Catalytic Site-Directed γ-Secretase Complex Inhibitors Do Not Discriminate Pharmacologically between Notch S3 and β-APP Cleavages

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ABSTRACT: The generation of γ-secretase inhibitors which block the release of β-amyloid peptide (Aβ) has long been an attractive therapeutic avenue for treatment or prevention of Alzheimer's disease (AD). Such inhibitors would reduce levels of Aβ available for aggregation into toxic assemblies that lead to the plaque pathology found in affected brain tissue. Cumulative evidence suggests that the S3 cleavage of Notch is also dependent on presenilins (PS) and is carried out by the multimeric PS-containing γ-secretase complex. It is therefore possible that Notch function could be affected by γ-secretase inhibitors. To assess the relationship between the cleavage of these substrates in the same system, Western blot cleavage assays have been established using a human cell line stably expressing both the β-amyloid precursor protein (β-APP) and the truncated Notch1 receptor fragment NotchΔE. Thus, a direct correlation may be made, following inhibitor treatment, of the decrease in the levels of the cleavage products, Aβ peptide and the Notch intracellular domain (NICD), as well as the increase in stabilized levels of both substrates. This analysis has been performed with a range of selected γ-secretase inhibitors from six distinct structural classes. Changes in all four species usually occur in concert and with remarkably good agreement. A significant cleavage window is not clearly apparent in any case. Thus, these Notch and β-APP cleavages cannot be dissected apart easily since they show the same pharmacological profile of inhibition. Whether this translates into proportionally reduced Notch signaling in vivo, however, remains to be seen.

One approach for the treatment or prevention of Alzheimer's disease (AD)[1] involves the development of small molecule inhibitors of γ-secretase, one of the enzymes responsible for the excision of Aβ peptide from the amyloid precursor protein (β-APP). Unlike the well-characterized β-secretase enzyme (reviewed in ref *1*), γ-secretase activity remains an elusive therapeutic target. Activity has been shown to be dependent on the expression of presenilins (*2*), and photoaffinity cross-linking studies (*3*) have revealed that presenilins are the primary targets for most of the structurally diverse classes of γ-secretase inhibitor reported to date. Recently, additional members of the γ-secretase assembly [nicastrin, Aph-1, and Pen-2 (*4*, *5*)] have been identified, understanding of the macromolecular complex is progressing, and a number of γ-secretase inhibitors have been reported (reviewed by ref *6*). However, dosing paradigms for these molecules will have to address issues arising from the discovery that the presenilin-containing γ-secretase complex is also responsible for intramembranous cleavage of a number of additional proteins. These include the Notch receptor (*7*), Notch ligands Delta and Jagged (*8*), receptor tyrosine kinase ErbB4 (*9*), low density lipoprotein receptor-related protein (LRP) (*10*), extracellular matrix protein CD44 (*11*), adhesion molecule E-cadherin (*12*), the immunoglobulin-like adhesion receptor nectin-1α (*13*), and the β-APP homologues β-APP-like protein-1 and -2 (*14*). In addition, γ-secretase has been proposed to cleave the cell-surface heparan sulfate proteoglycan syndecan 3 (*15*), while nectin-3 and -4, but not nectin-2, may also prove to be substrates for this enzyme (*13*).

The best understood of these alternative substrates are the Notch receptors. These type I integral membrane proteins are precleaved by a furin-like convertase to give the heterodimeric receptor that is responsive to the presence of any of the DSL (delta, serrate, or lag-2) family of ligands. Following binding of ligand, the receptor undergoes a conformational change which results in S2 cleavage by TACE (tumor necrosis factor α converting enzyme) to yield NEXT (Notch extracellular truncation), which is the substrate for γ-secretase. This third cleavage releases the Notch intracellular domain (NICD) which stimulates transcriptional derepression of key genes involved in development. Additionally, it is clear that a number of processes still active in maturity also rely on Notch signaling, including T cell development (*16*) and maintenance of hematopoietic stem

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[1] Abbreviations: AD, Alzheimer's disease; NICD, Notch intracellular domain; β-APP, β-amyloid precursor protein; Aβ, β-amyloid; β-APP CTF, β-amyloid precursor protein C-terminal fragment; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *tert*-butyl ester; DSL, delta, serrate, or lag-2; PS, presenilin; HES, hairy/enhancer of split.

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cells (*17*), and Notch has also been suggested to play a role in neurite outgrowth and synaptic plasticity (*18*). In an attempt to address these issues, this work describes an investigation of the existence of a therapeutic window between γ-secretase cleavage of β-APP and Notch.

EXPERIMENTAL PROCEDURES

Generation of Cell Line. A HEK293 cell line stably coexpressing β-APP 695 and NotchΔE has been described previously (*19*).

Western Blotting Assays. The HEK293/APP695/NotchΔE cells were plated at a density of 1.8×10^6 cells in a 3 cm dish and allowed to settle overnight. Compound was added from a 1000-fold concentrated DMSO stock to each dish for 5 h. Medium was collected for measurement of Aβ levels, and the cells were solubilized in TBS containing 1% Triton X-100, 0.5% NP-40, 0.2% SDS, and 1× protease inhibitors (Complete, Roche Diagnostics Ltd.). The lysates were normalized for protein content using a BCA protein assay kit (Pierce) according to the manufacturer's instructions. Aliquots of lysates containing 20 μg of protein were loaded onto both 7% Tris−glycine gels and 10−20% Tris−tricine gels (for separation of Notch and β-APP species, respectively) and blotted onto Hybond-N membranes (Schleicher & Schuell). Following detection, the Western blot films were subjected to densitometry as previously described (*19*).

Antibodies. Monoclonal antibody 9E10 for detection of the myc epitope on the NotchΔE construct was purchased from Oncogene. The rabbit polyclonal R7734 (raised against β-APP 659−694) was a gift from MRL San Diego. 4G8 (which recognizes residues 17−24 of Aβ) was obtained from Signet Laboratories. A hybridoma secreting the monoclonal antibody G2-10 (*20*) was licensed from the University of Heidelberg.

Origen Assay. Aβ peptide measurements were made by adapting the Origen ECL technology (Igen) for detection of Aβ(40) using biotinylated 4G8 and ruthenylated G2-10. Medium was removed from cells after the 5 h incubation with compound and diluted 1:1 with PBS/2% BSA/0.2% Tween-20 containing EDTA-free protease inhibitor cocktail (Complete, Roche Diagnostics Ltd.).

Synthetic Chemistry. Compound **1** was prepared by standard chemical techniques as described in WO 0019210 and WO 9828268 (both to Elan). The peptide scanning library of dipeptide ureas (Table 2) was synthesized using standard peptide coupling techniques.

RESULTS

Validation of the Cleavage Assays Using a Well-Characterized γ-Secretase Inhibitor. To investigate the existence of a window between cleavage of Notch and β-APP substrates, HEK293 cells stably expressing the wt 695 amino acid isoform of β-APP were transfected with DNA encoding the NotchΔE truncation of murine Notch1 (*21*), as described previously (*19*). The NotchΔE construct was chosen because it lacks the ectodomain of the receptor and therefore undergoes constitutive S3 cleavage to generate the NICD fragment even in the absence of DSL ligand. This assay allows a direct correlation to be made, following inhibitor treatment, of the changes in the levels of the cleavage products, NICD and secreted Aβ peptide, as well as in the levels of both substrates.

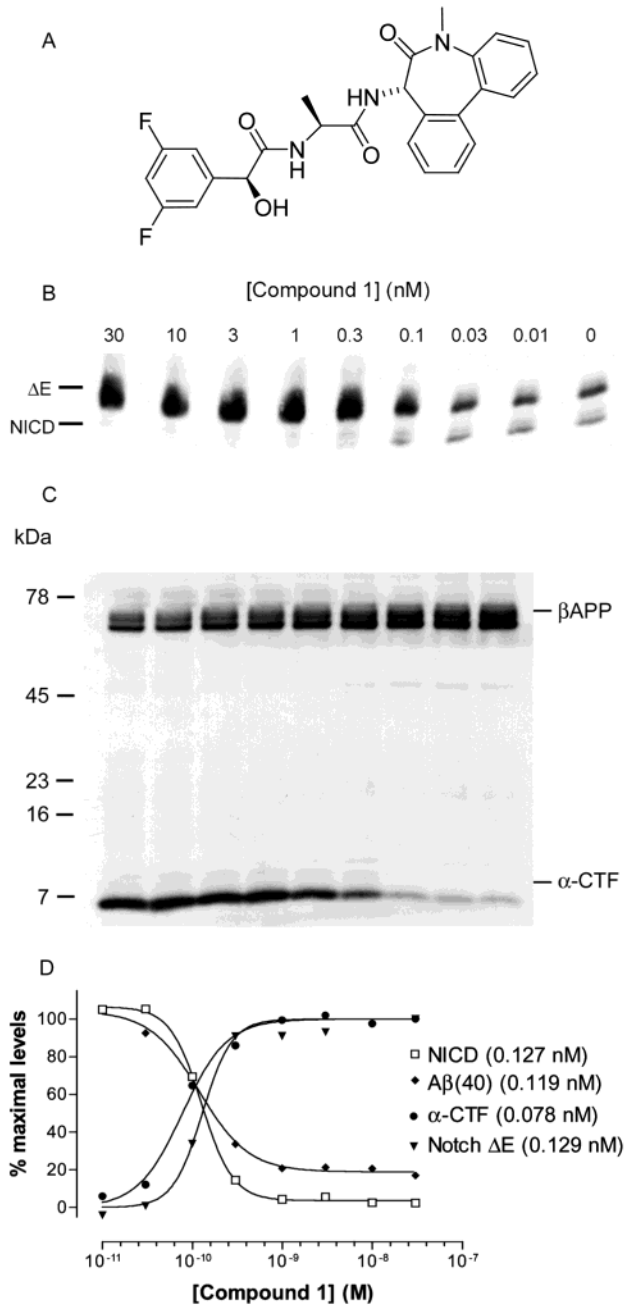


FIGURE 1: Effect of a well-characterized γ-secretase inhibitor on the cleavage of β-APP and Notch. (A) Structure of compound **1** (Audia presentation, Gordon Conference on Medicinal Chemistry, Aug 2001). (B) Detection of myc-tagged Notch species using antibody 9E10 following blotting of 7% Tris−glycine gels onto nitocellulose membranes. (C) Detection of β-APP CTF species on a blotted 10−20% Tris−tricine gel with polyclonal antibody R7334 (raised against residues 659−694 of β-APP). Compound concentrations as for panel B. (D) Quantitation of changes in β-APP-derived and Notch species.

The action of a specimen compound in this system is shown in Figure 1. Compound **1** (Figure 1A) is structurally related to the well-characterized γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *tert*-butyl ester [DAPT (*22*)], previously shown to inhibit γ-secretase in vitro and in vivo. Compound **1** was incubated with the HEK293 cells stably overexpressing β-APP 695 and NotchΔE for 5 h prior to removal of the media and lysis of the cells.

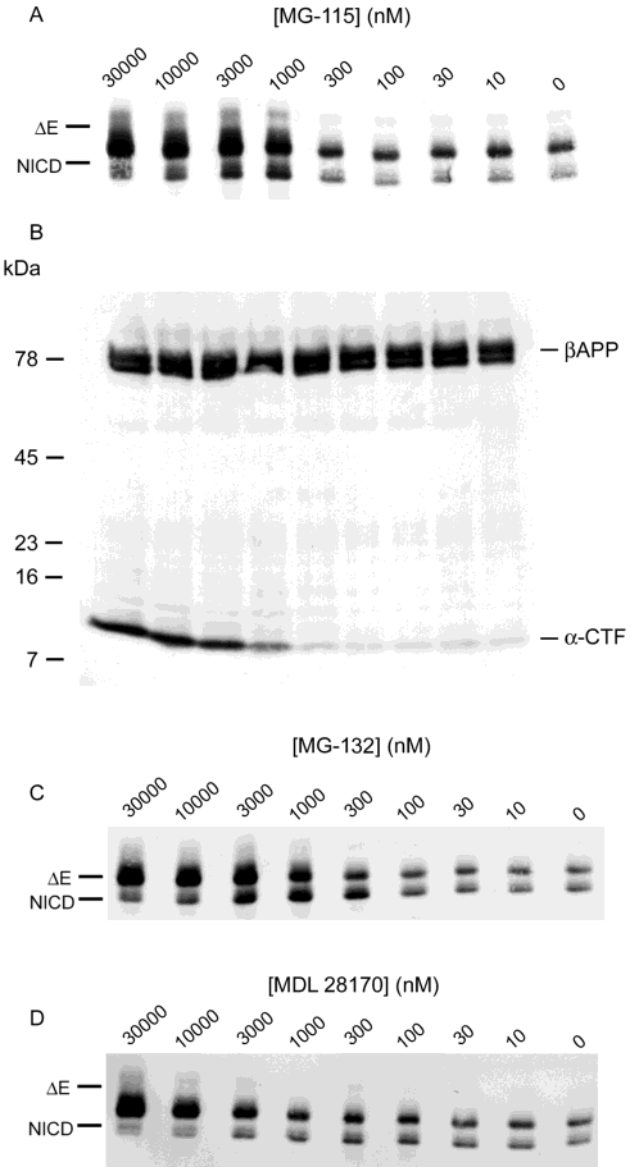
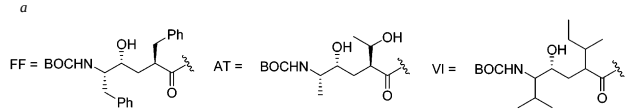


FIGURE 2: Effect of proteasome/calpain inhibitors on the cleavage of β-APP and Notch (blots detected as described in Figure 2). (A) Dose−response effect of MG-115 on cleavage of NotchΔE to NICD. (B) Stabilization of β-APP CTF species by MG-115. (C) Dose−response effect of MG-132 on cleavage of NotchΔE to NICD. (D) Dose−response effect of MDL28170 cleavage of NotchΔE to NICD.

Initial experiments showed that similar potencies of inhibitor action are seen after incubation for both 5 and 23 h so the shorter incubation time was routinely used in this work. As can be seen in the rightmost lanes of each blot (Figure 1B), there is significant steady-state turnover of the NotchΔE substrate to its NICD fragment in the absence of compound. As inhibitor is added and the concentration increased (moving leftward), the conversion of NotchΔE is inhibited so the cleaved NICD product disappears and the substrate accumulates, both in a dose-dependent manner. Detection of Aβ in the media was also inhibited (as assessed by ECL immunoassay), and similar accumulation of β-APP C-terminal fragments (CTFs) can be seen using an antibody raised against the C-terminus of β-APP (Figure 1C). This band has been shown to be the α-secretase cleavage product, since this is the predominant processing activity in HEK293 cells (*19*) and the band is not immunoprecipitated by WO-2 antibody, which recognizes residues 5−8 of Aβ (*20*), but is captured by 4G8, raised against residues 17−24 (D. Beher, unpublished observations). All the changes in bands are quantifiable by densitometry of the Western blots. As can be seen in the accompanying quantitation (Figure 1D), the IC_{50}s for the changes in all four species are in good agreement with each other.

Table 1: Incubation of HEK293/NotchΔE/APP Cells with β-APP Cleavage Site Mimetics (Isostere-AA1-AA2)

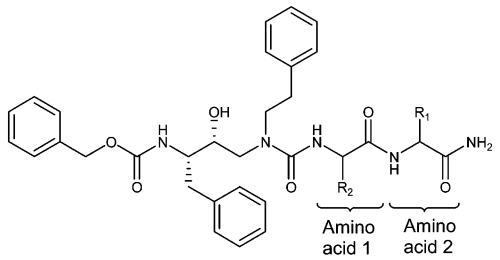
compound	isostere[a]	AA1	AA2	IC_{50} Aβ(40) (nM)	IC_{50} Notch (nM)
L-685458	FF	L	F	6	18
Merck D	VI	A	T	>3000	>3000
Merck E	AT	V	I	>3000	>3000
control 1	FF	A	T	206	167
control 2	FF	V	I	7	4

[a] 

Effect of Broad-Spectrum Protease Inhibitors. To characterize further the profile of action of γ-secretase inhibitors, a selection of broad spectrum protease inhibitors was evaluated in this assay. MG-115 is a peptidic inhibitor of the chymotrypsin-related activity of the proteasome that has been shown to cause apoptosis in Rat-1 and PC12 cell lines (*23*) and is sometimes used experimentally as a weak γ-secretase inhibitor. Notch/β-APP-expressing HEK293 cells were incubated with increasing concentrations of MG-115 for 5 h (a time point with minimal cytotoxicity). As shown in Figure 2, MG-115 causes a stabilization of both substrate species, NotchΔE and β-APP α-CTF, and reduced Aβ peptide secretion into the media. However, in contrast to γ-secretase inhibitor action (Figure 1), levels of the NICD product were stabilized rather than reduced (Figure 2A). This profile is consistent with that expected to result from inhibition of degradation by the proteasome. Furthermore, these changes occur at markedly higher concentrations than those at which many potent, bona fide γ-secretase inhibitors operate. Two additional broad-spectrum inhibitors were also evaluated in this system, MG-132 and MDL28170. MG-132 is a more potent proteasomal inhibitor shown to provoke cell-wide apoptosis via activation of the JNK signaling cascade (*24*) while MDL28170 is a calpain inhibitor capable of protection against excitotoxicity (*25*). A similar profile to MG-115 is seen in the Notch cleavage assay with MG-132. In contrast, the profile seen with MDL28170 is less pronounced and more reminiscent of γ-secretase inhibition, albeit with low potency.

Attempts To Design Derivatives of the Peptidic Inhibitor L-685458 Which Show a Window for Notch Inhibition. In contrast to DAPT-like structures, L-685458, a Merck γ-secretase inhibitor previously characterized (*26*), has a slightly more peptidic nature (Table 1). Derivatives of this molecule were synthesized to mimic the β-APP amino acid sequence around the cleavage sites that might give rise to the Aβ(40) and Aβ(42) C-termini (VIAT and ATVI, respectively, Merck D and E). Although it had been thought possible that these derivatives might show increased potency against γ-secretase, they instead lost the activity shown by the parent FF hydroxyethylene isostere (*27*). In addition, when these

Table 2: Potencies of Peptide Scanning Urea Derivatives of L-685458 Assessed for Differential Notch and β-APP Cleavage



compound	AA1	AA2	IC_{50} Aβ(40) (nM)	IC_{50} NICD (nM)	IC_{50} NotchΔE (nM)	IC_{50} α-CTF (nM)
Merck F	Leu	Phe	89	112.1	81.4	114.5
Merck G	Nle	Phe	51.4	13.8	26.5	25.6
Merck H	Phg	Phe	79.2	72.3	35.8	94.6
Merck I	Val	Phe	44.1	80.6	129.8	50.4
Merck J	Leu	Nle	139.5	160	183.4	146.7
Merck K	Leu	Phg	103.6	111.7	30.7	50.7

compounds were tested in the HEK293 cells stably expressing both β-APP and NotchΔE, their parallel lack of effect against both γ-secretase substrates was maintained, and no window between the cleavages was apparent at the concentrations tested (Table 1). In contrast, control mimic compounds retaining the FF hydroxyethylene isostere (controls 1 and 2) regained most activity.

A further attempt to create a window between β-APP and Notch cleavage employed the generation of a peptide scanning library of inhibitors based on the structure of L-685458. To simplify the chemistry, it was decided to modify the central amide linking group to a phenethylurea (compare L-685458 in Table 1 with Merck F in Table 2). This alteration causes only a slight change in IC_{50}s for Aβ or Notch species (Table 2), and it is noteworthy that Esler and co-workers have generated a very similar series of compounds, with methyl esters instead of amides (*28*). The Notch/β-APP cleavage activities of a representative sample of structures from the described peptide scanning library were tested in the Notch/β-APP cleavage assay. Although changes in potency of cleavage were seen relative to the parent compound (Merck F), the correlation between cleavage of both substrates and generation of both products was maintained (Table 2).

Absence of a Pharmacological Window between Cleavage of NotchΔE and β-APP with Diverse γ-Secretase Inhibitors. To date, this analysis has been done on a range of representative compounds exemplifying six different structural classes of γ-secretase inhibitors. When the IC_{50}s for the concentrations corresponding to 50% of the vehicle levels of both products, NICD and Aβ, were plotted against each other, a good diagonal correlation ($r^2 = 0.8371$, $p < 0.0001$) was obtained (Figure 3). Similar analysis of the potencies obtained for accumulation of both substrates, NotchΔE and β-APP, in the presence of inhibitor yields the same finding ($r^2 = 0.8659$, $p < 0.0001$). There are slight deviations from the true diagonal correlation plots, but these do not consistently affect both β-APP-derived species relative to both Notch proteins, suggesting that the deviations are within the error of the densitometry analysis. In addition, this excellent agreement in the IC_{50}s for all species is further reflected by the finding of the same rank order of potency of the inhibitors tested.

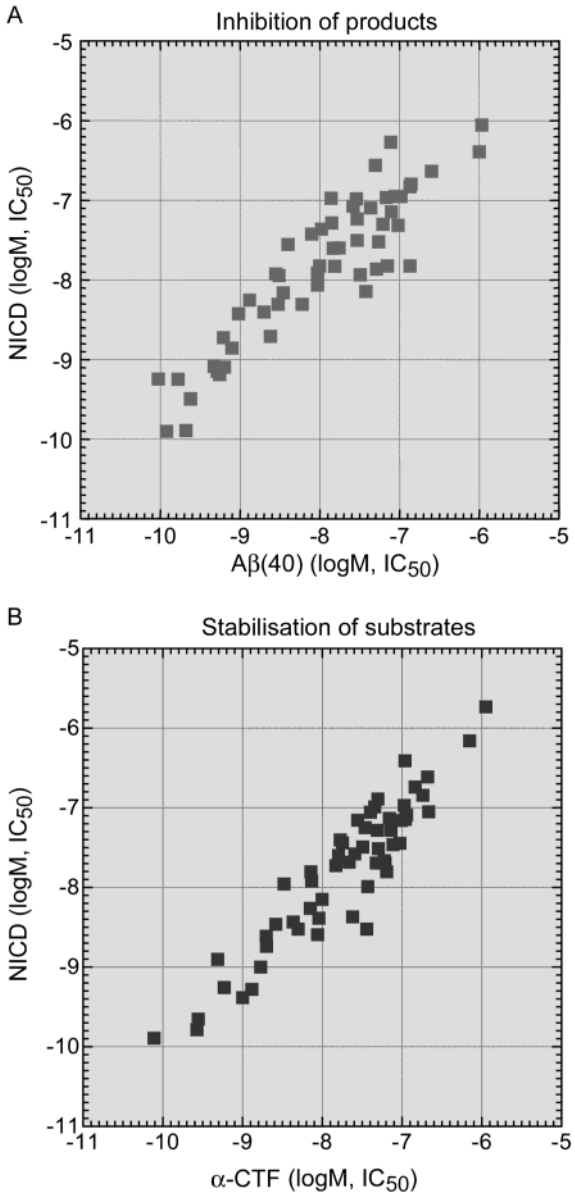


FIGURE 3: Comparison of IC_{50}s for γ-secretase cleavage of β-APP and Notch S3 cleavage.

DISCUSSION

This work describes a set of assays designed to characterize γ-secretase inhibitors by assessing their effects on the cleavage of β-APP and Notch. Using HEK293 cells expressing both substrates, dose-dependent effects consistent with the inhibition of both cleavages (loss of cleaved fragments and the accumulation of substrates) are clearly seen. This profile was compared with that generated with broad-specificity protease inhibitors such as MDL28170, MG-115, and MG-132. The calpain inhibitor MDL28170 shows weak γ-secretase inhibition (>3 μM). However, both of the proteasomal inhibitors (MG-115 and MG-132) show a markedly different inhibition profile in this system, since the NICD fragment is seen to accumulate in parallel with the stabilization of NotchΔE substrate. Such compounds have previously been used as tools to inhibit, and thereby characterize, γ-secretase (*2*, *29*). However, such use may be

inappropriate since recent findings suggest that γ-secretase is an aspartyl protease (in contrast to the serine protease classification of the trypsin- and chymotrypsin-like activities of the proteasome) and these compounds' action in this system is more consistent with an inhibition of cellular degradation. Any weak γ-secretase inhibition these compounds may show is probably indirect and secondary to their dominant interference with proteasomal degradation. It should also be noted that the γ-secretase inhibitors characterized in this work are distinct from the NSAID-related compounds proposed preferentially to modulate Aβ(42) release at concentrations below those at which Aβ(40), and possibly Notch, might be affected (*30*).

This work also describes two attempts to tease out a pharmacological window between the possible beneficial inhibition of β-APP cleavage and any likely interference with Notch signaling. In the first instance, derivatives of the peptidic inhibitor L-685458 mimicking the VIAT and ATVI sequences around positions 40 and 42 within the Aβ region of β-APP failed to favor cleavage of the substrate they mimicked over Notch. The unexpected loss of potency of these molecules confirmed that the nature of the lipophilic groups of the hydroxyethylene isostere of these structures was a far more critical component for potency since the control compounds FFAT and FFVI regained most of the original activity. These findings collectively suggest that β-APP may not be the optimal substrate for γ-secretase or, alternatively, that L-685458 is not a mimetic of this substrate. In the second instance, a library of dipeptide ureas based on the structure of L-685458 failed to identify compounds which show preferential inhibition of β-APP γ-cleavage. In the six examples shown in Table 2 typical levels of agreement between potencies were achieved for the Notch and β-APP species, showing that the activities once again tracked each other despite major changes to the peptidic structures.

These results provide strong support for the model of a single enzyme active site cleaving both β-APP and Notch, since there were no significant and consistent differences with any compound in potency for the disappearance of the substrates and the appearance of the cleavage products Aβ and NICD. Given the semiquantitative nature of densitometry analyses, slight discrepancies of 2−3-fold between species are sometimes observed. These experiments provide their own internal controls: these windows are unlikely to be real since they seldom affect both β-APP-derived species relative to both Notch species and never hold up consistently in repeat experiments (data not shown). The data therefore fail to show any significant window between Notch and β-APP cleavage. They complement and extend the finding that three diverse γ-secretase inhibitors show similar inhibition of C100Flag and N100Flag in a cell-free system (*31*). A substantial number of representative γ-secretase inhibitors (exemplifying six structural classes) that have been tested in this paper have similar IC_{50}s for both substrates and show similar rank order of potency of inhibition. A number of compounds, including L-685458, have been shown to capture presenilin (*3*) and may act as aspartyl protease transition state mimetics (*26*). Interestingly, the ability of analogous, immobilized active site-directed γ-secretase inhibitors to capture the direct γ-secretase substrate C99 along with the PS-containing secretase complex (*28*) has suggested that γ-secretase contains a substrate binding site that is distinct from its catalytic site. This is consistent with the demonstration that structurally diverse inhibitors have been shown to inhibit the presenilin complex noncompetitively (*32*). In a recent report, transition-state analogues and nontransition analogue small molecule inhibitors have been proposed to interact noncompetitively with respect to each other at distinct (catalytic versus allosteric?) sites on the presenilin complex (*49*). Given these interesting observations, it is hoped that continued characterization of the γ-secretase complex and its inhibitors will yield further insights into the structural basis for the lack of discrimination between NotchΔE and β-APP cleavage noted in this work.

The similarity between cleavage of both γ-secretase substrates examined is in agreement with a report (*33*) that the cleavages of β-APP and Notch may compete with one another, since overexpression of β-APP decreased endogenous Notch signal transduction while Delta-mediated activation of Notch processing reduced Aβ production. In contrast, another similar study found the two cleavages to be non-identical (*34*). However, it has been suggested that such experiments should be conducted under saturating conditions (R. Kopan, manuscript in preparation). Direct competition has also recently been reported under cell-free conditions (*31*).

A number of recent reports document a potential separation between cleavage of β-APP and Notch in special circumstances: in a cell-free system (*35*) and in cell lines expressing the Asp257Ala (*36*), Cys92Ser (*37*), Leu166Pro (*38*) or the Leu286Glu and Leu286Arg (*39*) mutations of PS1. In addition, a recent report (*40*) shows that PS1-hypomorphic mice crossed to give a spectrum of Notch phenotype severity showed a good correlation between survival and NICD generation, although γ-secretase cleavage of β-APP seemed to be similarly inhibited in all cases. This suggests that separation of the cleavages could perhaps be achieved. However, apart from these physiologically atypical situations, there are few cases of separation resulting directly from inhibitor treatment. Nonpeptidic isocoumarin inhibitors have been reported, at high concentrations, to block Aβ production with little effect on NICD (*41*). However, these findings are controversial (*42*), and these compounds may not interact directly with γ-secretase. The bulk of evidence therefore supports a common enzymatic activity that processes both β-APP and Notch with little discrimination (*43*), although it will be interesting to see how the discovery of the NSAID-related modulators (*30*) might refine this view. Additionally, γ-secretase action on β-APP may inadvertently downregulate Notch activity through the signaling effects on its product, β-APP intracellular domain (AICD). AICD upregulates the expression of Numb, in turn a negative regulator of Notch signaling (*44*). This finding also exemplifies the remaining complexity underlying the signaling pathways involved in β-APP and Notch processing, although the recent discovery of Nβ peptide analogous to Aβ (*45*) reinforces the similarity between these cleavages.

In conclusion, this work provides evidence that inhibition of β-APP and NotchΔE cleavage occurs pharmacologically without preference in a cell line overexpressing both substrates. It remains to be seen, however, how this relates to the situation in a complex organism. In man, such equivalence could complicate dosing regimes for any γ-secretase inhibitor, although it is not known to what extent this

will translate into inhibited Notch signaling per se. A PS-independent pathway of Notch signaling, proposed since 16% of control levels of HES1 transcription is still seen in PS1/2 null cells (*46*), may minimize these complications. The discovery of a Notch-processing activity that is distinct from the presenilins (*47*) and a recent report that intracellular Aβ-(42) is still generated in the early secretory pathway in the absence of presenilins (*48*) also suggest that there are additional layers of complexity awaiting discovery. Suitable therapeutic dose selection to achieve partial or intermittent γ-secretase inhibition may be tolerated, and the accompanying partial reduction in Aβ peptide levels could bring significant therapeutic value accumulatively over a prolonged course of treatment. These questions are probably best addressed in model systems or in a controlled clinical setting. As long as the major challenge to medical science represented by AD remains poorly met by currently available treatments, γ-secretase inhibitors remain a therapeutic target of great potential.

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REFERENCES

1. Vassar, R. (2001) *J. Mol. Neurosci. 17*, 157−170.
2. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) *Nature 398*, 518−522.
3. Li, Y. M., Xu, M., Lai, M. T., Huang, Q., Castro, J. L., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduvelil, J. G., Register, R. B., Sardana, M. K., Shearman, M. S., Smith, A. L., Shi, X. P., Yin, K. C., Shafer, J. A., and Gardell, S. J. (2000) *Nature 405*, 689−694.
4. Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L. S., Sorbi, S., Bruni, A., Fraser, P., and George-Hyslop, P. (2000) *Nature 407*, 48−54.
5. Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) *Dev. Cell 3*, 85−97.
6. Josien, H. (2002) *Curr. Opin. Drug Discov. Dev. 5*, 513−525.
7. Mumm, J. S., and Kopan, R. (2000) *Dev. Biol. 228*, 151−165.
8. Ikeuchi, T., and Sisodia, S. S. (2003) *J. Biol. Chem.* (in press).
9. Lee, H. J., Jung, K. M., Huang, Y. Z., Bennett, L. B., Lee, J. S., Mei, L., and Kim, T. W. (2002) *J. Biol. Chem. 277*, 6318−6323.
10. May, P., Reddy, Y. K., and Herz, J. (2002) *J. Biol. Chem. 277*, 18736−18743.
11. Lammich, S., Okochi, M., Takeda, M., Kaether, C., Capell, A., Zimmer, A. K., Edbauer, D., Walter, J., Steiner, H., and Haass, C. (2002) *J. Biol. Chem. 277*, 44754−44759.
12. Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z., Wisniewski, T., and Robakis, N. K. (2002) *EMBO J. 21*, 1948−1956.
13. Kim, D. Y., Ingano, L. A., and Kovacs, D. M. (2002) *J. Biol. Chem. 277*, 49976−49981.
14. Scheinfeld, M. H., Ghersi, E., Laky, K., Fowlkes, B. J., and D'Adamio, L. (2002) *J. Biol. Chem. 277*, 44195−44201.
15. De Strooper presentation (no. 1043), 8th International Conference on Alzheimer's Disease and Related Disorders, July 2002.
16. Doerfler, P., Shearman, M. S., and Perlmutter, R. M. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 9312−9317.
17. Varnum-Finney, B., Purton, L. E., Yu, M., Brashem-Stein, C., Flowers, D., Staats, S., Moore, K. A., Le, R., I, Mann, R., Gray, G., Artavanis-Tsakonas, S., and Bernstein, I. D. (1998) *Blood 91*, 4084−4091.
18. Berezovska, O., McLean, P., Knowles, R., Frosh, M., Lu, F. M., Lux, S. E., and Hyman, B. T. (1999) *Neuroscience 93*, 433−439.
19. Beher, D., Wrigley, J. D., Nadin, A., Evin, G., Masters, C. L., Harrison, T., Castro, J. L., and Shearman, M. S. (2001) *J. Biol. Chem. 276*, 45394−45402.
20. Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Forstl, H., Sandbrink, R., Masters, C. L., and Beyreuther, K. (1996) *J. Biol. Chem. 271*, 22908−22914.
21. Kopan, R., Schroeter, E. H., Weintraub, H., and Nye, J. S. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1683−1688.
22. Dovey, H. F., John, V., Anderson, J. P., Chen, L. Z., de Saint, A. 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., and Audia, J. E. (2001) *J. Neurochem. 76*, 173−181.
23. Lopes, U. G., Erhardt, P., Yao, R., and Cooper, G. M. (1997) *J. Biol. Chem. 272*, 12893−12896.
24. Meriin, A. B., Gabai, V. L., Yaglom, J., Shifrin, V. I., and Sherman, M. Y. (1998) *J. Biol. Chem. 273*, 6373−6379.
25. Rami, A., Ferger, D., and Krieglstein, J. (1997) *Neurosci. Res. 27*, 93−97.
26. Shearman, M. S., Beher, D., Clarke, E. E., Lewis, H. D., Harrison, T., Hunt, P., Nadin, A., Smith, A. L., Stevenson, G., and Castro, J. L. (2000) *Biochemistry 39*, 8698−8704.
27. Nadin, A., Owens, A. P., Castro, J. L., Harrison, T., and Shearman, M. S. (2003) *Bioorg. Med. Chem. Lett. 13*, 37−41.
28. Esler, W. P., Kimberly, W. T., Ostaszewski, B. L., Ye, W., Diehl, T. S., Selkoe, D. J., and Wolfe, M. S. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 2720−2725.
29. Skovronsky, D. M., Pijak, D. S., Doms, R. W., and Lee, V. M. (2000) *Biochemistry 39*, 810−817.
30. Weggen, S., Eriksen, J. L., Das, P., Sagi, S. A., Wang, R., Pietrzik, C. U., Findlay, K. A., Smith, T. E., Murphy, M. P., Bulter, T., Kang, D. E., Marquez-Sterling, N., Golde, T. E., and Koo, E. H. (2001) *Nature 414*, 212−216.
31. Kimberly, W. T., Esler, W. P., Ye, W., Ostaszewski, B. L., Gao, J., Diehl, T., Selkoe, D. J., and Wolfe, M. S. (2003) *Biochemistry 42*, 137−144.
32. Tian, G., Sobotka-Briner, C. D., Zysk, J., Liu, X., Birr, C., Sylvester, M. A., Edwards, P. D., Scott, C. D., and Greenberg, B. D. (2002) *J. Biol. Chem. 277*, 31499−31505.
33. Berezovska, O., Jack, C., Deng, A., Gastineau, N., Rebeck, G. W., and Hyman, B. T. (2001) *J. Biol. Chem. 276*, 30018−30023.
34. Petit, A., George-Hyslop, P., Fraser, P., and Checler, F. (2002) *Biochem. Biophys. Res. Commun. 290*, 1408−1410.
35. Ikeuchi, T., and Sisodia, S. S. (2002) *Neuromol. Med. 1*, 43−54.
36. Capell, A., Steiner, H., Romig, H., Keck, S., Baader, M., Grim, M. G., Baumeister, R., and Haass, C. (2000) *Nat. Cell Biol. 2*, 205−211.
37. Zhang, D. M., Levitan, D., Yu, G., Nishimura, M., Chen, F., Tandon, A., Kawarai, T., Arawaka, S., Supala, A., Song, Y. Q., Rogaeva, E., Liang, Y., Holmes, E., Milman, P., Sato, C., Zhang, L., and George-Hyslop, P. (2000) *Neuroreport 11*, 3227−3230.
38. Moehlmann, T., Winkler, E., Xia, X., Edbauer, D., Murrell, J., Capell, A., Kaether, C., Zheng, H., Ghetti, B., Haass, C., and Steiner, H. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 8025−8030.
39. Kulic, L., Walter, J., Multhaup, G., Teplow, D. B., Baumeister, R., Romig, H., Capell, A., Steiner, H., and Haass, C. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 5913−5918.
40. Rozmahel, R., Mount, H. T., Chen, F., Nguyen, V., Huang, J., Erdebil, S., Liauw, J., Yu, G., Hasegawa, H., Gu, Y., Song, Y.

Q., Schmidt, S. D., Nixon, R. A., Mathews, P. M., Bergeron, C., Fraser, P., Westaway, D., and George-Hyslop, P. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 14452−14457.
41. Petit, A., Bihel, F., Alves, d. C., Pourquie, O., Checler, F., and Kraus, J. L. (2001) *Nat. Cell Biol. 3*, 507−511.
42. Esler, W. P., Das, C., Campbell, W. A., Kimberly, W. T., Kornilova, A. Y., Diehl, T. S., Ye, W., Ostaszewski, B. L., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2002) *Nat. Cell Biol. 4*, E110−E111.
43. Fortini, M. E. (2002) *Nat. Rev. Mol. Cell Biol. 3*, 673−684.
44. Roncarati, R., Sestan, N., Scheinfeld, M. H., Berechid, B. E., Lopez, P. A., Meucci, O., McGlade, J. C., Rakic, P., and D'Adamio, L. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 7102−7107.
45. Okochi, M., Steiner, H., Fukumori, A., Tanii, H., Tomita, T., Tanaka, T., Iwatsubo, T., Kudo, T., Takeda, M., and Haass, C. (2002) *EMBO J. 21*, 5408−5416.
46. Berechid, B. E., Kitzmann, M., Foltz, D. R., Roach, A. H., Seiffert, D., Thompson, L. A., Olson, R. E., Bernstein, A., Donoviel, D. B., and Nye, J. S. (2002) *J. Biol. Chem. 277*, 8154−8165.
47. Taniguchi, Y., Karlstrom, H., Lundkvist, J., Mizutani, T., Otaka, A., Vestling, M., Bernstein, A., Donoviel, D., Lendahl, U., and Honjo, T. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 4014−4019.
48. Wilson, C. A., Doms, R. W., Zheng, H., and Lee, V. M. (2002) *Nat. Neurosci. 5*, 849−855.
49. Tian et al. (2003) *J. Biol. Chem*. (in press).

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