Inhibition and Modulation of γ-Secretase for Alzheimer's Disease

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Summary: The 4-kDa amyloid β-peptide (Aβ) is strongly implicated the pathogenesis of Alzheimer's disease (AD), and this peptide is cut out of the amyloid β-protein precursor (APP) by the sequential action of β- and γ-secretases. γ-Secretase is a membrane-embedded protease complex that cleaves the transmembrane region of APP to produce Aβ, and this protease is a top target for developing AD therapeutics. A number of inhibitors of the γ-secretase complex have been identified, including peptidomimetics that block the active site, helical peptides that interact with the initial substrate docking site, and other less peptide-like, more drug-like compounds. To date, one γ-secretase inhibitor has advanced into late-phase clinical trials for the treatment of AD, but serious concerns remain. The γ-secretase complex cleaves a number of other substrates, and γ-secretase inhibitors cause *in vivo* toxicities by blocking proteolysis of one essential substrate, the Notch receptor. Thus, compounds that modulate γ-secretase, rather than inhibit it, to selectively alter Aβ production without hindering signal transduction from the Notch receptor would be more ideal. Such modulators have been discovered and advanced, with one compound in late-phase clinical trials, renewing interest in γ-secretase as a therapeutic target. **Key Words:** Alzheimer's disease, amyloid β-protein, amyloid precursor protein, Notch receptor, secretase, γ-secretase.

INTRODUCTION

The two characteristic pathological features of Alzheimer's disease (AD) found in the brain are extracellular amyloid plaques and intraneuronal fibrillary tangles, the former being composed primarily of the 4-kDa amyloid β-peptide (Aβ) and the latter containing paired helical filaments of the microtubule-associated protein tau.[1] Although dominant mutations in the tau gene (MAPT on chromosome 17) can cause other forms of dementia,[2] missense mutations in the Aβ precursor protein gene (*APP*) alter Aβ production and cause familial, early-onset AD.[3] APP is a single-pass membrane protein that is sequentially cleaved in the luminal and extracellular region by β-secretase and within the transmembrane domain by γ-secretase to release Aβ (FIG. 1). Because a portion of Aβ is derived from the transmembrane domain, this peptide is prone to aggregation into neurotoxic assemblies. Heterogeneous proteolysis by γ-secretase leads to the formation of Aβ peptides of varying length at the C-terminus, ranging from 38 to 43 residues, with the longer forms being much more prone to aggregation.[4] Indeed, even though the longer forms are a minority of all Aβ peptides produced, they are the major species in the diffuse plaques that represent the earliest stage of Aβ deposition.[5]

The AD-associated missense mutations in APP are found in three different regions: 1) near the β-secretase cleavage site, leading to elevated Aβ, 2) within the Aβ sequence, changing the biophysical properties of the peptide, or 3) near the γ-secretase cleavage site, increasing the proportion of Aβ that is 42 residues long ($A\beta_{42}$) and much more prone to aggregation than the predominant 40-residue peptide ($A\beta_{40}$). These findings have provided strong support for the amyloid hypothesis of AD pathogenesis.[6] In 1995, missense mutations in the two presenilin genes were discovered to be associated with familial AD as well.[7–9] These genes encode multipass membrane proteins, and the role these mutant presenilins might play in AD was initially unclear. However, it was soon determined that the disease-associated mutations increased the proportion of $A\beta_{42}$ relative to $A\beta_{40}$,[10–13] suggesting that presenilins could modulate the transmembrane proteolysis performed by γ-secretase. Other studies demonstrated that presenilins are endoproteolytically processed into an N-terminal fragment (NTF) and C-terminal fragment (CTF) (FIG. 2), that these fragments remain associated, have a long biological half-life,

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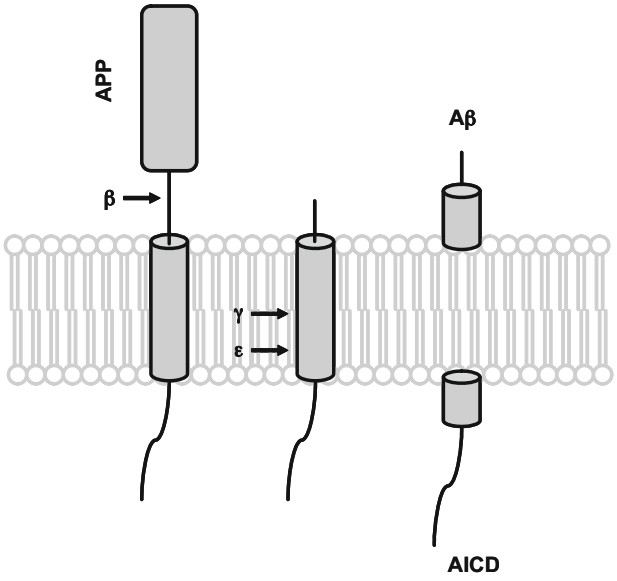


FIG. 1. Processing of amyloid β-protein precursor (APP) by β- and γ-secretases and the formation of amyloid β-protein (Aβ).

and their formation is gated by limiting cellular factors.[14–16] In contrast, full-length presenilin is rapidly degraded. All this pointed to the NTF/CTF heterodimer as the biologically active form of presenilin.

FOUR MEMBRANE PROTEINS COMPRISE THE γ-SECRETASE COMPLEX

Subsequently, two major clues pointed to the biochemical function of presenilin. The first was the finding that knockout of presenilin-1 dramatically reduces Aβ production at the level of γ-secretase.[17] Later studies demonstrated that knockout of both presenilin-1 and -2 completely eliminated γ-secretase activity.[18,19] Thus, presenilins are required for this protease activity. The second important clue was the observation that aspartyl protease transition-state mimics can likewise inhibit γ-secretase activity in cultured cells.[20] These findings suggested that, whatever the identity of γ-secretase, it would likely be an aspartyl protease. Connecting these clues led to the hypothesis that presenilin might be a novel membrane-embedded aspartyl protease, and the discovery that two conserved transmembrane aspartates in the presenilins are indeed critical for γ-secretase activity[21,22] (FIG. 2). Moreover, these two aspartates were also essential for presenilin NTF and CTF formation, suggesting that the protein undergoes autoproteolysis upon interaction with the limiting cellular factors noted above.

Further support for this hypothesis soon followed. First, the aspartyl protease transition-state mimicking inhibitors of γ-secretase were found to directly interact with presenilin-1.[23,24] Conversion of these inhibitors, designed to interact with the active site of γ-secretase, into affinity labeling reagents led to covalent attachment to presenilin NTF and CTF, suggesting that the catalytic site of the enzyme resides at the interface between these two subunits. Consistent with this notion, each subunit possesses one of the two essential aspartates. Second, presenilin consistently came along with γ-secretase ac-

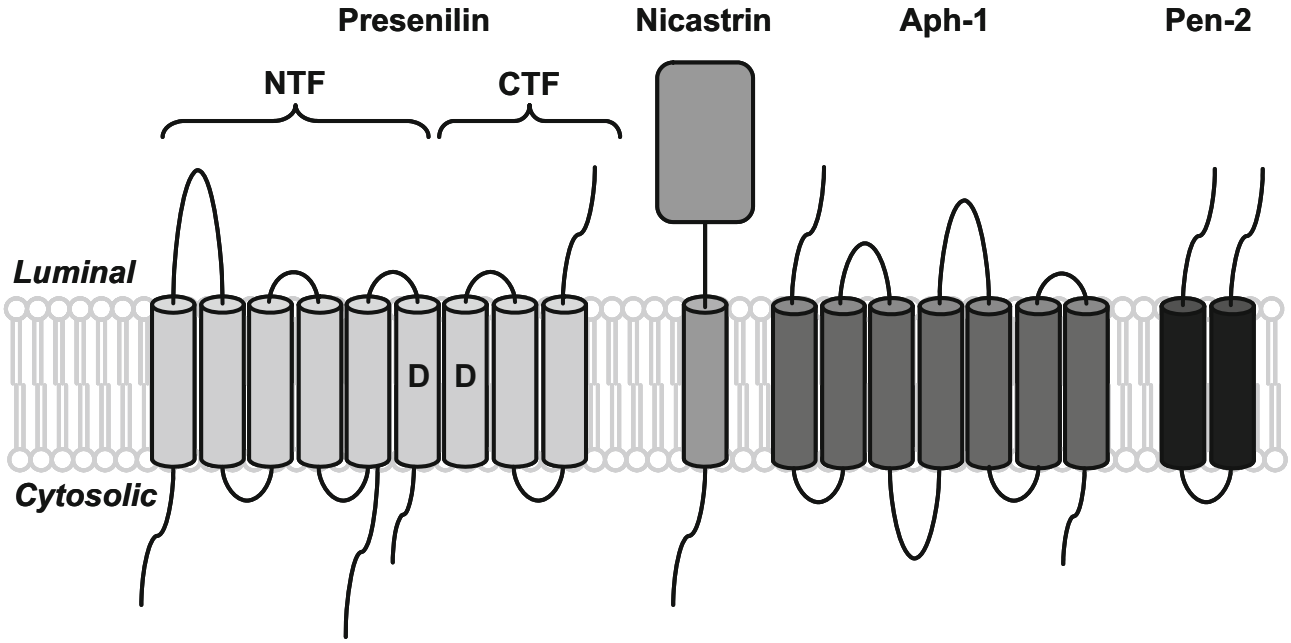


FIG. 2. Components of the γ-secretase complex. γ-Secretase is composed of four different integral membrane proteins: presenilin, nicastrin, Aph-1, and Pen-2. Presenilin undergoes endoproteolysis into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remain associated. Two conserved aspartates within adjacent transmembrane domains are essential for both presenilin endoproteolysis and γ-secretase activity.

tivity through biochemical purification steps as part of a high molecular weight complex.[23,25,26]

Presenilin did not appear to be a protease on its own; rather, it required other protein partners. A combination of genetic screening and biochemical isolation led to the discovery of three such partners, nicastrin, Aph-1, and Pen-2 (FIG. 2), which together also turned out to be the limiting cellular factors.[27–29] Expression of these three proteins along with presenilin results in presenilin endoproteolysis and increased γ-secretase activity.[26,30,31] Removal of any of the components prevents both events. The protease has now been purified by several groups,[32–34] and all five proteins are present (counting presenilin NTF and CTF as separate proteins). Together, these findings demonstrate that presenilin, nicastrin, Aph-1, and Pen-2 are all necessary and sufficient for γ-secretase activity.

Finally, a distantly related presenilin homolog was discovered to be signal peptide peptidase (SPP).[35] Affinity labeling with a transition-state analog inhibitor of SPP led to its identification, and sequence analysis revealed that SPP contains two conserved aspartates embedded within transmembrane motifs with close sequence similarity to the aspartate-containing motifs of presenilin. SPP does not require other protein factors for proteolytic activity; thus, by analogy, the catalytic component of the γ-secretase complex is presenilin.

The biochemical roles of the other protein factors are largely unknown. Nicastrin has recently been demonstrated to be important in substrate recognition, its ectodomain binding to the N-terminus of membrane-bound substrates.[33] This finding is consistent with the sequence similarity between nicastrin and aminopeptidases: critical catalytic residues are absent in nicastrin, allowing it to interact with N-termini but not cleave them itself. Rather, nicastrin aids substrate recognition for eventual proteolysis by presenilin. Aph-1 is thought to be a scaffolding protein:[30] it enters into a subcomplex with nicastrin during assembly.[36–39]

Presumably, presenilin then enters the complex, with addition of Pen-2 providing the trigger for presenilin endoproteolysis and the formation of active γ-secretase. Pen-2 interacts with the NTF region of presenilin,[40] specifically with the fourth transmembrane domain,[41,42] but how Pen-2 induces the presumed conformational change that leads to presenilin NTF and CTF formation is unknown.

MECHANISM OF γ-SECRETASE AND ROLE IN NOTCH SIGNALING

How γ-secretase accomplishes hydrolysis within the hydrophobic environment of the lipid bilayer is an intriguing biochemical question. Because the active site contains water and two aspartates, it is likely sequestered from the hydrophobic lipids.[22] Indeed, the enzyme apparently contains an initial docking site for the transmembrane region of the substrate that is distinct from the active site: endogenous APP substrate can be copurified with the γ-secretase complex using an affinity column with immobilized transition-state analog inhibitor.[25] Because the active site should be occupied by the immobilized inhibitor, the transmembrane domain of the substrate is presumably bound to an exosite on the protease complex.

Helical peptides designed to mimic the APP transmembrane domain are potent inhibitors of γ-secretase,[43] with IC_{50} values as low as 140 pmol/L in biochemical assays,[44] and these peptides interact with a site distinct from that of transition-state analog inhibitors.[45] Affinity labeling with helical peptides identified both presenilin NTF and CTF as contributors to the binding site; the other three components of γ-secretase were not labeled at all.[46] These findings suggest that the docking site, like the active site, is at the interface between the two presenilin subunits and implies that substrate passes, in whole or in part, between these subunits to access the internal active site (FIG. 3).

In parallel with the discoveries connecting presenilin to APP processing and AD were studies revealing a role of presenilin in the Notch signaling pathway of developmental biology.[47] This revelation proved critical for identifying the other members of the protease complex, two of which were discovered via genetic screens using Notch-deficient phenotypes as a readout.[28,29] Notch, like APP, was found to be cleaved within its transmembrane domain, and this proteolysis is necessary for Notch signaling and cell fate determinations.[48] Presenilin is necessary for this transmembrane cleavage,[49] and knockout of presenilin-1 results in a lethal phenotype similar to that seen upon knockout of Notch1.[50,51] These findings began to raise concerns about γ-secretase as a target for AD: inhibition of this protease, although lowering Aβ production, might also cause severe toxicities by blocking critical cell differentiation events. The remainder of this review provides a current assessment of the therapeutic potential of targeting γ-secretase, especially strategies for lowering Aβ without affecting Notch signaling.

THERAPEUTIC POTENTIAL OF γ-SECRETASE INHIBITORS FOR AD

The first reported in vivo testing of a γ-secretase inhibitor involved the dipeptidic compound DAPT, developed by Elan and Eli Lilly[52] (FIG. 4). This compound potently inhibited Aβ production in cells, with an IC50 of 115 nmol/L in human primary neuronal cultures; however, high single oral doses (100 mg/kg) were needed to observe 50% Aβ lowering in the brains of young APP transgenic mice. In a subsequent study,[53] subcutaneous

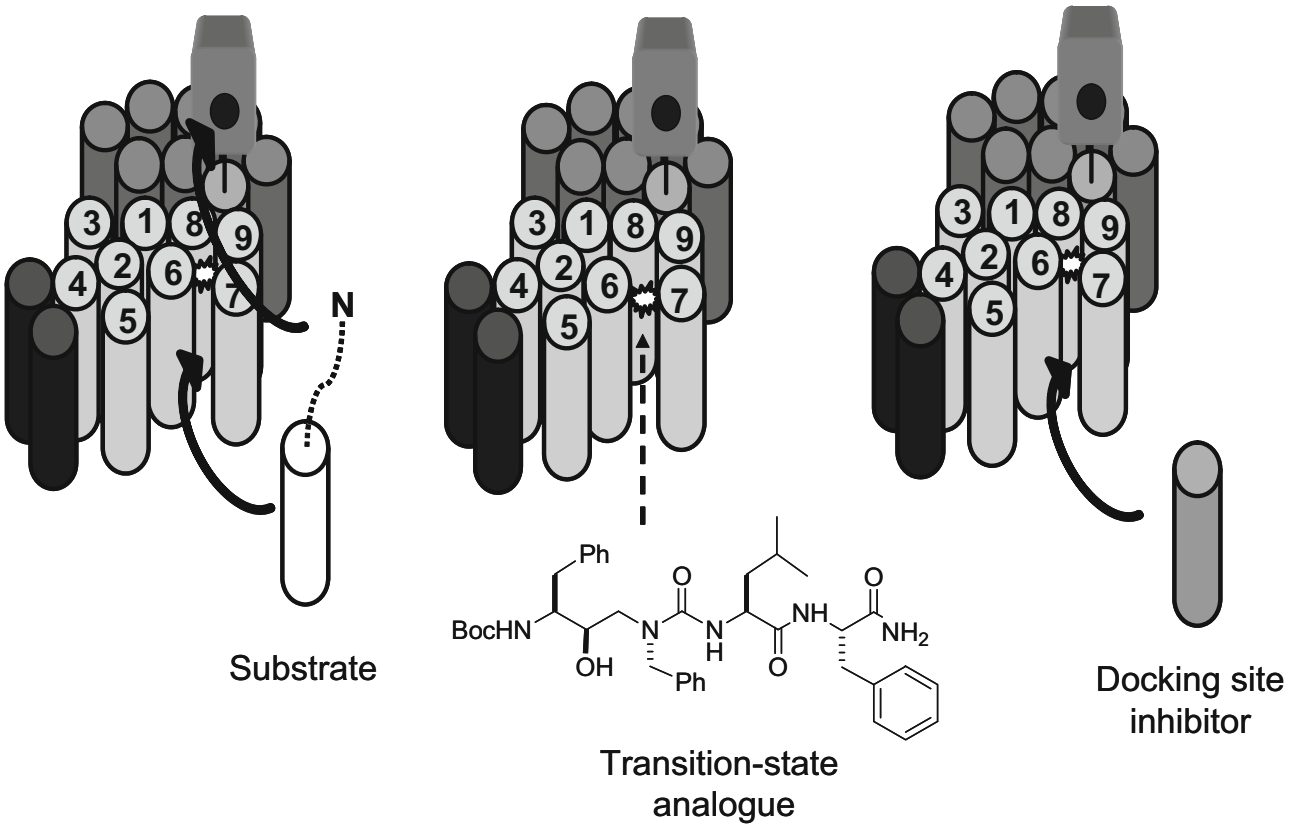


FIG. 3. General mechanism of γ-secretase and interaction with a transition-state analog inhibitor and docking site inhibitor. Substrate interacts with both presenilin and the ectodomain of nicastrin before entry into the internal active site. Transition-state analogs bind the active site directly, and helical peptides that mimic the substrate transmembrane domain bind to the external substrate docking site on presenilin.

doses of 10, 30 and 100 mg/kg resulted in a dose-dependent decrease in plasma and CSF Aβ levels. However, total brain Aβ was reduced only in young mice, with deposition of Aβ seen in the older mice. Similar results were seen after 14 days of administration, but lower drug levels over time and increased liver weight suggested induction of P450 enzymes and breakdown of the compound.

Bristol-Myers Squibb (BMS) and the former SIBIA developed sulfonamide inhibitor BMS-299897,[54,55] a compound with an IC_{50} of 7 nmol/L for inhibiting Aβ production in HEK293 cells stably overexpressing APP (FIG. 4). BMS-299897 is purported to be selective for inhibiting the processing of APP over Notch, although the use of different assays in this study does not allow simple comparisons. Single oral administration of this compound into APP transgenic mice gave an ED_{50} value of 30 mg/kg for lowering brain Aβ and 16 mg/kg for lower plasma Aβ at 3 h after dosing. Intraperitoneal administration in guinea pigs, which naturally produce high levels of Aβ that is identical in sequence to the human peptide, reduced brain, plasma, and CSF Aβ with an ED_{50} of 30 mg/kg at 3 h after dosing.

Benzodiazepine analog LY-411575 and benzolactam LY-450139, developed at Eli Lilly, are highly potent γ-secretase inhibitors that have been tested extensively *in vivo*[56–58] (FIG. 4). LY-411575 is one of the most potent γ-secretase inhibitors yet reported, with an IC_{50} of 119 pmol/L for inhibiting Aβ production in APP-overexpressing HEK293 cells. Upon administration of single doses in rats, this compound gave an ED_{50} of 1.3 mg/kg for reducing CSF and brain Aβ after 4 h. Remarkably, after a 10 mg/kg dose, brain and CSF Aβ levels were completely knocked down and did not recover 24 h after dosing. LY-411575 also lowered brain, CSF, and plasma Aβ in an APP transgenic mouse model, although reduction in brain Aβ levels lagged behind that of CSF and plasma.

The related compound, benzolactam LY-450139, is two orders of magnitude less potent, with an IC_{50} of 15 nmol/L; however, this compound has moved into clinical trials, and so far it is the only γ-secretase inhibitor for which human trials have been reported. LY-450139 was chronically administered for 5 months to young APP transgenic mice, leading to reduced total brain Aβ and slower formation of Aβ plaques. In initial human trials in healthy volunteers,[58,59] single doses of LY-450139 of up to 140 mg were apparently safe and reduced plasma Aβ levels by up to 72.6%. However, steady-state Aβ in the cerebral spinal fluid was not affected, and it is unclear if

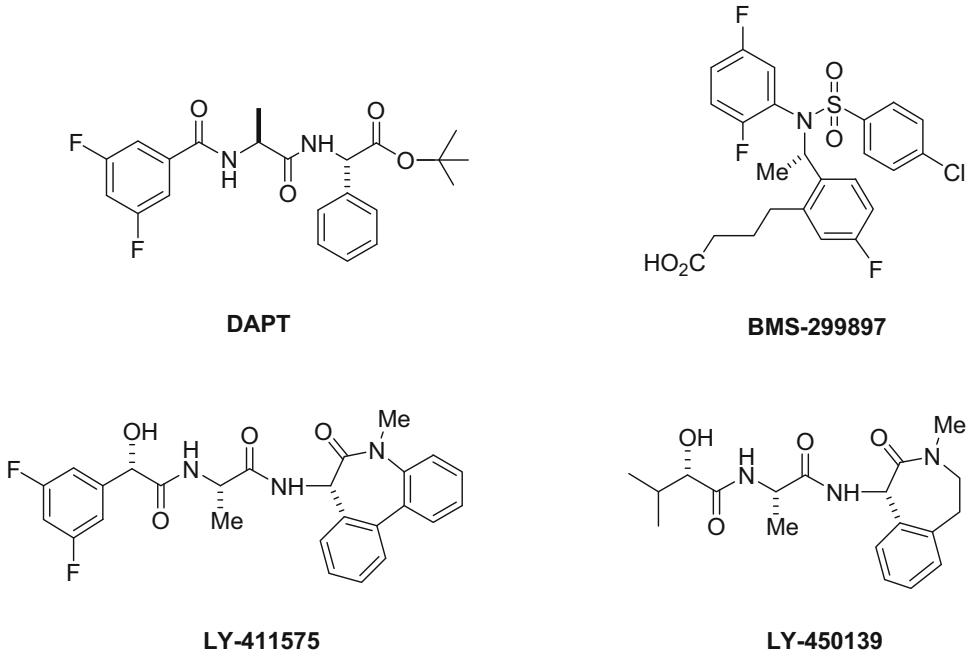


FIG. 4. γ-Secretase inhibitors tested *in vivo*.

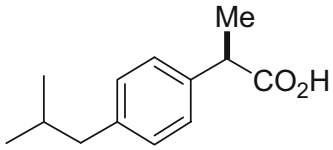
higher doses can lower brain Aβ and without Notch-related side effects.

THERAPEUTIC POTENTIAL OF γ-SECRETASE MODULATORS

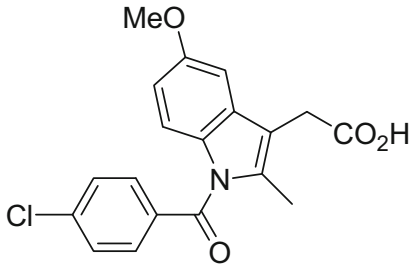
Although γ-secretase has in many ways been an attractive target for AD therapeutics, interference with Notch processing and signaling may lead to toxicities that preclude clinical use of inhibitors of this protease. Knockout of Notch1 or presenilin-1 is embryonic-lethal in mice,[50,51] but Notch signaling and γ-secretase activity are crucial in adulthood as well, because Notch plays a critical role in many cell differentiation events.[47] Indeed, treatment of mice with γ-secretase inhibitor LY-411575 at 10 mg/kg/day for 15 days caused severe gastrointestinal toxicity and, at 10 mg/kg/day, also interfered with the maturation of B- and T-lymphocytes, effects that are indeed due to inhibition of Notch processing and signaling.[60,61] Nevertheless, hope remains that a γ-secretase inhibitor might lower Aβ production in the brain enough to prevent Aβ oligomerization and fibril formation but also leave enough Notch signaling intact to avoid toxic effects. It is this hope that has stimulated the continuing clinical studies of LY450139, even though compounds in this general structural class have not displayed selective inhibition of APP processing with respect to that of Notch.[61]

In contrast, compounds that can modulate the enzyme to alter or block Aβ production with little or no effect on Notch would bypass this potential roadblock to therapeutics. Recent studies suggest that the protease complex contains allosteric binding sites that can alter substrate selectivity and the sites of substrate proteolysis. Certain nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, indomethacin, and sulindac sulfide (FIG. 5), can reduce the production of the highly aggregation-prone $A\beta_{42}$ peptide and increase a 38-residue form of Aβ.[62] This pharmacological property is independent of inhibition of cyclooxygenase (COX): the Aβ modulating effects are seen in COX-1/2 knockout cells and with structurally related compounds that do not inhibit COX. The alteration of the proteolytic cleavage site is observed with isolated or purified γ-secretase,[32,63] indicating that the compounds can interact directly with the protease complex to exert these effects. Enzyme kinetic studies and displacement experiments suggest the selective NSAIDs can be noncompetitive with respective to APP substrate and to a transition-state analog inhibitor, which also suggests interaction with a site distinct from the active site and the docking site.[64]

Although the biochemical mechanism of these NSAID γ-secretase modulators is unclear, the effect on APP proteolysis to lower $A\beta_{42}$ and increase $A\beta_{38}$ has been well documented. The site of cleavage within the Notch transmembrane domain is similarly affected, but this subtle change does not inhibit the release of the intracellular domain and thus does not affect Notch signaling.[65] For this reason, these agents may be safer as Alzheimer therapeutics than are inhibitors that block the active site or the docking site. Indeed, one compound, *R*-flurbiprofen (formerly flurizan, now called tarenflurbil; FIG. 5), has recently advanced to Phase III clinical trials in the



ibuprofen



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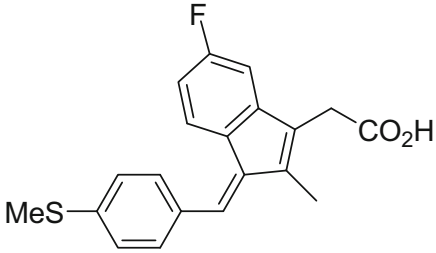
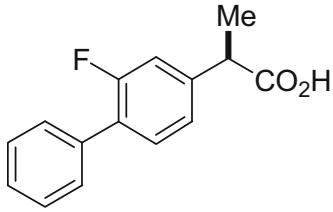


sulindac sulfide



R-flurbiprofen

FIG. 5. Selected nonsteroidal anti-inflammatory drugs (NSAIDs) that modulate Aβ production.

United States. The enantiomer, *S*-flurbiprofen, is a COX inhibitor and the active isomer of this NSAID. *R*-flurbiprofen modulates Aβ production but is not a COX inhibitor, illustrating that these are pharmacologically distinct effects. However, the potency of this drug candidate[66] and other NSAIDs toward Aβ_{42} lowering raises questions about efficacy, and it will likely be important to better understand how these compounds work and develop second- and third-generation agents.

In an APP transgenic mouse model, tarenflurbil was administered at 10 mg/kg/day for 4 months in young mice and was shown to attenuate spatial learning deficits. In older mice, drug administration over 2 weeks reduced Aβ plaque pathology but did not improve spatial learning.[67] In initial human trials,[68] tarenflurbil was administered at doses up to 1600 mg per day for 3 weeks. The drug was well tolerated and apparently lowered plasma Aβ_{42} levels at peak plasma drug concentrations. Although the drug could penetrate into the CNS, its ability to lower Aβ in the brain is not known. Recently, a cyclopropyl analog of tarenflurbil has been shown to effectively reduce Aβ pathology in APP transgenic mice over the course of 17 weeks with no sign of Notch-mediated toxicities.[69] In addition, a nitric oxide-releasing version of tarenflurbil was found to stimulate clearance of amyloid deposits through microglial activitation.[70]

Another type of allosteric modulator is compounds that resemble kinase inhibitors and interacts with a nucleotide binding site on the γ-secretase complex. The discovery that ATP can increase Aβ production in membrane preparations prompted the testing of a variety of compounds known to interact with ATP binding sites on other proteins.[71] In this focused screen, the Abl kinase inhibitor imatinib (Gleevec) emerged as a selective inhibitor of Aβ production in cells without affecting the proteolysis of Notch. In light of these findings, ATP and other nucleotides were tested for effects on purified γ-secretase preparations and found to selectively increase the proteolytic processing of a purified recombinant APP-based substrate without affecting the proteolysis of a Notch counterpart.[72] Furthermore, certain compounds known to interact with ATP binding sites were found to selectively inhibit APP processing vis-à-vis Notch in purified protease preparations. This and other evidence suggest that the γ-secretase complex contains a nucleotide binding site, and that this site allows allosteric regulation of γ-secretase processing of APP with respect to Notch. Whether this regulation is physiologically important is unclear, but the pharmacological relevance is profound and may lead to new therapeutic candidates for AD.

This hope is tempered by the fact that γ-secretase cleaves numerous other type I membrane protein stubs that result from ectodomain shedding.[73] Agents selective for APP *versus* Notch may reveal new long-term toxicities due to blocking proteolysis of these other substrates, toxicities masked by the severe Notch-related effects with nonselective inhibitors. To address this important

issue, the development of more potent analogs that work by this mechanism will be critical.

CONCLUSIONS

Our knowledge of γ-secretase and its role in AD and in biology has increased dramatically in the past 10 years. This enzyme is a complex of four different integral membrane proteins with a membrane-embedded active site. At present, a detailed structure of this complex is not even on the close horizon, and almost nothing is known about the shape and character of any of the drug binding sites. Also a hindrance to the development of clinically useful γ-secretase inhibitors is the critical importance of this protease in the Notch signaling pathway. It will likely be necessary to avoid interfering with Notch proteolysis by γ-secretase. Despite such hurdles, γ-secretase continues to be pursued as a top therapeutic target for AD and, in many respects, has advantages over the more classical aspartyl protease β-secretase. Indeed, γ-secretase inhibitors and modulators are well into human trials, but β-secretase inhibitors are only now moving beyond preclinical testing. Answers should soon be forthcoming about the safety and efficacy of the first-generation compounds, but whatever the result, it will be critically important to continue development of more potent and selective agents with better pharmaceutical properties.

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