional groups. The resulting pools of aldehydes were sufficiently pure, as determined by electrospray ionization mass spectrometry, so that further purification of the products was not necessary prior to assaying. Differences in sample purity as judged by HPLC and mass spectrometry were minimal and did not account for differences in activity. The most active pool of aldehydes contained the 3,5-dimethoxycinnamamide amino-terminal blocking group. The hydrophobic nature of this blocking group suggests that compounds with enhanced hydrophobicity inhibit γ-secretase to a greater extent than more hydrophilic compounds. This might be due to enhanced cell penetration introduced by the more hydrophobic amino-terminus or, alternatively, to enhanced molecular interactions between the hydrophobic amino-terminus of the inhibitor and the target protease. Hydrophobicity, however, did not correlate perfectly with activity since some pools of aldehydes (pools #3, #7, and #9) were considerably less active even though they contained fairly hydrophobic amino-terminal blocking groups. In addition, pool #10 containing the 3,5-dichlorocinnamamide amino-terminal blocking group was significantly less active despite a close structural similarity to the 3,5-dimethoxycinnamamide amino-terminal functional group of the active pool #5. The difference in the activities of these two pools is difficult to readily rationalize. The possibility exists of effects arising from the presence of multiple species that could interact with each other as well as the target protein, thus affecting the activity of the pool. We chose to go ahead and deconvolute the most active pool in order to identify the individual members with the best activities.

Deconvolution of the most active pool (#5) containing the 3,5-dimethoxycinnamamide amino-terminal blocking group showed that the overall activity of this active pool was a sum of the 10 partially active members of this pool. The relative activities of these active aldehydes identified the preferred P2 amino acid residues as Ile and Leu when the P1 amino acid was Phe. Substitution with Val at the P2 position also resulted in fairly active aldehydes, indicating the preference for a small aliphatic amino acid side chain at the P2 position. However, removal of the P2 side chain with the Gly substitution resulted in a less active inhibitor, suggesting that a side chain at the P2 position fulfills a minimal steric requirement to be active. Substitution of the P2 residue with 4-aminobutyric acid or with Pro likewise resulted in poor activity most likely due to the size of these large amino acid side chains. Substitutions with the D-stereoisomer of either Val or Phe did not enhance the activity; thus, there are no additional chiral constraints at the P2 position.

Keeping the amino-terminal blocking group (3,5-dimethoxycinnamamide) and the P2 residue (Ile) constant, the P1 residue was then optimized. The most active aldehyde observed (compound **1**) contained a Leu at the P1 position. This aldehyde had an IC_{50} of 9.6 μM which was approximately 10-fold lower than that reported for the carbobenzoxyl-valinyl-phenylalanal lead.[14] Placing a bulky tBuAla or a large Phe side chain at the P1 position also yielded fairly active aldehydes, consistent with the structure of the carbobenzoxyl-valinyl-phenylalanal lead at this position; however, substitution of the P1 Leu with either a Val or an Ile resulted in a less active compound. This finding is surprising since Val and Ile are hydrophobically and sterically very similar to Leu. The molecular basis for this difference in activity has yet to be determined. Like compound **1**,

other active peptide aldehydes described to date: calpain inhibitor I, carbobenzoxyl-leucinyl-leucinyl-leucinal, and calpeptin, all have Leu or the sterically similar norLeu amino acid at the P1 position. Thus, active peptide aldehydes show a consistent preference for Leu at the P1 position. The three commercially available aldehydes have Leu in the P2 position, which, based on our findings, is also a suitable although slightly less than optimum amino acid residue for this position.

The results described here are in agreement with previous studies showing that, through mutagenesis of the transmembrane region of βAPP, γ-secretase does not have a specific amino acid sequence requirement for substrate recognition but rather has a loosely defined preference for hydrophobic amino acid sequences.[39] More recent mutagenesis studies that introduced the Val46Phe familial point mutation into βAPP resulted in an increase in the 42/40 ratios for both Aβ and p3 production but did not alter the total amount of Aβ or p3 formed.[40,41] These results not only suggest that Aβ and p3 are produced by the same activity but also imply that there is a single γ-secretase activity responsible for the formation of Aβ1−40 and Aβ1−42. The question of whether Aβ1−40 and Aβ1−42 are produced by the same or different enzymes is still open to debate.

A mechanism for γ-secretase cleavage specificity was recently proposed based on results from phenylalanine-scanning mutagenesis studies.[42] The proposed model suggests that the γ-secretase site of βAPP adopts an α-helical conformation and the amino acid composition of this region of βAPP dictates where γ-secretase binds and cleaves. Interestingly, this model is consistent with either γ-40- and γ-42-secretase being different enzymes with similar active sites or γ-secretase being a single enzyme with the ability to bind to and cleave either the 40 or 42 site.[42]

The ability of compound **1** to preferentially inhibit the production of Aβ1−40 versus Aβ1−42 is consistent with the inhibitory activity of carbobenzoxyl-valinyl-phenylalanal and three other peptide aldehydes reported previously.[28,29] Thus, all active peptide aldehydes observed to date appear to function through a similar mechanism. If this differential inhibition is due to the presence of two distinct γ-secretases as previously proposed,[8,28,30] it is not clear, based on amino acid sequence alone, why compound **1** preferentially inhibits the Aβ1−40 γ-secretase since the amino acid sequence of this dipeptide inhibitor more closely resembles the 42 cleavage site of βAPP. It could be that the γ-secretase that produces Aβ1−40 belongs to a class of proteases that is more readily inhibited by peptide aldehyde inhibitors than the γ-secretase responsible for the formation of Aβ1−42. A recent report describing a peptide aldehyde-sensitive serine carboxypeptidase that generates Aβ1−40 by removing 2−3 amino acids from the carboxyl-terminus of the longer forms of Aβ is support for this mechanism of preferential Aβ inhibition.[43] Alternatively, the Aβ1−40 and Aβ1−42 γ-secretase activities may reside in distinct intracellular compartments as previously proposed.[25] If this is the case, compound **1** may be more readily accessible to the intracellular compartment possessing the Aβ1−40 γ-secretase activity. Further studies are necessary not only to establish the molecular basis for this preferential inhibition but also to better define the class of proteases to which γ-secretase belongs.

The combinatorial approach to synthesizing peptide aldehydes described in this report rapidly identified new inhibitors of γ-secretase. These inhibitors have helped to determine the preference of this important enzyme for hydrophobic amino acid sequences and provide further clues as to the mechanism of γ-secretase cleavage of βAPP.

Conclusions

A combinatorial approach was used to identify pools of dipeptide aldehydes capable of inhibiting the production of Aβ. Deconvolution of one pool of active aldehydes and systematic optimization of the most active member of the set resulted in the identification of a series of dipeptide aldehydes with enhanced potencies relative to carbobenzoxyl-valinyl-phenylalanal. The most active dipeptide aldehydes possessed hydrophobic amino acids at both the P1 and P2 positions. The most potent compound identified in this study was 3,5-dimethoxy-cinnamamide-isoleucinyl-leucinal (compound **1**) with an IC_{50} of 9.6 μM, approximately 10-fold more active than carbobenzoxyl-valinyl-phenylalanal. Compound **1** preferentially inhibited the production of Aβ1−40, whereas the production of Aβ1−42 was not as strongly inhibited. These results suggest that dipeptide aldehydes related to carbobenzoxyl-valinyl-phenylalanal inhibit Aβ through similar mechanisms and demonstrate the utility of a combinatorial synthesis approach to identify potent inhibitors of Aβ production.

Experimental Section

A. Abbreviations. Abbreviations of the common amino acids follow the recommendations of IUPAC−IUP. Additional abbreviations: 4-amino-BA, 4-aminobutyric acid; Fmoc, fluorenylmethoxycarbonyl; SDS, sodium dodecyl sulfate; tButAla, *tert*-butylalanine.

B. Preparation of 3-(*N*-methoxyamino)propanoyl Methoxybenzhydrylamine (MBHA) Resin. The synthesis of peptide aldehydes using (*N*-methoxyamino)propanoyl MBHA resin is illustrated in Scheme 1. MBHA resin was swollen by treatment with dimethylformamide (DMF), 10 mL g^{-1}, 5 times, 30 s. The resin was then treated with 10% diisopropylethylamine (DIEA) in dichloromethane (DCM), 10 mL g^{-1}, 2 times, 2 min. The resin was finally washed with DMF and treated with the active ester of Fmoc-3-(*N*-methoxyamino)propanoic acid (5 equiv) in DMF, generated by treating the acid with equivalent amounts of 1-hydroxybenzotriazole hydrate (HOBt) and diisopropylcarbodiimide (DIPC) in DMF for 30 min. The reaction was allowed to continue until no further free amine was detected on the resin, using a qualitative ninhydrin analysis. The resin was then washed with DMF, methanol, DCM, and ethyl acetate and dried under vacuum to a constant weight. The substitution of the free amine on the resin prior to treatment with Fmoc-3-(*N*-methoxyamino)propanoic acid is considered as the degree of substitution of the resin.

C. Cleavage of Peptide Aldehydes from 3-(*N*-Methoxyamino)propanoyl MBHA Resin. All side chain protecting groups on the solid-phase constructs were removed using 45% trifluoroacetic acid in DCM, 2 times, 2 min, 30 min. The resin was then washed with DCM, methanol, and ethyl acetate and dried under vacuum to a constant weight. An aliquot of the resin was suspended in anhydrous tetrahydrofuran (THF), 5 mL g^{-1}. The reaction assembly was purged with nitrogen and 5 equiv (with respect to the degree of substitution on the initial MBHA resin) of 1 M lithium aluminum hydride (LAH) was added under nitrogen at 0 °C. The reaction mixture was allowed to stir at 0 °C for 30 min after which the reaction was

quenched with 5% aqueous potassium bisulfate ($KHSO_4$). The spent resin was removed by filtration and the peptide aldehydes were extracted from the aqueous filtrate with ethyl acetate (4 × 25 mL). The ethyl acetate extract was washed with a saturated sodium chloride solution (2 × 25 mL) and dried over anhydrous $MgSO_4$. Volatile solvents were removed on a rotary evaporator and the residue was suspended in acetonitrile:water (1:9), frozen, and lyophilized to give white powders.

D. Synthesis of Fmoc-3-(*N*-methoxyamino)propanoic Acid. *tert*-Butyl acrylate (8.8 mL 60.07 mmol) was added to a solution of methoxyhydroxylamine hydrochloride (5.0 g 59.88 mmol) in the presence of diisopropylethylamine (50 mL) in acetonitrile (50 mL) as shown in Scheme 2. After refluxing for 60 h in an inert atmosphere, the reaction mixture was concentrated on a rotary evaporator and the residue taken up in ethyl acetate (50 mL) and washed with saturated sodium bicarbonate (3 × 50 mL) and saturated sodium chloride (3 × 50 mL). After drying over anhydrous magnesium sulfate, the organic layer was concentrated on a rotary evaporator to give 4.81 g of a colorless nonviscous oil. This material (3.53 g) was treated with Fmoc-Cl (5.19 g) in 10% sodium carbonate (35 mL) and dioxane (55 mL) for 12 h. The reaction mixture was poured into water (200 mL) and washed with ether (3 × 50 mL). Following acidification to pH 4 with 1 N hydrochloric acid, the aqueous layer was extracted with ethyl acetate (3 × 50 mL). The ethyl acetate layer was washed with 0.1 N hydrochloric acid (2 × 30 mL) and saturated sodium chloride (2 × 30 mL), dried over anhydrous magnesium sulfate, and concentrated to give 7.22 g of a viscous colored oil. This material was treated with trifluoroacetic acid:dichloromethane (1:1) (60 mL) for 2 h and upon concentration on a rotary evaporator, 7.36 g of a dark viscous oil was obtained. TLC: silica gel ethyl acetate:hexane (4:6), R_f 0.3. Upon trituration with ether a white solid ensued which was recrystallized from ethyl acetate/hexane to give 3.50 g of a white solid. Combustion analysis: C 66.85 calcd, found 66.59; H 5.61 calcd, found 5.73; N 4.10 calcd, found 4.02. ^{1}H NMR ($CDCl_3$): δ 2.58 *t* (2H), δ 3.64 *s* (3H), δ 3.75 *t* (2H), δ 4.24 *t* (1H), δ 4.49 *d* (2H), δ 7.30 *t* (2H), δ 7.38 *t* (2H), δ 7.59 *d* (2H), δ 7.74 *d* (2H).

E. Synthesis of Peptide Aldehydes. Following standard solid-phase synthesis, on the modified MBHA resin, the peptides were cleaved from the resin using LAH (5 equiv) in THF for 30 min at 0 °C. The reaction was quenched with treatment with 5% potassium hydrogen sulfate solution (aqueous). Following filtration to removed the resin, the product was extracted with methylene chloride or chloroform. The chloroform layer was dried over anhydrous sodium sulfate and concentrated to give crude product. This crude product was then redissolved in acetonitrile/water (0.1% trifluoroacetic acid, TFA) and purified using reverse-phase HPLC. Purifications were carried out on a preparative C_{18} Vydac column, using a gradient of water/acetonitrile (0.1% TFA), 5−45% acetonitrile. Fractions were collected across the main peaks and analyzed using analytical reverse-phase HPLC. Homogeneous fractions were pooled, concentrated, and lyophilized to give the final products as powders. The homogeneity of the products was determined by reverse-phase HPLC, and their identity was confirmed by elecrospray ionization mass spectroscopy. In the case of certain individual compounds, NMR spectra were obtained to confirm structure.

F. Monoclonal Antibodies. The hybridoma cell lines secreting monoclonal antibodies (mAbs) 1101.1, 108.1, or 1702.1 were generated from fusions of FOX-NY murine myeloma cells (American Type Culture Collection, Manassus, VA) with spleen cells from Balb/c mice immunized with Aβ peptides conjugated to bovine serum albumin (BSA). The peptides were synthesized to include a terminal cysteine for coupling to the carrier protein using the heterobifunctional cross-linker, *N*-maleimido-6-aminocaproyl-(2′-nitro-4′-sulfonic acid)phenyl ester (Bachem, Torrance, CA). Hybridoma 1101.1 was derived from a mouse immunized with Aβ residues 13−22, Ac-HHQKLVFFAE(C). By enzyme-linked immunosorbent assay (ELISA), mAb 1101.1 was shown to have no binding to Aβ peptides 1−17 or 17−29 but high-affinity binding to 1−28, 1−40, and 1−42 (data not shown). Since the peptide used to generate 1101.1 spans the α-secretase site of Aβ, this mAb recognizes all forms of Aβ and the carboxyl-terminal fragments from β-secretase cleavage. The hybridoma line 108.1[44] was obtained by immunizing with the Aβ 1−42 carboxyl-terminal peptide 35−42, (C)VGGVVIA. The 1702.1 hybridoma was obtained from a mouse immunized with Aβ 34−43, (C)MVGGVVIAT. The antiserum from this mouse unexpectedly showed better binding to 1−40 than to 1−42, and the derived monoclonal 1702.1 was found to have high affinity for 1−40 and no binding to 1−42. Polyclonal antibody BC-1 was prepared as described previously.[14]

G. Cell Culture. The N9 cell line expressing βAPP was created by transfecting the human cDNA encoding βAPP 695, driven by the human cytomegalovirus (CMV) promoter into chinese hamster ovary (CHO) cells using standard transfection protocols. Transfectants were selected with G418, isolated, and subcloned and βAPP expression was determined by immunoprecipitation. N9 cells were propagated in Dulbecco's modified eagle medium (DMEM)/Ham's F-12 (Life Technologies, Gaithersburg, MD) with 15 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.2, supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 units/mL penicillin, and 50 μg/mL streptomycin. Cells were seeded 24 h prior to assay, either in 96-well plates at 7.5×10^4 cells/well for enzyme immunoassay (EIA) or in 6-well plates at 1.4×10^6 cells/well for immunoprecipitations (IP).

H. EIA and IP Assays. A competitive enzyme-linked immunoassay (EIA) was used to quantitate Aβ secretion from N9 cells grown in 96-well plates. Confluent cells were rinsed twice with Hank's buffered saline solution (HBSS) containing Ca^{2+} and Mg^{2+} and then incubated with 150 μL/well of serum-free N9 cell growth medium supplemented with 0.2% BSA containing dilutions of test compounds (0−100 μM) at a final dimethyl sulfoxide (DMSO) concentration of 1% (v/v). Control cells were incubated in serum-free medium plus 1% DMSO. After 5 h at 37 °C, the conditioned media were transferred to EIA wells. The EIA plates were precoated with 1 μg/mL goat anti-mouse IgG Fc, washed, blocked with 1% BSA (Bayer Corp., Kankakee, IL) in phosphate-buffered saline (PBS), and emptied. A 100-μL volume of Aβ-containing medium (either the N9-conditioned media or 1−40 peptide standard diluted in serum-free medium) and a 100-μL volume of mAb 1101.1 diluted to 2.5 ng/mL in EIA buffer (PBS containing 0.05% Tween-20 and 0.1% BSA) were added to the wells. Wells with buffer only, without the mAb, served as controls for nonspecific binding. The plates were covered and kept overnight at 4 °C. Then, the "competitive probe", biotinylated Aβ1−28 (the 28 amino-terminal residues of Aβ synthesized with a biotinylated Asp^1), was added to the 200-μL contents of the EIA wells, at 50 μL/well, 18 ng/mL in EIA buffer. After 1 h at 4 °C, the plates were washed three times with PBS/Tween-20 and the wells were filled with 200 μL of a 1:10,000 dilution of streptavidin-horseradish peroxidase (HRP) (Zymed Laboratories, S. San Francisco, CA) in EIA buffer. The plates were again placed at 4 °C for 1 h then washed six times with PBS/Tween-20. The 3,3′,5,5′-tetramethylbenzidine (TMBZ) substrate solution (Sigma, St. Louis, MO) was added at 200 μL/well and after 30−45 min at room temperature the color development was quenched with the addition of 50 μL/well 2 N H_2SO_4. The absorbance at 450 nm was read using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA). The Aβ concentrations in the N9 wells were extrapolated from the standard curve. The compound-induced inhibition of Aβ secretion was calculated as a percentage relative to the secretion from control N9 cells treated with 1% DMSO alone. The concentration range of compound used to determine IC_{50} was 0−100 μM. IC_{50} values were determined by extrapolation of the compound concentration giving rise to 50% inhibition of Aβ production relative to a DMSO-treated (no compound) control.

For immunoprecipitation assays, confluent N9 cells in 6-well plates were washed twice with HBSS with calcium and

magnesium. Cells were incubated for 5 h at 37 °C in serum-free N9 medium without cysteine and methionine supplemented with 150 μCi ^{35}S-translabel (Amersham Life Sciences Inc., Arlington Heights, IL) and the appropriate concentration of test compound (1.5 mL/well). After the 5-h incubation period, the conditioned medium was harvested, precleared with a 5-μL aliquot of normal mouse serum and 25 μL of 10% rabbit anti-mouse conjugated protein A sepharose (PAS) (Pharmacia Biotech, Uppsala, Sweden), and immunoprecipitated overnight with 15 μL (1.6 mg/mL) of monoclonal antibody and 50 μL of 10% rabbit anti-mouse conjugated PAS. For immunoprecipitation of carboxyl-terminal fragments of βAPP, ^{35}S-labeled N9 cells were rinsed twice with HBSS and lysed in 1 mL of 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) containing a cocktail of protease inhibitors (Boehringer Mannheim, Indianapolis, IN). The cell lysates were precleared with 10 μL of normal rabbit serum and 25 μL of 10% PAS, then immunoprecipitated with 25 μL of antiserum BC-1 and 50 μL of 10% PAS. After immunoprecipitation, PAS pellets were washed three times with 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Nonidet P-40 and twice with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40. PAS pellets were finally rinsed twice with 10 mM Tris-HCl, pH 7.5, resuspended in 60 μL of 2X sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromphenol blue), and boiled 3 min.

I. Gel Electrophoresis. Immunoprecipitated Aβ was resolved on Tris/bicine gels containing 8 M urea as described previously.[45] Immunoprecipitated CTFs were resolved on 16.5% Tris/tricine gels as described previously.[14] Following electrophoresis, gels were fixed for 30 min in 20% methanol, 20% acetic acid (v/v), dried, exposed on a phosphor screen for 3 days to detect Aβ or overnight to detect CTFs, and quantitated by phosphorimage analysis on a PhosphorImager SF scanner (Molecular Dynamics, Sunnyvale, CA).

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