Rank-Order of Potencies for Inhibition of the Secretion of Aβ40 and Aβ42 Suggests That Both Are Generated by a Single γ-Secretase*

(Received for publication, March 3, 1999, and in revised form, April 20, 1999)

John T. Durkin§, Seetha Murthy, E. Jean Husten, Stephen P. Trusko, Mary J. Savage, David P. Rotella§, Barry D. Greenberg§, and Robert Siman‡

From Cephalon, Inc., West Chester, Pennsylvania 19380

The Alzheimer's disease amyloid peptide Aβ has a heterogeneous COOH terminus, as variants 40 and 42 residues long are found in neuritic plaques and are secreted constitutively by cultured cells. The proteolytic activity that liberates the Aβ COOH terminus from the β-amyloid precursor protein is called γ-secretase. It could be one protease with dual specificity or two distinct enzymes. By using enzyme-linked immunosorbent assays selective for Aβ40 or Aβ42, we have measured Aβ secretion by a HeLa cell line, and we have examined the dose responses for a panel of five structurally diverse γ-secretase inhibitors. The inhibitors lowered Aβ and p3 secretion and increased levels of the COOH-terminal 99-residue β-amyloid precursor protein derivative that is the precursor for Aβ but did not alter secretion of β-amyloid precursor protein derivatives generated by other secretases, indicating that the inhibitors blocked the γ-secretase processing step. The dose-dependent inhibition of Aβ42 was unusual, as the compounds elevated Aβ42 secretion at sub-inhibitory doses and then inhibited secretion at higher doses. A compound was identified that elevated Aβ42 secretion at a low concentration without inhibiting Aβ42 or Aβ40 at high concentrations, demonstrating that these phenomena are separable pharmacologically. Using either of two methods, IC50 values for inhibition of Aβ42 and Aβ40 were found to have the same rank-order and fall on a trend line with near-unit slope. These results favor the hypothesis that Aβ variants ending at residue 40 or 42 are generated by a single γ-secretase.

A hallmark feature of the neuropathology of Alzheimer's disease is the abundant deposition of amyloid into neuritic and diffuse plaques in the brain parenchyma. The predominant core constituent of amyloid plaques is a 40- to 42-residue amyloid peptide, Aβ. Aβ is generated from the β-amyloid precursor protein (APP) by two sequential proteolytic cleavages. First, β-secretase activity liberates the NH2 terminus of Aβ, generating a 99-residue COOH-terminal APP fragment (C99). Second, γ-secretase activity cleaves C99 to liberate the Aβ COOH terminus. APP is also processed in a nonamyloidogenic manner, being cleaved within the Aβ domain by an activity termed α-secretase. The terms α-, β-, and γ-secretase are conceptual terms for proteases that are not yet definitively identified (reviewed in Refs. 1–3).

The Aβ peptide is a constitutive secretory product of a variety of neuronal and non-neuronal cells, in which multiple NH2- and COOH-terminal cleavages generate several Aβ species. The most prevalent secreted forms of Aβ appear identical to neuritic plaque core amyloid Aβ and terminate either at residue 40 or 42 (4, 5). Although Aβ40 variants are the predominant component of soluble Aβ extracted from brain and secreted by cultured cells, Aβ42 forms are the predominant constituents of neuritic plaques (6–9).

In vitro, forms of Aβ that terminate at residue 42 form fibril faster than forms of Aβ that terminate at residue 40, by orders of magnitude (10). Mutations in the APP gene or in two unrelated genes, presenilin-1 and presenilin-2, are a leading cause of early-onset, inherited forms of Alzheimer's disease. Strikingly, all of the mutations linked to Alzheimer's disease increase the amount of Aβ42 that is secreted from cultured cells or extracted from the transgenic mouse brain and human plasma, whereas only some of the mutations are characterized by increased Aβ40 (reviewed in Refs. 3 and 11–13). These observations suggest Aβ42 has a central role in amyloid plaque formation and, hence, in the pathogenesis of disease.

The two major COOH-terminal variants of Aβ could be generated in several different ways. First, there may be a single γ-secretase capable of cleaving at both Aβ40 and Aβ42. Alternatively, two distinct endoproteases, with different substrate preferences and inhibitor sensitivities, could generate the two distinct species of Aβ. A third possibility is that a single γ-secretase generates Aβ42, which subsequently is processed by a carboxypeptidase to form Aβ40. The resolution of these alternatives will direct efforts to identify the protease, or proteases, responsible for formation of the Aβ variants and will pose different questions about molecular and cellular mechanisms of γ-secretase processing. Resolution of these alternatives may also have important implications for therapeutic strategies aimed at inhibiting Aβ production and interfering with amyloid deposition. If there are two distinct γ-secretases, the central role of Aβ42 in plaque formation argues for the importance of inhibiting the γ-secretase responsible for formation of Aβ42 and suggests that it may be possible to block production of Aβ42 without interfering with Aβ40. If, on the other hand, both species are generated by a single γ-secretase or if Aβ42 serves as a precursor for Aβ40, then inhibitors of Aβ42 production would be expected to block formation of Aβ40 as well.

Studies of the inhibition of cellular secretion of Aβ variants

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed: Cephalon, Inc., 145 Brandywine Pkwy., West Chester, PA 19380-4245. Tel.: 610-738-6160. Fax: 610-344-0065; E-mail: jdurkin@cephalon.com.

‡ Present address: Bristol-Myers Squibb Co., Princeton, NJ.

§ Present address: Astra Zeneca R & D Boston, Worcester, MA.

¶ Present address: University of Pennsylvania School of Medicine, Philadelphia.

1 The abbreviations used are: Aβ, β-amyloid peptide; APP, β-amyloid precursor protein; C99, the COOH-terminal 99 residues of APP; MEM, minimum essential medium; Ab, antibody; ELISA, enzyme-linked immunosorbent assay; Z, benzoxycarbonyl; Tricine, N-[(hydroxymethyl)ethyl]glycine.
have suggested that different γ-secretases may generate Aβ40 and Aβ42 (14–16). The existence of two γ-secretases has been suggested by the observation that a given concentration of peptidyl-aldehyde or difluoroketoamide protease inhibitors blocks secretion of Aβ40 but elevates Aβ42. Here we reconsider this question by a detailed examination of the dose responses for inhibition of Aβ40 and Aβ42 secretion by a panel of five structurally diverse γ-secretase inhibitors.

EXPERIMENTAL PROCEDURES

Materials—MEM and methionine-free MEM were from Mediatech. Opti-MEM® was from Life Technologies. Fetal bovine serum was from JRH Biosciences. [35S]Met was Trans-[35S]-label® from ICN. Cell culture-tested MeSO was from Sigma. Calpeptin was from Calbiochem.

Synthesis of Protease Inhibitors—Compounds A, D, and E are aldehydes and were synthesized by standard peptide chemistry methods. Compound B, a difluoroketoamide, is structurally similar to aspartyl protease inhibitors described in Refs. 17 and 18 and was synthesized as described therein. Compound N, a dihydroxyethylene, was synthesized as described in Ref. 19 (Compound 20a in that paper).

Measurement of APP Processing and Aβ Secretion in Cell Culture—M17 human neuroblastoma cells were stably transfected with an expression construct coding for human APP695 carrying the “Swedish” mutation R594N/MS596L (20). The cells were maintained in Opti-MEM plus 5% heat-inactivated fetal bovine serum. Metabolic labeling and immunoprecipitation were performed essentially as described (21). Briefly, subconfluent cells in 6-well dishes were incubated for 4 h in 1 ml of methionine-free MEM containing 200 μCi of [35S]Met. Aβ and p3 were immunoprecipitated from the medium with Ab1153, raised against Aβ1–28 (22), or with Ab58, raised against Aβ17–40 (1). COOH-terminal derivatives of APP were immunoprecipitated from cell lysates with Ab11, raised against APP696-685 (21). Immunoprecipitated proteins were separated by electrophoresis through Tris-Tricine gels and visualized by phosphorimaging.

HeLa-pNAN8 cells are a clone of HeLa cells transfected with the pNAN expression construct. This vector expresses a fusion protein consisting of the bovine growth hormone signal sequence, the COOH-terminal 103 residues of APP (beginning four residues upstream of the β-secretase cleavage site), and a tetrapeptide extension Tyr-Cys-Phe-Ala (23). The cells were maintained in MEM plus 10% fetal bovine serum. For each experiment, HeLa-pNAN8 cells were grown to ~90% confluence in 24-well plates. Cells were pretreated for 1 h and then treated for 4 h, with test compound or MeSO vehicle in 0.3 ml of medium per well. Each 24-well plate held three vehicle control wells and six doses of test compound in 0.3 ml of medium per dose. The dose spacing of the doses and the triplicate cultures were chosen to allow reliable determination of IC50 values despite the steepness of the dose-response curves (see Fig. 3). At the end of the conditioning period, the plates were centrifuged at 200 × g for 5 min, and the medium was loaded onto ELISA plates at appropriate dilutions for determination of Aβ40 and Aβ42. The Aβ40- and Aβ42-selective ELISAs have been described and characterized extensively (24). The compounds used in this study neither interfered with detection of Aβ40 or Aβ42 by ELISA nor enhanced detection of Aβ42.2 Dose-response curves were expressed as percent of control value and were fitted (by nonlinear least squares using the program GraphPad Prism) to sigmoidal dose-response curves with variable slope, the bottom fixed at zero and the top fixed to 100% or the observed maximum, whichever was greater.

Dose-response curves in the presence of 10 μM Compound N were measured as above, except that only six doses of test compound were included on each plate, allowing measurement of Aβ secretion by both untreated cells (no Compound N, no test compound) as well as Compound N-treated control (10 μM Compound N, no test compound).

RESULTS

The five inhibitors of Aβ secretion chosen for this work incorporate either aldehyde or difluoroketoamide isosteres as enzyme-reactive groups (Fig. 1). Compounds incorporating these groups, including Compound C, have been demonstrated previously to be inhibitors of γ-secretase processing (14–16, 25). Diagnostic of γ-secretase inhibition is blockade of both Aβ1–28 (22), or with Ab58, raised against Aβ17–40 (1). COOH-terminal derivatives of APP were immunoprecipitated from cell lysates with Ab11, raised against APP696-685 (21). Immunoprecipitated proteins were separated by electrophoresis through Tris-Tricine gels and visualized by phosphorimaging.

HeLa-pNAN8 cells are a clone of HeLa cells transfected with the pNAN expression construct. This vector expresses a fusion protein consisting of the bovine growth hormone signal sequence, the COOH-terminal 103 residues of APP (beginning four residues upstream of the β-secretase cleavage site), and a tetrapeptide extension Tyr-Cys-Phe-Ala (23). The cells were maintained in MEM plus 10% fetal bovine serum. For each experiment, HeLa-pNAN8 cells were grown to ~90% confluence in 24-well plates. Cells were pretreated for 1 h and then treated for 4 h, with test compound or MeSO vehicle in 0.3 ml of medium per well. Each 24-well plate held three vehicle control wells and six doses of test compound in 0.3 ml of medium per dose. The dose spacing of the doses and the triplicate cultures were chosen to allow reliable determination of IC50 values despite the steepness of the dose-response curves (see Fig. 3). At the end of the conditioning period, the plates were centrifuged at 200 × g for 5 min, and the medium was loaded onto ELISA plates at appropriate dilutions for determination of Aβ40 and Aβ42. The Aβ40- and Aβ42-selective ELISAs have been described and characterized extensively (24). The compounds used in this study neither interfered with detection of Aβ40 or Aβ42 by ELISA nor enhanced detection of Aβ42.2 Dose-response curves were expressed as percent of control value and were fitted (by nonlinear least squares using the program GraphPad Prism) to sigmoidal dose-response curves with variable slope, the bottom fixed at zero and the top fixed to 100% or the observed maximum, whichever was greater.

Dose-response curves in the presence of 10 μM Compound N were measured as above, except that only six doses of test compound were included on each plate, allowing measurement of Aβ secretion by both untreated cells (no Compound N, no test compound) as well as Compound N-treated control (10 μM Compound N, no test compound).

RESULTS

The five inhibitors of Aβ secretion chosen for this work incorporate either aldehyde or difluoroketoamide isosteres as enzyme-reactive groups (Fig. 1). Compounds incorporating these groups, including Compound C, have been demonstrated previously to be inhibitors of γ-secretase processing (14–16, 25). Diagnostic of γ-secretase inhibition is blockade of both Aβ1–28, 29). We measured dose responses for inhibition of Aβ40 and Aβ42 secretion by the HeLa-pNAN8 cell line. This cell line stably overexpresses C99 with tetrapeptide extensions, EYVM-(C99)-YCFA (23). A similar construct, with most of the NH2-terminal APP sequence deleted, has been used previously to

and p3, secreted species that have the γ-secretase cleavage in common, without concomitant effects on the secretion of APP fragments derived from the activity of other secretases (1). Both Compound A and Compound B inhibited secretion not only of Aβ but also of p3, as shown by metabolic labeling and immunoprecipitation following treatment with the inhibitors (Fig. 2, top panel). Comparable doses of the inhibitors did not alter APP synthesis or the secretion of APPα and APPβ, the NH2-terminal proteins of α- and β-secretases.3 Moreover, the compounds blocked γ-secretase processing of APP in a cell-free system, suggesting that they inhibit γ-secretase directly.3 To characterize further the effects of the five protease inhibitors on APP processing, levels of COOH-terminal derivatives of APP were measured. As shown in Fig. 2 (bottom panel), at a dose that blocked Aβ40 and Aβ42 secretion by more than 90%, compounds A and B elevated two [35S]Met-labeled COOH-terminal APP derivatives that serve as γ-secretase substrates, the ~12-kDa β-secretase-derived C99 fragment, and the ~9-kDa α-secretase-derived C83 fragment (5, 26, 27). The 6 kDa γ-secretase-derived C57 fragment was not detected in these experiments. Collectively, these data demonstrate that compounds used in the present analysis do not markedly reduce APP synthesis or block α- or β-secretase processing but instead inhibit the γ-secretase processing step.

Aβ40 and Aβ42 are generated physiologically by γ-secretase cleavage of C99, the 99 COOH-terminal residues of APP (5, 25, 28, 29). We measured dose responses for inhibition of Aβ40 and Aβ42 secretion by the HeLa-pNAN8 cell line. This cell line stably overexpresses C99 with tetrapeptide extensions, EVK-(C99)-YCFA (23). A similar construct, with most of the NH2-terminal APP sequence deleted, has been used previously to

3 E. J. Husten, S. P. Trusko, J. T. Durkin, M. J. Savage, J. R. Zysk, D. P. Rotella, J. K. Kawooya, R. Siman, and B. D. Greenberg, manuscript in preparation.

FIG. 1. Compounds (Cpds) used in the present study. Compound A is 1-menthoylcarbonyl-Leu-Leu-H. Compound B is Z-Leu-cyclohexyl-Ala-difluoroketone-2-methylbutylamide. Compound C is Z-Leu-norLeu-H, or calpeptin (41). Compound D is Z-Trp-Leu-H. Compound E is Z-pAla-Leu-H. Compound N is Boc-cyclohexyl-Ala-ile-(2S,3R,4S)-2-aminol-1-cyclohexyl-3,4-dihydroxy-6-methylheptane (see Ref. 19, Compound 20a in that paper).
Inhibitors of Aβ secretion also inhibit secretion of p3 and elevate steady-state levels of the precursor to Aβ, C99. Duplicate cultures of M17 cells were metabolically labeled with [35S]Met while being treated with the indicated compound. Top panel, Aβ and p3 were immunoprecipitated from the medium with Ab11. The C83 fragment was identified from the literature and by its co-migration with recombinant Met-C99. The C83 fragment was not detected in these experiments; the migration of recombinant Met-C57 indicated that it would run below the 6.5-kDa standard.2 These experiments were performed in M17 cells because HeLa-pNAN8 cells do not secrete appreciable amounts of p3.

Fig. 2. Inhibitors of Aβ secretion also inhibit secretion of p3 and elevate steady-state levels of the precursor to Aβ, C99. Duplicate cultures of M17 cells were metabolically labeled with [35S]Met while being treated with the indicated compound. Top panel, Aβ and p3 were immunoprecipitated from the medium with Ab11. The C83 fragment was identified from the literature and by its co-migration with recombinant Met-C99. The C83 fragment was not detected in these experiments; the migration of recombinant Met-C57 indicated that it would run below the 6.5-kDa standard.2 These experiments were performed in M17 cells because HeLa-pNAN8 cells do not secrete appreciable amounts of p3.

study sequence specificity of γ-secretase cleavage (30). Under the protocol described here, medium conditioned by HeLa-pNAN8 cells for 4 h accumulates 7.0 to 8.5 ng of Aβ40/ml and 0.9 to 1.5 ng of Aβ42/ml. Aβ42 constitutes ~14% of the total, as it does in medium conditioned by cell lines expressing full-length APP (e.g. Ref. 31). Aβ accumulation is linear with respect to time over 4 h. The high level of Aβ secretion by HeLa-pNAN8 cells facilitates measurement of dose-dependent alterations in secretion of both Aβ40 and Aβ42 by protease inhibitors. Discriminative electrophoretic and ELISA analyses indicate that Aβ40 and Aβ42 are the predominant Aβ forms being measured, although a minor contribution of Aβ forms whose NH2 termini are within 2–3 residues of Asp-1 cannot be ruled out.2

Fig. 3A illustrates the dose-response curves for inhibition of Aβ40 and Aβ42 secretion by the difluoroketoamide Compound B. The qualitative features of the dose-response curve are the same for all γ-secretase inhibitors tested. The most striking feature is the approximately 7-fold elevation of Aβ42 secretion at sub-inhibitory doses. Among the five γ-secretase inhibitors tested, elevations of Aβ42 ranged from 3- to 8-fold. At the peak of the elevation, Aβ42 constituted as much as 50% of the total secreted Aβ. Another feature of the dose-response curves is their steepness. Hill slopes ranged from 1.8 to 4.9 for Aβ40 and from 3.0 to 5.3 for Aβ42. The steepness of the dose-response curves dictated the close spacing of compound doses chosen in these experiments (six doses per log unit).

A compound was identified that elevated Aβ42 secretion at relatively low doses, without causing inhibition even at much higher doses. Fig. 4 shows dose-dependent effects on Aβ secretion of compound N, described previously as a potent inhibitor of a variety of aspartyl proteases (IC50 < 60 nM for cathepsin D, endothelin-converting enzyme, and renin; see Ref. 19). Compound N elevated Aβ42 secretion nearly 6-fold, with an estimated EC50 about 500 nM. At doses 100-fold higher, compound N did not inhibit secretion of either Aβ40 or Aβ42. In the presence of 10 μM compound N, a saturating concentration for elevation of Aβ42, dose-response curves for the five γ-secretase inhibitors showed little or no further elevation of Aβ42 (Fig. 3B), indicating that the γ-secretase inhibitors and compound N share a common mechanism for elevation of Aβ42 secretion. The presence of compound N did not interfere with inhibition of Aβ secretion by the γ-secretase inhibitors tested; indeed, the
IC$_{50}$ values in the presence of compound N are somewhat lower than in its absence. Consequently, the elevation of A$_{42}$ can be separated pharmacologically from inhibition of γ-secretase and blockade of A$_{42}$ and A$_{40}$ secretion.

Two methods were used to compare the rank-order of inhibitor potencies for A$_{40}$ and A$_{42}$. In the first, the IC$_{50}$ for A$_{42}$ was empirically defined as the dose at which A$_{42}$ secretion declines to half of its peak (elevated) value and was estimated by fitting the data to a sigmoidal dose-response with variable slope and the top fixed to the observed peak. Fig. 5A summarizes the relationship between the IC$_{50}$ values measured for A$_{40}$ and for A$_{42}$. For the five γ-secretase inhibitors tested, the IC$_{50}$ values fall near a trend line with unit slope. The rank-order of these compounds for inhibition of A$_{40}$ and A$_{42}$ is identical to their rank-order for inhibition of A$_{40}$. In the second method, IC$_{50}$ values for A$_{40}$ and A$_{42}$ were compared in the presence of 10 μM Compound N to saturate the elevation of A$_{42}$ at low γ-secretase inhibitor concentrations. Because the A$_{42}$ dose responses look like pure inhibition under this condition, determination of the A$_{42}$ IC$_{50}$ by this method is straightforward. IC$_{50}$ values measured by this method fall near a trend line with unit slope, exactly like the empirical IC$_{50}$ values (Fig. 5B). Table I summarizes IC$_{50}$ values determined by both methods. By both methods, the rank-order of γ-secretase inhibitors for inhibition of A$_{42}$ is identical to their rank-order for inhibition of A$_{40}$.

**DISCUSSION**

If both A$_{40}$ and A$_{42}$ are generated by a single γ-secretase, the rank-order of inhibitor potencies against these two cleavages should be the same. The potencies need not be identical, because in each case the inhibitor is competing with a distinct substrate sequence for which the protease may have different affinities. If, on the other hand, two different γ-secretases cleave the precursor at these two sites, there ought to be good inhibitors of one that are poor inhibitors of the other, and the rank-order of inhibitor potencies for the two Aβ species should differ. To distinguish between these possibilities, we have examined the dose-dependent inhibition of A$_{40}$ and A$_{42}$ secretion by a panel of five structurally diverse γ-secretase inhibitors. Previous studies using only one or two doses of protease inhibitor have demonstrated differential effects on A$_{40}$ and A$_{42}$ secretion, and these data have been used to argue for the existence of two distinct γ-secretases (14–16). We demonstrate here that comparison of inhibitor effects at only one or two concentrations is not straightforward, because the dose response for A$_{42}$ is the superposition of two effects as follows: elevation of A$_{42}$ production at relatively low doses and inhibition at higher doses.

Elevations in A$_{42}$ secretion have been observed previously, as synthetic intermediates of a difluoroketoamide γ-secretase inhibitor is inactive against A$_{40}$ secretion at 200 μM but elevate A$_{42}$ (Compounds 5–7 in Ref. 16). Peptide aldehydes, described as calpain inhibitors, have also been reported to elevate secretion of both A$_{40}$ and A$_{42}$ and to increase the ratio of A$_{42}$ to total Aβ (32). The elevation of A$_{40}$ reported there was not observed in the present work. We have found that the two effects, elevation of A$_{42}$ production at relatively low doses and inhibition at higher doses, are separable pharmacologically. Compound N potently and markedly elevates A$_{42}$ production with no inhibition even at a dose 100-fold higher (Fig. 4). Furthermore, a saturating concentration of Compound N for elevation of A$_{42}$ does not interfere with inhibition of A$_{42}$ by γ-secretase inhibitors.

We therefore adopt two approaches to separate elevation of A$_{42}$ secretion from its inhibition. In the first, we consider only the descending portion of the dose-response curve and fix the top of the fitted curve to the observed peak A$_{42}$. This approach supposes the elevation of A$_{42}$ to have gone to completion before inhibition begins, as is apparent from the dose-response curves (Fig. 3A). In the second approach, the elevation of A$_{42}$ is saturated by treatment of the cells with 10 μM Compound N. Because the γ-secretase inhibitors cause no further elevation of A$_{42}$ under this condition, their dose-response curves reflect pure inhibition. IC$_{50}$ values determined by both approaches rank the γ-secretase inhibitors in the same order for A$_{40}$ and for A$_{42}$ (Fig. 5).

The dose-response data presented here are consistent with previously published data on inhibition of Aβ secretion (14–16), in which inhibitors of γ-secretase processing, including compound C of the present report, were shown to have no effect or to elevate A$_{42}$ secretion at a dose that inhibited A$_{40}$ secretion. Inspection of Fig. 3A reveals how such a dose may be chosen. At higher doses, however, every γ-secretase inhibitor that inhibits A$_{40}$ secretion also inhibits A$_{42}$ secretion, and with the same rank-order potency.

The observation that certain protease inhibitors cause a marked and relatively selective increase in secretion of A$_{42}$ reveals a potentially important step in the regulation of A$_{42}$ levels, but at present we can only speculate as to the mechanism. One explanation would be that the protease inhibitors block, in addition to γ-secretase, a protease that degrades A$_{42}$. To test this hypothesis, cell-free conditioned medium was incubated for an additional 4 h (the usual conditioning period) in the presence or absence of the γ-secretase inhibitors.
at doses saturating for the elevation of Aβ42. No degradation of Aβ40 or Aβ42 was observed during the cell-free incubation, and no elevation of Aβ42 occurred on inclusion of the γ-secretase inhibitors. Note that this experiment does not address the possible intracellular degradation of Aβ42. But intracellular Aβ42 has been shown to be degraded more slowly than intracellular Aβ40, too slowly to contribute to the experiments here (33). Furthermore, Aβ is found in cultured cells at much lower levels than secreted Aβ (29, 34–36), suggesting that Aβ is rapidly secreted upon generation and, hence, that its intracellular degradation is unlikely to contribute to its turnover. Inhibition of Aβ42 degradation is therefore unlikely to account for the elevation of Aβ42 secretion reported here.

Another possible explanation for the elevation of Aβ42 would be the generation of Aβ40 by carboxypeptidase-mediated processing of Aβ42, with the protease inhibitors actually inhibiting the carboxypeptidase, resulting in an accumulation of Aβ42. Fig. 3 shows, however, that the elevation of Aβ42 begins at lower doses than the inhibition of Aβ40 and that modestly higher doses inhibit Aβ42 as well as Aβ40. Fig. 4 demonstrates that Compound N has no effect on secretion of Aβ40 at doses at which its elevation of Aβ42 has saturated. Neither of these observations is consistent with inhibition of carboxypeptidase-mediated processing of Aβ42 resulting in the elevation of Aβ42 secretion reported here.

Several lines of evidence suggest that C99, the immediate precursor of Aβ42, is available to γ-secretase in kinetically limiting amounts. That inhibition of γ-secretase increases the level of C99 (Fig. 2; see also Ref. 25) is inconsistent with the level of available C99 being saturating for γ-secretase processing and, instead, indicates that the availability of C99 is rate-limiting. A limiting role for C99 availability is further supported by the many reports that a double missense C99 is rate-limiting. A limiting role for C99 availability may be particularly rate-limiting for γ-secretase cleavage at concentrations below those necessary to perturb the membrane; in consequence, the elevation of Aβ42 will not be observed. Testing of this prediction awaits the discovery of more potent γ-secretase inhibitors.

Another mechanism is to hypothesize that the γ-secretase inhibitors also interfere with C99 turnover. The marked increase in Aβ42 secretion by relatively low doses of the peptidyl aldehydes, which act as inhibitors of cysteine, serine, and certain aspartyl proteases, and compound N, a potent broad-spectrum aspartyl protease inhibitor, may be due to their potent inhibition of a nonamyloidogenic degradation of C99, thereby increasing C99 availability and, consequently, Aβ42 formation. Preferential elevation of Aβ42 levels raises the possibility that C99 availability may be particularly rate-limiting for γ-secretase processing in the cellular compartment that generates Aβ42 (38, 39). According to this model, C99 elimination by an aspartyl protease plays a role in regulating Aβ42 production. An inhibitor of C99 elimination such as compound N may be a useful tool for identifying and characterizing a protease responsible for down-regulation of Aβ42.

The γ-secretase has received only limited prior characterization, although mutagenesis studies of APP-based substrates have suggested that the protease has a loose preference for hydrophobic residues in the vicinity of the cleavage site (30, 40). The inhibitor data presented here with four dipeptidyl aldehydes and a dipeptidyl difluoroketoamide are consistent with this model. Assuming that the aldehyde and difluoroketoamide moieties interact with critical active site residues, a variety of neighboring aliphatic and aromatic substituents appear to be well tolerated, leading to relatively minor alterations in affinity. Even introducing an additional spacer between the two amide functionalities, through the β-alanyl residue in Compound E, causes only a small decrease in potency, indicating that the enzyme has broad steric tolerance in the active site. Broad steric tolerance is consistent with a single enzyme capable of cleaving APP at two different sites to generate either Aβ40 or Aβ42.

We point out some qualifications to our suggestion that both Aβ40 and Aβ42 are generated by a single γ-secretase. The suggestion applies to secreted Aβ, as is the case for previous work on this question (14, 15), but does not address the nature of the protease or proteases responsible for intracellular formation of Aβ variants (38, 39). The possibility that secreted Aβ40 and Aβ42 are generated by two distinct but closely related proteases with very similar inhibitor sensitivities cannot be
Rank-Order Potency of γ-Secretase Inhibitors

ruled out but may be addressed by applying the approach described here to a larger and more structurally diverse set of more potent protease inhibitors.

In conclusion, we have determined the dose response for Aβ40 and Aβ42 secretion by HeLa-pNAN8 cells for a series of γ-secretase inhibitors. The inhibitors elevate Aβ42 secretion at relatively low doses but inhibit it at higher concentrations. The elevation of Aβ42 is likely due to a different mechanism than inhibition, as compounds exist that cause one without the other. Inhibition of Aβ secretion is accompanied by a reciprocal increase in levels of C99, its immediate precursor. Two different ways of extracting an IC₅₀ from the complex dose response for Aβ42 secretion result in the same rank-order for inhibition of Aβ42 as for inhibition of Aβ40. Taken together, these data suggest that Aβ40 and Aβ42 are generated by a single γ-secretase which uses a limiting amount of C99 as substrate.

Acknowledgments—We thank June L. Biedler (Sloan-Kettering) for the generous gift of M17 cells, Olaf S. Andersen (Cornell University Medical College) for penetrating discussions, M. Iqbal Cephalon Chem, and Mark Ator and Richard W. Scott for critical reading of the manuscript. We also thank Jeffry Vaught and Frank Baldino for their continued support and encouragement.

Note Added in Proof—The elevation of Aβ40 and Aβ42 secretion result in the same rank-order for inhibition of Aβ40 as for inhibition of Aβ40. Taken together, these data suggest that Aβ40 and Aβ42 are generated by a single γ-secretase which uses a limiting amount of C99 as substrate.

REFERENCES
1. Siman, R., Durkin, J. T., Husten, E. J., Savage, M. J., Murthy, S., Mistretta, S., Chatterjee, S., Botella, D. P., Dembrowsky, B., Poorman, B., and Greenberg, B. D. (1995) in Research Advances in Alzheimer’s Disease and Related Disorders (Iqbal, K., Mortimer, J. A., Winblad, B., and Wisniewski, H. M., eds) pp. 675–684, John Wiley & Sons Ltd., Chichester, UK
2. Schenk, D. B., Rydel, R. E., May, P., Little, S., Panetta, J., Lieberburg, I., and Mayeux, R. (1994) J. Biol. Chem. 269, 1743–1752
3. Suzuki, N., Cheung, T. K., Cai, W. X., Okada, A., Otvos, L., Jr., Eckman, C. W., Qu, X., Tabira, T., Greenberg, B. D., and Younkin, S. G. (1992) Science 255, 726–728
4. Reaume, A. G., Howland, D. S., Trusko, S. P., Savage, M. J., Lang, D. M., Greenberg, B. D., Siman, R., and Scott, R. W. (1996) J. Biol. Chem. 271, 23380–23388
5. Haass, C., Hung A. Y., Schlossmacher, M. G., Teplow, D. B., and Selkoe, D. J. (1993) J. Biol. Chem. 268, 3021–3024
6. Perez, R. G., Squazzo, S. L., and Koo, E. H. (1996) J. Biol. Chem. 271, 9100–9107
7. Lichtenthaler, S. F., Ida, N., Mulhaup, G., Masters, C. L., and Beyreuther, K. (1997) Biochemistry 36, 15396–15403
8. Suzuki, N., Cheung, T. K., Cai, W. X., Okada, A., Otvos, L., Jr., Eckman, C. W., Qu, X., Tabira, T., Greenberg, B. D., and Younkin, S. G. (1994) Science 264, 325–327
9. Cai, X. D., Golde, T. E., and Younkin, S. G. (1993) Science 260, 514–516
10. Iwatsubo, T., Okuda, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) Neuron 13, 45–53
11. Mak, K., Yang, F., Vinters, H. V., Frautschy, S. A., and Cole, G. M. (1994) Brain Res. 667, 138–142
12. Savage, M. J., Kwoyoo, J. K., Pinsker, L. R., Emmons, T. L., Mistretta, S., Siman, R., and Greenberg, B. D. (1995) Amyloid 2, 234–240
13. Mann, D. A., Iwatsubo, T., Cairns, N. J., Lantos, P. L., Nochlin, D., Sumi, S. M., Bird, T. D., Pourkaj, P., Hardy, J., Hutton, M., Prihara, G., Crook, R., Rossor, N. M., and Hallia, M. (1996) Ann. Neurol. 40, 149–151
14. Jefferis, P. J., Berger, E. P., and Lansbury, P. T., Jr. (1999) Biochemistry 38, 4691–4697
15. Younklin, S. G. (1995) Ann. Neurol. 37, 287–288
16. Hardy, J. (1997) Trends Neurosci. 20, 154–159
17. Hardy, J. W., Heinrikson, R. L., Greenberg, B. D., and Raub, T. J. (1996) Am. J. Pathol. 149, 86–99
18. Savage, M. J., Trusko, S. P., Howland, D. S., Pinsker, L. R., Mistretta, S., Reaume, A. G., Greenberg, B. D., Siman, R., and Scott, R. W. (1996) J. Neurosci. 16, 1743–1752
19. Higaki, J., Quon, D., Zhong, Z., and Cordell, B. (1995) Neuron 14, 651–659
20. Gotz, M., Hau, T., Haass, C., Lowry, D., Eising, M., Utsak, M., Qu X., Tabira, T., Greenberg, B. D., and Younkin, S. G. (1994) Science 255, 726–728
21. Reaume, A. G., Howland, D. S., Trusko, S. P., Savage, M. J., Lang, D. M., Greenberg, B. D., Siman, R., and Scott, R. W. (1996) J. Biol. Chem. 271, 23380–23388
22. Haass, C., Hung A. Y., Schlossmacher, M. G., Teplow, D. B., and Selkoe, D. J. (1993) J. Biol. Chem. 268, 1016–1020
23. Underwood, R. G., Squazzo, S. L., and Koo, E. H. (1996) J. Biol. Chem. 271, 9100–9107
24. Lichtenthaler, S. F., Ida, N., Mulhaup, G., Masters, C. L., and Beyreuther, K. (1997) Biochemistry 36, 15396–15403
25. Suzuki, N., Cheung, T. K., Cai, W. X., Okada, A., Otvos, L., Jr., Eckman, C. W., Qu, X., Tabira, T., Greenberg, B. D., and Younkin, S. G. (1994) Science 264, 325–327
26. Yamazaki, T., Haass, C., Saito, T. C., Omura, S., and Ihara, Y. (1997) Biochemistry 36, 8377–8383
27. Murdock, D. K., Kraus, M. F., and Glabe, C. G. (1997) Brain Res. 746, 275–284
28. Wernike, A. M., Turner, R. S., Pleasure, S. J., Golde, T. E., Younkin, S. G., Trojanowski, J. Q., and Lee, V. M.-Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9513–9517
29. Fuller, S. J., Storey, E., Li, Q.-X., Smith, A. I., Beyreuther, K., and Masters, C. L. (1995) Biochemistry 34, 8981–8988
30. Tienaar, J. P., Ida, N., Ikonen, E., Simons, M., Weidemann, A., Mulhaup, G., Masters, C. L., Dotti, C. G., and Beyreuther, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4125–4130
31. Haass, C., Lemere, C. A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D. J. (1995) Nat. Med. 1, 1291–1296
32. Hartmann, T., Bieger, S. H., Brühl, B., Tienaar, J. P., Ida, N., Allsop, D., Roberts, G. W., Masters, C. L., Dotti, C. G., Unicker, S., and Beyreuther, K. (1997) Nat. Med. 3, 1016–1020
33. Cook, D. G., Forman, M. S., Sung, J. C., Leight, S., Kolson, D. L., Iwatsubo, T., Lee, V. M.-Y., and Donn, H. W. (1997) Nat. Med. 3, 1021–1023
34. Tischer, E., and Cordell, B. (1996) J. Biol. Chem. 271, 21914–21919
35. Tsujinaka, T., Kajiwara, Y., Kambayashi, J., Sakon, M., Higuchi, N., Tanaka, T., and Mori, T. (1988) Biochem. Biophys. Res. Commun. 153, 1201–1208