



Published in final edited form as:

Curr Alzheimer Res. 2008 April ; 5(2): 158–164.

γ -Secretase Inhibition and Modulation for Alzheimer's Disease

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The β - and γ -secretases responsible for the formation of the amyloid β -peptide ($A\beta$) have been top targets for the development of Alzheimer therapeutics even before the identification of these proteases. Indeed, secretase inhibitors have been sought ever since the discovery of AD-causing missense mutations in APP [1-3] and the realization that these mutations alter $A\beta$ production [4-6]. Cell-based screens netted a number of compounds that blocked $A\beta$ formation, and virtually all of these initial leads appeared to work at the level of γ -secretase [7-11]. The identification of β - and γ -secretases ultimately revealed why this was the case. β -Secretase is a membrane-tethered pepsin-like aspartyl protease [12-15] that cleaves APP on the luminal/extracellular side at the juxtamembrane region. The enzyme has a long and shallow active site [16], and molecules that inhibit β -secretase are typically not very cell permeable. In contrast, γ -secretase cleaves within the transmembrane region of APP and contains a membrane-embedded active site [17]. Thus, compounds that inhibit γ -secretase are typically quite hydrophobic and readily traverse the cell membrane. For these reasons, γ -secretase inhibitors have advanced further along the drug development pipeline than β -secretase inhibitors, which are only just emerging from the preclinical stage.

γ -Secretase is nevertheless a difficult drug target in some other respects. In stark contrast to β -secretase, γ -secretase is a large protein complex composed of four different membrane proteins [18-20], and knowledge of structural details that would allow rational drug design is not available. Moreover, γ -secretase processes a number of different type I integral membrane proteins, so many that this protease has been dubbed “the proteasome of the membrane” [21]. Many of these processing events are part of critical pathways in cell biology that may have toxic consequences if blocked. Indeed, this is already well known to be the case with one particular γ -secretase substrate, the Notch receptor. Thus, modulation rather than inhibition of γ -secretase is preferred, to alter $A\beta$ production in a therapeutically relevant way without interfering with essential cellular processes. This minireview provides an overview of the various types of γ -secretase inhibitors and modulators and what is known about how they interact with the protease complex, their mechanisms of action, and their potential as disease-modifying therapies for AD.

Transition-State Analogues

Among the first types of specific γ -secretase inhibitors were peptide analogues containing classical transition-state mimicking moieties for aspartyl proteases. In aspartyl protease catalysis, two aspartates activate water for direct attack on the amide bond fated for cleavage, forming an unstable gem-diol intermediate (in which the carbonyl carbon becomes transiently attached to two hydroxyl groups upon attack by water). Transition-state analogue inhibitors of aspartyl proteases are chemically stable and contain one or two hydroxyl groups attached to a carbon, and this moiety replaces an amide bond in the peptide analogue. The net result is a

compound that interacts well with the two aspartates in the active site but is not susceptible to cleavage by the protease.

The first reported inhibitors of γ -secretase were peptide aldehydes originally developed for the proteasome [7]. These aldehydes are readily hydrated to form a stable gem-diol structure similar to that formed during aspartyl protease catalysis. Subsequently, difluoro ketone peptidomimetic inhibitors (e.g., DFK-167; Fig. 1) were developed that were specifically designed from the sequence of the APP transmembrane domain containing the γ -secretase site [8], and these difluoro ketones likewise become hydrated to form a chemically stable gem-diol. Unlike the peptide aldehydes, the difluoro ketone peptidomimetics contained residues corresponding to the P' side as well as on the P side (that is, on the C-terminal side relative to the cleavage site as well as on the N-terminal side). However, both types of compounds (aldehydes and ketones) can also react with serine or cysteine proteases, in which the nucleophilic serine or cysteine residue in the active site attacks the electrophilic carbonyl of the aldehyde or ketone functionality. Swapping the ketone with an alcohol moiety, a group unable to react with serine or cysteine proteases, retained inhibitory activity toward γ -secretase, evidence that γ -secretase might be an aspartyl protease [22]. Confirmatory results were soon reported with other alcohol-containing aspartyl protease peptidomimetics [9] (e.g., L-685,458; Fig.1).

These findings, along with the discovery that presenilins are essential for γ -secretase activity [23-25], prompted the identification of two conserved transmembrane aspartates in presenilin that are essential for activity and the initial suggestion that presenilin is a novel membrane-embedded aspartyl protease [26]. Indeed, the conversion of the alcohol-containing transition-state analogue inhibitors of γ -secretase to affinity labeling reagents led to covalent and specific binding to the two presenilin subunits (the N-terminal and C-terminal fragments, NTFs and CTFs, that form upon endoproteolysis of the presenilin holoprotein), strong evidence that the active site of γ -secretase resides at the subunit interface [27,28]. Indeed, each subunit contains one of the two conserved aspartates, suggesting that a catalytic pair of aspartates is formed at the interface between the two presenilin subunits.

The transition-state analogue inhibitors also proved useful for isolating the γ -secretase complex. Although presenilin appeared to be the catalytic component, it was clear that there were other unidentified components. First, presenilin did not possess proteolytic activity on its own. Second, the protein assembled into high molecular weight complexes [29,30]. Third, the endoproteolysis of presenilin into a stable heterodimeric form is tightly regulated by limiting cellular factors [31-33]. Thus, attempts were made to purify γ -secretase in the hope of identifying the missing members of what is now called the γ -secretase complex. Immobilization of a transition-state analogue inhibitor provided a means of affinity isolating the enzyme [34], and this purification step was one means by which the membrane proteins nicastrin, Aph-1 and Pen-2 were confirmed as *bona fide* γ -secretase complex members.

Docking Site Inhibitors

The isolation of γ -secretase using the immobilized transition-state analogue also helped understand how the protease interacts with substrate, because an endogenous APP substrate copurified with the enzyme [34]. Because the active site should be occupied by the immobilized transition-state analogue, the copurification of the substrate suggested the existence of a substrate binding site that is distinct from the active site. This other substrate binding site was dubbed the docking site, because this was envisioned to be where substrate initially interacts before entry into an internal, water-containing active site that is sequestered from the hydrophobic environment of the lipid bilayer.

Substrate-based peptides were designed to interact with this docking site. Peptides based on the transmembrane domain of APP and constrained in a helical conformation (by incorporation of the helix-inducing aminoisobutyric acid, or Aib; e.g., DSI-1, Fig. 1) can potently inhibit γ -secretase, apparently by interacting with this docking site [35]. Conversion of these helical peptide inhibitors into affinity labeling reagents led to the localization of the substrate docking site to the presenilin NTF/CTF interface [36]. As mentioned above, transition-state analogue inhibitors also bind directly to the NTF/CTF interface, but these bind at a site distinct from that of helical peptide inhibitors. These findings suggest a pathway for γ -secretase substrate from docking site to active site: upon binding to the outer surface of presenilin at the NTF/CTF interface, the substrate can pass, either in whole or in part, between these two presenilin subunits to access the internal active site. Interestingly, extension of a ten-residue helical peptide inhibitor by just three additional residues resulted in a potent inhibitor [37] apparently capable of binding both docking site and active site [36], suggesting that these two substrate binding sites are relatively close.

In Vivo Active Agents and Clinical Candidates

In addition to the transition-state analogues and helical substrate analogues, a number of other potent inhibitors of γ -secretase have been identified. Among the earliest was a dipeptide analogue called DAPT (Fig. 2), and this compound was the first reported *in vivo* active agent, inhibiting A β production in plasma and brain in an APP transgenic mouse model [11,38]. C-terminal modification of DAPT with benzodiazepine-like moieties led to the development of the highly potent inhibitor compound E [10] and related analogues with better *in vivo* properties, including LY-411,575 [39] and the clinical candidate LY-450,139 [40,41] (Fig. 2). In addition, certain sulfonamides displayed potent inhibition (BMS-299897, MRK-560; Fig. 2) [42,43] and had been considered as clinical candidates. All of these compounds (DAPT, benzodiazepines, sulfonamides) are apparently noncompetitive inhibitors of γ -secretase [44] and interact at a site distinct from docking and active site [36,45]. Nevertheless, the conversion of some of these inhibitors into affinity labeling reagents revealed that presenilin is the direct target [10,46,47]. To date, no inhibitors have been reported to interact directly with any of the other members of the γ -secretase complex. In principle though, it should be possible to target nicastrin, as the ectodomain of this type I integral membrane protein apparently plays a direct role in substrate recognition, binding to the luminal/extracellular N-terminus of substrates [48].

Reports of the effects of benzodiazepine-type compound LY-411-575 in mice show their ability to acutely and chronically lower A β in plasma and in brain. However, elevated doses result in severe gastrointestinal toxicity and interfere with the maturation of B- and T-lymphocytes in mice, effects that are due to inhibition of processing of another γ -secretase substrate, the Notch receptor [49,50]. These mechanism-based toxicities are the most serious stumbling block to the development of γ -secretase inhibitors as AD therapeutics. Clinical studies on only one γ -secretase inhibitor, LY-4150,139, have been reported in the literature [40,41]. These reports describe reduction of A β in plasma, but little or no effect in the CSF, suggesting that the doses might be too low. Increasing the dose must be done cautiously, however, with special attention toward gastrointestinal effects, such as severe cramps and diarrhea, and immunosuppressive effects.

Toward γ -Secretase Modulators

Although γ -secretase has in many ways been an attractive target for Alzheimer therapeutics, interference with Notch processing and signaling may lead to toxicities that preclude clinical use of inhibitors of this protease. However, compounds that can modulate the enzyme to alter or block A β production with little or no effect on Notch would bypass this potential roadblock

to therapeutics. Among the first reported modulators were isocoumarin JLK compounds (e.g., JLK2, Fig. 3), which blocked A β production at the γ -secretase level but did not affect Notch processing [51]. However, the direct target of these compounds remains unclear, as they do not affect γ -secretase activity in biochemical assays [52]. Nevertheless, other studies suggest that the protease complex contains allosteric binding sites that can alter substrate selectivity and the sites of substrate proteolysis. Certain non-steroidal anti-inflammatory drugs (NSAIDs; e.g., ibuprofen, indomethacin, and sulindac sulfide; Fig. 3) can reduce the production of the highly aggregation-prone A β 42 peptide and increase a 38-residue form of A β , a pharmacological property independent of inhibition of cyclooxygenase [53]. The alteration of the proteolytic cleavage site is observed with isolated or purified γ -secretase [54,55], suggesting that the compounds may interact directly with the protease complex to exert these effects. Enzyme kinetic studies and displacement experiments show that the selective NSAIDs can be noncompetitive with respect to APP substrate and to a transition-state analogue inhibitor, suggesting interaction with a site distinct from the active site [56]. 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 [57]. For this reason, these agents may be safer as Alzheimer therapeutics than inhibitors that block the active site or the docking site. Indeed, one compound, R-flurbiprofen (Fig. 3), has advanced to Phase III clinical trials.

Another type of modulator are compounds that resemble kinase inhibitors and interact with a nucleotide binding site on the γ -secretase complex. The discovery that ATP can increase A β production in membrane preparations prompted the testing of a variety of compounds that interact with ATP binding sites on other proteins [58]. In this focused screen, the Abl kinase inhibitor Gleevec (Fig. 3) emerged as a selective inhibitor of A β 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 [59]. 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 (e.g., naphthylketones 1367 and 1366; Fig. 3). The γ -secretase complex could be pulled down with beads containing immobilized ATP, and the presenilin-1 CTF was specifically photolabeled by 8-azido-ATP. This labeling was not blocked by a transition-state analogue inhibitor or by the recombinant APP- and Notch-based substrates; however, the APP-selective inhibitors could prevent photolabeling by 8-azido-ATP. Taken together, these results suggest that the γ -secretase complex contains a nucleotide binding site, to which the presenilin-1 CTF is at least a contributor, and that this site may allow allosteric regulation of γ -secretase processing of APP with respect to Notch. Whether such regulation is physiologically important is unclear, but the pharmacological relevance is profound and may lead to new therapeutic candidates for Alzheimer's disease. This hope is tempered by the fact that γ -secretase cleaves numerous other type I membrane protein stubs that result from ectodomain shedding. 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.

Structural Model for γ -Secretase

The design of more potent and selective modulators would be greatly facilitated by a detailed structure of the γ -secretase complex, ideally interacting with a modulator at a specific site. Elucidation of structure is quite difficult and represents the next frontier in understanding this biologically important and therapeutically relevant protease complex. A schematic structural model for the γ -secretase complex and how it interacts with substrate is offered in Figure 4. This model, while partially hypothetical, is consistent with the following observations. First,

dissociation into partial complexes using the detergent DDM revealed that Aph-1 interacts with nicastrin, Pen-2 interacts with the PS1 NTF, and the PS1 CTF associated separately with NTF and with Aph-1/nicastrin [60]. Second, nicastrin and Aph-1 as a subcomplex form an initial subcomplex during intracellular assembly of the protease [61], and is consistent with the nicastrin/Aph-1 partial complex observed upon detergent dissociation. Third, the finding that Pen-2 interacts with TMD4 of presenilin [62,63] confirmed the PS1 NTF/Pen-2 interaction seen upon detergent dissociation and identified a specific region of presenilin contributing to the interface. Fourth, evidence suggests that the C-terminus of presenilin interacts with the nicastrin TMD [64]. Fifth, cysteine cross-linking studies indicate that presenilin TMD1 and TMD8 are proximal [65]. Sixth, both the docking site and the active site lies at the interface between the presenilin NTF and CTF [26,28,66,67]. Seventh, studies with helical peptide inhibitors suggest that the docking and active sites are relatively close, within the length of three amino acid residues [67]. Eighth, the extracellular domain of nicastrin also plays a role in substrate recognition, binding to the N-terminus of the substrate [48]. This model for γ -secretase structure and function will undoubtedly prove to be imperfect but should provide a useful framework to ask specific questions and design appropriate experiments going forward. The ultimate goal, a high resolution structure, remains quite challenging, but already electron microscopy has provided the first glimpses of the protease complex, albeit at 10-15 Å resolution. Despite the low resolution, certain features can be visualized, including an interior chamber, which is likely where the active site resides, and two ports into this chamber, which may allow access for catalytic water.

Perspective

Our knowledge of γ -secretase and its role in AD and in biology has increased dramatically in the past ten years. This enzyme is a complex of four different integral membrane proteins with a membrane-embedded active site. At present, the possibility of a detailed structure of this complex seems like a distance dream, and little 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 these 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, while no β -secretase inhibitors have only just begun to pass through 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. Despite the difficulties, working toward a better structural and mechanistic understanding of the protease complex should ultimately aid drug discovery, allowing structure-based design of γ -secretase modulators.

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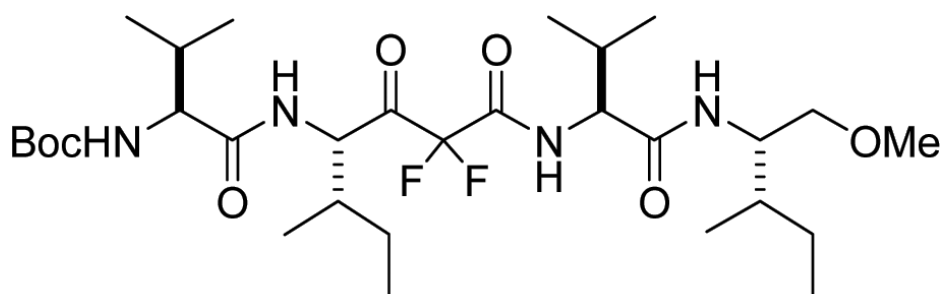
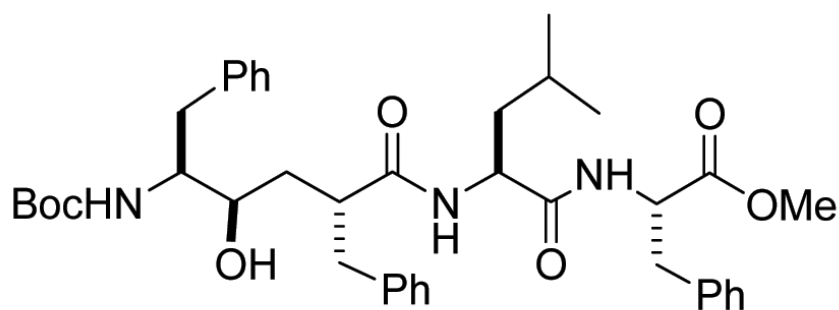
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**DFK-167****L-685,458**

Val-Gly-Aib-Val-Val-Ile-Aib-Thr*-Val-Aib-OMe

DSI-5

Figure 1. Chemical structures of difluoro ketone transition-state analogue γ -secretase inhibitor DFK-167, hydroxyl-containing transition-state analogue L-685,458, and aminoisobutyric acid (Aib)-containing helical peptide DSI-1.

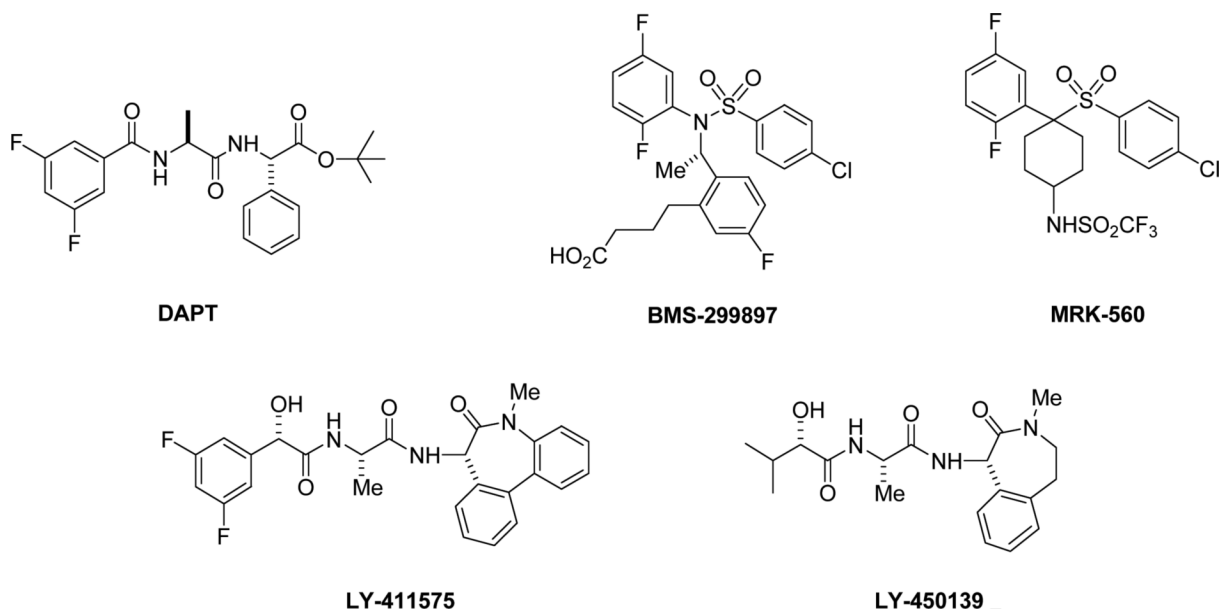


Figure 2.
Chemical structures of in vivo active γ -secretase inhibitors.

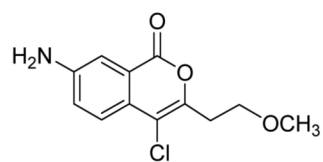
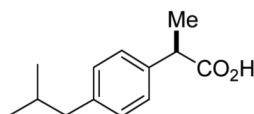
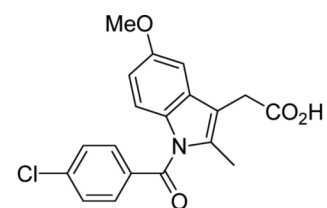
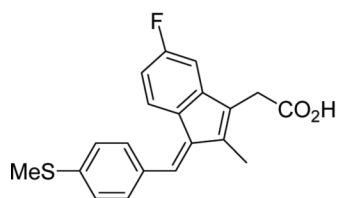
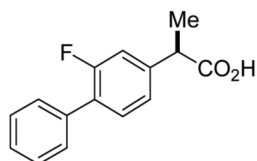
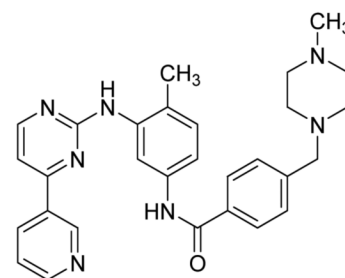
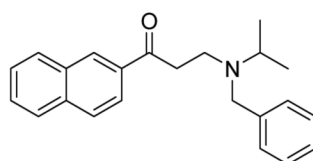
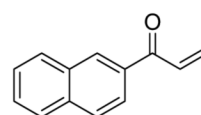
**JLK 2****ibuprofen****indomethacin****sulindac sulfide****R-flurbiprofen****Gleevec****1367****1366**

Figure 3.
Chemical structures of γ -secretase modulators.

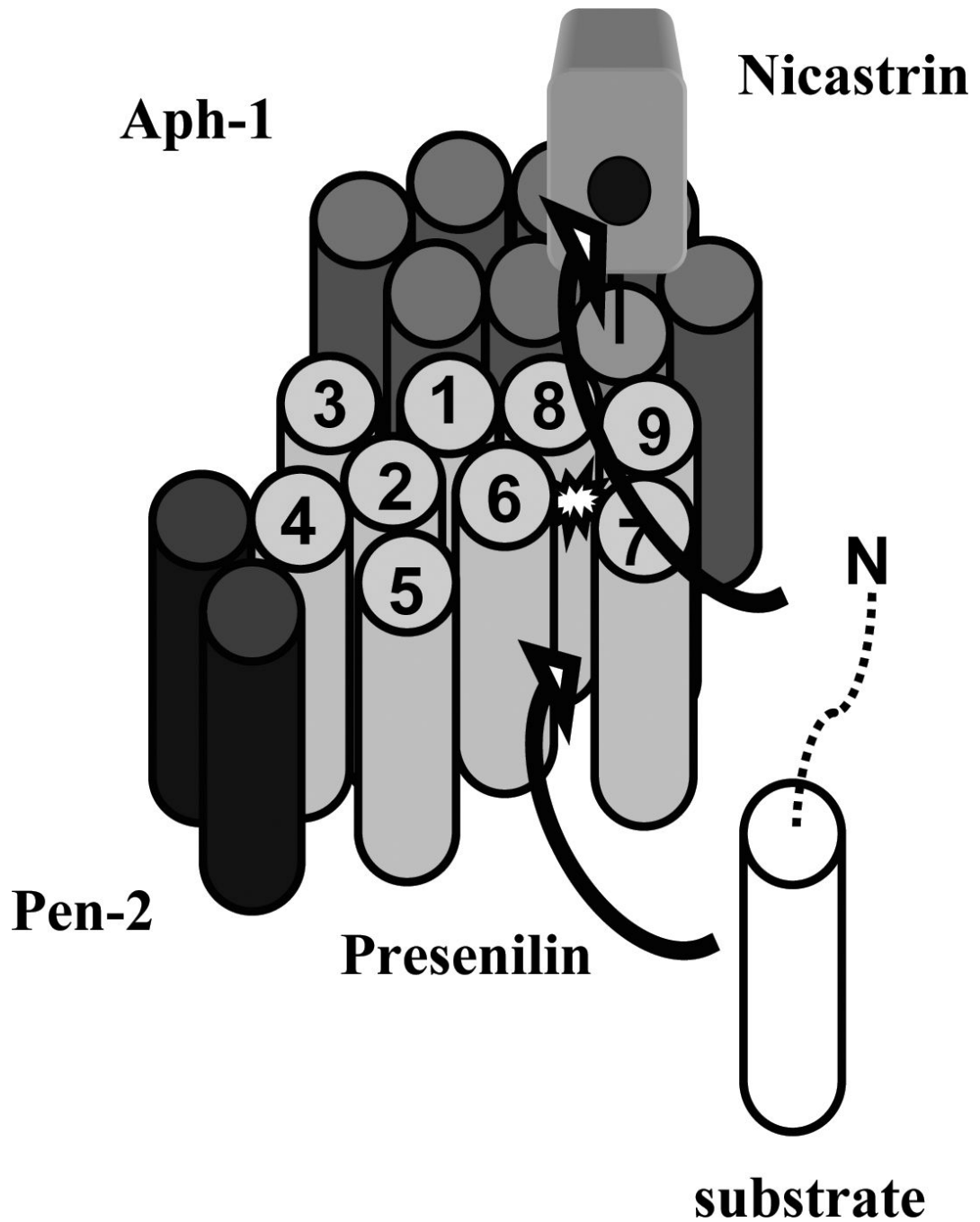


Figure 4.

Model for the γ -secretase complex and its interaction with substrate. The transmembrane region of the substrate initially docks at the presenilin NTF/CTF interface, while the N-terminus of the substrate interacts with the nicastrin ectodomain. The substrate, either in whole or in part, then accesses the internal active site, which contains water and two aspartates. The γ -secretase complex is drawn to take into account molecular and biochemical evidence as described in the text.