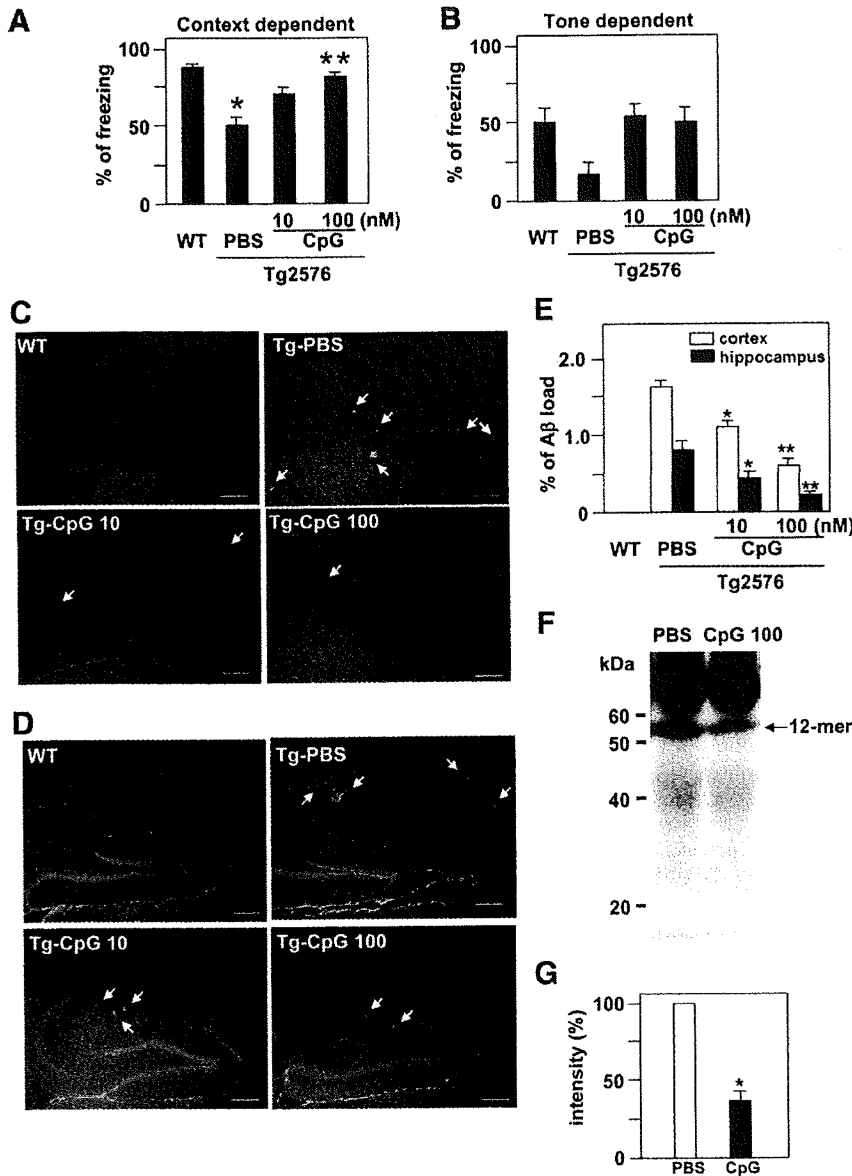


Figure 6. Effect of ICV injection of CpG on associative learning in the cued and contextual conditioning tests and $A\beta$ clearance in Tg2576 mice. The retention session was performed 24 hours after the training. Context-dependent (**A**) and tone-dependent (**B**) freezing times were measured at the age of 10 months. Each column indicates the mean ± SEM (wild-type (WT) mice, $n = 14$; vehicle-treated (PBS) Tg2576 mice, $n = 9$; CpG (10 nmol/L)-treated Tg2576, $n = 8$; CpG (100 nmol/L)-treated Tg2576, $n = 8$). $^{*}P < 0.05$ compared with wild type. $^{**}P < 0.05$ as compared with vehicle-treated Tg2576 mice. **C:** $A\beta$ deposits in the cortex. $A\beta$ was stained with 4G8 (green), microglia stained with anti-CD11b antibody (red), and cell nucleus stained with Hoechst 33342 (blue). **White arrows** indicate $A\beta$ deposits surrounding by microglia. Scale bar, 200 μm. **D:** $A\beta$ deposits in the hippocampus. Scale bar, 200 μm. **E:** Quantification of $A\beta$ deposits in **C** and **D**. The ratio between the $A\beta$ deposits area and the total area of the analyzed region was multiplied by 100. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with vehicle-treated Tg2576 mice. Each column indicates the mean ± SEM ($n = 3$). **F:** Western blot analysis of $oA\beta$ extracted from the hemi-forebrains of Tg 2576 mice. A 12-mer $oA\beta$ was detected in vehicle-treated Tg2576 mice and decreased in 100 nmol/L CpG-injected Tg2576 mice. **G:** Semi-quantification of 12-mer $oA\beta$ by densitometric analysis. The amount of 12-mer $oA\beta$1-42 in vehicle-treated Tg2576 mice was normalized to 100%. $^{*}P < 0.05$ as compared with vehicle-treated Tg2576 mice. Each column indicates the mean ± SEM ($n = 4$).

activated microglia nor induced the release of neurotoxic proinflammatory cytokines, NO, or glutamate. These results suggest that $oA\beta$1-42 is not acting to trigger of microglial neurotoxicity.

TLR signaling pathways contribute to phagocytosis of $A\beta$. TLR2 acts as an endogenous receptor for the clearance of $A\beta$ by bone marrow-derived microglia.[9] Interestingly, a TLR4 mutation exacerbates $A\beta$ burden in mouse models of AD.[39] Thus, we investigated whether microglia activated with TLR ligands exert neuroprotective effects against $oA\beta$1-42 toxicity. Consequently, we found that TLR9 ligand CpG enhanced microglial neuroprotection. Furthermore, CpG exerted the neuroprotective effect of BV-2 microglial cell line against $oA\beta$ toxicity (data not shown). TLR9, which detects single-stranded DNA containing unmethylated CpG, is located in intracellular endosomal-lysosomal compartment. We confirmed that microglia expressed TLR9 at a higher level, whereas astrocytes and neuronal cells expressed it at a lower level (data not shown). Thus, CpG mainly acts on microglia in the central nervous system. TLR7 and TLR8 are closely associated with TLR9. They are also located in endosomal-lysosomal compartment and detect single-stranded RNA. The ligands for TLR7 and TLR8 may also have some roles in microglial neuroprotection.

Western blot analysis revealed that microglia activated with CpG reduced the amount of $oA\beta$ present in the supernatant of treated cultures. Moreover, CpG was a potent inducer of antioxidant enzyme HO-1. The up-regulation of HO-1 in microglia by CpG treatment may lead to neuroprotection via suppression of ROS production by $oA\beta$. HO-1, a member of the heat-shock protein family, is a microsomal enzyme that oxidatively cleaves heme to produce biliverdin, carbon monoxide, and iron.[40] $A\beta$ binds to heme to promote a functional heme deficiency, mitochondrial dysfunction, and neurotoxicity.[41] Amyloid

precursor protein also binds to HO, and oxidative neurotoxicity is markedly enhanced in cerebral cortical cultures from amyloid precursor protein Swedish mutant transgenic mice.[42]

HO-1 is reported to be induced by the anti-inflammatory cytokine IL-10.[29] Although CpG induced IL-10 in microglia in the absence of oAβ, IL-10 production was inhibited in the presence of oAβ. Therefore, HO-1 may be induced by a discrete mechanism independent from IL-10 in AD.

MMP-9, a protease that degrades Aβ, is expressed at higher levels in the brains of AD patients and may play an important role in amyloid clearance by degrading both oAβ and fAβ.[10] MMP-9 expression is increased by serum amyloid A through formyl peptide receptor-like-1.[43] Although CpG stimulation induced MMP-9 in microglia, inhibiting MMP-9 pharmacologically did not affect neuroprotection by microglia. Thus, MMP-9 may not mainly contribute to the neuroprotection provided by CpG-activated microglia.

In the present study, CpG induced fewer neurotoxic molecules such as TNF-α, NO, and glutamate in microglia, whereas previous studies have reported that CpG-activated microglia produce TNF-α, IL-12, and NO[44] and induce neuronal damage.[45] The discrepancies between these studies and our experiments may be a consequence of differences in the concentrations of TLR ligands used. Higher concentration (10 μmol/L) of CpG have been used for microglial activation in previous reports, whereas here we have used lower concentrations (1 to 100 nmol/L) of CpG.

In addition, we observed that the neuroprotective effect differs among CpG ODN classes. The responses of microglia to the different classes of CpG have not been fully understood. Here, we showed for the first time that class A CpG did not activate microglia, whereas class B and C CpGs induced neuroprotection by microglia that was mediated by clearance of oAβ and induction of HO-1. Three major classes of CpG ODN are structurally distinct. The structures of class A CpG include poly-G motifs at the 5′ and/or 3′ ends that are capable of forming very stable but complex higher-ordered structures and a central phosphodiester region containing one or more CpG motifs in a self-complementary palindrome. Class B CpG has a completely phosphorothioate backbone and does not form typically higher-ordered structures. Class C CpG has a phosphorothioate backbone, and 3′ palindrome forms duplex.[46] These distinct structures of CpG ODN may reflect different microglial responses.

Finally, we examined the effect of CpG on oAβ1-42 neurotoxicity in two different *in vivo* studies. The ICV administration of Aβ25-35 is reported to cause cognitive impairment in a NORT. Oxidative stress contributes to the onset of this cognitive dysfunction.[23] We found that injection of oAβ1-42 also induced cognitive impairment as assessed by NORT. Surprisingly, one-time ICV injection of CpG improved both the cognitive impairment by oAβ1-42 in NORT. The impairment of associative learning in Tg 2576 mouse model of AD was also effectively suppressed by ICV injection of CpG. We also confirmed that CpG treatment decreased Aβ deposits and oAβ in Tg 2576 mice. Our results concur with the recent study that a total of 14 i.p. injection of CpG into Tg 2576 mice beginning at the age of 6 weeks, and once a month, ameliorates AD-related pathology.[28] Aβ plaques are reported to form extraordinarily quickly, over 24 hours. Within 1 to 2 days of a new plaque's appearance, microglia are activated and recruited to the site.[47] ICV injection of CpG may directly induce microglial activation via TLR9 and enhance microglial rapid uptake of oAβ through fluid-phase macropinocytosis as reported recently.[48] CpG may also enhance microglial phagocytosis of fAβ through formyl peptide receptor-like 2.[11] Such mechanisms of Aβ clearance by CpG can decrease Aβ plaque formation in Tg 2576 mice.

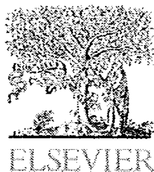
Recently, the therapeutic potential of CpG has generated great interest.[46] CpG offers a potent adjuvant activity that elicits a more effective immune response to infectious agents or tumors. A previous report[49] demonstrated that CpG strongly inhibits the effector phase of inflammatory arthritis. In addition, CpG can serve as a potent preconditioning stimulus and provide protection against ischemic brain injury.[50] Our findings suggest that CpG, especially class B and C, may also be effective therapeutic agents against oAβ1-42 neurotoxicity in AD.

References

1. Busciglio J, Lorenzo A, Yeh J, Yankner BA: β-Amyloid fibrils induce tau phosphorylation and loss of microtubule binding. Neuron 1995, 14:879–888
2. Grace EA, Rabiner CA, Busciglio J: Characterization of neuronal dystrophy induced by fibrillar amyloid β: implications for Alzheimer's disease. Neuroscience 2002, 114:265–273
3. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ: Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. Nature 2002, 416:535–539
4. De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, Klein WL: Aβ oligomers induce neuronal oxidative stress through an *N*-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. J Biol Chem 2007, 282:11590–11601
5. Deshpande A, Mina E, Glabe C, Busciglio J: Different conformations of amyloid β induce neurotoxicity by distinct mechanisms in human cortical neurons. J Neurosci 2006, 26:6011–6018
6. Meda L, Cassatella MA, Szendrei GI, Otvos L Jr, Baron P, Villalba M, Ferrari D, Rossi F: Activation of microglial cells by β-amyloid protein and interferon γ. Nature 1995, 374:647–650
7. McGeer PL, McGeer EG: Inflammation, autotoxicity and Alzheimer disease. Neurobiol Aging 2001, 22:799–809
8. Mizuno T, Kurotani T, Komatsu Y, Kawanokuchi J, Kato H, Mitsuma N, Suzumura A: Neuroprotective role of phosphodiesterase inhibitor Ibudilast on neuronal cell death induced by activated microglia. Neuropharmacology 2004, 46:404–411
9. Richard KL, Filali M, Préfontaine P, Rivest S: Toll-like receptor 2 acts as a natural innate immune receptor to clear amyloid β 1-42 and delay the cognitive decline in a mouse model of Alzheimer's disease. J Neurosci 2008, 28:5784–5793
10. Yan P, Hu X, Song H, Yin K, Bateman RJ, Cirrito JR, Xiao Q, Hsu FF, Turk JW, Xu J, Hsu CY, Holtzman DM, Lee JM: Matrix metalloproteinase-9 degrades amyloid-β fibrils in vitro and compact plaques in situ. J Biol Chem 2006, 281:24566–24574
11. Iribarren P, Chen K, Hu J, Gong W, Cho EH, Lockett S, Uranchimeg B, Wang JM: CpG-containing oligodeoxynucleotide promotes microglial cell uptake of amyloid β 1-42 peptide by up-regulating the expression of the G-protein-coupled receptor mFPR2. FASEB J 2005, 19:2032–2034

12. Chen K, Iribarren P, Hu J, Chen J, Gong W, Cho EH, Lockett S, Dunlop NM, Wang JM: Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid β peptide. J Biol Chem 2006, 281:3651–3659
13. Liu Y, Walter S, Stagi M, Cherny D, Letiembre M, Schulz-Schaeffer W, Heine H, Penke B, Neumann H, Fassbender K: LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. Brain 2005, 128:1778–1789
14. Xie Z, Wei M, Morgan TE, Fabrizio P, Han D, Finch CE, Longo VD: Peroxynitrite mediates neurotoxicity of amyloid β-peptide1-42- and lipopolysaccharide-activated microglia. J Neurosci 2002, 22:3484–3492
15. Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, Sonobe Y, Mizuno T, Suzumura A: Tumor necrosis factor-α induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. J Biol Chem 2006, 281:21362–21368
16. Suzumura A, Mezitis SG, Gonatas NK, Silberberg DH: MHC antigen expression on bulk isolated macrophage-microglia from newborn mouse brain: induction of Ia antigen expression by γ-interferon. J Neuroimmunol 1987, 15:263–278
17. Naiki H, Nakakuki K: First-order kinetic model of Alzheimer's β-amyloid fibril extension in vitro. Lab Invest 1996, 74:374–383
18. Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ: Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. J Biol Chem 2002, 277:32046–32053
19. Pollock JS, Forstermann U, Mitchell JA, Warne TD, Schmidt HHHW, Nakane M, Murad F: Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc Natl Acad Sci USA 1991, 88:10480–10484
20. Takeuchi H, Mizuno T, Zhang G, Wang J, Kawanokuchi J, Kuno R, Suzumura A: Neuritic beading induced by activated microglia is an early feature of neuronal dysfunction toward neuronal death by inhibition of mitochondrial respiration and axonal transport. J Biol Chem 2005, 280:10444–10454
21. Lesné S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH: A specific amyloid-β protein assembly in the brain impairs memory. Nature 2006, 440:352–357
22. Maurice T, Lockhart BP, Privat A: Amnesia induced in mice by centrally administered β-amyloid peptides involves cholinergic dysfunction. Brain Res 1996, 706:181–193
23. Alkam T, Nitta A, Mizoguchi H, Itoh A, Nabeshima T: A natural scavenger of peroxynitrites, rosmarinic acid, protects against impairment of memory induced by Aβ (25-35). Behav Brain Res 2007, 180:139–145
24. Kamei H, Nagai T, Nakano H, Togan Y, Takayanagi M, Takahashi K, Kobayashi K, Yoshida S, Maeda K, Takuma K, Nabeshima T, Yamada K: Repeated methamphetamine treatment impairs recognition memory through a failure of novelty-induced ERK1/2 activation in the prefrontal cortex of mice. Biol Psychiatry 2006, 59:75–84
25. Mizoguchi H, Takuma K, Fukakusa A, Ito Y, Nakatani A, Ibi D, Kim HC, Yamada K: Improvement by minocycline of methamphetamine-induced impairment of recognition memory in mice. Psychopharmacology 2008, 196:233–241
26. Mouri A, Noda Y, Hara H, Mizoguchi H, Tabira T, Nabeshima T: Oral vaccination with a viral vector containing Aβ cDNA attenuates age-related Aβ accumulation and memory deficits without causing inflammation in a mouse Alzheimer model. FASEB J 2007, 21:2135–2148
27. Franklin KBJ, Paxinos G: The mouse brain: in stereotaxic coordinates. San Diego, Academic 1997, pp. 69–89
28. Scholtzova H, Kascsak RJ, Bates KA, Boutajangout A, Kerr DJ, Meeker HC, Mehta PD, Spinner DS, Wisniewski T: Induction of Toll-like receptor 9 signaling as a method for ameliorating Alzheimer's disease-related pathology. J Neurosci 2009, 29:1846–1854
29. Lee TS, Chau LY: Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 2002, 8:240–246
30. Krug A, Rothenfusser S, Hornung V, Jahrsdorfer B, Blackwell S, Ballas ZK, Endres S, Krieg AM, Hartmann G: Identification of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells. Eur J Immunol 2001, 31:2154–2163
31. Selkoe DJ: Alzheimer's disease is a synaptic failure. Science 2002, 298:789–791
32. Demuro A, Mina E, Kayed R, Milton SC, Parker I, Glabe CG: Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. J Biol Chem 2005, 280:17294–17300
33. Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL: Natural oligomers of the Alzheimer amyloid-β protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci 2007, 27:2866–2875
34. Knobloch M, Farinelli M, Konietzko U, Nitsch RM, Mansuy IM: Aβ oligomer-mediated long-term potentiation impairment involves protein phosphatase 1-dependent mechanisms. J Neurosci 2007, 27:7648–7653
35. Townsend M, Mehta T, Selkoe DJ: Soluble Aβ inhibits specific signal transduction cascades common to the insulin receptor pathway. J Biol Chem 2007, 282:33305–33312
36. Manelli AM, Bulfinch LC, Sullivan PM, LaDu MJ: Aβ42 neurotoxicity in primary co-cultures: effect of apoE isoform and Abeta conformation. Neurobiol Aging 2007, 28:1139–1147
37. Lue LF, Walker DG, Rogers J: Modeling microglial activation in Alzheimer's disease with human postmortem microglial cultures. Neurobiol Aging 2001, 22945–956
38. Combs CK, Karlo JC, Kao SC, Landreth GE: β-Amyloid stimulation of microglia and monocytes results in TNF α-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. J Neurosci 2001, 21:1179–1188
39. Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K: Role of Toll-like receptor signalling in Aβ uptake and clearance. Brain 2006, 129: 3006–3019
40. Maines MD: The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997, 37:517–554
41. Atamna H, Frey WH II: A role for heme in Alzheimer's disease: heme binds amyloid β and has altered metabolism. Proc Natl Acad Sci USA 2004, 101:11153–11158
42. Takahashi M, Doré S, Ferris CD, Tomita T, Sawa A, Wolosker H, Borchelt DR, Iwatsubo T, Kim SH, Thinakaran G, Sisodia SS, Snyder SH: Amyloid precursor proteins inhibit heme oxygenase activity and augment neurotoxicity in Alzheimer's disease. Neuron 2000, 28:461–473
43. Lee HY, Kim MK, Park KS, Bae YH, Yun J, Park JI, Kwak JY, Bae YS: Serum amyloid A stimulates matrix-metalloproteinase-9 up-regulation via formyl peptide receptor like-1-mediated signaling in human monocytic cells. Biochem Biophys Res Commun 2005, 330:989–998
44. Dalpke AH, Schäfer MK, Frey M, Zimmermann S, Tebbe J, Weihe E, Heeg K: Immunostimulatory CpG-DNA activates murine microglia. J Immunol 2002, 168:4854–4863
45. Iliev AI, Stringaris AK, Nau R, Neumann H: Neuronal injury mediated via stimulation of microglial Toll-like receptor-9 (TLR9). FASEB J 2004, 18:412–414
46. Krieg AM: Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov 2006, 5:471–484
47. Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M: de Calignon A, Rozkalne A, Koenigsknecht-Talboo J, Holtzman DM, Bacskai BJ, Hyman BT: Rapid appearance and local toxicity of amyloid-β plaques in a mouse model of Alzheimer's disease. Nature 2008, 451:720–724
48. Mandrekar S, Jiang Q, Lee CY, Koenigsknecht-Talboo J, Holtzman DM, Landreth GE: Microglia mediate the clearance of soluble Aβ through fluid phase macropinocytosis. J Neurosci 2009, 29:4252–4262
49. Wu HJ, Sawaya H, Binstadt B, Brickelmaier M, Blasius A, Gorelik L, Mahmood U, Weissleder R, Carulli J, Benoist C, Mathis D: Inflammatory arthritis can be reined in by CpG-induced DC-NK cell cross-talk. J Exp Med 2007, 204:1911–1922
50. Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule JL, Lessov NS, Simon RP, Stenzel-Poore MP: Toll-like receptor 9: a new target of ischemic preconditioning in the brain. J Cereb Blood Flow Metab 2008, 28:1040–1047

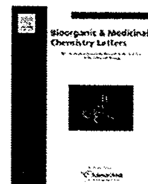
Bioorganic & Medicinal Chemistry Letters 19 (2009) 6869–6871



Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl



Development of photoaffinity probes for γ-secretase equipped with a nitrobenzenesulfonamide-type cleavable linker

Satoshi Yokoshima [a,b], Yuzo Abe [a], Naoto Watanabe [a], Yoichi Kita [a], Toshiyuki Kan [c], Takeshi Iwatsubo [a,b,d], Taisuke Tomita [a,b], Tohru Fukuyama [a,b,*]

[a] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[b] Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[d] Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 18 September 2009
Revised 19 October 2009
Accepted 20 October 2009
Available online 23 October 2009

Keywords:
γ-Secretase
Photoaffinity labeling
Probe
Cleavable linker

ABSTRACT

We have developed photoaffinity probes for γ-secretase with a nitrobenzenesulfonamide-type linker that can be cleaved with 2-mercaptoethanol under physiological conditions.

© 2009 Elsevier Ltd. All rights reserved.

Photoaffinity labeling is an efficient method for studying the interactions between biologically active compounds (ligands) and their target molecules.[1] The method facilitates the identification of both the target molecule and the binding domain. A photoaffinity probe is usually composed of a ligand, a photoreactive labeling group, and an indicator. A biotin tag is widely employed as an indicator because the biotinylated molecule can be easily detected by several immunological methods and isolated through binding to avidin beads.[2] One drawback to this approach is that harsh conditions are required to disrupt the strong interaction between the biotin and the avidin. Thus, to liberate the biotinylated molecule, the beads are typically boiled with detergents to denature the avidin. These conditions may result in degradation of the target molecules as well as contamination of the other proteins that bind to the beads with non-specific manner. To overcome this drawback, a variety of cleavable linkers, which can be cleaved under mild conditions to specifically release the labeled target molecules, have been developed.[3]

We have focused on the functional analysis of γ-secretase, which is one of the important therapeutic targets for Alzheimer's disease.[4] During the course of investigation of the potent γ-secretase inhibitor DAPT (**1**), we synthesized the photoaffinity probe DAP-BpB (**2**) and found that the major target of DAPT is presenilin 1 C-terminal fragment (PS1 CTF) (Fig. 1).[5] However, further detailed examinations of the DAPT-PS1 CTF interaction could not be conducted because of the low purity of the labeled proteins, which is caused by contaminated proteins bound to the avidin beads. We have prepared the other photoaffinity probes with disulfide moieties as cleavable linkers. However, they failed to effectively probe the PS1 CTF (data not shown). These results prompted us to develop photoaffinity probes equipped with a novel cleavable linker.

We envisioned that 2-nitrobenzensulfonamide would be an effective cleavable linker because this molecule can be cleaved under mild conditions using thiolate to generate an amine unit and a

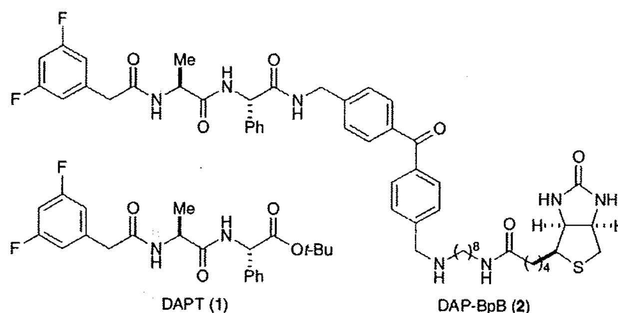
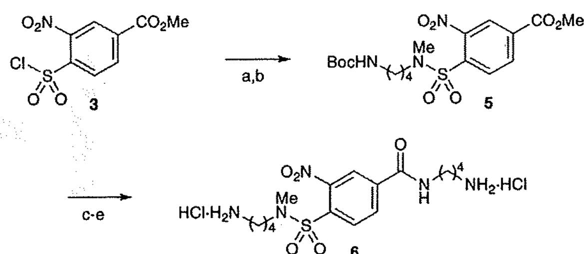


Figure 1. Structures of DAPT and DAP-BpB.

* Corresponding author. Tel.: +81 3 5841 4777; fax: +81 3 5802 8694.
E-mail address: fukuyama@mol.f.u-tokyo.ac.jp (T. Fukuyama).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.10.086



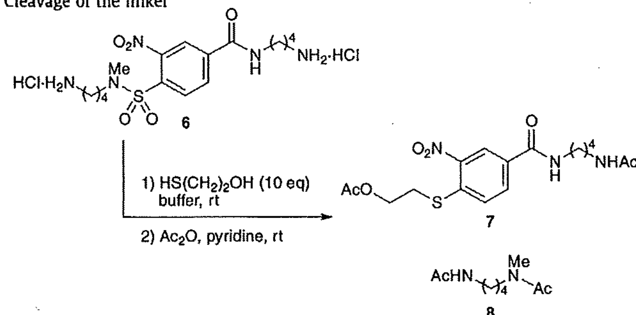
Scheme 1. Reagents and conditions: (a) $BocHN(CH_2)_4NH_2$ (**4**), Et_3N, CH_2Cl_2, rt; (b) MeI, Cs_2CO_3, DMF, reflux; (c) $LiOH{\cdot}H_2O$, THF–MeOH, rt; (d) $BocHN(CH_2)_4NH_2$ (**4**), WSCD·HCl, HOBt, DMF, rt, 68% (four steps); (e) $SOCl_2$, MeOH, rt.

3-nitrobenzene unit.[6] It has been reported that 3-nitro-4-sulfamoylbenzamides can be used as linkers for solid-phase synthesis.[7] To examine whether this unit can be used for photoaffinity labeling, we synthesized a model compound bearing 3-nitro-4-sulfamoylbenzamide and evaluated its cleavability under physiological conditions.

To access the model compound, known sulfonyl chloride **3**[7] was treated with *N*-Boc-butanediamine (**4**) and the resulting sulfonamide unit was methylated to afford **5** (Scheme 1). Hydrolysis of the methyl ester in **5**, coupling of the resultant carboxylic acid with amine **4**, and final deprotection of the Boc groups furnished the requisite model compound **6**.

With model sulfonamide **6** in hand, we attempted to cleave the sulfonamide unit under physiological conditions. Sulfonamide **6** was treated with 10 equiv of 2-mercaptoethanol in two buffers. The reaction progress was determined by isolation of **7** after acetylation of the reaction mixture. As shown in Table 1, the cleavage reactions proceeded smoothly in aqueous media to afford **7** in excellent yield. The reaction rates depended on the concentrations of both the substrate and 2-mercaptoethanol. The cleavage reaction proceeded even in almost neutral phosphate buffer (pH 7.4) although the time needed for completion of the reaction was longer than the reaction time required at higher pH. Both of the reaction conditions were much milder than the conditions required for breaking the avidin–biotin interaction.

Table 1
Cleavage of the linker

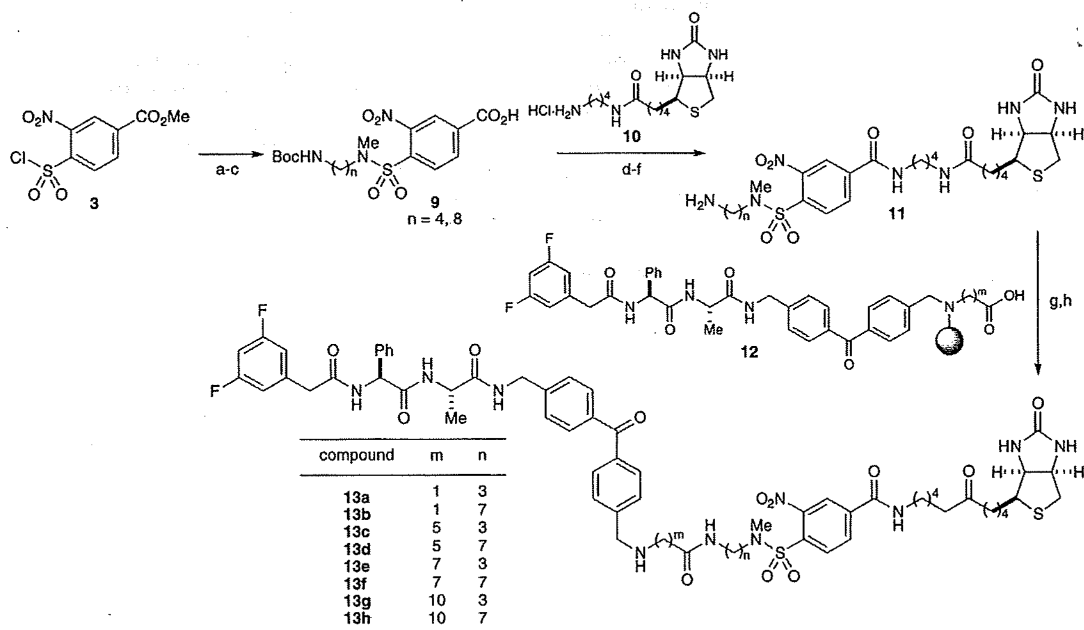


Buffer	pH	Concentration of **6** (mM)	Time (h)	Yield (%, **7**)
$NaHCO_3$–Na_2CO_3	9.2	3.0	7	93
		1.5	12	97
KH_2PO_4–$NaHPO_4$	7.4	3.0	24	93
		1.5	33	100

With a suitable cleavable unit in hand, we focused on incorporation of the linker unit into photoaffinity probes (Scheme 2). Two variants of carboxylic acids **9** with different chain lengths ($n = 4$ or 8) were prepared following the same procedures described in the synthesis of the model compound. Condensation of **9** with biotin unit **10** proceeded smoothly. After removal of the Boc group with methanolic hydrochloric acid, the resulting salt was liberated with Amberlite IR-400 (OMe form). Condensation of the resulting amines **11** ($n = 4$, 8) with polymer-bound carboxylic acid units **12** ($m = 1$, 5, 7, 10),[8] followed by acidic treatment, afforded photoaffinity probes **13a–h** with different lengths.

Using an in vitro γ-secretase assay, we found that these probes had inhibitory activities at almost equal levels to that of DAPT (Table 2).

We next investigated the labeling abilities of probes **13a–h**. CHAPSO-solubilized lysates of HeLa cells (~150 μg) mixed with the



compound	m	n
13a	1	3
13b	1	7
13c	5	3
13d	5	7
13e	7	3
13f	7	7
13g	10	3
13h	10	7

Scheme 2. Reagents and conditions: (a) $BocHN(CH_2)_nNH_2$, Et_3N, CH_2Cl_2, rt; (b) MeI, Cs_2CO_3, MeCN, reflux; (c) $LiOH{\cdot}H_2O$, THF–MeOH–H_2O, rt; (d) **9**, PyBoP, HOBt, DMF, rt; (e) $SOCl_2$, MeOH, rt; (f) Amberlite IR-400 (OMe form), MeOH; (g) **11**, WSCD·HCl, HOBt, *i*-Pr_2NEt, NMP, rt; (h) TFA, CH_2Cl_2, rt.

Table 2
Inhibitory activities of probes **13a–h** for Aβ40 and 42[a]

Compound	Aβ40 (%)	Aβ42 (%)
13a	81.9	75.9
13b	81.9	77.0
13c	54.9	60.2
13d	78.2	59.3
13e	62.8	66.2
13f	62.3	89.3
13g	74.5	86.3
13h	55.0	69.2
DAPT (**1**)	77.4	76.3

[a] Percentages of inhibition of the Aβ generation in the presence of 1 μM of compounds (DMSO = 0%). Detailed experimental conditions are described in Ref. 3b.

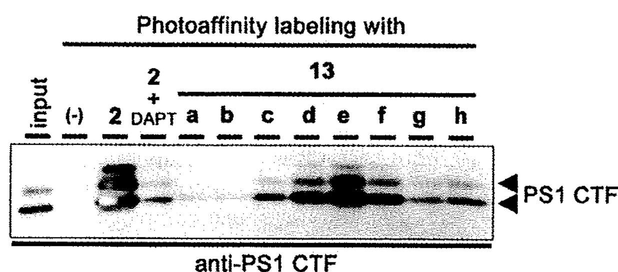


Figure 2. Photoaffinity labeling with probes **13a–h**.

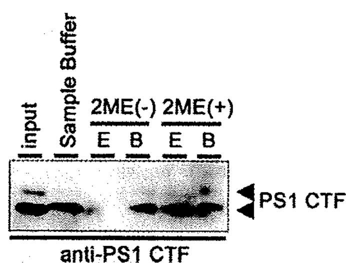


Figure 3. Photoaffinity labeling with probe **13e**. 2ME: 2-mercartoethanol. E and B indicate the eluted proteins and the material remaining on the beads, respectively.

probes (**2** or **13a–h**, 0.1 μM) were irradiated with long-wave near-UV light for 1 h. The biotinylated proteins were then captured with streptavidin sepharose beads. The beads were boiled in buffer containing 2% SDS and 1% 2-mercaptoethanol to liberate the total biotinylated PS1 CTF separated by SDS–PAGE using 12.5% Tris–Glycine gel. Proteins were then transferred on nitrocellulose membrane and detected by western blotting using chemiluminescence method as previously described.[5] The probes **13a–h** labeled PS1 CTF in a similar manner to that by DAP-BpB, while the levels of the biotinylated proteins varied (Fig. 2). No clear relationship between the labeling and the inhibitory activities of the probes was observed. Among them, probe **13e** showed the best labeling ability and was used in further investigations.

After capturing the biotinylated proteins with probe **13e**, the streptavidin sepharose beads were treated in carbonate buffer (pH 9.2) containing 2% SDS in the presence or absence of 3% 2-mercaptoethanol. To reduce non-specific degradation and/or aggregation of the peptide, the cleavage reaction was performed for 4 h at 20 °C. The eluates were analyzed by western blotting (E in Fig. 3). To evaluate the efficiency of the cleavage, the residual beads were subsequently boiled and the biotinylated PS1 CTFs remained on the beads was analyzed (B in Fig. 3). In the presence of 2-mercaptoethanol, the linker was cleaved to elute PS1 CTF from the beads, while no PS1 CTF was detected in the eluates without 2-mercaptoethanol. However, some amount of the biotinylated proteins still remained on the beads. Although the cleavage was expected to occur more efficiently at higher pH, amount of the captured PS1 CTF was lower under these conditions (data not shown). Efforts to optimize the cleavage reaction (i.e., the reaction times, the type of buffer, and the linker length) are currently underway.

In summary, we found that a nitrobenzenesulfonamide-type linker could be cleaved under physiological conditions in the presence of 2-mercaptoethanol. Using this linker unit, we successfully developed photoaffinity probes for the γ-secretase.

Acknowledgements

This work was financially supported in part by a Grant for the 21th Century COE Program, Grants-in-Aid for Young Scientists (S) from Japan Society for the Promotion of Science (JSPS) (T.T.), by the Targeted Proteins Research Program of the Japan Science and Technology Corporation, and Scientific Research on Priority Areas from The Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. N.W. was a research fellow of JSPS.

References and notes

1. For reviews, see: (a) Dorman, G.; Prestwich, G. D. *Biochemistry* **1994**, *33*, 5661; (b) Kotzyba-Hirbrt, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed.* **1995**, *34*, 1296; (c) Tomohiro, T.; Hashimoto, M.; Hatanaka, Y. *Chem. Rec.* **2005**, *5*, 385.
2. Hofmann, K.; Kiso, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3516.
3. (a) Herman, T.; Levy, J.; Shimkus, M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2593; (b) Olejnik, J.; Sonar, S.; Krzymanska-Olejnik, E.; Rothschild, K. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7590; (c) Fang, K.; Hashimoto, M.; Jockusch, S.; Turo, N.; Nakanishi, K. *J. Am. Chem. Soc.* **1998**, *120*, 8543; (d) Li, H.; Liu, Y.; Fang, K.; Nakanishi, K. *Chem. Commun.* **1999**, 365; (e) Fang, S.; Bergstrom, D. *Nucleic Acid Res.* **2003**, *31*, 708; (f) Park, J.; Sadakane, Y.; Masuda, K.; Tomihiro, T.; Nakano, T.; Hatanaka, Y. *ChemBioChem* **2005**, *6*, 814; (g) Verhelst, S. H. L.; Fonovic, M.; Bogyo, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 1284; (h) Bongo, N. B.; Tomohiro, T.; Hatanaka, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 80.
4. Tomita, T. *Expert Rev. Neurother.* **2009**, *9*, 661.
5. (a) Kan, T.; Tominari, Y.; Morohashi, Y.; Natsugari, H.; Tomita, T.; Iwatsubo, T.; Fukuyama, T. *Chem. Commun.* **2003**, 2244; (b) Morohashi, Y.; Kan, T.; Tominari, Y.; Fuwa, H.; Okamura, Y.; Watanabe, N.; Natsugari, H.; Fukuyama, T.; Iwatsubo, T.; Tomita, T. *J. Biol. Chem.* **2006**, *281*, 14670.
6. (a) Fukuyama, T.; Jow, C.-K.; Cheung, M. *Tetrahedron Lett.* **1995**, *36*, 6373; (b) Fukuyama, T.; Cheung, M.; Jow, C.-K.; Hidai, Y.; Kan, T. *Tetrahedron Lett.* **1997**, *38*, 5831; (c) Kan, T.; Fukuyama, T. *J. Synth. Org. Chem. Jpn.* **2001**, *59*, 779; (d) Kan, T.; Fukuyama, T. *Chem. Commun.* **2004**, 353.
7. (a) Kay, C.; Murray, P. J.; Sandow, L.; Holmes, A. B. *Tetrahedron Lett.* **1997**, *38*, 6941; (b) Congreve, M. S.; Kay, C.; Scicinski, J. J.; Ley, S. V.; Williams, G.; Murray, P. J.; McKeown, S. C.; Watson, S. P. *Tetrahedron Lett.* **2003**, *44*, 4153.
8. Kan, T.; Kita, Y.; Morohashi, Y.; Tominari, Y.; Hosoda, S.; Tomita, T.; Natsugari, H.; Iwatsubo, T.; Fukuyama, T. *Org. Lett.* **2007**, *9*, 2055.

For reprint orders, please contact reprints@expert-reviews.com

EXPERT REVIEWS

Secretase inhibitors and modulators for Alzheimer's disease treatment

Expert Rev. Neurother. 9(5), 661–679 (2009)

Taisuke Tomita
Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo, Tokyo 113-0033, Japan
Tel.: +81 358 414 868
Fax: +81 358 414 708
taisuke@mol.f.u-tokyo.ac.jp

Genetic and biological studies provide strong evidence that the deposition of amyloid-β peptide (Aβ) contributes to the etiology of Alzheimer's disease (AD). Aβ is generated from amyloid-β precursor protein by β- and γ-secretases, which are plausible molecular targets for AD treatment. Thus, drugs that regulate the production of Aβ by inhibiting or modulating secretase activity could provide effective therapeutics for AD. Both secretases are transmembrane proteases: β-site amyloid-β precursor protein cleaving enzyme 1, the main neuronal β-secretase, is a single span transmembrane aspartyl protease; γ-secretase is a multiprotein complex comprising four core subunits that are all transmembrane proteins: presenilin, nicastrin, anterior pharynx-defective 1 and presenilin enhancer 2. Molecular biochemical, enzymological and genetic analyses reveal the molecular mechanisms of these secretases in the generation of Aβ. Moreover, extensive drug screening and development have enabled some secretase inhibitors and modulators to advance into late-Phase clinical trials. This review focuses on recent progresses in β- and γ-secretase biology, including the proteolytic mechanism, regulation and composition of these enzymes. Moreover, this review discusses the recent development of inhibitors, and provides a direction for the effective treatment of AD through inhibition/modulation of β- and γ-secretase activities.

KEYWORDS: Alzheimer's disease • amyloid-β protein • amyloid precursor protein • Notch • secretase • lipid raft • inhibitor • modulator • vesicle trafficking

Alzheimer's disease (AD) is a progressive, dementing neurodegenerative disorder affecting over 27 million people worldwide in 2006 [1,2]. As the average age of the population increases, the number of AD patients is expected to quadruple by 2050. This fatal brain disease was first described by German psychiatrist Alois Alzheimer in 1906. He reported the deposition of two abnormal structures, termed senile plaques and neurofibrillary tangles, in the brain of Auguste D, who suffered from dementia. Now these abnormalities are thought to be prime suspects in damaging and killing neurons in affected patients. Senile plaques, which are deposited in the extracellular space of brain parenchyma, primarily contain amyloid-β peptides (Aβ) that are proteolytically produced from amyloid-β precursor protein (APP) (FIGURE 1) [2]. Neurofibrillary tangles are formed within the neuronal cytoplasm and are composed of filaments of hyperphosphorylated forms of the microtubule-associated protein tau [3]. Immunohistochemical studies suggest that the primary pathological event in AD brains is the deposition of Aβ as senile plaques [2]. By contrast, the appearance of neurofibrillary tangles correlates with the neuronal loss [2,3]. Moreover, the deposition of the patients with aggregated tau is observed in brains of other types of dementia (e.g., frontotemporal dementia [FTD]). Thus, both Aβ and tau are thought to be crucial to the etiology of AD and neuronal death, respectively.

Pathomechanisms of AD

Identification and analyses of the genes causative to familial AD (FAD) accelerated the understanding of the molecular pathology of AD [4]. The majority of newly synthesized APP is proteolytically processed by α-secretase to release a soluble large extracellular domain (soluble APPα) and small C-terminal stub (C83). To date, several A disintegrin and metalloprotease domain protein (ADAM) proteases have been found as α-secretase: as TNF-α converting enzyme (TACE or ADAM17), ADAM9 and ADAM10 [5]. However, some fraction of APP is cleaved

10.1586/ERN.09.24
© 2009 Expert Reviews Ltd
ISSN 1473-7175

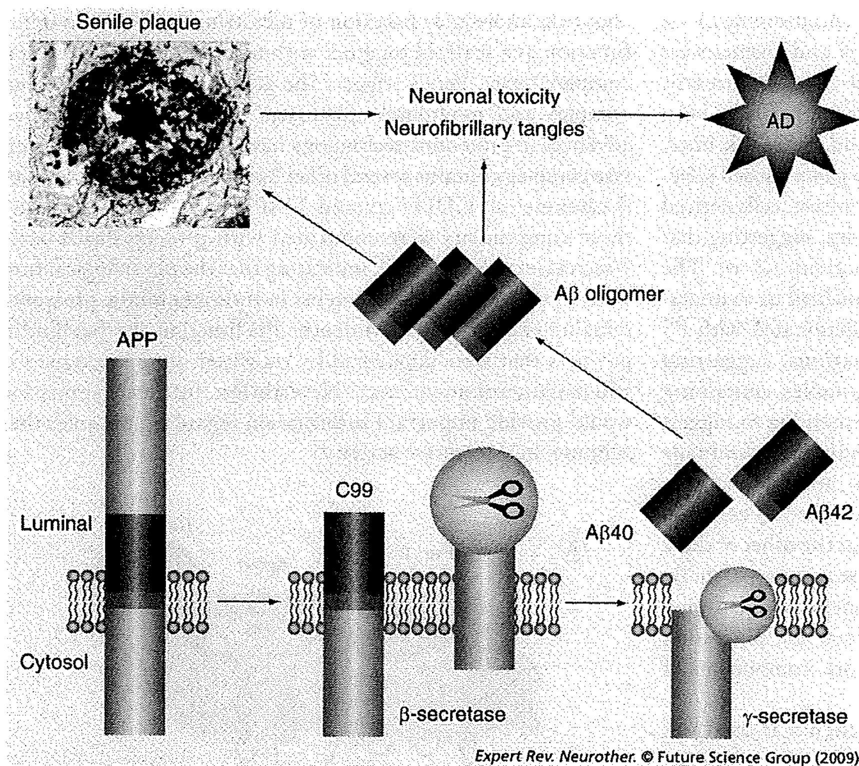


Figure 1. Amyloidogenic processing of APP and the amyloid hypothesis. The ectodomain of APP is first shed by β-secretase. Alternatively, APP can be cut within the Aβ sequence by α-secretase (not shown). The C-terminal stub (C99) is then cleaved by γ-secretase to release Aβ. Aggregated Aβ oligomers form senile plaques and/or mediate neuronal toxicity to cause AD.
Aβ: Amyloid-β peptide; AD: Alzheimer's disease; APP: Amyloid-β precursor protein.

by β-secretase at more distal sites to the membrane to generate soluble APPβ and a longer C-terminal stub, C99. The remaining C-terminal stubs are subsequently cleaved by γ-secretase within the transmembrane domain (TMD). γ-secretase-mediated cleavage of C99 resulted in the generation of Aβ, while C83 is processed to release the shorter nontoxic peptide, p3. The C-terminal length of Aβ generated by γ-secretase is heterogeneous: Aβ40, composed of a 40-amino acid peptide is the major species of the Aβ secreted from cells; while Aβ42 with two more amino acids is a relatively minor molecular species [6,7]. However, Aβ42 is the initially and predominantly deposited Aβ species in AD brains [8], and aggregates much faster than the more common Aβ40 species [9]. Importantly, all genetic mutations in the *APP* gene that cosegregate with affected members in FAD pedigrees increase production of the amyloidogenic Aβ42 species or aggregability of Aβ peptides [2,4]. The Swedish mutation located at most N-terminal sides of Aβ caused a drastic overproduction in Aβ generation by the increase in the β-secretase-mediated cleavage [10,11]. Furthermore, several mutations located near the C-terminus of Aβ caused a selective increase in the generation of Aβ42 by affecting γ-secretase activity [6]. By contrast, point mutations located within the Aβ sequence showed no effect on the production, but increased the aggregability of this amyloidogenic peptide [12]. Finally, genetic studies of gene copy number variation revealed that the duplication of *APP* gene is linked to FAD, suggesting that the levels of aggregated Aβ in the brain is the rate-limiting factor for the onset of AD [13]. By contrast, mutations in the *tau* gene are linked to familial FTD without the deposition of senile plaques [3], supporting the notion, based on pathology, that tau is directly involved in neurodegeneration. These genetic and biochemical data indicate that Aβ is the real molecular culprit that underlies the pathogenesis of AD (the 'amyloid hypothesis') [14].

Molecular identity of the secretases for amyloidogenic processing

Based on the amyloid hypothesis, drugs that can prevent production, aggregation and deposition of Aβ are thought to be promising therapeutics for AD [2,15]. Much attention has been focused on the inhibition or modulation of activities of β- and γ-secretases as disease-modifying therapies based on pathological mechanisms [16–21]. In particular, the amyloidogenic pathway in neurons is initiated by β-secretase cleavage. Thus, the elucidation of the molecular identity of β-secretase had been one of the key issues for the development of AD therapeutics [22,23]. In fact, identification of β-site APP cleaving enzyme (BACE)1 was reported mainly by pharmaceutical companies with different strategies [24–28]. All results led to the same conclusion: a single span transmembrane glycolprotein, BACE1, that harbors an aspartic protease domain at the extracellular side, is responsible for β-secretase activity. Genetic ablation of *BACE1* caused complete loss of Aβ and C99, as well as sAPPβ generation in mouse brains, supporting the notion that BACE1 itself is β-secretase [29–31]. To date, several proteins have been implicated as BACE1-binding partners. However, the recombinant extracellular domain of BACE1 harbors β-secretase activity in *in vitro* assays, implicating that BACE1 functions as a protease by itself. Growing evidence suggests that the levels and/or activity of BACE1 are increased in AD patients, yet no FAD-linked mutation in the *BACE1* gene has been reported [32–35]. A link between BACE1 levels, Aβ load and AD pathology has been reported [36], suggesting that increased BACE1 expression is indeed an important risk factor for sporadic AD. Intriguingly, recent analyses revealed that the levels of BACE1 protein are regulated at multiple levels in the biosynthetic pathway (see later).

Compared with β-secretase, γ-secretase has remained a mystery for a long time, as γ-secretase endoproteolyzes a scissile bond within the hydrophobic membrane-embedded TMD of APP [37]. Historically, clues regarding the molecular identity of γ-secretase were first obtained from genetic studies of FAD patients. After

the finding that mutations in the *APP* gene at chromosome 21 are minor in total FAD pedigree, presenilin (*PS*)*1* and *2* genes were identified as major causative genes for FAD [38–40]. Currently, over 150 point mutations in the *PS1* and *PS2* genes have been linked to FAD [401]. Using cell-based assays and transgenic mice, it was found that FAD-linked mutation in *PS* genes caused selective increase in Aβ42 production [41–45]. By contrast, cells derived from *PS* knockout mice lost γ-secretase activity, suggesting that PS is a pivotal component for γ-secretase activity [46–49]. The development of an *in vitro* γ-secretase assay enabled us to understand that this proteolytic activity was cofractionated with PS polypeptides in high-molecular-weight fractions, suggesting that γ-secretase represents a large protein complex containing PS [50]. Finally, several lines of evidence are emerging to suggest that γ-secretase is a high-molecular-weight, multipass membrane protein complex comprised of PS, nicastrin [51], anterior pharynx-defective 1 (Aph-1) [52,53] and presenilin enhancer 2 (Pen-2) [53] (Figure 2A). Most importantly, ablation of one or the other of these genes resulted in the complete loss of γ-secretase activity [53–61]. By contrast, overexpression of these four components reconstituted γ-secretase activity in heterologous systems [59,62–65], suggesting that these four membrane proteins are the 'core' components of the γ-secretase complex (Figure 2A) [37].

Extensive cellular, molecular and biochemical analyses revealed that PS functions as a catalytic center of γ-secretase, the two intramembranous aspartates within TMD6 and 7 of PS being indispensable for proteolytic activity [66]. Sequence alignments demonstrated that these aspartates are highly conserved among species. Moreover, a γ-secretase inhibitor (GSI) carrying the transition-state analogue moiety directly targeted the PS polypeptides [67,68]. Finally, a discovery of signal peptide peptidase (SPP), which also cleaves membrane-embedded polypeptides with the catalytic aspartates within TMDs [69], enabled classification of these atypical proteases as 'GxGD-type' intramembrane-cleaving aspartic protease [70]. To date, other intramembrane-cleaving proteases with different catalytic sites (i.e., site-2 protease [S2P] as metalloprotease and rhomboid as serine protease) have also been identified [71,72]. These discoveries established the existence of an intramembrane cleaving protease-mediated cellular signaling mechanism termed regulated intramembrane proteolysis [73,74].

In contrast to PS, the functional roles of the other components still remain obscure. Nicastrin may play a role in substrate recognition as an exosite of γ-secretase: its ectodomain can directly interact with the N-terminus of γ-secretase substrates [75], although this view has been challenged [76]. Rather, cell biological analyses indicate that the conformational maturation of the extracellular domain of nicastrin is one of the major determinants for the stabilization and activity of the γ-secretase complex [77]. We recently developed a novel monoclonal antibody targeting the functional conformation of the extracellular domain of nicastrin (patent number WO2007129457 [301]) [78]. Intriguingly, this antibody is able to inhibit γ-secretase activity *in vitro* as well as *in vivo*. Identification of the modes of action of this neutralizing antibody will provide important information about the molecular function of nicastrin. Aph-1 is thought to function as a scaffold together with nicastrin for the γ-secretase complex [79,80]. Pen-2 triggers the activation of the γ-secretase complex and modulates enzymatic activity [59,81]. Moreover, advances in proteomic technology have revealed that the γ-secretase complex contains several other binding partners (e.g., α- and β-catenins [51], CD147 [82] and TMP21 [83]). However, none of these components were copurified with proteolytically active γ-secretase complex [84,85], indicating that the physiological functions of these 'accessory' proteins in intramembrane proteolysis remain to be elucidated. Moreover, the function of other binding partners that were copurified by transition-state analogue GSI still remain unknown [86,87]. Nevertheless, further examinations would provide important information regarding the roles these subunits in γ-secretase activity.

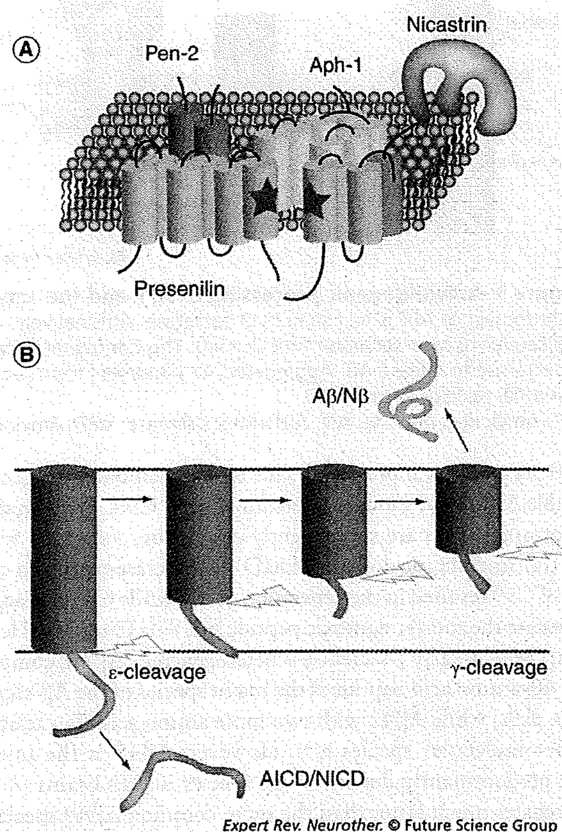


Figure 2. Schematic depiction of the γ-secretase complex and a model of intramembrane cleavage. (A) The core complex of γ-secretase is comprised of Presenilin, Nicastrin, Aph-1 and Pen-2. Presenilin harbors catalytic aspartates (stars) within its transmembrane domains. **(B)** γ-secretase executes intramembrane proteolysis of several single-span membrane proteins, including amyloid β precursor protein and Notch proteins to generate Aβ/AICD and Nβ/NICD, respectively. After ε-cleavage occurred to liberate AICD/NICD, successive cleavage within the transmembrane domain renders to release Aβ/Nβ peptides.
Aβ: Amyloid-β peptide; AICD: Amyloid-β precursor protein intracellular domain; Nβ: Notch-β peptide; NICD: Notch intracellular domain.

Lessons from genetic models for AD therapeutics

The identification of *BACE1* as the protease required for Aβ production in the brain was met with great enthusiasm, particularly when the original analysis of BACE1 knockout mice did not reveal any undesired phenotype with complete loss of brain Aβ [29–31]. However, this enthusiasm has been challenged by thorough analyses of these genetic models. Several investigators identified that *BACE1* knockout mice showed cognitive deficits [88–92], premature death [93] and hypomyelination [94,95]. The hypomyelination phenotype in particuar was caused by a reduction in proteolytic processing of neuregulin-1, the other substrate of BACE1, as well as γ-secretase [96,97]. Moreover, recently dysregulation of neuregulin-1 processing has been shown to cause schizophrenia-like behavioral traits in *BACE1* knockout mice [97,98]. These data suggest that complete inhibition of BACE1 activity might increase the risk for psychological disorder as an adverse effect. However, pharmacological study has suggested that the modulation of BACE1 activity in adults without affecting neuregulin-1 is possible [99]. Moreover, amyloid deposition in the brains of APP transgenic mice carrying a BACE1$^{+/-}$ genotype was significantly decreased [100], indicating that a partial inhibition of BACE1 activity might have a benefit for AD therapeutics.

All FAD-linked mutations in *PS* genes examined so far increased the ratio of Aβ42 that comprises total Aβ. This effect was first observed in human patients [41]. Later, the same effect was observed in *in vitro*, as well as *in vivo*, experimental settings [42–45]. Finally, generation of compound mice expressing FAD-linked *APP* and *PS* genes resulted in an accelerated deposition of amyloid plaques in mice brains [42–44]. By contrast, *PS* knockout in mice brains caused the loss of production of Aβ peptides [46–49]. These data strongly support the amyloid hypothesis: the pathogenic role of Aβ42 and PS/γ-secretase in the deposition of amyloid plaques in brains, thereby causing AD.

Moreover, genetic models provided us with unexpected findings regarding the physiological function of γ-secretase. At first, *PS* mutant *Caenorhabditis elegans* showed significant developmental defects related to Notch signaling [101]. Subsequently, Notch signaling defects were reported in *PS* mutant mice and *Drosophila* [102,103]. The genetic significance of all γ-secretase components in Notch signaling has been found in all genetic models examined so far [53–61,102,103]. Finally, GSI treatment in animals showed a phenocopy of the genetic inhibition of Notch signaling [104–111]. Biochemical studies suggested that Notch, a single-span membrane receptor, is directly cleaved by γ-secretase activity within its TMD (Figure 2B) [112]. The Notch signaling pathway plays a variety of roles in cell fate decisions during development [113,114]. Upon ligand binding from neighboring cells, Notch receptors are proteolytically cleaved by an ADAM/TACE family metalloprotease followed by PS-dependent γ-secretase cleavage to release the Notch intracellular domain (NICD) [115,116]. NICD then translocates into the nucleus and activates transcription of its downstream targets. Up until now, over 50 single-span membrane proteins have been reported as substrates for γ-secretase activity. Some substrates, including Notch, utilize intracellular domains (ICDs) as a transcriptional regulator [117,118]. Notably, the overexpression of FAD-linked mutant human *PS1* rescued the majority of the Notch-related phenotype in *PS1* knockout mice [119], suggesting that the most apparent and conserved 'physiological' function of γ-secretase activity is an essential Notch signaling component. Intriguingly, loss of *PS* genes in mouse brains resulted in the loss of dendritic spines and brain atrophy [120,121]. In addition, some FAD-linked PS mutations decreased the generation of the ICDs and some point mutations on *PS* genes linked to FTD have been found [401]. These data suggest that γ-secretase activity has a seminal role in synaptic maintenance irrespective of Aβ deposition. Importantly, γ-secretase cleaves multiple neuronal substrates to transduce their signals (e.g., ErbB4, p75NTR neurotrophin receptor, N-cadherin and sodium channel β4 subunit) [117,118]. Moreover, recently it was found that FAD-linked mutant *PS1* impairs adult neurogenesis in transgenic mice [122–124]. Nevertheless, the pathological significance of FAD-linked PS mutations on AD through these substrates still remains unknown [125–127].

Development of β-secretase inhibitors

β-secretase is a membrane-bound protease with motifs containing the highly conserved signature sequence of aspartic proteases, D(T/S)G(T/S), within which the aspartic acid residue is essential for proteolytic activity. The crystal structure of BACE1 confirms that the active site is comprised of a long cleft for substrate recognition located between the N- and C-terminal lobes of BACE1 [128]. The side chain pockets located at the surface of the cleft interact with amino acid residues of the substrates, resulting in hydrolysis. Moreover, pH-dependent conformational switching occurs in the active site cleft [129]. These structural features are used for the 'rational design' of β-secretase inhibitors (BSIs) (Figure 3) [17,130]. The first generation of BSIs were designed as transition-state analogues for the aspartyl protease (e.g., OM99-2) (Figure 3 [1]) [128]. In fact, to date, the majority of commercially available potent BSIs are peptide-based compounds containing a transition-state moity (e.g., hydroxyethylene, statin and hydroxymethylcarbonyl isostere). Based on the structural information of BACE1 and transition-state analogue binding, extensive structural modifications aiming at size reduction were performed. Finally, Tang's group at the University of Oklahoma reached a new generation of inhibitors containing hydroxyethylamine isostere and isophthalamide moiety, GRL-8234 (Figure 3 [2]) [131]. This compound potently inhibited BACE1 activity *in vitro* as well as *in vivo*. Kiso's group at Kyoto Pharmaceutical University also generated a small potent BSI containing a 2,6-pyridinedicarboxylic, chelidamic or chelidonic residue at the P_2 position together with hydroxymethylcarbonyl isostere (e.g., KNI-1027) (Figure 3 [3]) [132].

Considering the usage of BSIs as drugs, making less peptidic compounds is mandatory to obtain sufficient oral absorption and penetration of the BBB. In fact, these two issues have hampered progress in development of BSIs for AD therapeutics for a long time. After extensive structure–activity relationship analyses, a hydroxyethylamino isostere-based inhibitor, GSK188909 (Figure 3 [4]), was described as the first orally bioavailable, nonpeptidic BSI that is able to reduce brain Aβ levels in APP transgenic mice [133]. Importantly, a P-glycoprotein inhibitor is required for a

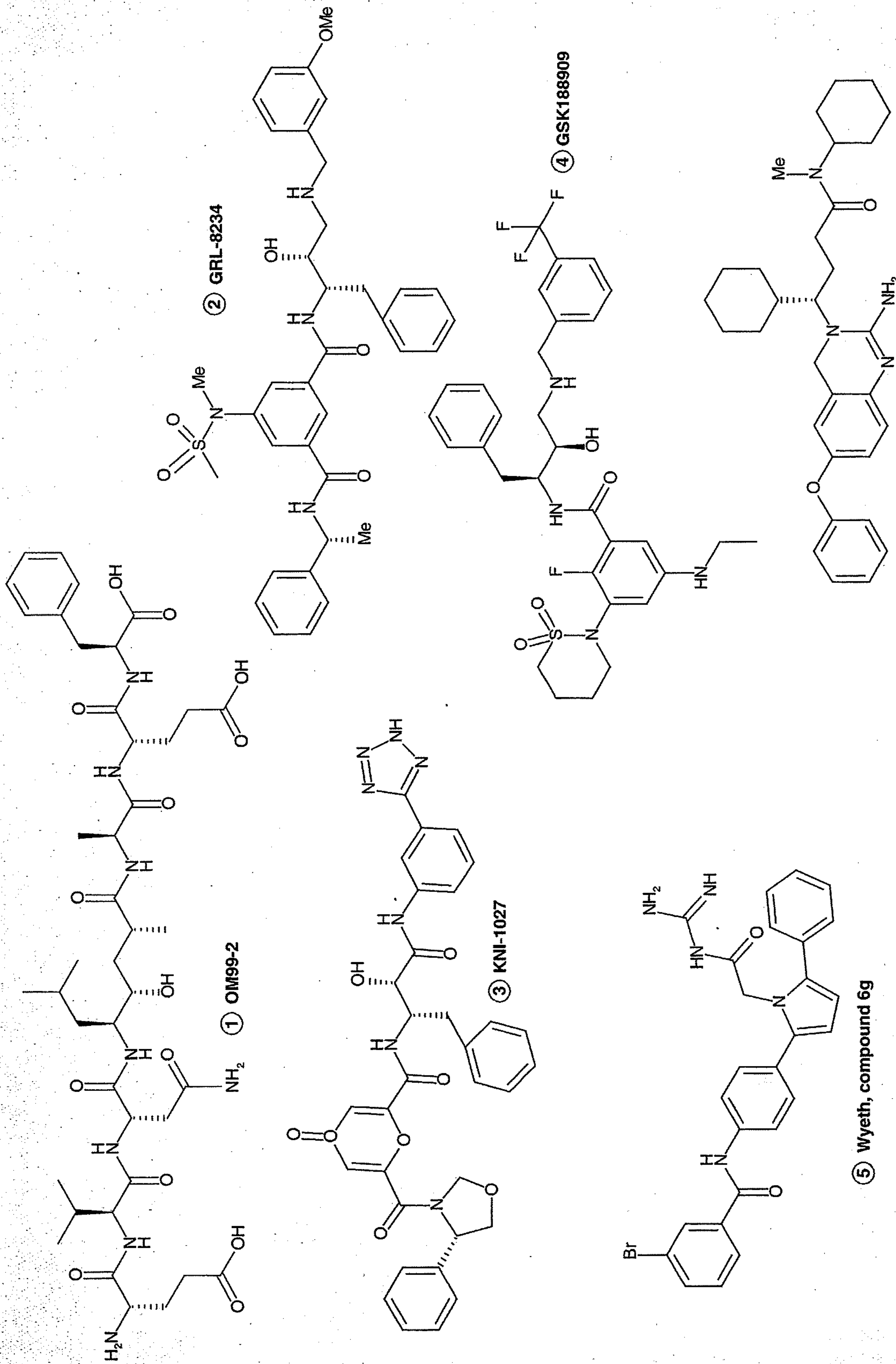


Figure 3. Chemical structures of β-secretase inhibitors.

significant reduction in the levels of Aβ in the brain in an acute oral administration paradigm. However, encouragingly, subchronic dosing of GSK188909 in the absence of a P-glycoprotein inhibitor also lowers brain Aβ. Moreover, high throughput screening using a large compound library by Wyeth led to the discovery of a novel, low-molecular-weight acylguanidine-type BSI (Figure 3 [5]) [134]. The cocrystal structure revealed that the acylguanidine moiety forms hydrogen bonds with the catalytic aspartates of BACE1. Moreover, this inhibitor stabilizes the enzyme in an open conformation, while most peptidomimetic BSIs bind BACE1 in a closed-flap form. Other library screening by Johnson & Johnson also revealed that aminoquinazoline-type compounds (Figure 3 [6]) are able to function as BSIs in a similar manner to that of acylguanidine-type BSIs [135]. Nonetheless, significant advances have been made in the last few years for development of BSI. Most importantly, a drug candidate BSI, CTS-21166, developed by CoMentis and Asteras Pharma has completed a Phase I clinical trial (clinicaltrials.gov identifier NCT00621010).

Development of γ-secretase inhibitors & modulators

Despite the intricacy of γ-secretase and its proteolytic mechanism, several cell-permeable GSIs have been identified by cell-based, high-throughput screening and combinatorial chemistry (Figure 4) [18–21]. Some of the potent peptidic inhibitors include aspartyl protease transition-state mimic moieties (e.g., L-685,458, 31C-III; Figure 4 [7] & [8], respectively), which directly targets the active site of the enzyme [67,68]. They block the cleavage of not only APP but other substrates, such as Notch. However, these transition-state analogues are used only for the discovery stage because of instability and inefficacy in *in vivo*. Importantly, discovery and chemical biological application of these transition-state analogues led to the conclusion that PS is a catalytic subunit in the γ-secretase complex, that is aspartyl protease. Dipeptidic GSIs (e.g., N-[N-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester [DAPT]; Figure 4 [9]), the potent and cell-permeable compounds, also inhibit Notch signaling. In fact, administration of potent GSIs (e.g., compound E [Figure 4; 10] and LY411575 [Figure 4; 11]) caused significant thymic and gastrointestinal toxicity in mice and rats [104,105,109–111,136]. However, upon extensive investigations by organic chemistry and pharmacological analyses, one of the dipeptidic compounds, called LY450139 (Semagacestat) (Figure 4 [12]) was established by Eli Lilly [137–140]. LY450139 is a potent GSI that shows a good pharmacokinetic profile *in vivo*. LY450139 is now is being tested in a Phase III study (the Interrupting Alzheimer's Dementia by Evaluating Treatment of Amyloid Pathology [IDENTITY] trial: clinicaltrials.gov identifier NCT00762411) to see if it can slow the progression of amyloid pathology and neurodegeneration.

The other clue for the modulation of γ-secretase activity by small compounds was recognized from epidemiological studies. A population-based cohort study provided strong evidence that the long-term use of NSAIDs significantly reduced the risk of AD [141]. Disappointingly, a placebo-controlled, randomized trial using naproxen, rofecoxib and cerecoxib, which are US FDA-approved NSAIDs (the Alzheimer's Disease Anti-Inflammatory Prevention Trial [ADAPT]: clinicaltrials.gov identifier NCT00007189), revealed that the anti-inflammatory effect of NSAIDs is not sufficient for the treatment of AD [142,143]. However, molecular and biochemical study revealed that a subset of FDA-approved NSAIDs (e.g., sulindac sulfide (Figure 5 [13]), ibuprofen and indomethacin) directly modulate γ-secretase activity to selectively decrease the secretion of Aβ42 accompanied by an increase in Aβ38 generation, whereas the generation of ICDs was not significantly affected [144–148]. Such compounds are now called γ-secretase modulators (GSMs) (Figure 5) and are expected to be useful as therapeutics for AD without any Notch-based adverse effects. Interestingly, some NSAID- and lipid metabolism-related compounds (e.g., fenofibrate; Figure 5 [14]) caused a significant increase in Aβ42 levels accompanied with a decrease in Aβ38 generation [149,150]. These compounds are called Aβ42-raising GSMs or inverse GSMs. Structure and activity relationship analyses indicate that small modifications to the GSM reverses its character to an Aβ42 raiser, implicating very stringent regulation in the mode of Aβ38 and Aβ42 generation [151,152]. The NSAID-derived Aβ42-selective lowering reagent, *R*-Flurbiprofen (Tarenflurbil) (Figure 5 [15]) [145,146,153] was tested in a Phase III trial (clinicaltrials.gov identifier NCT00322036) by Myriad pharmaceuticals. However, recently this trial was terminated because the drug failed to achieve significance in this study, and the development of this compound was discontinued. However, there are several upcoming potent orally available GSM candidates: CHF5074 (Figure 5 [16]) [154], NGX series compound (Figure 5 [17]) by TorreyPines Therapeutics and GSM-1 (Figure 5 [18]) by Merck [155].

γ-secretase inhibitors with substrate or γ-cleavage site specificity (i.e., second-generation GSIs) might be good candidates for AD drugs that overcome the adverse effects associated with first-generation GSIs *in vivo* (Figure 6). Several pharmaceutical companies published patents regarding compounds containing the sulfonamide moiety as GSIs. The sulfonamide derivative BMS-299897 (Figure 6 [19]) inhibited γ-secretase activity *in vitro* as well as *in vivo* [156], and did not compete with transition-state analogues [157–159], suggesting that sulfonamide-type compounds directly affect the γ-secretase complex at a distinct site from those in which transition-state analogues interact. Notably, BMS-299897 showed no effect on T-cell differentiation, while it reduced Aβ levels in the brains and plasma of transgenic models [110,156], and as a result was classed as a Notch-sparing GSI. Thus, the molecular target/mechanism of Notch-sparing GSIs might make them good candidates for AD treatment. Recently, the other sulfonamide-type Notch-sparing GSI, GSI-953 (Figure 6 [20]) (Begacestat), was developed by Wyeth [160,161], and they announced that they have started Phase I studies (clinicaltrials.gov identifiers NCT00441987, NCT00479219 and NCT00547560).

Alternative approaches for modulation of β- & γ-secretase activities

As BACE1 cleaves APP at the extracellular side, antibody-based novel therapeutic interventions were reported. Administration or overexpression of antibodies against the β-secretase cleavage site of APP caused a significant reduction in Aβ generation [162,163]. These antibodies masked the cleavage site at the ectodomain of APP and caused a steric hindrance of APP–BACE1 recognition.

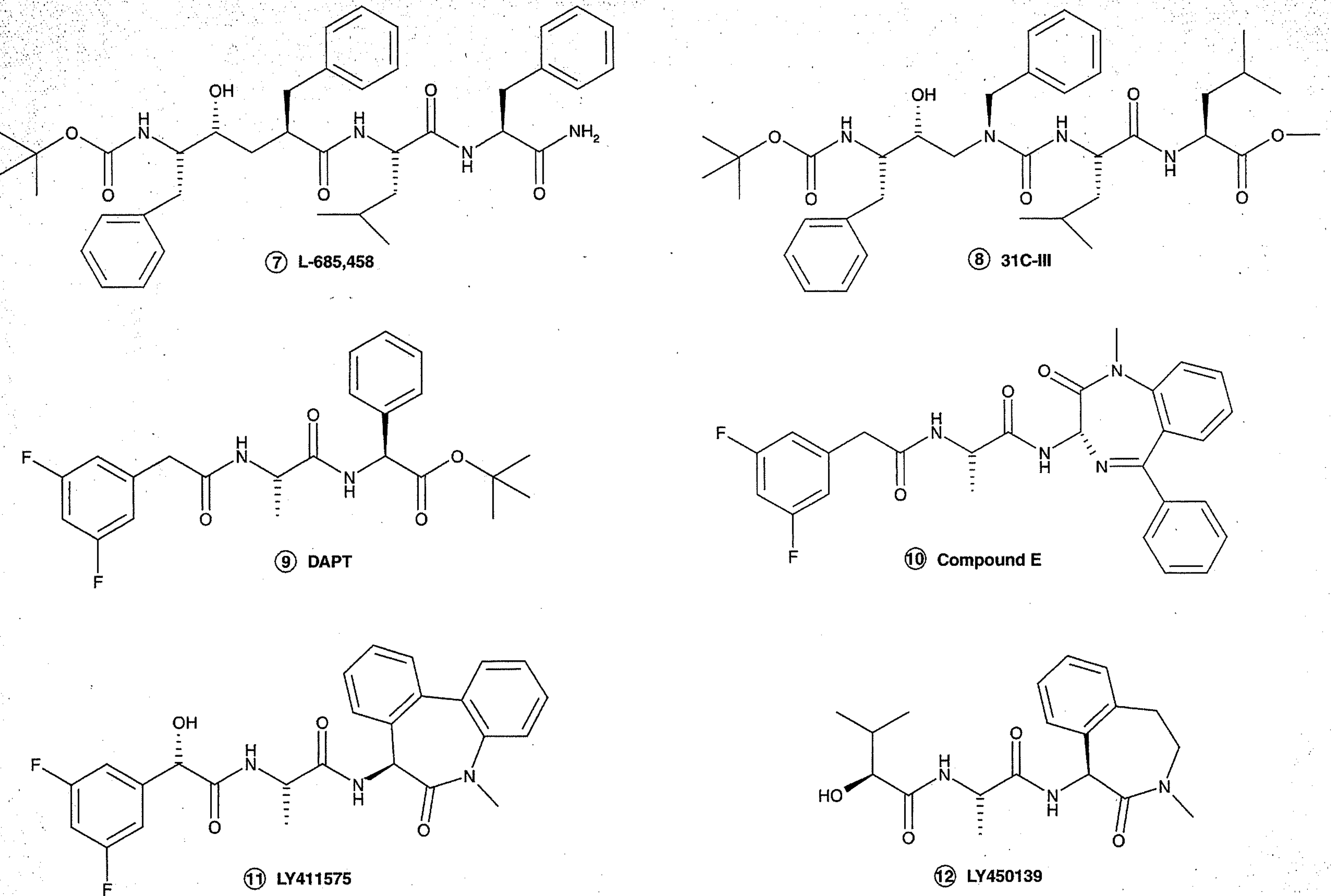


Figure 4. Chemical structures of first-generation γ-secretase inhibitors.

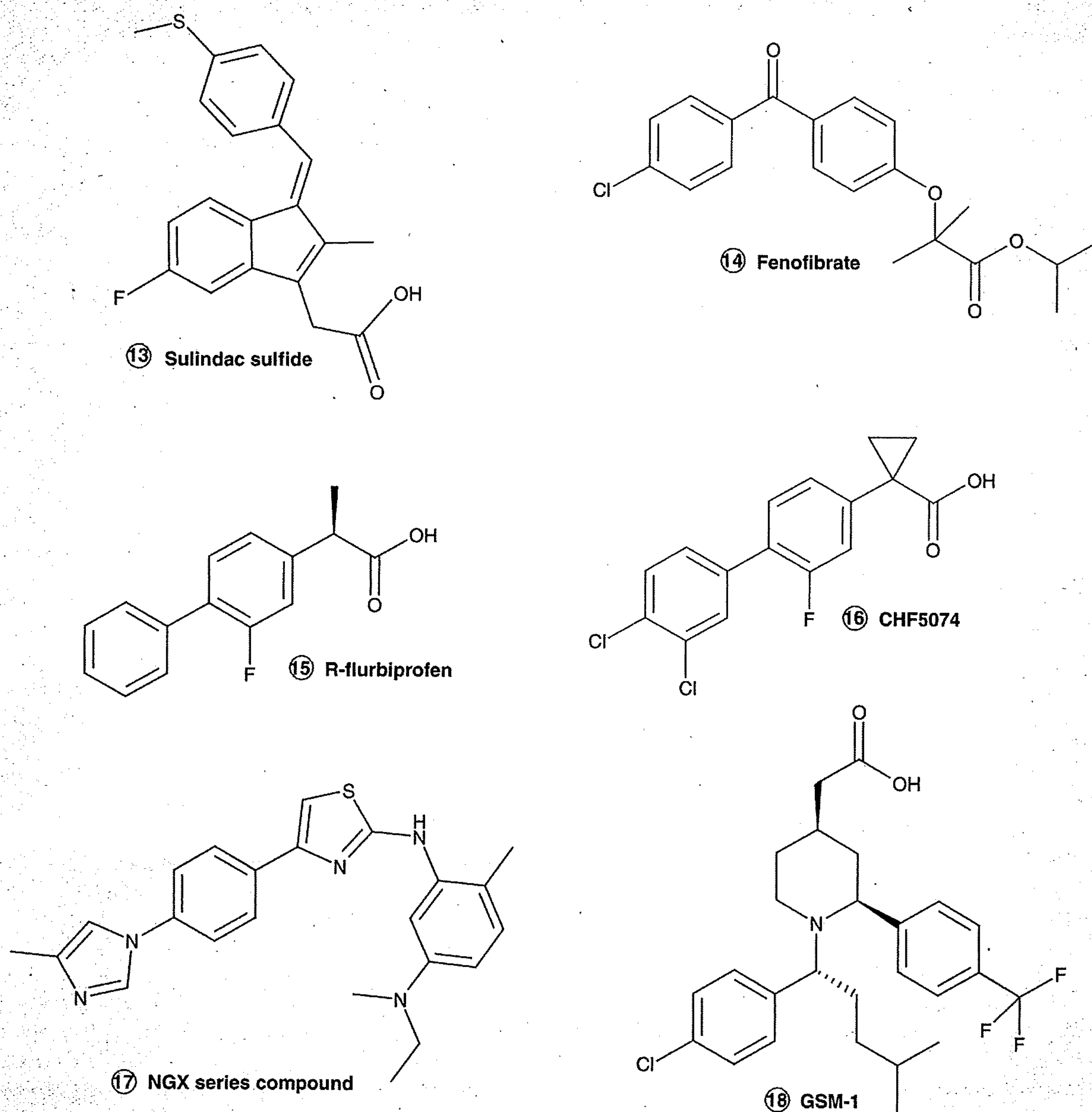


Figure 5. γ-secretase modulators.

Success of this strategy suggests that a compound targeting the β-secretase cleavage site may have a therapeutic benefit for AD. Moreover, Tang's group reported that immunization of BACE1 recombinant protein in an AD mouse model reduced Aβ generation and deposition of amyloid in the mice brains [164]. The neutralizing antibody in this approach specifically bound to BACE1 and serves as a BSI. Identification of the modes of action of this functional antibody would provide novel molecular information regarding the inhibition or modulation of β-secretase activity.

Pathologically, BACE1 protein levels and/or activity have been found to be increased in sporadic AD patients [32–36], while the number of neurons that are responsible for BACE1 expression is low. Thus, direct reduction of BACE1 protein is one of the plausible approaches for therapeutics. The mechanisms by which the expression levels of BACE1 are increased in the AD brain still remain obscure. Importantly, the expression of BACE1 protein is regulated by multiple biological processes in several cell/animal models. In the translation process, energy deprivation induces phosphorylation of the initiation factor eIF2α, which increases translation of BACE1 mRNA and elevates Aβ production [165]. In addition, noncoding RNAs, including BACE1 antisense transcript and several miRNAs, are reported as positive/negative regulators for BACE1 expression via the control of mRNA levels or translation of BACE1 [166–169], suggesting this complicated mechanism

would work in the regulation of BACE1 protein levels. Contrary to BACE1, little contribution of the translational/transcriptional system on the γ-secretase complex is reported. Biochemical studies indicate that γ-secretase is a highly stable protein complex (half-life > 48 h) [170,171]. Moreover, augmentation of γ-secretase activity requires simultaneous overexpression of the four core subunits [59,62–65]. In fact, overexpression of *PS* resulted in the accumulation of PS holoprotein, an inactive form, as the levels of other subunits are limited [172]. Finally, cells derived from compound knockout mice $PS1^{+/-}$ and $PS2^{-/-}$ showed similar levels of γ-secretase activity to that from wild-type mice, suggesting that a single *PS1* gene might be enough for full activity [48,49,173,174]. However, the challenge would shed light on the way to discover a novel molecular target for the effective reduction of Aβ in the brains.

Each of BACE1, γ-secretase and APP extensively undergo intracellular trafficking during APP proteolysis [175,176]. Essentially, acidic pH is the optimal condition for BACE1. Thus, the endocytic process of APP and BACE1 would be required for β-secretase cleavage. This endocytic process of BACE1 required the participation of a family of adaptor Golgi-localizing, γ-adaptin ear homology domain, ARF-binding (GGA) proteins, and reduction of the GGA proteins caused the increase in Aβ production [177]. By contrast, the subcellular localization of functional γ-secretase complex remains unknown. Initial reports using an overexpression paradigm of PS raised a lengthy argument called 'spatial paradox'; overexpressed PS mainly localized at the endoplasmic reticulum, whereas γ-secretase-mediated cleavage would occur distal to this organelle [178]. However, after the identification of nicastrin, which is heavily glycosylated and detected at the cell surface [179], it is now widely accepted that the major functional form of the γ-secretase complex localizes at late secretory and endocytic pathways [180]. Of note, γ-secretase activity showed a distinct cleavage profile at neutral and acidic pH in an *in vitro* assay [181]. In addition, recently, the orphan G protein-coupled receptor (GPR)3 was identified as a genetic modulator of Aβ production [182]. The overexpression of *GPR3* caused an increase in the formation and cell-surface localization of the mature γ-secretase complex without an effect on Notch processing. Thus, subcellular trafficking machinery might modulate the proteolytic activity of the γ-secretase complex.

Moreover, biochemical analyses revealed that APP, BACE1 and γ-secretase were fractionated in lipid raft fractions [183]. The lipid raft is a specialized membrane microdomain that is rich in cholesterol and sphingolipids, and participates in a variety of important biological functions [184,185]. Growing evidence suggests that lipid rafts might be the principal membrane domains in which amyloidogenic processing of APP occurs [175]. Thus, regulating the trafficking mechanisms that enable APP, BACE1 and γ-secretase to localize lipid rafts might provide a novel approach for the reduction of Aβ levels. Recently, it was found that the syntaxin1-X11/Mint-Munc18 axis is involved in the localization of APP in lipid rafts [186]. Moreover, the metabolism of cholesterol, which is an important component of lipid rafts, seems to be involved in the regulation of Aβ generation via direct effects on the amyloidogenic processing. Nevertheless, identification of the components involved in the elusive trafficking mechanisms as well as lipid metabolism responsible for raft localization of APP, BACE1 and γ-secretase could provide a strong impetus for the development of novel therapeutics based on the targeting of APP processing.

As the nonamyloidogenic pathway by α-secretase precludes Aβ formation, an upregulation of α-secretase activity is an alternative strategy to decrease Aβ levels in the brain. In fact, overexpression or stimulation of ADAM10 in mice successfully decreased the Aβ deposition in the brain [187]. Moreover, recently etazolate, a selective $GABA_A$ receptor modulator, was reported as a stimulator for α-secretase activity in neurons [188]. Cell biological study indicates that etazolate showed a neuroprotective effect through increased production of sAPPα. Clinical development of this compound is now underway by ExonHit Therapeutics (Paris, France).

Expert commentary: rational design of GSIs/GSMs

Despite a growing understanding of PS biology, critical questions still remain: how does PS select and transfer the substrates into its active site? How does PS 'hydrolyze' a peptide bond within a hydrophobic environment? How do GSIs/GSMs affect γ-secretase activity to alter Aβ production? All these fundamental questions about intramembrane proteolysis should be answered for rational design of GSIs/GSMs. Biochemical analyses using cell-based and *in vitro* assays provide important

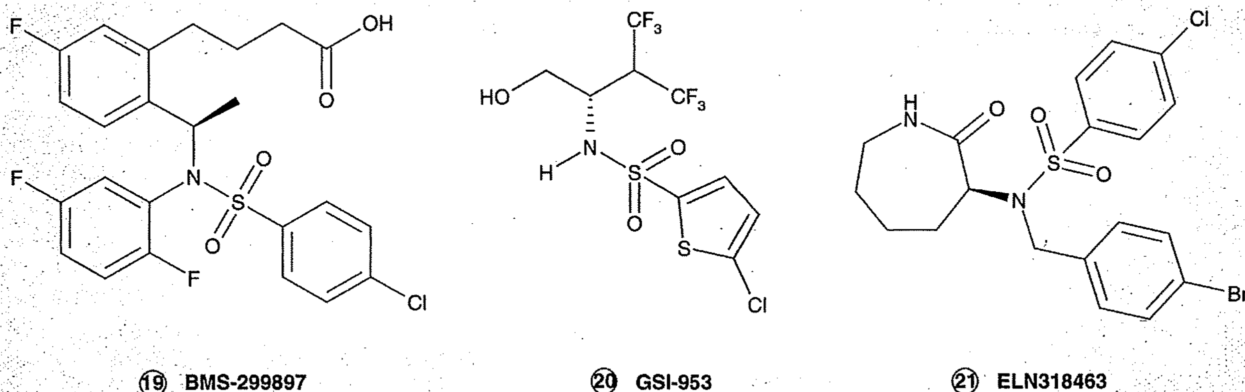


Figure 6. Second-generation γ-secretase inhibitors.

information about the nature of this atypical cleavage. The first finding was that most N-terminals of NICD correspond to an intramembrane site proximal to the cytoplasmic side [115]. Next, APP ICD (AICD) generation from APP was confirmed in a similar manner to that in Notch [189–192]. However, the cleavage site for AICD, called the ε-cleavage site, also locates near the cytoplasmic region where it is distal to the C-terminus of Aβ. Subsequently, γ-secretase cleavage of Notch near the middle of the TMD to release Aβ-like fragments (Notch β) was found, suggesting that both APP and Notch are cleaved at γ- and ε-cleavage sites with different topologies [193]. Genetic and pharmacological analyses indicated that both cleavages are executed by γ-secretase. However, it was very difficult to envision the molecular mechanism of this dual cleavage by a single enzyme, as there is no homology of primary sequence around the γ- and ε-cleavage sites. Key to this unifying idea on dual cleavage is the finding that γ-secretase apparently cuts at the ε-cleavage site first, to produce either a 48- or 49-residue Aβ [194,195]. Subsequent cuts every 3–4 residues (i.e., every helical turn of the substrate) ultimately produce the 38–43 residue Aβ peptides that are secreted from the cell. This mechanism is now conceived as the 'tripeptide hypothesis' of γ-secretase-mediated cleavage (Figure 2B), while the successive model of intramembranous cleavage remains suppositional. Intriguingly, recent biochemical analyses revealed that SPP and SPP-like proteins (SPPLs), that are crossinhibited by a transition-state analogue type GSI, also cleaved at multiple scissile bonds in the substrate [196,197]. This cleavage profile was affected by GSM as well as point mutations in SPP at the conserved residues corresponding to the FAD-linked mutations in PS [196,198,199]. These data indicate that endoproteolysis at several positions in the substrate TMD might be one of the enzymatic features of GxGD-type intramembrane-cleaving aspartic proteases.

Both γ-secretase and SPP showed a broad spectrum for the substrate; no requirement in the primary amino acid sequence was identified [117,118,200–202]. However, existence of helix-breaking residues is required for SPP-mediate cleavage [201]. In addition, helix-dimerization motifs (i.e., GXXXG) significantly affected the property of the γ-secretase cleavage [203–205]. Importantly, the substrate specificities of rhomboid and S2P, the intramembrane-cleaving serine- and metalloproteases, respectively, were also governed by helix-breaking residues [206,207]. These data suggest that the structural conformation of the substrate TMD plays an important role endoproteolysis by intramembrane proteases. However, γ-secretase activity failed to process the substrate for rhomboids in an *in vitro* assay [208]. Moreover, APP processing in cells lacking S2P was unaffected [209]. Finally, sensitivities against protease inhibitors are dependent on the type of catalytic site, suggesting that the cleavage specificity of the intramembrane-cleaving proteases is determined by the structural codes of both substrates and enzymes. Nevertheless, such basic issues require continued attention to acquire a deeper understanding of the various physiological and pathophysiological events mediated by γ-secretase and the other intramembrane-cleaving enzymes.

Identification of the target protein/domain of the GSIs/GSMs by chemical biological strategy would shed light on the molecular function of the subunits in the catalytic process. Photoaffinity probes based on the transition-state analogue type GSI directly targeted PS [67,68], facilitating our understanding that PS itself is a catalytic subunit of the γ-secretase complex. Importantly, the transition-state analogue type GSI copurified the substrate with γ-secretase [86]. Together with enzymological and pharmacological studies [157,158], it is now conceived that PS harbors sites for substrate binding, substrate entry and proteolysis [18,37]. Additionally, a peptide-based approach revealed that TMD1/2 region [210] and the C-terminus of PS, the latter of which is indispensable for γ-secretase complex formation [211–213], have been implicated in the binding of the substrate (patent number WO2004026331) [302]. Understanding the mechanism of substrate recognition and the capture system of γ-secretase is an important issue, as rational development of substrate-specific GSIs is needed for AD therapeutics. It has been demonstrated that the extracellular domain of nicastrin binds to the N-terminus of membrane-bound substrates [75]. However, nicastrin itself has no proteolytic activity. Rather, nicastrin aids substrate recognition for eventual proteolysis by PS as an exosite, although this view has been challenged [76]. Recent genetic analysis using the reconstitution system in yeast also supported an accessory, but not an essential role of nicastrin in γ-secretase-mediated cleavage [214]. Nevertheless, the identification of a γ-secretase neutralizing antibody that targets the extracellular domain of nicastrin suggests that nicastrin might be a novel target for the modulation of γ-secretase activity. Aib-containing helical peptide, which is a TMD mimetic, is able to inhibit γ-secretase activity [215]. Photoaffinity labeling experiments using Aib-containing photoprobe revealed that PS1 N-terminal fragment (NTF) and C-terminal fragment exhibit the helix-binding region, which seems to be a substrate recognition domain in PS [216]. We recently found that a β-peptide-based foldamer that mimics α-helix structure also functions as a potent GSI and targets PS, supporting the notion that γ-secretase recognize the substrate structure (Imamura Y, Watanabe N, Umezawa N *et al.*, Unpublished Data). Further fine protein chemical analysis of the substrate recognition domain might provide useful information for the development of substrate-specific GSIs/GSMs.

We have found that dipeptidic-type GSIs also directly targeted PS [217,218]. Pharmacological experiments suggest that dipeptidic-type GSI inhibits the substrate entry process from the hydrophobic to the hydrophilic environment within the lipid bilayer; the latter corresponds to the catalytic site [108,157–159,216,219]. Intriguingly, even among the dipeptidic-type GSI, DAPT directly targeted the PS C-terminal fragment, but compound E and dibenzazepine bound to PS NTF, while the binding of these compounds completely competed [218]. In addition, pharmacological analyses of these compounds revealed that DAPT is a specific inhibitor for γ-secretase, but compound E and dibenzazepine are able to cross-inhibit SPP and SPPLs [219–221]. No adverse effect caused by the inhibition of the SPP family by

treatment of GSI *in vivo* have thus far been reported. However, proteolytic activities of the SPP family are involved in immune response signals [222], viral and parasite infection [223,224], and inflammatory responses [197,225]. Moreover, SPP mutant animals showed developmental defects [226,227]. Structural analysis of the γ-secretase complex and SPP with these dipeptidic inhibitors would be needed in order to understand the molecular basis of the enzyme specificity.

Currently, much attention is focused on the molecular mechanisms of Notch-sparing GSI. It was reported that sulfonamide-type GSI with different structures (BMS-299897 and ELN318463 [Figure 6; 21]) showed different inhibitory potencies against PS1- and PS2-containing γ-secretase complex, while L-685,458 and DAPT inhibited at similar potencies [228]. Considering the results of competition assay [157–159] and photoaffinity labeling experiments [217,218], sulfonamide-type GSI also targets PS in a similar manner to that by transition state analogues and dipeptidic-type GSI, but the molecular effect(s) might be different. In particular, the PS1-specific property of Notch-sparing GSIs seems to be important for avoiding Notch-based adverse effects. This specificity depends on the primary sequence in TMD3 and the exon 8 region of PS1 [228]. However, the physiological function of this region in the catalytic process still remains unknown. We have developed a sulfonamide-based photoprobe [229] and also found that this compound directly targets PS1 NTF. Investigation of the precise effect of the compound on catalytic processes is now underway.

Finally, recent progress indicates that NSAID-type GSMs as well as an Aβ42-raising GSM, fenofibrate, directly target APP (substrate targeting GSM [stGSM]) [151]. Notably, stGSM interaction was localized to residues 28–36 of Aβ, a region critical for Aβ42 generation as well as the aggregation profile of the peptide [203–205,230]. The compounds known to interact with this region (i.e., Aβ aggregation inhibitors) act as GSMs, and some stGSMs alter the production of cell-derived Aβ oligomers. A similar compound has also been reported by Merck [231]. However, even though the molecular targets of GSMs have been identified, the modes of action of these compounds still remain controversial [81,232].

For mechanism-based rational design of the compounds, understanding the structure–function relationship of the target protein is mandatory. Compared with β-secretase, the γ-secretase complex is one of the most difficult proteins to resolve the structure by conventional structural biological techniques (i.e., x ray crystallography and nuclear MRI), because of the nature of the high-molecular-weight membrane protein complex. However, other approaches are now emerging for the elucidation of the structure and the function of γ-secretase. Substituted cysteine accessibility method (SCAM) is one of the practical approaches to identify the structure of the membrane protein embedded within the lipid bilayer [233,234]. Using SCAM, we and others have identified the existence of a hydrophilic 'catalytic pore' connecting the luminal/extracellular milieu with the catalytic aspartates [235,236]. Moreover, together with the crosslinking and the inhibitor competition assays, we found that TMD9 of PS functions as a lateral gate for the substrate entry from the hydrophobic membranes into the hydrophilic catalytic pore [237,238]. Further examinations by SCAM pave the way for the understanding of the structure of membrane-embedded γ-secretase in an active state.

The other method for understanding the whole structure of the γ-secretase complex is single particle analysis by electron microscopy (EM) of the purified enzyme. Although the resolution of these images is still low, EM analyses have allowed us to recognize an interior pore that is reminiscent of the proteasome and which mediates the entry of water molecules [78,239,240]. Ongoing studies using cryo-EM and/or 2D crystallization may provide fine structural information with higher resolution. In addition, recent progress in structural analyses of intramembrane proteases, rhomboid and S2P protease [241], encourage us to attempt x-ray crystallographic analysis of γ-secretase. Further chemical biological studies, together with structural biology, would be required for the fine understanding of the catalytic mechanisms of γ-secretase and the molecular action of GSIs/GSMs on this atypical enzyme.

Five-year view

Secretases are attractive as molecular targets for AD therapeutics. Significant progress of an effective small molecule-based BSI and GSIs/GSMs have been achieved. Currently, clinical trials using these compounds are ongoing. These proof-of-concept studies using BSIs and GSIs/GSMs lend support to the idea that *in vivo* Aβ reduction can be achieved with this approach, and that the modulation of Aβ levels in the brain is a disease-modifying therapeutic for AD. To make a final conclusion, it may take more than 5 years. However, I strongly believe that we will steadily progress to overcome AD.

Genetic analyses of β- and γ-secretases also provide novel pathological information of other diseases, such as psychiatric disorders and cancer. In particular, the obligatory role of γ-secretase activity in Notch signaling is considered as an obstacle in developing inhibitors for AD therapeutics. However, this problem should be an advantage in chemotherapy of certain cancers, because aberrant Notch signaling has been implicated in several types of cancer [242,243]. Notch signaling also functions in angiogenesis, suggesting that modulation of Notch signaling is applicable to angiogenesis-based cancer therapy [244]. Moreover, Notch signaling is involved in stem cell signaling, including cancer stem cells [245,246]. Notably, it was revealed that combination therapy with a GSI plus glucorticoids can reduce gut toxicity *in vivo* [247]. Thus, the same caveats regarding the significant adverse effects associated with GSIs may be overcome by the development of certain specific compounds and/or effective regimens.

Molecular identification of the secretases has also opened the door for a new basic science: proteolysis of transmembrane proteins around and within the membrane. Common themes have begun to emerge among the evolutionarily diverse class of intramembrane proteases. Complex subcellular traffic and movement between membrane microdomains, following which the formerly separated protease and substrate come into contact, are involved in this

proteolytic event [175,180,248,249]. Proteolytic cleavage is not merely degradative in nature, but initiates a signaling cascade that can impact on genomic expression, cell–cell communication, cellular homeostasis and pathological processes [74]. Nevertheless, further extensive efforts in both academic and pharmaceutical laboratories may raise high hopes for the establishment of a feasible therapeutic approach for AD, psychiatric disease and possibly cancers by the inhibition and modulation of β- and γ-secretase activities.

Acknowledgements

I would like to thank Takeshi Iwatsubo (The University of Tokyo) and several collaborators for supporting my research over a long period of time. I also acknowledge the previous and current members of our department for helpful discussions and technical assistance.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- The two characteristic pathological feature of Alzheimer's disease (AD) are amyloid plaques and neurofibrillary tangles, the former being composed of amyloid-β peptide (Aβ).
- Multiple rare mutations that cause familial AD lead to increased production or aggregation of Aβ. Aβ is strongly implicated in the pathogenesis of AD.
- Aβ is generated from its precursor protein, amyloid precursor protein, by the sequential action of β- and γ-secretases.
- Inhibition and modulation of β- and γ-secretases to reduce the amount of Aβ in the brain are plausible therapeutics against AD.
- β-site amyloid precursor protein cleaving enzyme, a single membrane-spanning aspartic protease, is responsible for β-secretase activity.
- The γ-secretase complex is an aspartic intramembrane protease comprised of presenilin as a catalytic subunit, nicastrin, anterior pharynx-defective 1 and presenilin enhancer 2.
- Significant progress towards clinical trials has been made in the selective inhibition/modulation of both proteases, regardless of structural information for γ-secretase.
- Several biological aspects and regulatory mechanisms of both secretases have been found, implicating that alternative methods to modulate Aβ-generating activity could be possible.

References

1 Brookmayer R, Johnson E, Ziegler-Graham K, Arrighi HM. Forecasting the global burden of Alzheimer's disease. *Alzheimers Dement.* 3(3), 186–191 (2007).

2 Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide. *Nat. Rev. Mol. Cell Biol.* 8(2), 101–112 (2007).

3 Ballatore C, Lee VM, Trojanowski JQ. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat. Rev. Neurosci.* 8(9), 663–672 (2007).

4 Bertram L, Tanzi RE. Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses. *Nat. Rev. Neurosci.* 9(10), 768–778 (2008).

5 Deuss M, Reiss K, Hartmann D. Part-time α-secretases: the functional biology of ADAM 9, 10 and 17. *Curr. Alzheimer Res.* 5(2), 187–201 (2008).

6 Suzuki N, Cheung TT, Cai XD *et al.* An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (βAPP717) mutants. *Science* 264(5163), 1336–1340 (1994).

7 Asami-Odaka A, Ishibashi Y, Kikuchi T, Kitada C, Suzuki N. Long amyloid β-protein secreted from wild-type human neuroblastoma IMR-32 cells. *Biochemistry* 34(32), 10272–10278 (1995).

8 Iwatsubo T, Odaka A, Suzuki N *et al.* Visualization of Aβ 42(43) and Aβ 40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is Aβ 42(43). *Neuron* 13(1), 45–53 (1994).

9 Jarrett JT, Berger EP, Lansbury PT Jr. The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* 32(18), 4693–4697 (1993).

10 Citron M, Oltersdorf T, Haass C *et al.* Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production. *Nature* 360(6405), 672–674 (1992).

11 Cai XD, Golde TE, Younkin SG. Release of excess amyloid β protein from a mutant amyloid β protein precursor. *Science* 259(5094), 514–516 (1993).

12 Klein WL, Stine WB Jr, Teplow DB. Small assemblies of unmodified amyloid β-protein are the proximate neurotoxin in Alzheimer's disease. *Neurobiol. Aging* 25(5), 569–580 (2004).

13 Rovelet-Lecrux A, Hannequin D, Raux G *et al.* APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nat. Genet.* 38(1), 24–26 (2006).

14 Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297(5580), 353–356 (2002).

15 Barten DM, Albright CF. Therapeutic strategies for Alzheimer's disease. *Mol. Neurobiol.* 37(2–3), 171–186 (2008).

16 Evin G, Kenche VB. BACE inhibitors as potential therapeutics for Alzheimer's disease. *Recent Patents CNS Drug Discov.* 2(3), 188–199 (2007).

17 Ghosh AK, Gemma S, Tang J. β-secretase as a therapeutic target for Alzheimer's disease. *Neurotherapeutics* 5(3), 399–408 (2008).

18 Wolfe MS. Inhibition and modulation of γ-secretase for Alzheimer's disease. *Neurotherapeutics* 5(3), 391–398 (2008).

19 Tomita T. At the frontline of Alzheimer's disease treatment: γ-secretase inhibitor/modulator mechanism. *Naunyn Schmiedebergs Arch. Pharmacol.* 377(4–6), 295–300 (2008).

20 Imbimbo BP. Therapeutic potential of γ-secretase inhibitors and modulators. *Curr. Top. Med. Chem.* 8(1), 54–61 (2008).

21 Evin G. γ-secretase modulators: hopes and setbacks for the future of Alzheimer's treatment. *Expert Rev. Neurother.* 8(11), 1611–1613 (2008).

22 Stockley JH, O'Neill C. Understanding BACE1: essential protease for amyloid-β production in Alzheimer's disease. *Cell. Mol. Life Sci.* 65(20), 3265–3289 (2008).

23 Cole SL, Vassar R. The role of amyloid precursor protein processing by BACE1, the β-secretase, in Alzheimer disease pathophysiology. *J. Biol. Chem.* 283(44), 29621–29625 (2008).

24 Hussain I, Powell D, Howlett DR *et al.* Identification of a novel aspartic protease (Asp 2) as β-secretase. *Mol. Cell. Neurosci.* 14(6), 419–427 (1999).

25 Vassar R, Bennett BD, Babu-Khan S *et al.* β-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286(5440), 735–741 (1999).

26 Sinha S, Anderson JP, Barbour R *et al.* Purification and cloning of amyloid precursor protein β-secretase from human brain. *Nature* 402(6761), 537–540 (1999).

27 Yan R, Bienkowski MJ, Shuck ME *et al.* Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* 402(6761), 533–537 (1999).

28 Lin X, Koelsch G, Wu S *et al.* Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proc. Natl Acad. Sci. USA* 97(4), 1456–1460 (2000).

29 Cai H, Wang Y, McCarthy D *et al.* BACE1 is the major β-secretase for generation of Aβ peptides by neurons. *Nat. Neurosci.* 4(3), 233–234 (2001).

30 Luo Y, Bolon B, Kahn S *et al.* Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. *Nat. Neurosci.* 4(3), 231–232 (2001).

31 Roberds SL, Anderson J, Basi G *et al.* BACE knockout mice are healthy despite lacking the primary β-secretase activity in brain: implications for Alzheimer's disease therapeutics. *Hum. Mol. Genet.* 10(12), 1317–1324 (2001).

32 Fukumoto H, Cheung BS, Hyman BT, Irizarry MC. β-secretase protein and activity are increased in the neocortex in Alzheimer disease. *Arch. Neurol.* 59(9), 1381–1389 (2002).

33 Sun A, Koelsch G, Tang J, Bing G. Localization of β-secretase memapsin 2 in the brain of Alzheimer's patients and normal aged controls. *Exp. Neurol.* 175(1), 10–22 (2002).

34 Holsinger RM, McLean CA, Beyreuther K, Masters CL, Evin G. Increased expression of the amyloid precursor β-secretase in Alzheimer's disease. *Ann. Neurol.* 51(6), 783–786 (2002).

35 Yang LB, Lindholm K, Yan R *et al.* Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat. Med.* 9(1), 3–4 (2003).

36 Li R, Lindholm K, Yang LB *et al.* Amyloid β peptide load is correlated with increased β-secretase activity in sporadic Alzheimer's disease patients. *Proc. Natl Acad. Sci. USA* 101(10), 3632–3637 (2004).

37 Selkoe DJ, Wolfe MS. Presenilin: running with scissors in the membrane. *Cell* 131(2), 215–221 (2007).

38 Sherrington R, Rogaev EI, Liang Y *et al.* Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375(6534), 754–760 (1995).

39 Levy-Lahad E, Wasco W, Poorkaj P *et al.* Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* 269(5226), 973–977 (1995).

40 Rogaev EI, Sherrington R, Rogaeva EA *et al.* Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature* 376(6543), 775–778 (1995).

41 Scheuner D, Eckman C, Jensen M *et al.* Secreted amyloid β-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* 2(8), 864–870 (1996).

42 Borchelt DR, Thinakaran G, Eckman CB *et al.* Familial Alzheimer's disease-linked presenilin 1 variants elevate Aβ1–42/1–40 ratio *in vitro* and *in vivo*. *Neuron* 17(5), 1005–1013 (1996).

43 Duff K, Eckman C, Zehr C *et al.* Increased amyloid-β 42(43) in brains of mice expressing mutant presenilin 1. *Nature* 383(6602), 710–713 (1996).

44 Citron M, Westaway D, Xia W *et al.* Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice. *Nat. Med.* 3(1), 67–72 (1997).

45 Tomita T, Maruyama K, Saido TC *et al.* The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid β protein ending at the 42nd (or 43rd) residue. *Proc. Natl Acad. Sci. USA* 94(5), 2025–2030 (1997).

46 De Strooper B, Saftig P, Craessaerts K *et al.* Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 391(6665), 387–390 (1998).

47 Naruse S, Thinakaran G, Luo JJ *et al.* Effects of PS1 deficiency on membrane protein trafficking in neurons. *Neuron* 21(5), 1213–1221 (1998).

48 Herreman A, Serneels L, Annaert W *et al.* Total inactivation of γ-secretase activity in presenilin-deficient embryonic stem cells. *Nat. Cell Biol.* 2(7), 461–462 (2000).

49 Zhang Z, Nadeau P, Song W *et al.* Presenilins are required for γ-secretase cleavage of β-APP and transmembrane cleavage of Notch-1. *Nat. Cell Biol.* 2(7), 463–465 (2000).

50 Li YM, Lai MT, Xu M *et al.* Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state. *Proc. Natl Acad. Sci. USA* 97(11), 6138–6143 (2000).

51 Yu G, Nishimura M, Arawaka S *et al.* Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing. *Nature* 407(6800), 48–54 (2000).

52 Goutte C, Tsunozaki M, Hale VA, Priess JR. APH-1 is a multipass membrane protein essential for the Notch signaling pathway in *Caenorhabditis elegans* embryos. *Proc. Natl Acad. Sci. USA* 99(2), 775–779 (2002).

53 Francis R, McGrath G, Zhang J *et al.* aph-1 and pen-2 are required for Notch pathway signaling, γ-secretase cleavage of βAPP, and presenilin protein accumulation. *Dev. Cell* 3(1), 85–97 (2002).

54 Chung HM, Struhl G. Nicastrin is required for presenilin-mediated transmembrane cleavage in *Drosophila*. *Nat. Cell Biol.* 3(12), 1129–1132 (2001).

55 Hu Y, Ye Y, Fortini ME. Nicastrin is required for γ-secretase cleavage of the *Drosophila* Notch receptor. *Dev. Cell* 2(1), 69–78 (2002).

56 Lopez-Schier H, St Johnston D. *Drosophila* nicastrin is essential for the intramembranous cleavage of notch. *Dev. Cell* 2(1), 79–89 (2002).

57 Li J, Fici GJ, Mao CA *et al.* Positive and negative regulation of the γ-secretase activity by nicastrin in a murine model. *J. Biol. Chem.* 278(35), 33445–33449 (2003).

58 Li T, Ma G, Cai H, Price DL, Wong PC. Nicastrin is required for assembly of presenilin/γ-secretase complexes to mediate Notch signaling and for processing and trafficking of β-amyloid precursor protein in mammals. *J. Neurosci.* 23(8), 3272–3277 (2003).

59 Takasugi N, Tomita T, Hayashi I *et al.* The role of presenilin cofactors in the γ-secretase complex. *Nature* 422(6930), 438–441 (2003).

60 Ma G, Li T, Price DL, Wong PC. APH-1a is the principal mammalian APH-1 isoform present in γ-secretase complexes during embryonic development. *J. Neurosci.* 25(1), 192–198 (2005).

61 Serneels L, Dejaegere T, Craessaerts K *et al.* Differential contribution of the three Aph1 genes to γ-secretase activity *in vivo*. *Proc. Natl Acad. Sci. USA* 102(5), 1719–1724 (2005).

62 Edbauer D, Winkler E, Regula JT *et al.* Reconstitution of γ-secretase activity. *Nat. Cell Biol.* 5(5), 486–488 (2003).

63 Kim SH, Ikeuchi T, Yu C, Sisodia SS. Regulated hyperaccumulation of presenilin-1 and the "γ-secretase" complex. Evidence for differential intramembranous processing of transmembrane subatrates. *J. Biol. Chem.* 278(36), 33992–34002 (2003).

64 Kimberly WT, LaVoie MJ, Ostaszewski BL *et al.* γ-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proc. Natl Acad. Sci. USA* 100(11), 6382–6387 (2003).

65 Hayashi I, Urano Y, Fukuda R *et al.* Selective reconstitution and recovery of functional γ-secretase complex on budded baculovirus particles. *J. Biol. Chem.* 279(36), 38040–38046 (2004).

66 Wolfe MS, Xia W, Ostaszewski BL *et al.* Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity. *Nature* 398(6727), 513–517 (1999).

67 Esler WP, Kimberly WT, Ostaszewski BL *et al.* Transition-state analogue inhibitors of γ-secretase bind directly to presenilin-1. *Nat. Cell Biol.* 2(7), 428–434 (2000).

68 Li YM, Xu M, Lai MT *et al.* Photoactivated γ-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature* 405(6787), 689–694 (2000).

69 Weihofen A, Binns K, Lemberg MK, Ashman K, Martoglio B. Identification of signal peptide peptidase, a presenilin-type aspartic protease. *Science* 296(5576), 2215–2218 (2002).

70 Haass C, Steiner H. Alzheimer disease γ-secretase: a complex story of GxGD-type presenilin proteases. *Trends Cell Biol.* 12(12), 556–562 (2002).

71 Urban S, Freeman M. Intramembrane proteolysis controls diverse signalling pathways throughout evolution. *Curr. Opin. Genet. Dev.* 12(5), 512–518 (2002).

72 Wolfe MS, Kopan R. Intramembrane proteolysis: theme and variations. *Science* 305(5687), 1119–1123 (2004).

73 Brown MS, Ye J, Rawson RB, Goldstein JL. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 100(4), 391–398 (2000).

74 Hass MR, Sato C, Kopan R, Zhao G. Presenilin: RIP and beyond. *Semin. Cell Dev. Biol.* 20(2), 201–210 (2009).

75 Shah S, Lee SF, Tabuchi K *et al.* Nicastrin functions as a γ-secretase-substrate receptor. *Cell* 122(3), 435–447 (2005).

76 Chavez-Gutierrez L, Tolia A, Maes E *et al.* Glu(332) in the nicastrin ectodomain is essential for γ-secretase complex maturation but not for its activity. *J. Biol. Chem.* 283(29), 20096–20105 (2008).

77 Shirotani K, Edbauer D, Capell A *et al.* γ-secretase activity is associated with a conformational change of nicastrin. *J. Biol. Chem.* 278(19), 16474–16477 (2003).

78 Ogura T, Mio K, Hayashi I *et al.* Three-dimensional structure of the γ-secretase complex. *Biochem. Biophys. Res. Commun.* 343(2), 525–534 (2006).

79 LaVoie MJ, Fraering PC, Ostaszewski BL *et al.* Assembly of the γ-secretase complex involves early formation of an intermediate subcomplex of Aph-1 and nicastrin. *J. Biol. Chem.* 278(39), 37213–37222 (2003).

80 Niimura M, Isoo N, Takasugi N *et al.* Aph-1 contributes to the stabilization and trafficking of the γ-secretase complex through mechanisms involving intermolecular and intramolecular interactions. *J. Biol. Chem.* 280(13), 12967–12975 (2005).

81 Isoo N, Sato C, Miyashita H *et al.* Aβ42 overproduction associated with structural changes in the catalytic pore of γ-secretase: common effects of Pen-2 *N*-terminal elongation and fenofibrate. *J. Biol. Chem.* 282(17), 12388–12396 (2007).

82 Zhou S, Zhou H, Walian PJ, Jap BK. CD147 is a regulatory subunit of the γ-secretase complex in Alzheimer's disease amyloid β-peptide production. *Proc. Natl Acad. Sci. USA* 102(21), 7499–7504 (2005).

83 Chen F, Hasegawa H, Schmitt-Ulms G *et al.* TMP21 is a presenilin complex component that modulates γ-secretase but not ε-secretase activity. *Nature* 440(7088), 1208–1212 (2006).

84 Beher D, Fricker M, Nadin A *et al. In vitro* characterization of the presenilin-dependent γ-secretase complex using a novel affinity ligand. *Biochemistry* 42(27), 8133–8142 (2003).

85 Winkler E, Hobson S, Fukumori A *et al.* Purification, pharmacological modulation, and biochemical characterization of interactors of endogenous human γ-secretase. *Biochemistry* DOI: 10.1021/bi801204g (2009) (Epub ahead of print).

86 Esler WP, Kimberly WT, Ostaszewski BL *et al.* Activity-dependent isolation of the presenilin-γ-secretase complex reveals nicastrin and a γ substrate. *Proc. Natl Acad. Sci. USA* 99(5), 2720–2725 (2002).

87 Fraering PC, Ye W, Strub JM *et al.* Purification and characterization of the human γ-secretase complex. *Biochemistry* 43(30), 9774–9789 (2004).

88 Ohno M, Sametsky EA, Younkin LH *et al.* BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. *Neuron* 41(1), 27–33 (2004).

89 Laird FM, Cai H, Savonenko AV *et al.* BACE1, a major determinant of selective vulnerability of the brain to amyloid-β amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *J. Neurosci.* 25(50), 11693–11709 (2005).

90 Ohno M, Chang L, Tseng W *et al.* Temporal memory deficits in Alzheimer's mouse models: rescue by genetic deletion of BACE1. *Eur J. Neurosci.* 23(1), 251–260 (2006).

91 Ma H, Lesne S, Kotilinek L *et al.* Involvement of β-site APP cleaving enzyme 1 (BACE1) in amyloid precursor protein-mediated enhancement of memory and activity-dependent synaptic plasticity. *Proc. Natl Acad. Sci. USA* 104(19), 8167–8172 (2007).