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Perspective

Secretase Targets for Alzheimer's Disease: Identification and Therapeutic Potential†

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Only a decade ago, the attempt to develop therapeutic agents for the effective long-term treatment and cure of neurodegenerative disorders such as Alzheimer's disease (AD) might have been likened to attacking a windmill with a lance. What biochemical or signaling processes should be modulated? What proteins should be targeted? What cellular and animal models would be appropriate? How would potentially useful agents be assessed clinically? The answers to these questions were not obvious, but by the close of the century, the molecular events leading to the major neurodegenerative diseases were clearly unraveling, laying the foundation for pharmaceutical intervention. As a result, today's efforts to realize effective agents against these scourges are not considered so quixotic.

In general, the essential knowledge base for realizing therapeutic agents for neurological disorders is built in the progression shown in Figure 1. First, the pathology must be carefully studied to clearly define the disease.

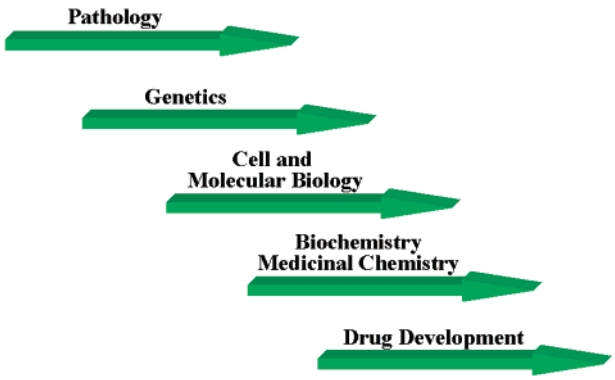

Figure 1. Research phases toward realizing therapeutics for neurological diseases.

What are its essential characteristics, and how does it differ from a disease that on the surface may appear very similar? A detailed description of the disease then allows the search for genetic linkages in families with substantially higher incidences, and the gene hunters have been spectacularly successful at identifying specific genes responsible for specific human diseases, including neurological disorders. The task is far from finished with the genetics, however, because it is often not obvious what the normal function of the encoded protein might be or why an aberration might cause a specific disease. Cell and molecular biological approaches are needed at this juncture. Knockout animals can offer important clues to normal function, and transgenic animals not only allow investigation of the pathological role of mutant genes but can also provide important models for drug development. Clues to normal as well as pathological function can come from identifying inter-

† Abbreviations: Aβ (amyloid-β protein), Aβ_{40} (40-residue C-terminal variant of amyloid-β), Aβ_{42} (42-residue C-terminal variant of amyloid-β), ACE (angiotensin converting enzyme), AD (Alzheimer's disease), APP (amyloid-β precursor protein), α-APP$_s$ (soluble APP product of α-secretase cleavage), β-APP$_s$ (soluble APP product of β-secretase cleavage), BACE (β-site APP-cleaving enzyme), C83 (83-residue APP C-terminal fragment; product of α-secretase cleavage), C99 (99-residue APP C-terminal fragment; product of β-secretase cleavage), ER (endoplasmic reticulum), FAD (familial Alzheimer's disease), NICD (Notch intracellular domain), PS1 (presenilin-1), PS2 (presenilin-2), PS1 ΔE9 (splice variant of presenilin-1 with exon 9 deleted), S2P (site 2 protease in SREBP processing), SREBP (sterol regulatory element binding protein), TACE (tumor necrosis factor-α converting enzyme), TM (transmembrane domain).

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Table 1. Neurological Disorders with Aberrant Protein Deposition

disorder	protein
Alzheimer's disease	Aβ, tau
Parkinson's disease	α-synuclein
Huntington disease	huntingtin
prion diseases	PrP
amylotrophic lateral sclerosis	SOD1

acting proteins and affected signaling pathways. Such clues allow the testing of specific hypotheses concerning biochemical functions and the design of small molecules that specifically modulate those functions. The search is then on to identify agents that modulate these functions optimally in vivo. The phase arrows in Figure 1 are purposely staggered: one process begins before the preceding ones end, and the latter phases can feed back on the earlier ones to provide fresh insight.

AD Pathology

The pathology must take precedence, and in terms of pathology, the common theme of aberrant protein deposition is emerging with respect to a number of different neurodegenerative diseases (Table 1).[1] In AD, one finds in the cerebral cortex the extraneuronal plaques and intraneuronal tangles first described by Alois Alzheimer nearly a century ago. The major protein component of the plaques is the amyloid-β protein (Aβ), and the tangles are composed of filaments of the microtubule-associated protein tau. In Parkinson's disease, neuronal deposits called Lewy bodies containing α-synuclein are found in the substantial nigra, the subregion of the brain in which dopaminergic neurons are selectively lost. In Huntington's disease, the mutated huntingtin protein possesses a long string of glutamates that leads to the formation of intracellular inclusions in disease-affected neurons. In the prion diseases, such as Creutzfeldt−Jakob disease in humans, scrapie in sheep, and mad cow disease in cattle, the prion protein not only aggregates but also the aggregated protein is apparently infectious in the absence of genetic material. In amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), mutations in superoxide dismutase lead to astrocytic inclusions in transgenic mice. These protein aggregates are thought to lead to the degeneration of a specific subpopulation of neurons either because these aggregates are formed primarily in certain regions of the brain (i.e., where conditions are conducive to aggregate formations) or because certain neuronal subtypes are more sensitive to these toxic aggregates than other neurons.

The amyloid plaques and neurofibrillary tangles of the AD brain have been subjected to rigorous pathological analysis at the cytological and biochemical levels, with the hope that further clues to disease etiology might be gleaned. In AD, dense deposits containing fibrillar forms of Aβ are intimately associated with dystrophic (degenerating) axons and dendrites (together referred to as neurites),[1b] and these neuritic plaques are found in cerebral and midbrain regions associated with cognition and memory.[2−4] Within and surrounding the neuritic plaques are activated microglia, a type of brain inflammatory cell, as well as reactive glial cells called astrocytes.[5,6] Aβ-specific antibodies reveal less dense "diffuse" plaques that are not associated with dystrophic neurites, activated microglia, or reactive astrocytes.[7−9] These plaques contain amorphous, nonfibrillary Aβ and are found in areas of the brain generally not implicated in clinical AD. Moreover, the diffuse plaques are often found in abundance in elderly, cognitively normal people, leading to the suggestion that these diffuse plaques may be the precursors to pathogenic dense plaques. Neurofibrillary tangles are also found in the brain regions critical to higher brain function. Biochemical analysis reveals that the filamentous form of the tau protein found in these tangles is hyperphosphorylated. Tau hyperphosphorylation renders insoluble this otherwise highly soluble cytosolic protein, and this modified form of tau is also found in many plaque-associated dystrophic neurites. Interestingly, tau-containing neurofibrillary tangles occur in a number of other, uncommon neurodegenerative diseases. In contrast, the amyloid-containing neuritic plaques are unique to AD.

Table 2. Genetics of Alzheimer's Disease

chromosome	gene defect	age of onset	Aβ phenotype
21	APP mutations	50s	↑ Aβ or Aβ_{42}
14	presenilin-1 mutations	20s−50s	↑ Aβ_{42}
1	presenilin-2 mutations	50s	↑ Aβ_{42}
19	apoE4 polymorphism	60s and older	↑ Aβ plaques and vascular deposits

Despite the intriguing commonality between the otherwise disparate neurodegenerative disorders noted in Table 1, the central question of whether the associated abnormal protein aggregates themselves cause disease or are simply byproducts of the disease process has not been completely settled. Indeed, Alzheimer's original observations spurred an ongoing and often rancorous century-long debate about the pathological role of the amyloid plaques and neurofibrillary tangles. Are these lesions causative, or are they merely "tombstones" or markers of regions that have degenerated due to unknown pathogenic events? As discussed below, the identification of genes associated with AD clearly demonstrates that alterations in the proteolytic processing that produces amyloid-β protein can cause AD. Thus, Aβ is either the molecular culprit or an intimately linked epiphenomenon. In either event, blocking the responsible proteases would be a reasonable approach to treating AD.

AD Genetics

Defining the disease in terms of pathology allows the pinning down of its genetic basis. In Alzheimer's disease (AD), three genes have been clearly identified as causative and one gene as a risk factor (Table 2).[10] Major clues came from families suffering from autosomal dominant, early-onset forms of AD (familial AD or FAD). Other than the fact that FAD is clearly hereditary and manifests itself at earlier ages (<60 years), it is indistinguishable from the sporadic form of AD (e.g., with respect to behavioral patterns, disease progression, plaque deposition, tangle formation).

The first identified FAD-causing mutations were found in the gene encoding the amyloid-β precursor protein (APP) on chromosome 21.[11−13] At least five such mutations have been identified.[14] These are found near

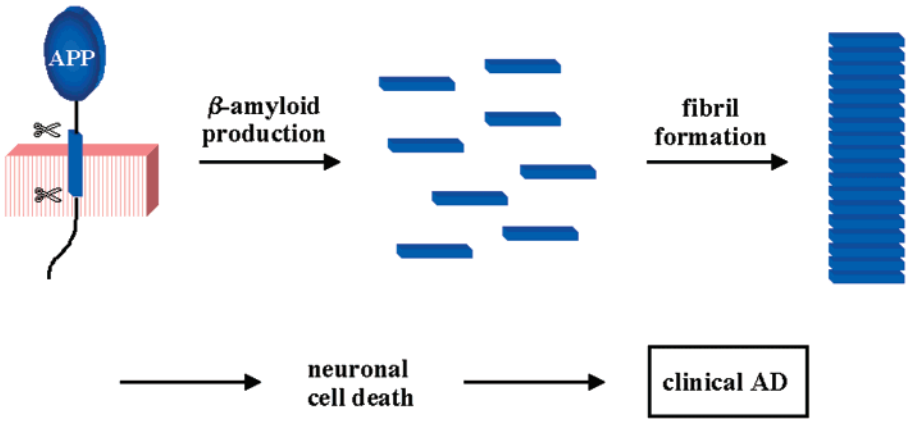


Figure 2. The amyloid hypothesis of Alzheimer's disease.

the proteolytic cleavage sites in APP that result in Aβ, and all lead to increased production of Aβ in general or specifically a 42-residue form of Aβ (Aβ_{42}) in transfected cells,[15−19] in transgenic mice,[20,21] and in the plasma of mutant carriers.[22] Two other disease-causing mutations are found within the Aβ region, near an alternate proteolytic processing site; however, the resultant mutant Aβ aggregates in the cerebral vasculature and causes hereditary cerebral hemorrhage with amyloidosis, either with or without features of AD.[23]

A related genetic clue that Aβ is involved in the early molecular events leading to AD is the fact that all Down syndrome (trisomy 21) patients invariably develop AD by age 50. Down syndrome patients carry an extra copy of the APP gene, located on chromosome 21, and they produce more Aβ from birth and develop amyloid plaques as early as age 12.[24,25] These early plaques are of the diffuse kind and contain Aβ_{42} almost exclusively. Because victims of Down syndrome are fated to develop AD at early ages, the observance of Aβ_{42}-specific diffuse plaques suggest that these plaques could be the precursors of the dense, neuritic plaques found in the AD brain.

APP mutations, however, account for only about 10% of FAD cases and only 2% of all incidences of AD. Most FAD is caused by missense mutations in the genes for presenilin-1 (PS1) and presenilin-2 (PS2), located on chromosomes 14 and 1, respectively.[26,27] These genes encode multi-transmembrane proteins, the normal biological roles of which were completely unknown upon discovery. More than 70 different mutations in the presenilins have been identified that lead to FAD.[28] Virtually all are missense mutations, and these are located in various regions of the primary sequence (see below). Remarkably, all such mutations analyzed to date lead to specific increases in Aβ_{42} production, implicating this particular Aβ species in the etiology of AD.[21,22,29,30]

Considerable effort has gone into identifying genes associated with the more common late-onset, sporadic form of AD. Such searching led to the pinpointing of the apoE gene. ApoE is a lipid transport protein that comes in three allelic variants: E2, E3, and E4. The ApoE4 allele is a major risk factor for late-onset AD: those who carry one or two copies of this allele may not necessarily develop AD, but these carriers are at substantially increased risk.[31] As a function of age, the risk increases with the number of ApoE4 alleles inherited, with the mean age of onset being some 15 years earlier and the incidence of AD 10 times more likely in individuals who inherit two E4 rather than two E3 alleles. Inheritance of ApoE2 decreases risk of the disease and increases age of onset (i.e., it appears to be a protective factor).[32] Yet some individuals homozygous for E4 show no AD symptoms in their 90s, illustrating the principle that this allele is a risk factor, not a determinant of whether AD will develop.[33] Apparently, the apoE variants differentially affect Aβ deposition and resulting neurodegeneration. A dose-dependent increase in the density of neuritic Aβ plaques and vascular Aβ deposits are associated with ApoE4.[34,35] More recently, studies with transgenic mice have demonstrated that apoE is required for amyloid formation and glial activation caused by FAD-mutant APP.[36]

The Amyloid Hypothesis and Therapeutic Strategies

The common theme of Aβ production and deposition has bolstered the amyloid hypothesis of AD pathogenesis (Figure 2), which states that production and deposition of Aβ in the form of fibrils leads to neuronal cell death and eventually to the clinical presentation and progression of AD.[37] The fact that Aβ_{42} is increased in all forms of early-onset AD is very intriguing because this form of Aβ is particularly prone to fibril formation,[38] and Aβ fibrils are toxic to cultured neurons.[39,40] Moreover, Aβ_{42} fibrils can serve as templates that induce fibrillization of other Aβ species that would otherwise remain soluble.[38] While the tau-containing neurofibrillary tangles are also characteristic of AD, they are now thought to develop downstream of Aβ plaque formation.[41] As mentioned earlier, tau-mediated tangle formation is not unique to AD, and mutations in tau that result in paired helical filaments and tangle formation are associated with other, more rare neurodegenerative disorders.[41] However, no AD-causing tau mutations have been found, and postmortem analysis of young Down syndrome brains show that Aβ_{42} deposition occurs in the absence of tangle formation.[42,43] Moreover, human neurons in AD-vulnerable brain regions specifically accumulate Aβ_{42}, suggesting that intracellular Aβ_{42} accumulation is an early event in neuronal dysfunction.[44] Thus, while tau deposition or dysfunction can apparently lead directly to neurodegeneration, the formation of paired helical filaments of tau may be one way that the brain responds to a variety of toxic events,

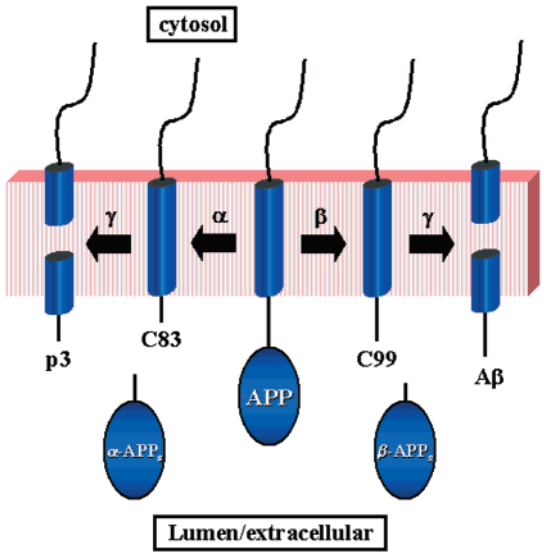


Figure 3. Proteolytic processing of the amyloid-β precursor protein (APP).

including Aβ_{42} deposition and subsequent amyloid plaque formation.

Because the weight of evidence from pathology, genetics, cell biology, and molecular biology heavily favor the amyloid hypothesis, strategies that modulate the production, deposition, or toxicity of Aβ might be considered reasonable therapeutic approaches. The use of antioxidants or antiinflammatory agents has been proposed as a means of intercepting downstream toxic events resulting from Aβ deposition.[45] The availability of archives of such agents within major pharmaceutical companies and the potential for crossover into other therapeutic domains might make this an attractive approach, although it is not clear that such agents alone could prevent or treat AD. Blocking Aβ aggregation and fibril formation is considered a more direct approach because this aims for a known target. Inhibiting Aβ aggregation could be accomplished directly with compounds that bind Aβ monomers or soluble aggregates of Aβ^{46-48} or might be achieved indirectly with compounds that induce the cellular production of endogenous agents that bind or degrade Aβ. Currently, there is no obvious way to develop agents that block Aβ aggregation indirectly (although the aforementioned experiments with ApoE knockout mice do suggest one target), and the direct inhibition of aggregation may require stoichiometric in vivo concentrations to be effective (although an example of a 1:10 inhibitor:Aβ ratio raises hopes that binding higher-order aggregates could prevent fibrillogenesis[49]). Of course, if Aβ fibrils per se do not cause onset or progression of the disease, then this approach would not be appropriate.

Currently, the third approach, reducing Aβ production, is widely thought to be the most likely to succeed. Indeed, the two proteases that cut Aβ out of its precursor protein, APP, have emerged as top targets for developing therapeutic agents for AD (Figure 3). The first cut is carried out by β-secretase, which was recently identified as a membrane-bound aspartyl protease.[50] This proteolysis takes place just outside the membrane, releasing soluble APP (β-APP$_s$) and leaving behind a 99-residue membrane-associated C-terminal fragment, C99. Alternatively, APP is cut by α-secretase, a membrane-bound metalloprotease,[51,52] to produce α-APP$_s$ and an 83-residue membrane-associated C-terminal fragment, C83. Both C99 and C83 are substrates for γ-secretase, a mysterious enzyme that carries out an unusual proteolysis in the middle of the transmembrane domain of APP,[53] resulting in formation of the 4 kDa Aβ from C99 and p3, a 3 kDa N-terminally truncated form of Aβ, from C83. As mentioned above, FAD-mutant presenilins are known to modulate γ-secretase activity to increase Aβ_{42} production.

After the initial characterization of Aβ as the principal component of amyloid plaques, the locating of its precursor protein APP, and the pinpointing of FAD-causing APP mutations, the AD research field began working intensely to identify α-, β-, and γ-secretases because of the keen belief that these would be tractable targets for treating AD that would strike at the fundamental molecular mechanism of the disease. Although these proteases proved elusive for quite some time, the APP-cleaving secretases have all been unmasked to various degrees in the past two years, revealing some fascinating basic biology in the process. In the meantime, the search for agents that reduce Aβ production progressed even in the absence of defined enzymological targets. This strategy was made possible by the seminal finding that Aβ is not just produced in the brain under pathological conditions but is formed by virtually all cell types and can be overproduced by transfecting APP into immortalized cell lines.[54-57] Indeed, a number of inhibitors of γ-secretase activity have been identified even though the responsible enzyme has not been purified and has only recently begun to yield its secrets.

The remainder of this Perspective will discuss the biology of these integral membrane proteases and their substrates, present current pharmacological approaches to modulating their actions, and offer an assessment of the potential of these targets for the treatment of AD.

Membrane Protein Sorting and Trafficking

Understanding the secretases and their substrates requires some appreciation of how membrane proteins are formed, segregated, and shuttled to various sites within the cell. Integral membrane proteins are synthesized in the rough endoplasmic reticulum (ER), with insertion into the membrane occurring synchronously with translation from the ribosome. Portions of the newly synthesized protein destined to face the extracellular milieu are first found in the lumen of the ER, and those that will face the intracellular environment are oriented toward the cytosol. The folding of membrane proteins also takes place primarily in the ER, often with the help of specific foldases as well as chaperone proteins that prevent untoward interactions that would otherwise interfere with the folding process.[58,59] Misfolded proteins are branded with ubiquitin, a protein that serves as a signal for degradation by the proteosome, a large, complex protease with multiple catalytic functions.[60]

After translation, insertion, and folding, membrane proteins are routed to particular subcellular locations.[61] The destination of a given protein is determined, in many cases, by a specific localization sequence, a short stretch of cytosolic primary sequence that serves as a molecular "zip code". Examples of localization sequences

include C-terminal KDEL and KK motifs for type I proteins and N-terminal RR motifs for type II proteins, all of which ensure ER localization. Unless the protein contains a specific ER retention sequence, it will be transported to the Golgi apparatus, a multilamellar organelle that is the site of important post-translational modifications such as glycosylation. The type of glycosylation that the protein undergoes can also help determine its final destination. For instance, incorporation of mannose-6-phosphate tags proteins for the lysosome,[62] and glycosylphosphatidyl inositol (GPI) anchors proteins to the membranes and targets them to the cell surface.[63,64] In many cases, specific transport proteins within the Golgi have been identified that recognize these glycosylation tags and primary sequence "zip codes" and shuttle the tagged proteins to specific subcellular locales, a process generally termed protein transport or trafficking. Proteins destined for the cell surface (plasma membrane) or for secretion are transported through what is termed the secretory pathway. Others will be transported to specific organelles (e.g., lysosomes, nuclear envelope).

Proteins at the cell surface undergo considerable endocytosis, in which patches of protein-containing membranes get pinched off into vesicles called endosomes. These endosomes can then return to the plasma membrane or they may fuse with lysosomes, targeting the membrane proteins that they contain for degradation. The rate of endosomal recycling is surprisingly fast: the entire surface of the cell turns over every few hours (macrophages completely turnover their plasma membrane in about 0.5 h).[65] Thus, membranes within and on the surface of the cell are in constant flux, and secretases or "sheddases" in general (not just those that process APP) are critical players in this regard.[66]

β-Secretase

β-Secretase generates the N-terminus of A*β*, cleaving APP on the lumenal/extracellular side at approximately 30 residues from the transmembrane domain.[67] As mentioned earlier, most cell types produce A*β*, indicating broad expression of *β*-secretase. However, considerably more A*β* is generated in primary brain cultures than in peripheral cells,[68] and neurons display more *β*-secretase activity than astrocytes[69] (perhaps partly explaining why A*β* selectively aggregates in the brain). *β*-Secretase activity has been associated with several different membrane compartments in the cell: endosomes, lysosomes, Golgi, and endoplasmic reticulum (ER).[70−77] These observations, combined with the fact that *β*-secretase releases the ectodomain of APP, suggested that *β*-secretase is likely a membrane-bound protease similar to other sheddases (e.g., TNF-α secretase, ACE secretase, TGF-*β* secretase).[66] *β*-secretase cuts at the sequence EVKM*DAEF, where the asterisk denotes the cleavage site (see Figure 4). However, a KM-to-NL double mutation immediately adjacent to the *β*-secretase cleavage site causes FAD in a Swedish family and leads to increased A*β* production by enhancing proteolysis at the *β*-site.[15] Whether the same *β*-secretase processed both wild-type and Swedish mutant APP was not initially clear, especially because *β*-secretase otherwise appears to have a fairly restricted sequence specificity.[78]

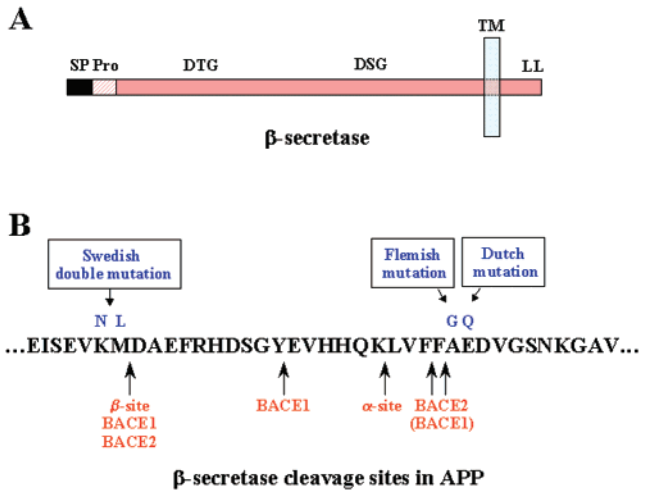


Figure 4. (A) Structure of *β*-secretase. (B) Sequence of APP in and around the α- and *β*-secretase cleavage sites and disease-causing missense mutations.

This enzyme eluded vigorous attempts at identification until very recently. Although a number of different *β*-secretase candidates had been previously nominated for consideration, none fulfilled all the criteria or matched the characteristics noted above. Last year, however, four different pharmaceutical companies and one academic laboratory each independently reported the identification of *β*-secretase as a novel membrane-tethered aspartyl protease.[79−83] Interestingly, several very different approaches were taken to arrive at the same answer. A team at Amgen used an expression cloning strategy:[79] a cDNA library was derived from a cell line known to possess *β*-secretase activity ,and pools of these cDNAs were transfected into cells overexpressing Swedish mutant APP. cDNA pools that produced elevated levels of A*β* were then subdivided to single cDNA clones. Of the positive clones identified, one shared homology with members of the pepsin family of aspartyl proteases. Researchers at Elan Pharmaceuticals used a more classical biochemical approach,[80] developing a substrate analogue inhibitor suitable for affinity chromatography. Subsequent N-terminal sequencing led to successful isolation of the cDNA clone encoding *β*-secretase. Meanwhile, scientists at Pharmacia & Upjohn employed a genomic approach.[81] As the sequence of the *Caenorhabditis elegans* genome neared completion, these researchers identified the complete set of aspartyl proteases encoded in this worm, then searched for human counterparts via the expressed sequence tag (EST) database. Several new aspartyl proteases were identified, one of which possessed *β*-secretase activity and was highly expressed in the brain. Similarly, research groups at SmithKline Beecham Pharmaceuticals[82] and at the University of Oklahoma[83] both directly searched for novel aspartyl proteases in EST databases, with subsequent cloning and purification of candidate *β*-secretases.

In all five of these reports, *β*-secretase (also named *β*-site APP-cleaving enzyme or BACE, BACE1, asp2, memapsin 2 by the various research groups) was identified as a novel aspartyl protease of 501 amino acids containing a single transmembrane domain near the C-terminus as well as a signal sequence and pro-peptide region at the N-terminus (Figure 4). Consistent with

observations from cell-based APP mutagenesis studies, the enzyme processes Swedish mutant APP or short peptides based on this mutant much better than the corresponding wild-type protein or peptide,[79,81,83] but it does not cleave a peptide containing a Met-to-Val change at P1.[79] Two aspartates, D93 and D289, are required for activity. Mutation of either aspartate, however, does not affect removal of the pro-peptide region in transfected cells, indicating that β-secretase does not autoproteolyze.[84] Indeed, installation of an ER retention sequence demonstrated that pro-peptide cleavage occurs after exit from this organelle.[85] The responsible β-secretase-activating protease appears to be a furin-like convertase.[86] β-Secretase shows a pH optimum of 4−4.5 for the cleavage of both wild-type and Swedish mutant peptides.[79,80,83] Interestingly, although clearly an aspartyl protease, β-secretase is not blocked by the broad-spectrum aspartyl protease inhibitor pepstatin A at 30−50 μM.[79,80] However, incorporation of statine into peptide substrates provided a potent inhibitor[80] (see below).

β-Secretase mRNA is expressed in various human tissues, as expected, with highest expression in the pancreas and all analyzed brain subregions.[79,81,83] An analysis of β-secretase activity in different tissues, however, revealed essentially no activity in the pancreas.[80] The mRNA levels are higher in neurons than in glia, consistent with β-secretase activity observed in cell culture.[79] The β-secretase protein is N-glycosylated[87,88] and is expressed primarily in the Golgi and in endosomes, although the enzyme could be detected at the plasma membrane as well. A cytoplasmic dileucine motif is apparently necessary for the recycling of β-secretase into endosomes.[85] Sequencing of APP fragment C99 generated from overexpressed β-secretase[79] confirmed that the major cleavage site is at the expected Asp1 (Aβ numbering); however, β-secretase expression also induced the formation of another protein, slightly smaller and beginning at Glu11 (Figure 4). Glu11-Aβ is also formed naturally from APP, and apparently β-secretase initiates the formation of this alternative truncated Aβ species as well. Inhibition of the endogenous β-secretase gene was accomplished using antisense oligonucleotides.[79] Both Aβ and β-APP$_s$ levels were substantially reduced by antisense oligos but not by reverse sequence oligos. Treatment with β-secretase antisense oligos also raised α-APP$_s$ levels, presumably because blocking β-secretase provides more substrate APP for α-secretase proteolysis.

The gene for β-secretase (BACE1) has been localized to chromosome 11, but no FAD-causing mutation in this gene has yet been identified.[89] However, a β-secretase homologue, BACE2, maps to the Down syndrome chromosome 21, raising the possibility that this protease contributes to the development of AD in Down patients, because they carry an extra copy of chromosome 21.[89] BACE1 and BACE2 exhibit 52% amino acid sequence identity and 68% similarity, and BACE2 cleaves APP and short peptides in a β-secretase-like manner: activity is similarly increased at Asp1 by the Swedish double mutation and prevented by the P1 Met-to-Val mutation.[90] However, BACE2 is not expressed well in the brain, suggesting that it may contribute little or nothing to AD neuritic plaque formation. Moreover, BACE2 generates little Glu11-Ab but efficiently performs an additional proteolysis in the middle of the Aβ region, resulting in the production of Phe20-Aβ and Ala21-Aβ (Figure 4), with the implication that BACE2 might limit the production of pathogenic forms of Aβ. If such were the case, the ideal β-secretase inhibitor would block BACE1 selectively, leaving BACE2 active.

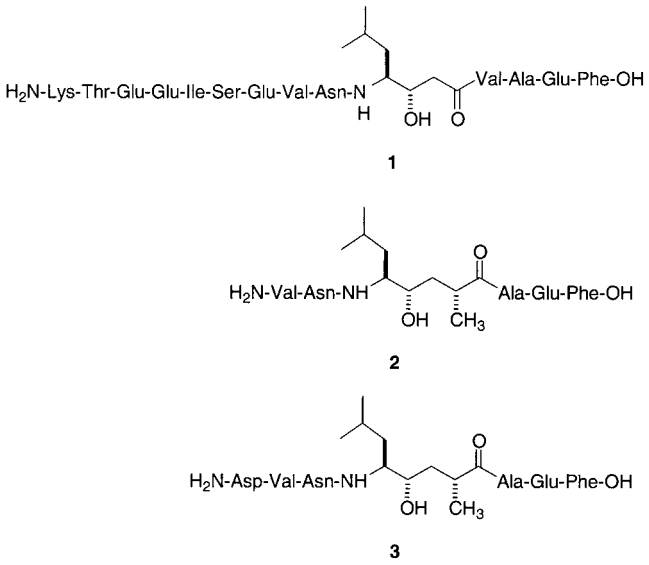


Figure 5. β-Secretase inhibitors.

Despite the keen interest, few inhibitors of β-secretase activity have been described. As mentioned above, Elan reported substrate-based inhibitors that ultimately were used to affinity purify the protease.[80] The first compound was based on the $P_{10}-P_{4'}$ residues of the Swedish mutant APP, replacing the P1 leucine with statine. This peptide analogue inhibited β-secretase activity, albeit poorly, with an IC_{50} of $\sim$40 μM in solubilized extracts of human brain membranes. Acetylation of the statine hydroxyl or replacement of *S*- with the *R*-statine diastereomer led to loss of inhibitory activity, indicating that, as in other statine-containing inhibitors of aspartyl proteases, inhibitory potency is dependent on an unmodified hydroxyl residue in the appropriate configuration. However, replacement of the P1′ aspartate with valine resulted in a substantial increase in potency (IC_{50} $\sim$ 30 nM), providing a suitable reagent (**1**, Figure 5) for affinity purification of β-secretase. Not surprisingly though, given its size and the presence of hydrophilic residues, this compound displays no inhibition of Aβ production in whole cells.

Ghosh and colleagues[91] have recently reported peptidomimetics based on the sequence VNL*AAEF. This peptide likewise contains the preferred Swedish NL double mutation at P2−P1, but also contains alanine at P1′, which was determined to be preferred over aspartate. The scissile Leu-Ala bond was replaced with a hydroxyethylene transition-state analogue isostere, a now classical strategy for inhibiting aspartyl proteases.[92−94] The new compounds, OM99-1 and OM99-2, (**2** and **3**, respectively, Figure 5) inhibited recombinant human β-secretase with K_i values of 68 nM and 10 nM, respectively. However, the selectivity of these prototype inhibitors with respect to other human aspartyl proteases was poor: the K_i of OM99-2 for cathepsin D was only 5-fold higher than for β-secretase. Presumably, the size of these compounds can be whittled down to an

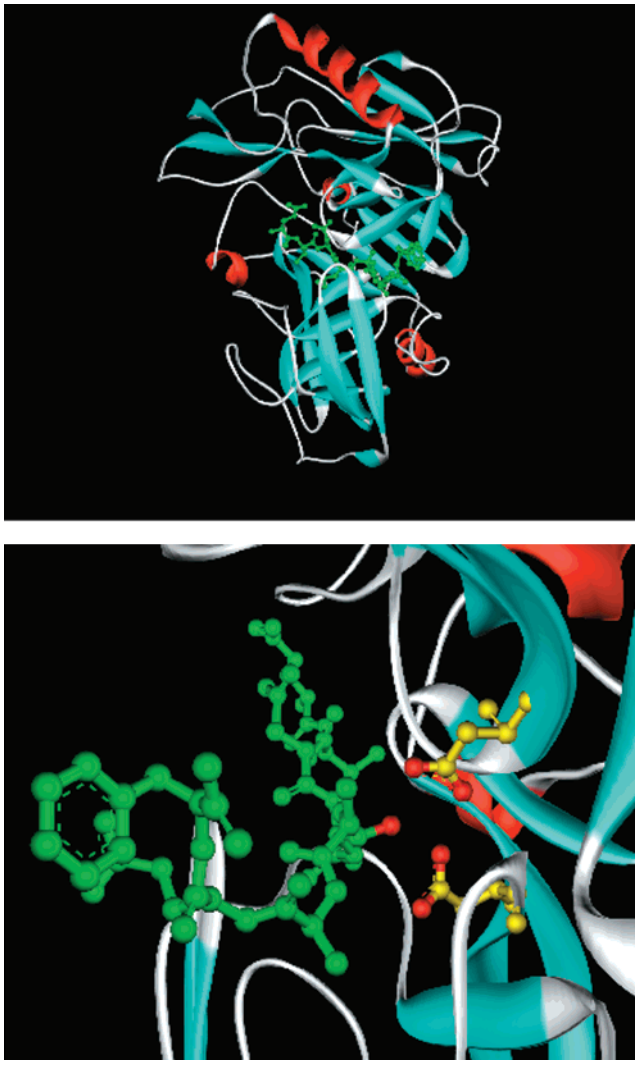


Figure 6. X-ray crystal structure of β-secretase bound to inhibitor **3**. (Top) The inhibitor (green) resides in the active site located in the cleft of the bilobal structure. (Bottom) Close-up view showing the interaction of the inhibitor hydroxyl group with the two catalytic aspartates (yellow).

effective pharmacophore, and the selectivity could be enhanced by systematic incorporation of various residues at P4 through P2′ (these side chains are well-defined in the crystal structure; see below).

Already a crystal structure of β-secretase bound to **3** at 1.9 Å resolution has been determined (Figure 6).[95] The bilobal structure of β-secretase has the conserved general folding of aspartyl proteases. Indeed, the backbone structure could be overlapped quite well onto that of pepsin. The inhibitor is located in the substrate binding cleft between the amino- and carboxy-terminal lobes, and as expected the transition-state mimicking hydroxyethyl moiety is coordinated with the two active site aspartates (Asp32 and Asp228). As with other aspartyl proteases, β-secretase possesses a "flap" that partially covers the cleft, and the backbone of the inhibitor is mostly in an extended conformation. However, β-secretase does display some structural differences, at least compared with pepsin. Four insertions are located on the molecular surface near the amino-terminus of the inhibitor, and together these insertions significantly enlarge the molecular boundary of β-secretase compared with pepsin. Two other insertions are located at the surface near the inhibitor C-terminus. In general, the β-secretase active site is more open and accessible than that of pepsin, and most of the hydrogen bond interactions between the enzyme and the backbone of the inhibitor are highly conserved among eukaryotic and HIV aspartyl proteases. However, the S2 and S4 subsites are relatively hydrophilic and open to solvent, and the hydrophilic character of these subsites is not conserved in the corresponding subsites of other human aspartyl proteases, such as pepsin, gastricsin, and cathepsins D and E, suggesting that these differences could be exploited for the design of selective inhibitors. In contrast, the P3′ and P4′ inhibitor side chains **3** point toward the molecular surface and have little interaction with the protease. The backbone of residues P2′−P4′ deviates from the regular extended conformation, with a kink at P2′, another unusual feature for an aspartyl protease that might be turned to advantage in inhibitor design.

β-Secretase would appear to be an optimal therapeutic target for the prevention and treatment of AD. The protease catalyzes the initial step in Aβ production, and Aβ is strongly implicated in the pathogenesis of the disease. Moreover, shunting APP into the α-secretase pathway might be beneficial as well, as APP_s is thought to be neuroprotective[96] and to enhance memory and prevent learning deficits.[97] Targeting β-secretase would also seem worthwhile based on the wealth of experience and information on inhibiting other aspartyl proteases, especially cathepsin D, renin, and HIV protease.[92,93,98] Furthermore, the determination of the crystal structure of β-secretase with **3** now makes structure-based design possible, a strategy that will no doubt benefit from the many crystal structures of other inhibitor-aspartyl protease complexes.

Nevertheless, there are too many unknowns at this point to say with confidence that effective β-secretase inhibitors can be readily developed or that β-secretase is necessarily a good target. For agents to work effectively in vivo, the compounds must not only cross the blood-brain barrier, but they must also penetrate neurons. Because they must work intracellularly (where Aβ is generated), it is all the more important to work toward highly selective agents that will not interfere with other intracellular proteases and with critical signaling pathways. Does β-secretase process other substrates besides APP? Given the fact that other membrane secretases have multiple substrates (e.g., TNF-α converting enzyme or TACE[99]), β-secretase may cleave other membrane proteins besides APP. Answers to this question will soon be forthcoming with the development of β-secretase knockout mice, which display no obvious phenotype except a dramatic reduction in Aβ levels.[100,101] On the other hand, even if β-secretase does play an important role in normal human adult physiology, only partial inhibition may be needed for a therapeutic effect (i.e., reduction of Aβ formation, not abolition). What is the normal role of BACE2? This enzyme is markedly expressed in heart, kidney, and placenta, suggesting that it may have an important function in highly vascularized systemic tissues.[90] If so, it might be critical to develop agents that selectively block BACE1 over BACE2. Again, knockout animals can address this issue. How is this enzyme itself processed

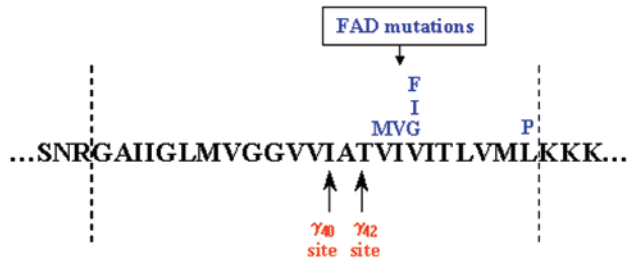


Figure 7. Sequence of APP in and around the γ-secretase cleavage site and disease-causing missense mutations. Dashed lines represent membrane boundaries.

and otherwise regulated? The identification of the activating protease that converts pro-BACE to BACE may reveal another worthwhile target, as would elucidating signaling events that lead to BACE processing of APP.

γ-Secretase

The other major protease target for the development of AD therapeutics is γ-secretase.[102] This enzyme has been considered central to understanding the etiology of AD because it determines the proportion of the highly fibrillogenic Aβ_{42} peptide. After either α- or β-secretase release the APP ectodomain, the resulting C83 and C99 APP C-terminal fragments are clipped in the middle of their transmembrane regions by γ-secretase (Figure 7).[67] Normally, about 90% of the proteolysis occurs between Val40 and Ile41 (Aβ numbering) to give Aβ_{40}; roughly 10% takes place between Ala42 and Thr43 to produce Aβ_{42}. Minor proportions of other C-terminal variants, such as Aβ_{39} and Aβ_{43}, are formed as well. γ-Secretase has been of interest not only because of its key role in AD pathogenesis but also because it presents an intriguing biochemical problem: how does this enzyme catalyze a hydrolysis at a site apparently located within a membrane?

During the search for the major FAD-causing genes on chromosomes 14 and 1, many thought the encoded proteins would reveal at least one, if not both, of the proteases involved in Aβ production. When the search identified the presenilins in 1995,[26,27] it was far from clear what the normal function of these proteins might be and why mutant forms might lead to AD. The two proteins, presenilin-1 (PS1) and presenilin-2 (PS2), are 65% identical, but the only homology they had to anything else known at the time was to a set of otherwise obscure proteins in worms involved in egg-laying and spermatogenesis.[103,104] Remarkably, these proteins were the sites of dozens of FAD-causing missense mutations.[105] Over 70 such mutations have now been identified, with all but six occurring in PS1.[28] These mutations are found in many regions of the linear sequence, although they tend to cluster in certain areas (Figure 8).

How could all these different PS mutations cause AD? Some mutations are extremely deleterious, a single copy of the mutant gene resulting in the onset of dementia as early as 25 years old.[106] Because of the amyloid plaque pathology of AD and because FAD-causing APP mutations are near β- and γ-secretase cleavage sites and affect Aβ production, investigating the effects of FAD-causing PS mutations on Aβ production, deposition, or toxicity seemed reasonable. Indeed, all PS mutations

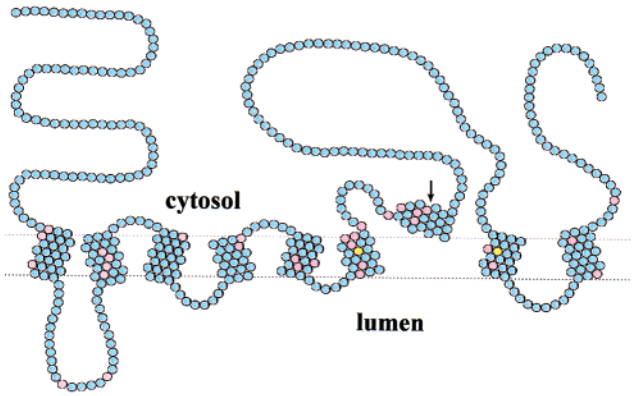


Figure 8. Topology of presenilin-1. Sites of FAD-causing missense mutations are in lavender, and the two conserved transmembrane aspartates essential for γ-secretase activity are in yellow. Presenilins undergo proteolytic processing in the hydrophobic portion of the large cytosolic loop (arrow) to form stable heterodimeric complexes.

examined to date have been found to cause specific increases in Aβ_{42} production, and this effect can be observed in transfected cells, in transgenic mice, and in blood plasma or media from cultured fibroblasts of FAD patients.[21,22,29,30] Thus, presenilins could somehow modulate γ-secretase activity to enhance cleavage of the Ala42−Thr43 amide bond. A major clue to the function of the presenilins came via PS1 knockout mice: deletion of PS1 in these mice was lethal in utero, indicating the clear requirement of this gene for proper development of the organism.[107,108]

Unfortunately, the embryonic lethality resulting from deleting PS1 did not suggest reasons why PS mutations might cause AD relatively late in life. Nevertheless, fibroblasts from PS1-deficient embryos could be cultured, and transfection of these cells with APP revealed that γ-secretase activity was markedly reduced.[109] The maturation and distribution of APP was not affected by the deletion of the PS1 gene, nor was the release of α- or β-APP$_s$ altered. However, γ-secretase substrates C83 and C99 were dramatically elevated, and Aβ production was substantially lowered. Formation of total Aβ and Aβ_{42} was reduced to similar degrees (to roughly 20% of levels seen in fibroblasts from PS1 +/+ littermates), indicating that PS1 plays a role in the production of both Aβ_{40} (which makes up 90% of all Aβ) and Aβ_{42}. The remaining γ-secretase activity was thought to be due to PS2. Indeed, the recent development of PS1/PS2 double knockout mice[110,111] allowed the culturing of embryonic stem cells, and transfection of APP demonstrated the complete absence of γ-secretase activity.[112,113] Thus, presenilins are absolutely required for the γ-secretase cleavage of APP.

Meanwhile, the development of γ-secretase inhibitors began providing information about the characteristics of this mysterious protease. The first reported inhibitors were short, hydrophobic peptide aldehydes originally designed as calpain inhibitors (**4**−**6**, Figure 9), and these compounds blocked Aβ production at the γ-secretase level in APP-transfected cells with IC$_{50}$s of 5−200 μM.[114,115] The fact that these were off-the-shelf inhibitors of calpain suggested that γ-secretase might likewise be a cysteine protease. However, peptide aldehydes can inhibit serine and aspartyl proteases as well,[116−118] so the ability of these compounds to block γ-secretase

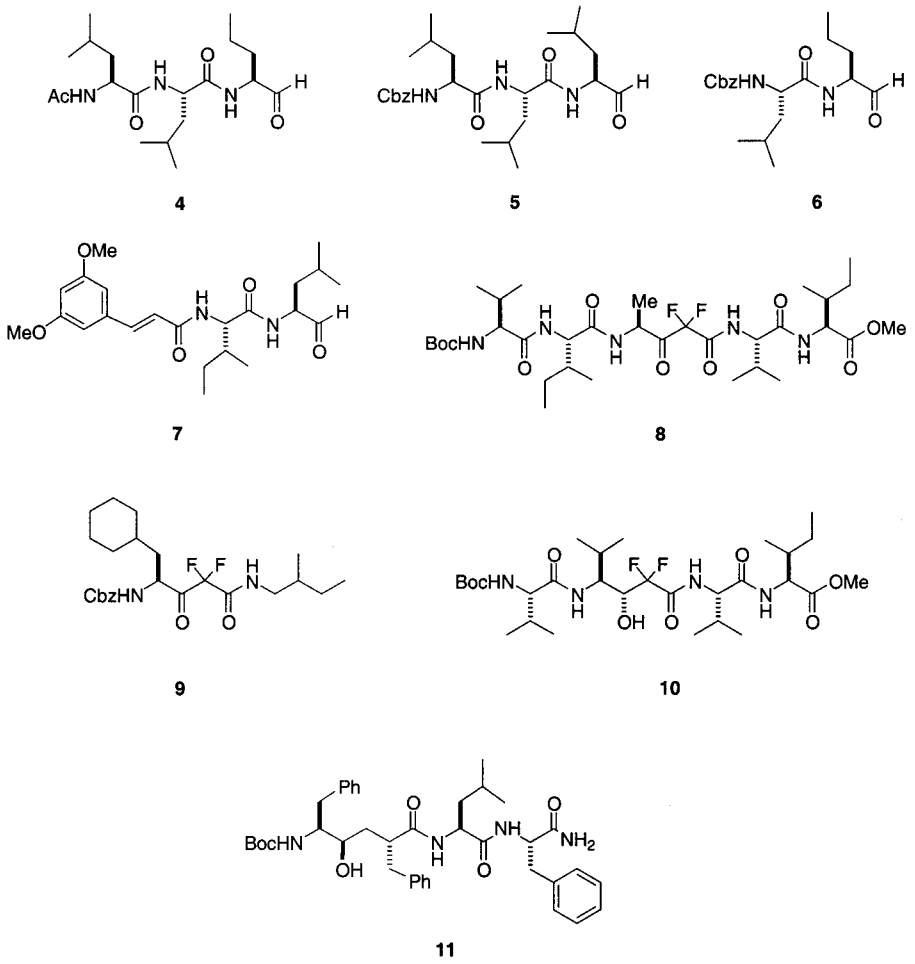


Figure 9. γ-Secretase inhibitors.

activity did not necessarily point to a specific mechanism. Still, these compounds served as useful molecular tools: the compounds blocked Aβ_{40} production selectively over that of Aβ_{42}, suggesting that pharmacologically distinct γ-secretases could be responsible for the formation of these two Aβ species.[115,119] This finding offered hope that compounds with the reverse selectivity could be identified, affording agents that would lower the more deleterious Aβ_{42}. Higaki et al. used a combinatorial approach to improve γ-secretase inhibitory properties of a peptide aldehyde lead.[120] Although compounds such as **7** with 10-fold greater potency over the lead were identified, these compounds likewise inhibited Aβ_{40} selectively over Aβ_{42}.

In 1998, our laboratory reported the first substrate-based inhibitor for γ-secretase (**8**, Figure 9; also referred to as MW167 in the literature).[121] Compound **8** was designed from the cleavage site in APP that results in Aβ_{42} formation, VIA*TVI, with replacement of the Ala-Thr cleavage site by a difluoro ketone moiety. This compound blocked Aβ production in APP-transfected cells (IC_{50} of $\sim$13 μM) at the level of γ-secretase: treatment with MW167 dramatically elevated γ-secretase substrates C83 and C99 but did not inhibit α- or β-APP_s levels. Surprisingly, although **8** was designed from the cleavage site leading to Aβ_{42}, this compound, like the peptide aldehydes, was a more effective inhibitor of Aβ_{40} formation than of Aβ_{42}. In fact, treatment of cells with low concentrations of compound led to counterintuitive increases in Aβ_{42} production, a phenomenon also observed with the peptide aldehyde inhibitors.[115,119] Compound **8** also did not offer specific information about the possible mechanism of γ-secretase action. Difluoro ketones are readily hydrated in aqueous solution, and these hydrates closely mimic the gem-diol intermediate in aspartyl protease catalysis.[122−125] However, difluoro ketones can also inhibit serine proteases, with the active site serine attacking with the ketone to form a covalent enzyme−inhibitor complex,[126] and cysteine proteases might be envisioned to interact by a similar mechanism.

Despite the poor potency, the lack of selectivity toward Aβ_{42}, and the inability to provide immediate mechanistic information, **8** was nevertheless the first reported compound specifically designed for γ-secretase, and this peptidomimetic served as a starting point to develop various analogues as molecular probes for the unidentified protease. Modification of **8** provided a panel of compounds that blocked Aβ_{40} and Aβ_{42} production, although again, none were selective for reducing Aβ_{42} and all increased Aβ_{42} at subinhibitory concentrations.[127] Yet this panel of difluoro ketone peptidomimetics inhibited Aβ_{40} and Aβ_{42} with virtually identical rank orders of potency, suggesting that the active sites of putative γ_{40}- and γ_{42}-secretases are closely similar. Because the ability of compounds to increase Aβ_{42} production in APP-transfected cells closely correlated with their inhibitory potency against γ-secretase activity, it seems plausible that partial inhibition of Aβ_{40} production increases the availability of C99 for Aβ_{42}

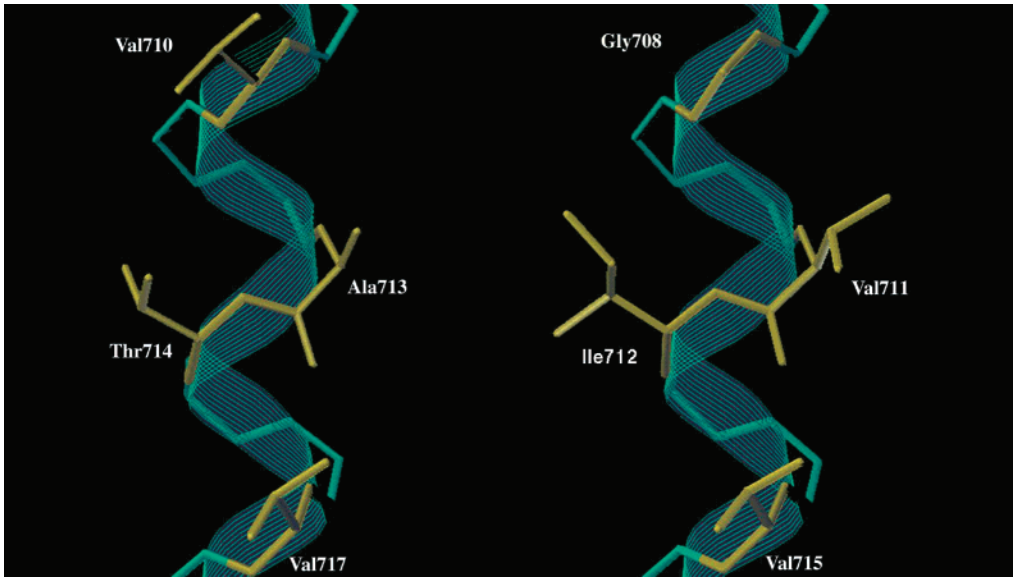


Figure 10. Helical model of the APP γ-secretase cleavage sites. (Left) Ala713−Thr714 is the amide bond processed to produce Aβ_{42}. Val717 is a site of several different FAD-causing missense mutations, and these mutations specifically increase Aβ_{42} production. The side chain of this residue is immediately adjacent to the Ala713−Thr714 amide bond. (Right) Rotating this model 180° shows the Val711−Ile712 bond cleaved to produce Aβ_{40}. Thus, the two major γ-secretase cleavage sites are on opposite sides of the helix.

production. That is, a separate γ_{40}-secretase would have a higher affinity for substrate than γ_{42}-secretase.[128]

A related study by Durkin and colleagues[129] observed similar effects using a set of compounds that inhibited Aβ at the γ-secretase level (all peptide aldehydes except for difluoro ketone **9** with a cyclohexylmethyl substituent in P1). Although the compounds raised Aβ_{42} levels at subinhibitory concentrations, they inhibited Aβ_{40} and Aβ_{42} with a similar rank order of potency. Interestingly, a dihydroxyethylene peptidomimetic was identified that did not inhibit γ-secretase, but even so was capable of increasing Aβ_{42}, demonstrating that the two phenomena could be separated. However, the reverse observation, inhibiting γ-secretase activity in cell culture without enhancing Aβ_{42} production, has not yet been reported. Apparently, increasing C99, either by partially blocking γ_{40}-secretase activity or by otherwise stabilizing C99 against other catabolic processes, can augment Aβ_{42} production.[128]

Surprisingly, γ-secretase tolerated a number of structural alterations in the substituents of difluoro ketone peptidomimetics, suggesting loose sequence specificity for inhibitors.[127] Indeed, γ-secretase had already been shown to possess loose sequence specificity for substrates, as a number of mutations in APP near the γ-secretase cleavage site still allow Aβ production in transfected cells.[130−133] Difluoro alcohol counterparts to these ketones (e.g., **10**) likewise blocked Aβ production, albeit with reduced potency.[127] Because difluoro alcohols are only known to inhibit aspartyl proteases,[122,124,134] the ability of these compounds to inhibit Aβ production suggested that γ-secretase falls into this mechanistic class of proteases.

More recently, we made a series of related difluoro ketones with various hydrophobic P1 substituents to explore the steric boundaries of the complementary S1 pocket and identify selective inhibitors of Aβ_{40} or Aβ_{42}.[135] Surprisingly, the more sterically demanding substituents (e.g., cyclohexylmethyl, *s*-butyl) increased γ-secretase inhibitory potency, suggesting a large S1 pocket in the protease that can accommodate these substituents and providing further evidence for loose sequence specificity. Moreover, this set of compounds blocked Aβ_{40} and Aβ_{42} production with essentially the same rank order of potency, further supporting the close similarity between the responsible γ-secretase active sites. Installation of the bulky cyclohexylmethyl group at P1 allowed N-terminal truncation (removal of P2) without loss of potency, suggesting that optimal S1 binding may allow the development of potent compounds with molecular weights below 600 Da. The presence of cyclohexylmethyl at P1 likewise led to the identification of a difluoro alcohol equipotent with its difluoro ketone counterpart, providing further support for an aspartyl protease mechanism for γ-secretase (see also below).

Does γ-secretase catalyze a hydrolysis within the boundaries of the lipid bilayer? To address this question, we modeled the γ-secretase cleavage site in APP as an α-helix, a conformation typical of transmembrane domains, in the hope that this might provide some insight into the nature of the substrate/γ-secretase interaction.[127] Interestingly, the two major cleavage sites, one leading to Aβ_{40} and the other to Aβ_{42}, are on opposite faces of the helix (Figure 10), and FAD-causing APP mutations are immediately adjacent to the scissile amide bond that leads to Aβ_{42}. Thus, the helical model provides a simple biochemical explanation for why these FAD mutations result in selective increases in Aβ_{42} production. Lichtenthaler et al. provided important experimental support for this model via a phenylalanine scanning mutagenesis study.[133] Systematic replacement of each APP transmembrane residue after the γ_{42} cut site with phenylalanine resulted in periodic changes in effects on Aβ_{40} and Aβ_{42} production that were consistent with a helical conformation of substrate upon initial interaction with the protease. These findings are consistent with γ-secretase being a novel intramembrane-

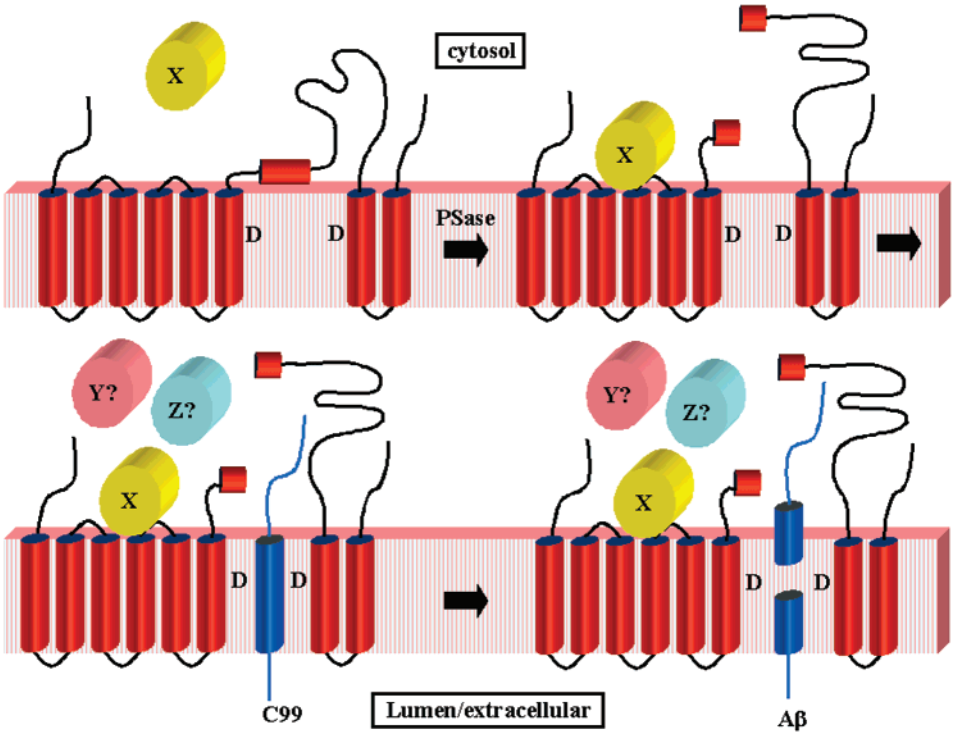


Figure 11. Model for the role of presenilins in γ-secretase processing of APP. Full-length presenilins interact with limiting cellular factors (protein X) to produce PS heterodimers by the action of an unidentified presenilinase (PSase). The requirement of the two transmembrane aspartates for PSase activity suggests that presenilins undergo autoproteolysis upon interaction with X. Other factors may also be needed to form the γ-secretase complex, in which the two aspartates are the catalytic residues of the active site. Binding of substrate C99 leads to Aβ production.

cleaving protease (I-CLiP), which hydrolyzes its substrates within the confines of the membrane.[136]

γ-Secretase does not appear to be the only example of an I-CLiP: another protease involved in cholesterol metabolism shows some similar characteristics. The sterol regulatory element binding protein (SREBP) is a basic-helix-loop-helix transcription factor that is initially synthesized in the ER as a precursor with two transmembrane domains.[137] When cholesterol levels are low, SREBP is cleaved between its transmembrane domains within the lumen of the ER by a site 1 protease. The transcription factor, though, is still ER-bound until a second cut by site 2 protease (S2P) releases it, allowing it to translocate to the nucleus and activate the expression of genes needed for cholesterol biosynthesis. S2P apparently cleaves four residues inside the boundary of the ER membrane,[138,139] and the cloning of S2P revealed that the protease itself has multiple transmembrane domains.[140,141] S2P contains a short HEXXH region found within the middle of a hydrophobic stretch of residues, and this sequence is a consensus motif for a number of metalloproteases. Indeed, these residues are essential for proteolysis, as is a critical aspartate that lies in the middle of another hydrophobic stretch, suggesting that the active site of S2P may be sequestered in the membrane.[141] A recent mutagenesis study near the site 2 cleavage of SREBP also revealed loose sequence specificity, although the requirement of two residues, Asn-Pro, downstream from the cleavage site suggested that helix instability after S1P proteolysis might result in a conformational change that exposes site 2 outside the membrane.[142]

What is the biochemical role of presenilins in mediating γ-secretase activity? Could presenilins be proteases? Presenilins bear no sequence homology with known proteases. Moreover, overexpression of presenilins does not lead to increased γ-secretase activity.[21] However, presenilins themselves undergo proteolytic processing within the hydrophobic region of the large cytosolic loop between transmembrane domain (TM) 6 and TM 7 (Figure 11) to form stable heterodimeric complexes composed of the N- and C-terminal fragments.[148,149] [Most studies support an eight-TM topology for the presenilins,[143−145] although six- and seven-TM topologies have also been suggested.[146,147]] These heterodimers are only produced to limited levels even upon overexpression of the holoprotein[148,150−152] and may be found at the cell surface.[173] Expression of exogenous presenilins leads to replacement of endogenous presenilin heterodimers with the corresponding exogenous heterodimers, indicating competition for limiting cellular factors needed for stabilization and endoproteolysis.[153]

FAD-causing presenilin mutants are likewise processed to stable heterodimers with one exception, a missense mutation in PS1 that leads to the aberrant splicing out of exon 9, a region that encodes the endoproteolytic cleavage site.[148,150] This PS1 ΔE9 variant is an active presenilin, able to partially rescue a loss of function presenilin mutation in the worm *C. elegans*,[154,155] and, like other FAD-causing presenilin mutants, causes increased Aβ_{42} production.[155,156] Upon overexpression, most PS1 ΔE9 is rapidly degraded similar to unprocessed wild-type presenilins; however, a small portion of this PS1 variant is stabilized in cells[150,157] and forms a high molecular weight complex like the N- and C-terminal fragments,[149,158] suggesting that it can interact with the same limiting cellular factors as wild-type presenilins. These observations are

consistent with the idea that the bioactive form of presenilin is the heterodimer and that the hydrophobic region in the large cytosolic loop is an inhibitory domain.

γ-Secretase displays the pharmacological profile of an aspartyl protease, appears to catalyze an intramembranous proteolysis, and requires presenilins for activity. Given these characteristics of γ-secretase and clues to presenilin function, could presenilins be γ-secretase? Indeed, presenilins contain two transmembrane aspartates (Figure 8), one found in TM6 and one in TM7, predicted to lie the same distance within the membrane (i.e., they could interact with each other) and roughly aligned with the γ-secretase cleavage site in APP (i.e., they might work together to cut C99 and C83). These two aspartates are completely conserved from worms and flies to mice and men and are even found in a recently identified plant presenilin.[159] Mutation of either TM aspartate to alanine did not affect the expression or subcellular location of APP, and the subcellular distribution of the mutant presenilins was also similar to the wild-type.[160] However, the mutant presenilins were completely incapable of undergoing endoproteolysis and acted in a dominant-negative manner with respect to γ-secretase processing of APP. Similar effects on APP processing were observed even when conservative mutations to glutamate[160] or asparagine[161,162] were made, indicating the crucial identity of these two key residues as aspartates and suggesting that the effects are not likely due to misfolding. These effects have been corroborated by several different laboratories and have been seen for both PS1 and PS2.[158,160−164]

The aspartates are critical for γ-secretase activity independent of their role in presenilin endoproteolysis: aspartate mutation in the PS1 ΔE9 variant still blocked γ-secretase activity, even though endoproteolysis is not required of this presenilin variant.[160] Together these results suggest that presenilins might be the catalytic component of γ-secretase (Figure 11): upon interaction with as yet unidentified limiting cellular factors, presenilin undergoes autoproteolysis via the two aspartates, and the two presenilin subunits remain together, each contributing one aspartate to the active site of γ-secretase. The issues of PS autoproteolysis and the role of PS endoproteolysis is controversial, especially in light of the identification of certain uncleavable artificial missense PS1 mutants that are still functional with respect to γ-secretase activity.[161] On the other hand, these mutations may disrupt the putative pro domain so that it no longer blocks the active site.

The presenilins are not only involved in the proteolytic processing of APP. They are also critical for processing of the Notch receptor, a signaling molecule crucial for cell-fate determination during embryogenesis.[165] After translation in the ER, Notch is processed by a furin-like protease, resulting in a heterodimeric receptor that is shuttled to the cell surface[166] (Figure 12). Upon interaction with a cognate ligand, the ectodomain of Notch is shed by a metalloprotease apparently identical to tumor necrosis factor-α converting enzyme (TACE).[167,168] Interestingly, metalloproteases such as TACE and ADAM-10 are among the identified α-secretases that shed the APP ectodomain[51,52] (see also below). The membrane-associated C-terminus is then cut within the postulated transmembrane domain to release the Notch intracellular domain (NICD), which then translocates to the nucleus where it interacts with and activates the CSL family of transcription factors.[169] NICD formation is absolutely required for signaling from the Notch receptor: knock-in of a single point mutation near the transmembrane cleavage site in Notch1 results in an embryonic lethal phenotype in mice closely similar to that observed upon knockout of the entire Notch1 protein.[170]

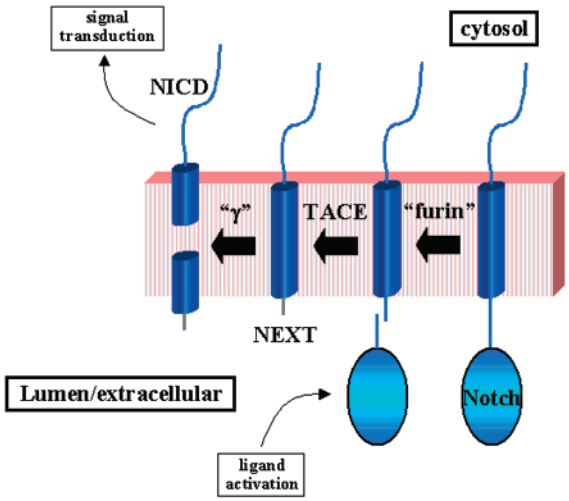


Figure 12. Proteolytic processing of the Notch receptor.

The parallels between APP and Notch processing are striking. Not only are both cleaved by TACE, but also the transmembrane regions of both proteins are processed by a γ-secretase-like protease that requires presenilins. Deletion of PS1 in mice is embryonic lethal, with a phenotype similar to that observed upon knockout of Notch1,[107,108] and the PS1/PS2 double knockout phenotype is even more similar.[110,111] Deficiency in PS1 dramatically reduces NICD formation,[171] and the complete absence of presenilins results in total abolition of NICD production.[112,113] Treatment of cells with γ-secretase inhibitors designed from the transmembrane cleavage site within APP (compound **8**) likewise blocks NICD production[171] and nuclear translocation[172] and reduces Notch signaling from a reporter gene.[172] Moreover, the two conserved TM aspartates in presenilins are required for cleavage of the Notch TM domain: as seen with γ-secretase inhibitors, expression of Asp-mutant PS1 or PS2 results in reduction of NICD formation, translocation, and signaling.[164,172−174] Thus, if presenilins are the catalytic components of the γ-secretases that process APP, they are also likely the catalytic components of the related proteases that clip the transmembrane region of Notch.

Effects of the various FAD-mutant presenilins on Notch processing are unclear. One FAD-mutant PS1 was able to rescue the PS1 null phenotype in mice,[175,176] indicating that it is still functional. On the other hand, several such mutant presenilins did not fully rescue Notch deficiencies in NICD formation, translocation, and signaling in PS1-deficient cells.[177] Moreover, FAD mutations only partially rescue or do not rescue at all an egg-laying deficiency in *C. elegans* caused by a mutation in sel-12, a worm presenilin,[154,178] and certain artificial mutations in the large loop of PS1 differentially inhibit NICD and Aβ production.[179]

Subcellular localization presents a potential problem for the idea that presenilins could be γ-secretases: presenilins are primarily found in the ER and early Golgi,[180] while γ-secretase activity apparently takes place on the cell surface as well as the ER and Golgi.[71,73,74,77,114,181] Very small amounts of presenilin heterodimers, however, have been found at the cell surface in complexation with the membrane-associated C-terminus of Notch,[173] and only small amounts of this presumably bioactive form should be needed for catalysis. Nevertheless, this "spatial paradox" still needs clear resolution. Another perplexing phenomenon is that the holoproteins of APP and Notch form stable complexes with presenilins in the ER.[182−184] APP and Notch themselves are not substrates for γ-secretase, so why do they interact with presenilins if presenilins are the proteases? However, as mentioned above, the membrane-associated C-terminus of Notch likewise forms stable complexes,[173] and APP-derived C83 and C99 can also complex with presenilins in the presence of a γ-secretase inhibitor or when either critical presenilin aspartate is mutated.[185] Perhaps presenilins mature together with APP or Notch: while complexed with APP or Notch, presenilins undergo endoproteolysis; then, after the ectodomain of APP or Notch is removed, the remaining membrane-bound C-terminus can access the active site of heterodimeric presenilin/γ-secretase.

Other unresolved issues are the site and specificity of Notch cleavage vis-à-vis that of APP. Although Notch appears to be cleaved within its transmembrane region, the site of cleavage is close to the cytosolic edge, while the APP γ-secretase site is apparently in the middle of the membrane. For either cleavage events, though, only one of the resulting proteolytic products has been identified; characterizing the other fragments may clarify this issue. As for sequence specificity, a single Val-to-Leu change dramatically reduces proteolysis of the Notch transmembrane region,[169] while a variety of mutations in the APP transmembrane region are tolerated by γ-secretase. On the other hand, mutagenesis of the Notch transmembrane region typically does not abolish proteolysis or the resultant transcriptional activation.[169,186] Examination of these proteolytic events in purified enzyme assays may offer new insights to resolve this issue.

Advancing the understanding of γ-secretase and the role of presenilins in this activity had been hampered by the lack of an isolated enzyme assay. Recently, Li et al. reported a solubilized γ-secretase assay that faithfully reproduces the properties of the protease activity observed in whole cells.[187] Isolated microsomes were solubilized with detergent, and γ-secretase activity was determined by measuring Aβ production from a C-terminally modified version of C99. Aβ_{40} and Aβ_{42} were produced in the same ratio as seen in living cells ($\sim$9:1), and peptidomimetics that blocked Aβ_{40} and Aβ_{42} formation in cells likewise inhibited production of these Aβ species in the solubilized protease assay. Choice of detergent was critical for Aβ production in the assay: CHAPSO was optimal, although CHAPS, a detergent known to keep presenilin subunits together,[149] was also compatible with activity, and Triton-X100 did not allow any Aβ formation. After separation of the detergent-solubilized material by size-exclusion chromatography, γ-secretase activity coeluted with the two subunits of PS1. Remarkably, immunoprecipitated PS1 heterodimers also produced Aβ from the artificial substrate, strongly suggesting that presenilins are part of a large γ-secretase complex.

More direct evidence that presenilins are the catalytic components of γ-secretases has recently come from affinity labeling studies using transition-state analogue inhibitors. Shearman et al. identified a peptidomimetic γ-secretase inhibitor **11** (Figure 9) by rescreening compounds originally designed against HIV protease.[188] The compound blocks γ-secretase activity with an IC_{50} of 0.3 nM in the solubilized protease assay[187] and contains a hydroxyethyl isostere, a transition-state mimicking moiety found in many aspartyl protease inhibitors. While the transition-state mimicking alcohol directs the compound to aspartyl proteases, flanking substructures determine specificity. Indeed, this compound does not inhibit aspartyl proteases cathepsin D and HIV-1 protease.[188] The stereochemistry of the hydroxyethyl isostere is critical for the inhibitory activity: the *S* isomer is 270 times more potent than the *R* isomer, an unexpected finding as the *R* isomer is typically more potent toward other aspartyl proteases (e.g., HIV protease). Oxidation of the *S* isomer to the ketone resulted in a 10-fold loss of activity in a competitive binding assay.

Photoactivatable versions of the potent *S* isomer bound covalently to presenilin subunits exclusively.[189] Interestingly, installation of the photoreactive group on one end of the inhibitor (**12**, Figure 13) led to labeling of the N-terminal presenilin subunit, while installation on the other end (**13**) resulted in the tagging of the C-terminal subunit. Moreover, while these agents did not label wild-type PS1 holoprotein, they did tag PS1 ΔE9, which as described above is not processed to heterodimers but nevertheless active. Similarly, Esler et al. identified peptidomimetic inhibitors containing a difluoro alcohol group, another type of transition-state mimicking moiety, and these compounds were developed starting from a substrate-based inhibitor designed from the γ-secretase cleavage site in APP.[190] Conversion of one such analogue to a reactive bromoacetamide provided an affinity reagent (**14**) that likewise bound covalently and specifically to PS1 subunits in cell lysates, isolated microsomes, and whole cells. Either PS1 subunit so labeled could be brought down with antibodies to the other subunit under coimmunoprecipitation conditions, demonstrating that the inhibitor bound to heterodimeric PS1. Seiffert and colleagues likewise identified presenilin subunits as the molecular target of novel peptidomimetic γ-secretase inhibitors. The affinity probe **15**, however, does not resemble known transition-state mimics (see below), so it is not clear whether this compound would be expected to bind to the active site of the protease.[191]

Taken together, these results strongly suggest that heterodimeric presenilins contain the catalytic component of γ-secretase: inhibitors in two of the three studies are transition-state analogues targeted to the active site. The wild-type presenilin holoprotein could be an inactive zymogen that requires cleavage into two subunits for activation. In any event, the active site is likely at the PS heterodimeric interface: both subunits are labeled

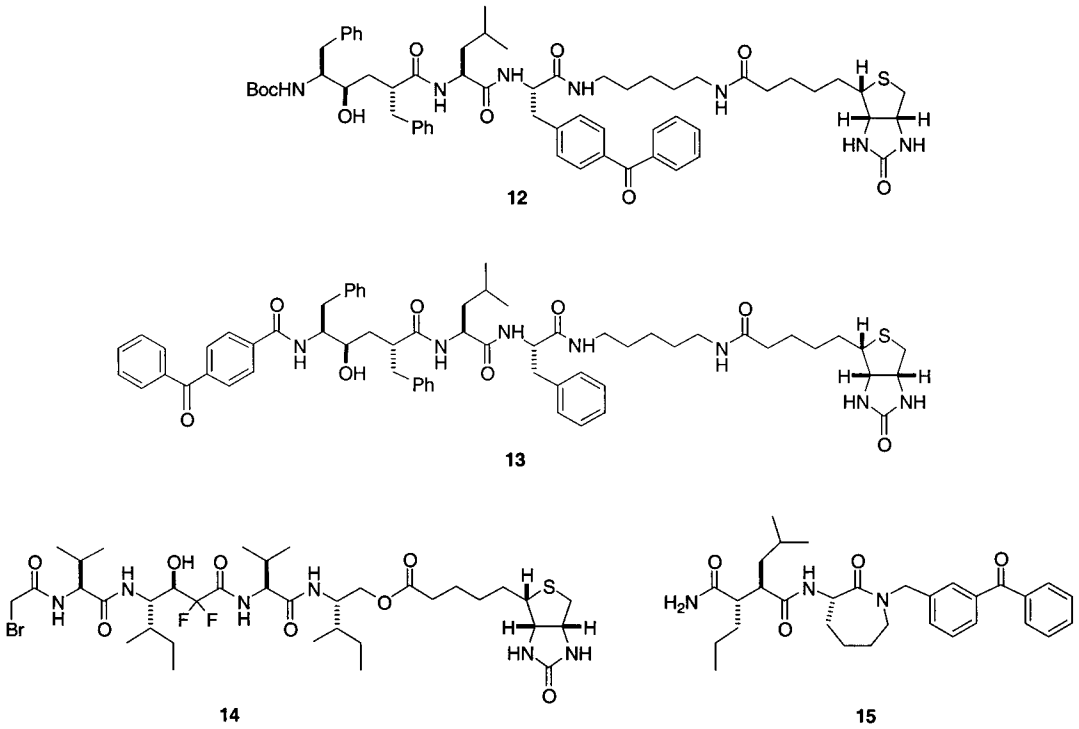


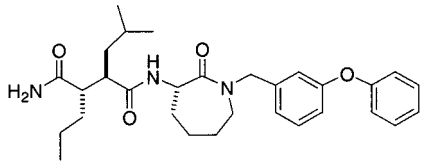
Figure 13. Affinity probes for γ-secretase.

by γ-secretase affinity reagents, and each contributes one critical aspartate. Whether a separate "presenilinase" converts the holoprotein to subunits or presenilins undergo autoproteolysis remains to be determined, although the absolute requirement of the two transmembrane aspartates for heterodimer formation suggests the latter.

The affinity labeling studies simultaneously identify the protease responsible for the transmembrane cleavage of Notch, because substrate-based γ-secretase inhibitors such as those used by Esler et al. also block this Notch proteolysis (see above). These findings considerably reinforce the amyloid hypothesis of AD pathogenesis: all FAD-causing mutations identified to date (accounting for ~60% of all FAD cases) are either in the precursor protein leading to Aβ (APP) or are in the proteases that catalyze the final step in Aβ generation (presenilins/γ-secretases). At the same time, an important target for drug development has been identified, although it is not yet clear whether toxic effects of blocking cleavage of other γ-secretase substrates (e.g., Notch) will negate therapeutic effects. Although presenilins are essential for proper embryonic development, the role of these proteins in aging adults is not known.

Clearly, the development of specific γ-secretase inhibitors has been critical to the elucidation of the role of presenilins and identifying Notch as an alternative substrate. Descriptions of the structural requirements of some of these compounds for inhibiting γ-secretase (the peptide aldehydes, difluoro ketones, difluoro alcohols, and hydroxyethyl analogues) were interleafed with complementary biological experiments described above. Other inhibitors include those described by Seiffert et al. that contain a 2,3-dialkylated succinamide moiety.[191] Removal of one of the succinamide alkyl substituents resulted in a dramatic reduction in potency: the IC_{50} for inhibiting Aβ production in a cell-based assay was 40 nM for **16** (Figure 14) and 52 μM without the *n*-propyl substituent. These compounds displayed comparable potencies in a displacement assay using isolated membranes. Replacement of the 3-phenoxybenzyl group with benzophenone reduced potency only slightly, and the resulting photoreactive compound **15** was used to affinity label presenilins as described above. The lactam substructure is apparently not necessary for activity, as replacement with a benzodiazepine moiety led to a somewhat more potent analogue, compound **17**. Indeed, with the benzodiazepine moiety in place, the succinamide moiety could be replaced by difluorophenylacetylalanine to give compound **18**, a subnanomolar inhibitor in whole cells. In addition, fenchylamine sulfonamide inhibitor **19** has been reported as an inhibitor of γ-secretase activity in whole cells, albeit at micromolar concentrations.[192] More recently, Dovey et al. reported that dipeptide analogue **20** was a potent inhibitor of γ-secretase that could lower brain Aβ levels acutely in APP-transgenic mice.[193]

The issue of whether γ-secretase is a good target for treating AD remains an open question, the primary concerns being the resultant increases in APP C-terminal stubs, C83 and C99, and inhibition of Notch signaling. Indeed, learning is impaired in transgenic mice expressing C99,[194] and knockout of γ-secretase activity is embryonic lethal due to interference with Notch.[107,108] However, the learning deficits in C99 transgenic mice may be due to resultant amyloid deposition.[194] Moreover, while γ-secretase inhibition can also interfere with Notch proteolysis, it may still be possible to selectively inhibit APP processing over that of Notch. This might require compounds that block γ-secretase through an allosteric effect, rather than through interaction with the active site. In any event, current evidence indicates that Notch processing can be blocked down to a certain threshold without affecting Notch signaling,[169,172] suggesting that the signaling mechanism may be saturated with NICD. If so, then



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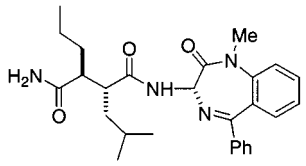

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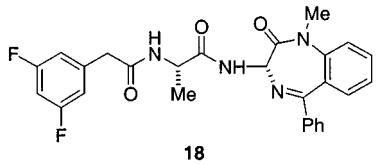

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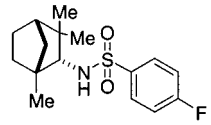

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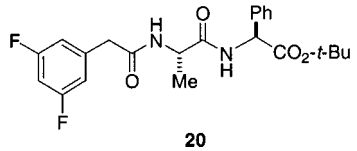

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Figure 14. γ-Secretase inhibitors.

Aβ production may be reduced to a substantial degree without untoward effects on Notch. Nevertheless, useful γ-secretase inhibitors will have to distribute to the brain effectively, or else peripheral toxicity upon chronic administration may be unavoidable.

α-Secretase

Alternative processing of APP by α-secretase precludes Aβ production, as this proteolysis occurs within the Aβ sequence.[195] α-Secretase cleaves APP at Lys686−Leu689 (APP770 numbering; see Figure 4), releasing α-APP$_s$ and concomitantly forming C83, the latter being further processed to the N-terminally truncated Aβ variant called p3. Two missense mutations in APP near the α-secretease cleavage site cause disease and increase Aβ production: A692G and E693Q.[18,196,197] These mutations occur in families with hereditary cerebral hemorrhage with amyloidosis,[198−200] in which the patients suffer recurrent and ultimately fatal cerebral hemorrhage due to amyloid deposition in the meningeal and cerebral vasculature. Carriers of these mutations may or may not develop early-onset AD. The increased Aβ deposition may be due to elevated Aβ production caused by decreased α-secretase processing (i.e., more APP is shunted into the β-secretase pathway);[18] however, a recent report suggested that the A692G mutation enhances C99 (and hence Aβ) production by preventing alternative processing near this site by BACE2.[90] The principal determinants of APP cleavage by α-secretase appear to be the distance of the hydrolyzed bond from the membrane (12 or 13 residues) and a local helical conformation.[201] Indeed, whereas a peptide substrate spanning the α-secretase cleavage site representing wild-type APP displays α-helical character, the corresponding substrate from A692G APP is random coiled.[52]

Although cells contain a certain level of basal α-secretase activity, this proteolysis can be substantially induced by classical protein kinase C activators such as phorbol esters.[202−205] Moreover, activation of receptors that work through protein kinase C can augment α-secretase cleavage of APP with concomitant reduction in β-secretase processing. For instance, agonists of the metabotropic glutamate receptors can lower Aβ by shunting APP toward the α-secretase pathway.[206] Muscarinic agonists (M1 and M3) can also decrease Aβ production by this means, and this effect has been observed in vitro as well as in vivo.[207−210] Because of this effect on Aβ production, M1 and M3 agonists may be useful agents for treating AD. In addition, evidence suggests that α-APP$_s$ may have a neuroprotective effect and enhance learning and cognition,[97] so augmenting α-secretase processing of APP may be beneficial in treating AD for this reason as well.

Pharmacological studies initially suggested that α-secretase may be a zinc-dependent metalloprotease, as the activity can be blocked by peptide hydroxamates.[211] Interestingly, a series of hydroxamates inhibited APP processing by α-secretase and angiotensin-converting enzyme (ACE) processing by ACE secretase with similar potencies and rank order of potencies.[211] In contrast, these compounds had a differential effect on TACE.[212] Nevertheless, knockout of TACE in mice resulted in elimination of inducible α-secretase activity, indicating that TACE plays an essential role in α-secretase processing of APP.[51] Moreover, TACE cleaved peptide substrates based on the α-secretase cleavage site in APP at Lys686−Leu689, suggesting that TACE itself may be one of the α-secretases. TACE apparently processes a spectrum of type I membrane proteins, including TNF-α, the p75 TNF receptor, L-selectin adhesion molecule, and transforming growth factor-α (TGF-α), and knockout of TACE is lethal, emphasizing the importance of ectodomain shedding during embryonic development.[99] As mentioned above, TACE appears to be the sheddase responsible for release of the Notch ectodomain after ligand binding,[167] a role that would likely be essential for embryonic development; however, the defects seen upon TACE knockout were similar to those of mice lacking TGFα.[99] In any event, manipulation of TACE as a therapeutic strategy for AD may lead to toxicity due to effects on the shedding of other membrane proteins.

Another metalloprotease, ADAM 10, also appears to process APP in an α-secretase-like manner.[52] Overexpression of ADAM-10 in human cells increased both basal and PKC-inducible α-secretase activity several-fold. Moreover, endogenous α-secretase activity was inhibited by a dominant-negative form of ADAM10 with a point mutation in the zinc binding site. A peptide substrate based on the α-secretase cleavage site in APP was processed by ADAM 10 at the Lys686−Leu689

bond, whereas a substrate based on the disease-causing A692G mutant APP is cleaved less efficiently. Interestingly, ADAM 10 is also implicated in the Notch signaling pathway.[213] Thus, TACE and ADAM 10 appear to have similar roles with respect to APP and Notch processing. Whether stimulation of ADAM 10 would be a better therapeutic strategy than stimulating TACE is presently unclear, as little is known about the role of ADAM 10 in shedding other membrane proteins.

Summary and Conclusions

In the past few years, key pieces in the puzzle of AD have been falling into place. In particular, the APP-processing secretases are no longer completely mysterious: specific proteins are now known to carry out these proteolytic events. β-Secretase is a novel membrane-tethered aspartyl protease, but because of its relation to the cathepsin D family and the fact that the business end is clearly located outside the membrane, this protease is amenable to rigorous structural biological studies and structure-based design. Given the ample experience of the drug industry in the area of aspartyl protease inhibition, potent β-secretase inhibitors with good pharmacokinetic properties may be soon forthcoming. BACE1 knockout mice are apparently normal and have dramatically reduced brain Aβ levels, validating this protease as a therapeutic target. However, agents selective for inhibiting BACE1 over BACE2 may be needed to avoid peripheral toxicity. Knockout of the BACE2 gene in mice should help answer this critical question, and the crystal structure of BACE2 may suggest strategies for designing selective BACE1 inhibitors.

While γ-secretase also appears to be an aspartyl protease, this enzyme is less tractable with respect to appreciating the details of its active site topology. The two key aspartates in presenilins essential for proteolytic activity are apparently embedded in the membrane, and other members of what is likely to be a larger γ-secretase complex have yet to be identified. The recent development of a cell-free solubilized enzyme assay should facilitate the discovery of new inhibitors and provide an essential means for beginning to understand the biochemical details of how this unusual enzyme works. In particular, the identification of other members of what is apparently a multi-protein complex should reveal new targets for inhibitor design. A crystal structure for this enzyme is not likely to be obtained any time soon. Thus, a combination of affinity labeling with transition-state analogue inhibitors and site-directed mutagenesis may be the most practical strategy toward characterizing the active site.

Despite the biochemical difficulties, effective γ-secretase inhibitors have already been identified through cell-based screens, and at least one of these can lower brain Aβ levels in mice.[193] Nevertheless, the fact that Notch signaling requires a presenilin-dependent protease similar or identical to γ-secretase raises concerns about mechanism-based toxicity. The development of an in vitro assay for this Notch processing event, along with purification of the γ-secretase complex, should help answer the question of whether the transmembrane-cleaving proteases for APP and Notch are identical. Furthermore, an understanding of the mechanism of γ-secretase specificity (i.e., Aβ_{40} versus Aβ_{42} production) may suggest strategies to selectively lower the more deleterious Aβ_{42}. No such agents have been reported, but their identification may lead to compounds that are therapeutically effective while preserving most γ-secretase activity.

α-Secretase activity appears to reside in the metalloproteases TACE and ADAM10, and stimulating this activity via PKC-activating receptors may lead to therapeutically valuable agents that shunt APP away from the amyloidogenic pathway and toward a neuroprotective pathway. Muscarinic agents in particular may prove useful toward this end. One caveat here, as with β- and γ-secretase inhibition, is that the α-secretases apparently process multiple substrates. Whether the activities of these Alzheimer secretases can be modified without untoward effects remains to be seen. The identification of cellular pathways that modulate the selectivity of these secretases may offer new targets with the potential to avoid mechanism-based toxicities, but whether such pathways even exist is completely unknown.

Despite the many remaining unknowns, this area of biomedical investigation has greatly matured, as can be appreciated by revisiting Figure 1. In the quest to understand the molecular underpinning of AD and develop treatments, the pathology pointed to the potential importance of amyloid plaques and neurofibrillary tangles. Genetics revealed that mutations in APP or presenilins alone could cause AD. The cell and molecular biology told us that presenilins were involved in APP and Notch processing as well as signaling from the Notch receptor, and the development of inhibitors helped characterize γ-secretase enough to suggest that the two transmembrane aspartates of presenilins might be part of an unusual intramembranous active site. We are now primarily in the biochemical phase: the problems have been brought down to the level of enzymology. Meanwhile, the search for inhibitors with ideal pharmaceutical properties continues. Such agents will undoubtedly feed back to help fill in the gaps in the preceding phases of understanding and may also help solve a major human health problem. Although substantial hurdles still remain, AD and other neurodegenerative diseases no longer seem like unbeatable foes, and the quest for therapeutic agents no longer like an impossible dream.

Biography

Michael S. Wolfe received his B.S. in chemistry in 1984 from the Philadelphia College of Pharmacy and Science and earned his Ph.D. in medicinal chemistry in 1990 from the University of Kansas under the direction of Ronald T. Borchardt. After two postdoctoral stints, with Jeffrey Aubé at the University of Kansas and Martin Zatz at the NIH, he joined the faculty of the University of Tennessee in Memphis in 1994. In 1999, he became Associate Professor of Neurology at Harvard Medical School, where his present work involves interfacing chemistry and biology toward understanding the molecular basis of Alzheimer's and other neurological diseases. He and his wife, Janet (also a PCPS and KU graduate), live in Newton, Massachusetts, with their three children, Daniel, 10, Julia, 9, and Christian, 8.

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