Structural and Chemical Biology of Presenilin Complexes

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The presenilin proteins are the catalytic subunits of a tetrameric complex containing presenilin 1 or 2, anterior pharynx defective 1 (APH1), nicastrin, and PEN-2. Other components such as TMP21 may exist in a subset of specialized complexes. The presenilin complex is the founding member of a unique class of aspartyl proteases that catalyze the γ , ϵ , ζ site cleavage of the transmembrane domains of Type I membrane proteins including amyloid precursor protein (APP) and Notch. Here, we detail the structural and chemical biology of this unusual enzyme. Taken together, these studies suggest that the complex exists in several conformations, and subtle long-range (allosteric) shifts in the conformation of the complex underpin substrate access to the catalytic site and the mechanism of action for allosteric inhibitors and modulators. Understanding the mechanics of these shifts will facilitate the design of γ -secretase modulator (GSM) compounds that modulate the relative efficiency of γ , ϵ , ζ site cleavage and/or substrate specificity.

The presenilin (PS) genes were first identified during searches for genes responsible for early onset, autosomal dominant forms of familial Alzheimer's disease (FAD) (Rogaev et al. 1995; Sherrington et al. 1995). The genes are highly conserved in evolution, but in vertebrates, there are two highly similar homologous presenilin genes: *PSEN1* (on chromosome 14 in humans, encoding PS1) and *PSEN2* (on chromosome 1, encoding PS2). Both PS1 and PS2 are ~50-kDa polytopic transmembrane (TM)

proteins that interact with nicastrin, PEN-2, and anterior pharynx defective 1 (APH1) to form a 1:1:1:1 heterotetrameric complex (Fig. 1) (Li et al. 2014). Presenilin complexes lacking any one of their subunits are destabilized and degraded (Takasugi et al. 2003). However, a subset of complexes may contain additional TM proteins such as TMP21, which may have a regulatory function (Chen et al. 2006). The biologically active complex performs regulated cleavage through the transmembrane domains

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Figure 1. A diagrammatic representation of the presenilin-complex subunit component proteins including presenilin, PEN-2, anterior pharynx defective 1 (APH1), and nicastrin. The orientations of the membrane proteins relative to the cytoplasmic and luminal faces of the membrane are depicted. Presenilin has nine predicted transmembrane (TM) domains, PEN-2 has two partial TM domains and a final fully spanning TM domain, APH1 has seven, and nicastrin has one TM domain.

of selected Type I TM proteins (Fig. 2) (De Strooper et al. 1999; Nishimura et al. 1999; Wolfe et al. 1999b; Francis et al. 2002; Yu et al. 2000; Chen et al. 2006).

TOPOLOGY AND STRUCTURE OF PRESENILIN-COMPLEX COMPONENTS

Presenilin

Presenilin PS1 (and PS2) are the catalytic subunits of the heterotetrameric presenilin 1 (or presenilin 2) complexes (Wolfe et al. 1999b; Li et al. 2000a; Chen et al. 2006; Ahn et al. 2010) and are the founding members of the GXGD family of intramembranous aspartyl proteases. Other members of this protease family include signal peptide peptidases (SPP) and a variety of archaeal homologs (Weihofen et al. 2002; Martoglio and Golde 2003; Friedmann et al. 2004; Fluhner et al. 2009; Lu et al. 2012a). These proteins will not be discussed in detail here except to note that they are monomeric or dimeric, and they have an opposite topology to the membrane relative to the presenilin proteins.

The presenilin proteins have nine helical TM domains arranged with the hydrophilic, flexible N terminus in the cytosol and the C terminus protruding into the lumen or extracellular space (Doan et al. 1996; Laudon et al. 2005; Wolfe 2013). TM6 and TM7 each contain one of two catalytic aspartyl residues (Wolfe et al. 1999b). During the assembly and maturation of presenilin complexes, the PS1 or PS2 subunits undergo endoproteolytic cleavage

into N- and C-terminal fragments, which then remain stably associated with each other (Thinakaran et al. 1996; Steiner et al. 2008; De Strooper and Annaert 2010). For PS1, the cleavage occurs near residue Met298 (encoded by exon 9) within the cytoplasmic peptide loop connecting TM6 and TM7 (Podlisny et al. 1997). Missense mutations in the presenilins alter the relative abundance of amyloid- β ($A\beta$) peptide species produced during presenilin-complex-mediated cleavage of APP-C100 following BACE cleavage of APP (Scheuner et al. 1996; Citron et al. 1997; Chávez-Gutiérrez et al. 2012).

PS1 and PS2 have highly similar amino acid sequences and topologies. One crucial difference appears to be a “[DIE]xxxL/I/M” motif in PS2 that interacts in a phosphorylation-dependent manner with AP-1 complexes and targets PS2 to the late endosome/lysosome complex. The absence of this sequence motif in PS1 allows for the wider subcellular location of PS1 in cell membranes (Sannerud et al. 2016).

During the two decades since the presenilin genes were first cloned, several attempts have been made to deduce the three-dimensional structure of the presenilin proteins using multiple methods including cross-linking (Watanabe et al. 2010) and solution nuclear magnetic resonance (NMR) of subfragments (PDB code 2KR6) (Sobhanifar et al. 2010). Additionally, a crystal structure has been generated for a distant and highly atypical presenilin homolog isolated from the archaeon *Methanoculleus marisnigri* JR1 (MCMJR1 or MmPSH) (PDB code 4HYG) (Li et al. 2013). MmPSH has a PS-like topology

Figure 2. A diagrammatic representation of the intramembranous cleavage sites within the transmembrane (TM) domain of substrate proteins is shown using the APP-C100 substrate released from full-length APP by prior β -secretase cleavage as the canonical substrate. The predominant and minor series of cleavages are depicted. The N-terminal fragment forms the amyloid- β (A β) peptide associated with neurotoxicity in Alzheimer's disease (AD). The C-terminal fragment is a putative signaling molecule that can be translocated to the nucleus. The two smaller internal fragments are rapidly degraded.

but has only weak presenilin-like γ -secretase activity and, paradoxically, significant signal-peptide peptidase activity (R Dodd, S Qamar, and C Bohm, unpubl.). The crystal structure of MmPSH must be interpreted with caution in light of three caveats. First, it was generated from a modified, catalytically inactive construct. Second, the two catalytic aspartate residues are not in close enough proximity for catalysis to occur, suggesting that the TM domains have moved during crystallization. Third, there are several other features to this structure that likely represent crystallization artifacts. For instance, the TM6 helices are displaced and insert among the TM helices of other nearby tetramers within the crystal arrays—an occurrence that is unlikely to be physiological.

Nicastrin

Nicastrin is a Type I integral membrane protein that consists of a large N-terminal extracellular glycosylated ectodomain that contains a con-

served DYIGS motif, a transmembrane helical domain, and a very short cytoplasmic C terminus (Yu et al. 2000). Nicastrin binds to A β 1 and forms an initial scaffolding molecule that may regulate intracellular trafficking of the nascent presenilin complex during its assembly (Shirotani et al. 2003; Zhang et al. 2005; Spasic et al. 2007). Within the assembled presenilin complex, nicastrin binds to the C terminus of PS1 (Bergman et al. 2004).

In addition to the role of nicastrin in regulating presenilin-complex assembly, the ectodomain of nicastrin, which contains a conserved DYIGS motif, may be involved in substrate selection and acquisition by detecting the lengths of extracellular N-terminal protrusions of substrate proteins and by excluding Type I membrane proteins that have not undergone a prior α - or β -secretase-like sheddase cleavage to release most of the N-terminal domain (Shah et al. 2005). However, nicastrin is not essential for γ -secretase activity (Ahn et al. 2010; Zhao et al. 2010). The ectodomain of nicastrin has

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a predicted aminopeptidase/transferrin receptor-like structure similar to that of the human transferrin receptor (PDB code 1CX8) and the glutamate carboxyl peptidase PSMA (PDB code 2XEF) (Fagan et al. 2001). This structural homology, which supports a role in substrate acquisition but not cleavage, was recently confirmed in a cryo-electron microscopy (cryo-EM) study (Lu et al. 2014) (PDB code 4UPC) and by crystallography of a distant *Dic-tyostelium* homolog (PDB code 4R12) (Xie et al. 2014).

PEN-2 and APH1

Relatively less is known about the structural biology and function of PEN-2 and APH1. Both were identified during genetic screens for enhancers and suppressors of Notch signaling (Francis et al. 2002).

PEN-2 is a 101-residue (12-kDa) membrane protein with three predicted transmembrane domains—the first two of which are re-entrant and only partially cross the bilayer and a third that is a complete TM helix (Lu et al. 2014; Bai et al. 2015a,b; Zhang et al. 2015). The N terminus is cytosolic, whereas the C terminus is luminal. PEN-2 binds to TM4 of PS1 (Kim and Sisodia 2005; Watanabe et al. 2005) and may stabilize the presenilin complex during its final assembly and maturation (Prokop et al. 2004; Holmes et al. 2014).

APH1 (Francis et al. 2002) is encoded in humans by two paralogous genes (APH1A on chromosome 1 and APH1B on chromosome 15), which produce two highly similar gene products of about 195–265 residues. A further duplication of the APH1B gene in mice gave rise to a third APH1 family gene, APH1C. Because any given presenilin complex has only a single APH1 protein, the existence of APH1A, APH1B, and APH1C allows two different types of each PS1 or PS2 complex in humans and three different types in mice. These different isoforms may have subtly different activities (Serneels et al. 2005, 2009). APH1 has a seven-TM topology with its N terminus in the lumen and its C terminus in the cytoplasm (Fortna et al. 2004). All human and mouse APH1 paral-

ogs contain a conserved GXXXG motif that may be involved in interactions with other subunits of the presenilin complex (Lee et al. 2004). As noted above, the APH1:nicastrin complex likely forms an initial scaffold onto which PS1/PS2 and PEN-2 are subsequently added (Lee et al. 2002; Yang et al. 2002; Gu et al. 2003, 2004; Spasic et al. 2007; Pardossi-Piquard et al. 2009; Mao et al. 2012), with the PS1 or PS2 C terminus abutting APH1 (Bergman et al. 2004).

Structure of the Presenilin Complex as a Whole Entity

Recently, despite the small size and lack of symmetry, great strides have been made in understanding the three-dimensional structure of the presenilin complexes through application of EM and electron density mapping methods. The initial EM reconstruction studies before 2014 generated a variety of three-dimensional models differing in shape and volume (Lazarov et al. 2006; Ogura et al. 2006; Osenkowski et al. 2009; Renzi et al. 2011). None of these were validated using independent biophysical methods, and they were also not consistent with each other. However, as the analytical and statistical methods improved, several more recent and accurate models were published.

In the first of these models, negative-stain single-particle three-dimensional EM data were combined with several complementary cross-validating biochemical, pharmacological, and biophysical methods, including size exclusion chromatography with multiangle light scattering (SEC-MALS) and Förster resonance energy transfer fluorescence lifetime imaging (FRET-FLIM) (Li et al. 2014). The resulting 17-Å model revealed that presenilin complexes have a bi-lobed shape, with a larger base ($93 \times 93 \times 60$ Å) and a smaller head ($65 \times 60 \times 55$ Å) (Fig. 3A). The ectodomain of nicastrin is located in the head domain. The base of the bi-lobed complex contains the TM domains of the PS1/PS2, PEN-2, nicastrin, and APH1 subunits (Fig. 3). Crucially, the base domain has a lateral cleft that communicates with the central cavity and that could potentially provide a route for sub-

Figure 3. Single-particle electron density maps of the human presenilin complex. (A) The initial low resolution (17 Å) map (left) and the subsequent higher resolution cryo-electron microscopy (cryo-EM) map (3.4 Å) show the essential features of the structure, with the nicastrin ectodomain in the smaller head and the transmembrane (TM) domains embedded in the larger base domain. Both structures show the existence of a lateral cleft, which opens into a central horseshoe-shaped cavity. (B) A 180° rotation of the model in A shows the relative positions of the two aspartate residues on TM6 and TM7. (C) Superimposition of the maps of the compound E-bound presenilin complex at 17 Å and the similar DAPT-bound complex at 3.4-Å resolution (left). Both show evidence of subtle conformational changes with movement of TM domains that result in closure of the substrate-binding pocket. In the higher resolution cryo-EM model (right), a conformational change at the end of TM6 can be visualized (red arrow) following DAPT (red spheres) binding, which likely alters acquisition and transfer of substrate to the active site.

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strate access to the catalytic PS1/PS2 subunit in the center of that horseshoe-shaped cavity.

More recently, the use of new detectors and image-processing methods has enabled further refinements of this model by increasing the image resolution to 4.5 Å (EMD-2677 and -3061, PDB code 4UPC and 5A63) (Lu et al. 2014; Bai et al. 2015b). This higher-resolution model confirmed the bi-lobed shape of human presenilin complexes as their native state (Fig. 3). Interestingly, several of the recent EM structural studies have reported that within any isolate of pure PS1 complexes, the complexes exist in multiple different conformations (Bai et al. 2015b; Elad et al. 2015). This agrees well with previous pharmacological studies, which suggest that at any given time only a small proportion of complexes are catalytically active (Behr et al. 2003; Lai et al. 2003).

Functional Anatomy of the Presenilin Complex

The increasing resolution of three-dimensional EM models of the presenilin complex has ushered in a new era to investigate how certain inhibitor drugs work. As described in greater detail below, numerous compound series have been identified through a variety of chemical screening strategies as inhibitors or modulators of the secretase function of the presenilin complex. Only a minority of these compounds actually work at the catalytic site. Many compounds have complicated mechanisms of action that likely involve binding at a site remote from the catalytic pocket, but then allosterically modifying the conformation of the complex. Some of these modifications alter substrate binding and access to the catalytic pocket. Others alter substrate preference and/or the efficiency of specific cleavage events.

The chemistry of these compounds is described in detail in the following sections. The structural effect of these compounds is now the topic of intense investigation. Two non-transition-state peptidomimetic γ -secretase inhibitors—namely, DAPT and compound E [(S,S)-2-[2-(3,5-difluorophenyl)-acetylamin]-N-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-

benzo[e][1,4]diazepin-3-yl)-propionamide, molecular mass = 490.5 Da]—have already been investigated using these methods (Li et al. 2014; Bai et al. 2015a). Compound E blocks substrate binding to the initial substrate-docking site (Li et al. 2014). Moreover, compound E binding can stabilize the PS1 complex and prevent its detergent-induced dissociation (Li et al. 2014), suggesting that inhibitor binding causes a significant conformational change that brings the complex components closer together. Intriguingly, there is a reciprocal allosteric effect in which substrate binding to the initial substrate docking site increases subsequent to compound E binding (Li et al. 2014). The existence of these inhibitor-induced conformational shifts has been robustly validated by two different types of experiments. First, in vitro intramolecular FRET-FLIM methods demonstrated a significant change in FRET-FLIM between tags placed on the PS1-NTF and PS1-CTE, indicating that the subcomponents of the complex had been brought closer together by compound E binding (Li et al. 2014). Second, the single-particle three-dimensional EM structure model for compound E-bound PS1 complexes was highly similar to the native complexes, with a bi-lobed overall shape. However, there were several critical differences, including (1) rotation of the nicastrin-containing head domain, and (2) compaction of the membrane-embedded base domain with closure of the lateral cleft (Fig. 3C) (Li et al. 2014). Similar changes at higher resolution have now been documented for DAPT, a similar peptidomimetic inhibitor (Fig. 3C) (Bai et al. 2015a). DAPT causes several conformational shifts including a shift in the presence of a tripeptide motif at the end of TM6, which may facilitate selection, acquisition, and transfer of substrate TMs to the catalytic site (R Dodd, S Qamar, and C Bohm, unpubl.).

Taken together, these observations suggest that the presenilin complexes are structurally dynamic complexes, and shifts in their conformation induced by ligand/substrate or inhibitor/modulator binding are reflected by changes in function. Below, we describe some of these compounds, many of which will be explored for

their effects on presenilin-complex conformation in the near future.

CHEMICAL BIOLOGY OF THE PRESENILIN COMPLEX

γ -Secretase Inhibitors (GSIs)

A variety of GSIs with distinct structures have been discovered (Josien 2002; D'Onofrio et al. 2012). Here, we focus on three types of GSIs: exosite-targeted GSIs, active site-directed GSI probes, and GSIs in clinical trials for treatment of AD and cancer.

Exosite-Targeted GSIs

The use of docking sites for substrate recognition represents a unique biological mechanism to achieve specificity in complex systems containing multiple component proteins. These docking sites, which are also referred to as exosites, are defined as specialized substrate binding sites located in separate domains outside the active site of the enzyme. They have been discovered in thrombin and matrix metalloproteinase and function to modulate and broaden substrate specificity (Overall 2002; Krishnaswamy 2005). γ -Secretase is a macromolecular complex that contains multiple exosites that are capable of interacting with substrates. Initially, Tian et al. (2002) demonstrated that at least two sites exist in the γ -secretase complex: the docking site and the active site. Moreover, kinetic studies using the TM domain of the APP substrate suggest that the TM domain does not interact with the docking site, and either the extracellular or intracellular region of β CTF binds to the docking site (Yin et al. 2007). It has been reported that the APP substrate interacts with γ -secretase at three other regions in addition to the active site. First, a docking site residing in the extracellular domain of nicastrin interacts with the N-terminal fragment of APP- β CTF (Shah et al. 2005), although the precise binding region on APP- β CTF has not been defined. Second, Kornilova et al. (2005) searched for substrate-binding sites by designing helical peptides that mimic the APP

transmembrane domain. This study suggested that this interaction site was located on PS, adjacent to the active site. Third, a substrate inhibitory domain (ASID) of the APP substrate interacts with a site that consists of at least PS1-NTE, PS1-CTF, and nicastrin (Tian et al. 2010). Although the exact binding sites have not yet been mapped, the existence of these exosites on γ -secretase is well documented in multiple studies (Tian et al. 2002, 2010; Kornilova et al. 2005; Yin et al. 2007). How these interaction regions coordinate to regulate γ -secretase activity and specificity is an exciting area of investigation. Accordingly, substrate-based peptidomimetic inhibitors and probes have been developed (Fig. 4A). Moreover, ASID-based inhibitors exhibit specificity for APP over Notch substrate (Tian et al. 2010). Because γ -secretase cleaves multiple substrates, it is of interest to investigate whether unique regulatory sequences are present in other γ -secretase substrates and function in the modulation of their own processing by interacting with specific ASID sites within γ -secretase, as observed with APP.

Active Site-Directed GSI Probes

Since Shearman et al. (2000) reported L-685,458 (L-458) as a potent γ -secretase inhibitor, molecular probes based on this molecule have been extensively developed (Li et al. 2000b; Xu et al. 2002; Behr et al. 2003; Chun et al. 2004; Placanica et al. 2009; Yang et al. 2009; Teranishi et al. 2010; Crump et al. 2012a; Ballard et al. 2014; Gertsik et al. 2014) and have offered valuable tools for investigating γ -secretase (Fig. 4B–D). L458 and its derivatives contain a hydroxyethylene dipeptide transition-state isostere that only interacts with the active form of aspartyl proteases. These probes have successfully identified PS1 as the catalytic subunit of γ -secretase (Li et al. 2000b) and have shown that PS1 is associated with active γ -secretase in the plasma membrane to address the issue of “spatial paradox” (Tarassishin et al. 2004). Moreover, these probes interact with the PS-NTE/PS-CTF heterodimer, the active form of γ -secretase, but not with PS full-length, suggesting that the PS full-length complex func-

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tions as a zymogen (Li et al. 2000b; Ahn et al. 2010). Importantly, these activity-based probes revealed that at least two types of PS complexes (the active and inactive) coexist in cells, and only a small fraction (<14%) of the PS1 complex is catalytically active (Behr et al. 2003; Lai et al. 2003). Therefore, separation of the active PS γ -secretase form from its inactive form is necessary for elucidating the function of PS1-engaged γ -secretase. Using these probes, some studies have shown that PS1 wild-type and FAD mutants differentially affect the equilibrium of the PS1- and PS2-containing active γ -secretase complexes, and the expression of Pen2 and PS1 mutants alter the stoichiometry of subunits within the γ -secretase complexes (Placanica et al. 2009). Consequently, these probes have been used for isolation of active γ -secretase complexes and identification of HIF1 α as a regulatory subunit of γ -secretase (Villa et al. 2014). An integrated approach with multiple photoprobes, referred to as photophore walking, has been developed for detection of conformational changes of the active site of γ -secretase by genetic mutations or small molecules (Shelton et al. 2009; Chau et al. 2012), offering a practical means for analyzing the active site of endogenous γ -secretase and insights into the mechanism of γ -secretase specificity. Photophore walking studies suggest that PS1 mutations alter the S2 subsite of the active site that allows for accommodation of larger P2 residues, leading

to preference of the enzyme for A β 42 cleavage (Chau et al. 2012). In contrast, the binding of acid GSMs to PS1 allosterically changes the S1 subsite within the active site and ultimately leads to a selective lowering of A β 42 (Crump et al. 2011).

Other transition-state inhibitors were developed by Wolfe and colleagues (Fig. 4E) including APP substrate-based difluoro ketone compounds and hydroxyethyl urea peptidomimetics. These probes have successfully been used for characterization, identification, and isolation of γ -secretase, as well as for investigating the active site of γ -secretase and the mechanism of reaction (Wolfe et al. 1999a,b; Esler et al. 2000, 2002, 2004; Kimberly et al. 2003; Kornilova et al. 2003, 2005) in which γ -secretase substrates dock to the initial binding site or exosite and then reach the active site for hydrolysis (Kornilova et al. 2005).

Clinical GSIs for AD

Semagacestat (Fig. 4F) was the first GSI to enter phase III clinical trials. Despite encouraging results obtained from earlier clinical investigations (Siemers et al. 2007), the phase III trial failed because of the unexpected observation that semagacestat caused worsening of memory in patients (Doody et al. 2013). There was also an increased risk of skin cancer, which probably stemmed from Notch inhibition (Xia et al.

A	Name	Structure	In vitro (nM)
	D-10	Boc-Val-Gly-Aib-Val-Val-Ile-Aib-Thr*-Val-Aib-OMe	100
	D-13	Ac-Val-Gly-Aib-Val-Val-Ile-Aib-Thr*-Val-Aib-Val-Val-Aib-NH ₂	30
	D-10-Bpa-Bt	Bt-Link-Val-Gly-Aib-Val-Val-Bpa-Aib-Thr*-Val-Aib-OMe	100
	D-13-Bpa-Bt	Bt-Link-Val-Gly-Aib-Val-Val-Bpa-Aib-Thr*-Val-Aib-Val-Val-Aib-NH ₂	10
	A β 17-23Bpa ²⁰	Leu-Val-Phe-Bpa-Glu-Asp	81
	A β 17-23Bpa ²⁰ -Bt	Leu-Val-Phe-Bpa-Glu-Asp-Bt	83

Figure 4. Structure of γ -secretase inhibitor compounds. (A) Substrate-based peptidomimetic inhibitors and probes. (Continued on following pages.)

Figure 4. (Continued) (B) L458 and photophore analogs used for photophore walking.

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Figure 4. (Continued) (C) Biotinylated L458 probes with different linker lengths.

Figure 4. (Continued) (D) Clickable L458 and CBAP probes.

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Figure 4. (Continued) (E) Difluoro ketone compounds and hydroxyethyl urea peptidomimetics.

Figure 4. (Continued) (F) Clinical γ -secretase inhibitors for Alzheimer's disease and cancer.

2001; Nicolas et al. 2003). The mechanism of cognitive decline, which remains unclear, has raised significant questions about the use of GSIs for AD treatment. Was the clinical trial suitably designed to avoid Notch-mediated toxicity (De Strooper 2014)? Does cognitive decline come from blockage of Notch cleavage or other γ -secretase substrates? Does the accumulation of APP β CTF cause neurotoxicity (Mitani et al. 2012)? Low concentrations of GSIs can cause A β elevation, and withdrawing GSIs leads to a rebound of A β production in the plasma. Do these facts underlie the neurotoxicity (Lanz et al. 2006)? Together, these data suggest that total inhibition of APP processing could actually aggravate AD pathology and lead to worsening cognition. Finally, a fundamental question is whether loss or gain of function of PS1 mutations leads to AD (Shen and Kelleher 2007; Xia et al. 2015), which significantly impacts the development of γ -secretase-based and even A β -based therapy for AD treatment.

Initially, avagacestat was reported as a "Notch-sparing" GSI (Gillman et al. 2010), which inhibits the processing of APP while leav-

ing Notch signaling intact. However, the mechanism of avagacestat is controversial (Chávez-Gutiérrez et al. 2012; Crump et al. 2012b). In addition to gastrointestinal and dermatologic complications associated with Notch inhibition, higher doses also led to cognitive decline (Coric et al. 2012), indicating that avagacestat may share a common mechanism of toxicity with semagacestat.

A number of GSIs, including RO4929097, MK-0752, and PF-3084014, are being evaluated for the treatment of leukemia, lymphoma, and advanced solid cancer (<https://clinicaltrials.gov/ct2/results?term=gamma-secretase+inhibitors+and+cancer&Search=Search>) (Andersson and Lendahl 2014). Earlier clinical studies of MK-0752 were linked with dose-limiting gastrointestinal toxicity (DeAngelo et al. 2006), which is likely because of inhibition of Notch1 and Notch2 in intestinal crypts (van Es et al. 2005). Recently, two phase I studies assessed safety, maximum-tolerated dose (MTD), pharmacokinetics (PK), pharmacodynamics (PD), and preliminary antitumor efficacy of MK-0752 and RO4929097 in patients

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with advanced solid tumors (Krop et al. 2012; Tolcher et al. 2012). Both GSIs were administered in three different schedules. RO4929097 was well tolerated at 270 mg on schedule A (3 days on, 4 days off during weeks 1 and 2, every 3 weeks; then 3 days on, 4 days off during subsequent cycles) and at 135 mg on schedule B (7 consecutive days, every 3 weeks) (Tolcher et al. 2012). Moreover, a weak and moderate PK/PD correlation was observed for A β 40 or VEGFR-2 in plasma as well as Hes-1 mRNA expression in hair follicles (Tolcher et al. 2012). MK-0752 was generally well tolerated at 3200 mg on schedule C (once per week) and resulted in strong modulation of a Notch gene signature (Krop et al. 2012). Moreover, clinical benefit was observed in both trials, which suggests that although GSI-based therapies have been unsuccessful to date for AD, they may have a clearer indication in the treatment of Notch-sensitive malignancies (Gounder and Schwartz 2012).

γ -Secretase Modulators (GSMs)

Because of the safety concerns with inhibition of γ -secretase, alternative approaches to modulate γ -secretase activity have attracted much attention. The notion of γ -secretase modulation was introduced in 2001 when it was discovered that a subset of nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, indomethacin, and sulindac sulfide, selectively lowered A β 42 levels but increased A β 38 production without inhibiting Notch1 cleavage (Weggen et al. 2001).

GSMs have a unique profile compared with GSIs. They (1) cause selective decrease of A β 42 or decrease of both A β 42 and A β 40; (2) cause an increase in shorter A β species (A β 38 and/or A β 37); (3) cause no significant effect on the total amount of A β produced; (4) cause no accumulation of APP- β CTF; (5) preserve ϵ cleavage of γ -secretase substrates, thus sparing APP- and Notch-ICD production; and (6) cause no plasma A β rebound (Crump et al. 2013). Not surprisingly, these desirable features garnered the attention of many pharmaceutical companies and have inspired the discovery of additional classes of GSMs as potential dis-

ease-modifying agents for the treatment of AD (Pettersson et al. 2011, 2013). These GSMs can be generally divided into three categories: NSAID-derived carboxylic acid GSMs, non-NSAID heterocyclic GSMs, and natural product-derived GSMs (Fig. 5A–C) (Wagner et al. 2012; Crump et al. 2013). Overall, the acid GSMs reduce the production of A β 42 and promote the generation of A β 38 and have little effect on A β 40 production, total A β levels, APP intracellular domain (AICD), and Notch1 processing (see below). The heterocyclic GSMs have a slightly different profile in that they lower the production of A β 42 and A β 40 while increasing the levels of A β 38 and A β 37 to differing degrees (see also below). The natural product-derived triterpene GSMs were discovered from the extract of the black cohosh plant and possess a distinct A β profile, with a reduction in A β 42 and A β 38 and an increase in A β 39 and A β 37. Although the implication of elevating A β 37 or A β 38 in AD is unknown, it is believed that the shorter forms are less prone to aggregation and less pathogenic than A β 42, and there is some evidence that suggests these shorter species could actually inhibit the aggregation of A β 42. The molecular basis for how GSMs spare the ϵ cleavage of APP and other substrates while decreasing the production of A β 42 is not known. Studies using photoaffinity probes have shown that the different classes of GSMs bind to PS1-NTF and have distinct allosteric binding sites (Pozdnyakov et al. 2013; Takeo et al. 2014). The precise binding sites on PS1-NTF are not known, but it is likely that binding causes conformational changes in the active site of presenilin, which alters the γ -cleavage sites, resulting in the different A β profiles depending on the GSM (Fig. 5A).

NSAID-Derived Acid GSMs

The first generation of NSAID GSMs includes sulindac sulfide, indomethacin, (R)-ibuprofen, and (R)-flurbiprofen (tarenflurbil) (Fig. 5B) (Weggen et al. 2001). The NSAID-derived GSMs selectively lower the formation of A β 42 with a concomitant increase in the generation of A β 38, albeit with weak potency. Importantly,

Figure 5. Allosteric γ -secretase modulators. (A) Cartoon showing allosteric interactions between the active site and the binding sites of heterocyclic and acid γ -secretase modulator compounds. (B) Structure of acid γ -secretase modulator compounds. (Continued on following page.)

these NSAIDs exhibited little to no effect on the proteolysis of other γ -secretase substrates such as Notch. *R*-flurbiprofen also selectively lowered A β 42 levels in a broken cell γ -secretase assay (A β 42 IC₅₀ $\sim$ 200–300 μ M), suggesting a direct but weak interaction with γ -secretase (Eriksen et al. 2003). Despite its weak potency

and poor brain penetration, tarenflurbil was advanced into clinical studies, but it did not slow cognitive decline in patients with mild AD in a phase 3 clinical trial. More recently, Chiesi developed a series of flurbiprofen analogs with improved potency for lowering A β 42, culminating in CHF5074 (A β 42 IC₅₀ = 3–

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Figure 5. (Continued) (C) Structure of heterocyclic γ -secretase modulator compounds.

41 μ M) (Peretto et al. 2005). The pharmacokinetics and pharmacodynamics of CHF5074 (200, 400, and 600 mg/d) were evaluated in healthy subjects, and it was found that the soluble CD40 ligand, a marker of microglia activation, was lowered in a dose-dependent fashion (Imbimbo et al. 2013). However, no lowering of A β levels in either plasma or cerebrospinal fluid (CSF) was detected, so the compound could act as a microglial modulator rather than as a GSM. Because of the weak potency and poor brain penetration, it has not been possible to test

the GSM mechanism in the clinic with this class of compounds. Therefore, a key goal has been to develop GSM compounds with improved potency and brain availability. To that end, Merck has replaced the core aryl ring with a piperidine ring and optimized the substituents to generate a potent series of piperidine acetic acid GSMs, exemplified by GSM-1. GSM-1 has become the prototypical acid GSM with an A β 42 IC₅₀ value of 120–348 nM (Fig. 5B) (Page et al. 2008, 2010). GSM-1 selectively reduced A β 42 and increased A β 38, whereas the levels of total

A β and AICD did not change. In addition, Astellas has reported the pharmacological profile of another piperidine acetic acid GSM referred to as GSM-2 (Mitani et al. 2012). GSM-2 selectively lowered A β 42 (IC_{50} = 65 nM), while increasing A β 38 (EC_{50} = 81 nM) with no effect on β CTF levels or Notch signaling. In contrast to the GSIs LY450139 and BMS-708,163, when administered for 8 days, GSM-2 ameliorated the cognitive deficit in 5.5-month-old Tg2576 mice as measured in the Y-maze test. Although all three drugs reduced hippocampal A β 42 levels, β CTF levels increased with the two GSIs but were unchanged with GSM-2. These data suggest that β CTF elevation could partially explain the cognitive impairment associated with GSI treatment.

EnVivo, Janssen, and Biogen have returned to the phenylacetic acid core of flurbiprofen and added additional substituents on the core aryl ring—namely, the para-trifluoromethylphenyl that is present in GSM-1—to generate compounds with A β 42 IC_{50} values less than 200 nM (Fig. 5B). EnVivo has introduced EVP-0015962, which decreased A β 42 with an IC_{50} of 67 nM while concomitantly increasing A β 38 (1.7-fold) (Rogers et al. 2012). Consistent with the profile of a GSM, A β total, APP-CTFs, and AICD/NICD generation were not changed. Chronic treatment in Tg2576 mice with EVP-0015962 (20 and 60 mg/kg/d, which results in brain concentrations of 2.5 and 8.3 μ M, respectively) reduced cytosolic, membrane-bound, and aggregated A β 42; amyloid plaque load in the hippocampus; and cognitive deficits in the contextual fear-conditioning test. Janssen added a second trifluoromethylphenyl substituent to give JNJ-40418677, a brain-penetrant phenyl acetic acid GSM that reduced A β 42 secretion in cells and primary rat cortical neuronal cultures with an IC_{50} of approximately 200 nM (Van Broeck et al. 2011). JNJ-40418677 did not change levels of total A β , and Notch processing and AICD production were not affected. Acute treatment with JNJ-40418677 selectively reduced levels of A β 42 in the brains of wild-type mice with a concomitant increase in A β 38; however, chronic administration in Tg2576 mice from 6 to 13 months of age resulted in

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dose-dependent reductions of all A β species in soluble and deposited fractions. In addition, a dose-dependent reduction in amyloid-plaque load was observed. The mechanism for the lowering of all A β species in the soluble fraction with chronic treatment of this GSM is not known. Biogen incorporated additional features of GSM-1 onto the core of flurbiprofen to create BIIB042, which lowered A β 42 in CHO cells with an IC_{50} of 170 nM and increased A β 38 with an EC_{50} of 150 nM (Peng et al. 2011). A reduction of brain A β 42 was observed when BIIB042 was administered to mice, rats, and cynomolgus monkeys. In all of these examples, the gain in potency came at the expense of lipophilicity, and it remains to be seen whether an acceptable safety profile with adequate brain exposure at the target can be attained.

Non-NSAID Heterocyclic GSMS

The first non-NSAID-derived class of GSMS was initially reported by NeuroGenetics and further advanced by Eisai. It is characterized by an A-B-C-D-type heterocyclic framework, wherein a majority of the earlier series incorporates an arylimidazole moiety as the A-B subunit. Optimization of a 15 μ M high-throughput screening hit led to identification of the NeuroGenetics GSM NGP-555, which has an A β IC_{50} of 10 nM (Fig. 5C) (Kounnas et al. 2010). Unlike NSAID-derived acid GSMS, which selectively reduce A β 42, the non-NSAID-derived heterocyclic GSMS reduce both A β 42 and A β 40 while increasing the levels of A β 38 and A β 37. NGP-555 was evaluated in Tg2576 mice at doses of 5–100 mg/kg administered orally for 3 days, and an $\sim$ 30% reduction of brain A β 42 was observed at the 50 mg/kg dose. Efficacy was also demonstrated in a 7-month plaque-reduction study (Kounnas et al. 2010). Despite challenges with low solubility and high lipophilicity in this series, NGP-555 entered phase 1 clinical testing in 2015 (<https://clinicaltrials.gov/ct2/results?term=NGP-555&Search=Search>, last accessed November 24, 2015).

A 2005 patent application from Eisai disclosed a related chemotype as exemplified by E2012 (Kimura et al. 2005). This compound

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incorporates a lactam as the central C-ring, and it became the prototypical heterocyclic GSM on which multiple organizations have initiated their own GSM programs. It has an A β 42 IC₅₀ value of 92 nM (Hashimoto et al. 2010), and robust in vivo activity was demonstrated in CSF biomarker studies in dogs and multiple rodent species (Portelius et al. 2010; Lu et al. 2012b). In 2006, E2012 became the first heterocyclic GSM to enter phase 1 clinical trials, and it was evaluated in a single ascending dose study with doses ranging from 1 to 400 mg, where an ~50% reduction of plasma A β 42 was reported at the 400-mg dose (Nagy et al. 2010). The development of E2012 was subsequently halted because of lenticular opacity (cataract) in a 13-week rat safety study (Nakano-Ito et al. 2014). Although this safety finding was eventually de-risked, further development of E2012 was not pursued in favor of the more potent clinical candidate E2212 (A β 42 IC₅₀ = 9 nM), which was reported to have a better safety margin (Yu et al. 2014). In vivo profiling in guinea pig and rat indicate robust reductions of brain A β 42 (Lu et al. 2012b). For example, a 15 mg/kg oral dose in rat resulted in a 47.7% reduction of brain A β 42, whereas a dose of 100 mg/kg afforded an ~70% reduction. E2212 was well tolerated in healthy human volunteers at doses up to and including 250 mg, and dose-dependent reduction of plasma A β 42 was observed with a 53.6% reduction at the 250-mg dose (Yu et al. 2014). The structure of E2212 has not been disclosed, but it is presumed to be as depicted in Fig. 5C.

Schering Plough (now Merck) has explored multiple scaffolds based on E2012, and this work resulted in the identification of oxadiazines as amide bioisosteres as exemplified by SP-53 (A β 42 IC₅₀ = 33 nM) (Huang et al. 2012; Sun et al. 2012). When dosed orally at 3 mg/kg in rats, SP-53 achieved a 37% and 35% reduction of A β 42 in the cortex and CSF, respectively. The GSMs NGP-555, E2012, E2212, and SP-53 contain an olefin as a rigid linker to connect the B- and C-rings. Several GSM programs throughout the industry have sought to replace the electron-rich olefin with an alternative linker, and the use of a secondary

amine has become increasingly prevalent (Pettersson et al. 2013). This strategy was explored by researchers at Janssen, resulting in JNJ-42601572 (A β 42 IC₅₀ = 16 nM), which reduced brain A β 42 by 51% when dosed in mice at 10 mg/kg orally (Bischoff et al. 2012). As with a majority of GSMs described in the literature up to this point, JNJ-42601572 maintained high aromaticity and lipophilicity (cLogP = 5.3), and this may explain the signs of liver toxicity that were observed in dogs when dosed at 20 mg/kg orally.

The strategy of incorporating an NH-linker has been used by several other companies including Hoffman-La Roche, AstraZeneca, and BMS. A common theme has been to utilize an aminopyrimidine as the C-ring hydrogen bond acceptor, and in some cases, fuse it to a second aliphatic ring to impart additional conformational control. Hoffman-La Roche has disclosed GSMs such as RO-02, which has an A β 42 IC₅₀ value of 15 nM (Ebke et al. 2011). AstraZeneca has published structurally related GSMs including AZ4800, which has an A β 42 IC₅₀ of 26 nM and lowered brain A β 42 by 46% when dosed orally at 130 mg/kg (Borgegard et al. 2012). BMS incorporated an aminotriazole core and a chloroimidazole A-ring in place of the commonly used 4-methylimidazole to afford potent GSMs such as BMS-869780 (A β 42 IC₅₀ = 5.6 nM) (Toyn et al. 2014). PK/PD modeling indicated that a dose of 700 mg would be required to achieve a 25% reduction of A β 42 in the human brain. The high projected human dose, taken together with observations of liver toxicity in rat, led to the discontinuation of BMS-869780 from further development.

In addition to connecting the B- and C-rings via an olefin or an NH-linker, direct attachment has been explored by several companies (Pettersson et al. 2011). Following observations of liver toxicity with JNJ-42601572, Janssen used this approach to make significant improvements with regard to physicochemical properties as exemplified by GSM JNJ-16 (Gijssen and Mercken 2012; Oehrich et al. 2013). This compound has an A β 42 IC₅₀ of 56 nM, but with significantly reduced lipophilicity relative to JNJ-42601572 (cLogP of 3.1 and 5.3

for JNJ-16 and JNJ-42601572, respectively). JNJ-16 was evaluated in dogs with an oral dose of 20 mg/kg, and a 30%–40% reduction in CSF A β 42 was achieved. This level of efficacy was accompanied by high plasma exposure (24 μ M), but no signs of liver toxicity were observed. Furthermore, JNJ-16 was evaluated in a 2-wk rat safety study at a daily dose of 200 mg/kg without observing overt liver toxicity. Although this is an encouraging advancement, the authors comment that the high exposures required to achieve robust A β -lowering activity in vivo may not be compatible with long-term treatment. Thus, further improvements in efficacy will be needed to identify a viable clinical candidate.

Although the A-B section of the A-B-C-D-type heteroaryl series has been highly conserved in the literature, a number of examples have emerged in which compounds differ significantly from earlier chemotypes. Pfizer has reported a novel series of GSMs incorporating a pyridopyrazine-1,6-dione heterocyclic core that allowed for improved alignment of potency, physicochemical properties, and ADME, ultimately leading to identification of PF-06442609 (A β 42 IC₅₀ = 6 nM, cLogP = 3.1) (Tran et al. 2013; Pettersson et al. 2014, 2015). This compound afforded a 41% reduction of brain A β 42 when dosed orally in guinea pig at 60 mg/kg. An alternative series has also been reported by Roche, in which the aromatic B-ring has been replaced with a saturated piperidine as exemplified in RO5506284 (A β 42 IC₅₀ = 25.7 nM in H4-APP Swe cells) (Brendel et al. 2015). Brain A β 42 was reduced by 48% when dosed orally at 30 mg/kg in transgenic mice (APP-Swe). RO5506284 was also shown to inhibit de novo plaque formation upon dosing for 6 months, as assessed by amyloid PET imaging.

A novel class of natural-product-derived GSMs was identified by Satori. The initial lead, a triterpene glycoside, was isolated from the extract of the black cohosh plant and has an A β 42 IC₅₀ value of 100 nM in H4 APP cells (Findeis et al. 2012). This new series has a unique A β profile in which A β 42 and A β 38 are reduced and A β 37 and A β 39 are increased, whereas A β 40 remains relatively unchanged.

Optimization of the initial lead ultimately afforded the preclinical candidate SPI-1865 (A β 42 IC₅₀ of 106 nM) (Hubbs et al. 2012, 2015; Loureiro et al. 2013). Following single oral doses of 10, 30, and 100 mg/kg in rat, SPI-1865 achieved brain A β 42 reduction of 21, 37, and 50%, respectively, at the 24-h time point following the final dose. However, further development of SPI-1865 was eventually halted because of adverse effects on adrenal function in monkeys (Hall and Patel 2014).

CONCLUSIONS

The recent structural studies on presenilin complexes, taken together with recent discoveries in the medicinal chemistry and chemical biology of the presenilin complexes that are reviewed here, represent a major advance toward understanding how the presenilin complexes work. The next steps for the field will be to build structural models of the complex associated with various inhibitors and modulators. By defining the consequent three-dimensional structural shifts in the architecture of the complex, it may eventually be possible to design compounds targeting specific substrates and/or specific cleavage products.

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