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Recent Advances in the Identification of γ-Secretase Inhibitors To Clinically Test the Aβ Oligomer Hypothesis of Alzheimer's Disease

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Introduction: The Growing Burden and Unmet Medical Need of Alzheimer's Disease

Alzheimer's disease (AD[a]) was first described over a hundred years ago by Alois Alzheimer as a dementia associated with the presence of extracellular insoluble β-amyloid plaques in the brain, intraneuronal neurofibrillary tangles, and extensive neuronal loss.[1] Because the incidence of AD increases with age and the proportion of the elderly in our population is also increasing, the projected number of new cases of AD threatens to overwhelm our health care system.[2] Currently available drugs to treat AD do not arrest the underlying disease process, instead, largely treating the symptoms.[3] What is urgently needed is a therapy to either slow or reverse the neuronal loss and the underlying cognitive decline, a so-called disease-modifying anti-Alzheimer's drug (DMAAD).[4−8]

Recent Progress in Understanding the Pathophysiology of AD: Evolution of the β-Amyloid Hypothesis into the Aβ Oligomer Hypothesis of AD

The key to the rational design of a DMAAD is an understanding of the underlying disease processes at the molecular level. Despite the fact that AD was described over a hundred years ago, it is only in the past ∼25 years that substantial progress has been made in identifying the AD pathophysiology of amyloid plaques and neurofibrillary tangles at the molecular level (for recent reviews, see Hardy,[9] Hodges,[10] and Goedart[11]). The extracellular insoluble β-amyloid plaques were shown to consist largely of an aggregated protein $A\beta_{42}$.

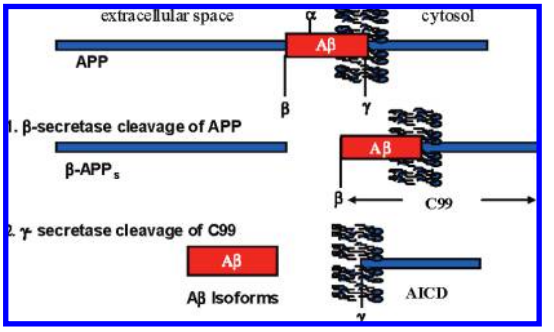

Figure 1. Biosynthesis of Aβ from APP through sequential processing by β-secretase and γ-secretase enzymes.

This protein is derived from a larger protein precursor APP (amyloid precursor protein) by the action of β-secretase, which generates soluble β-APP$_s$ (β-secretase cleaved APP soluble fragment) and membrane bound C99 (Figure 1). Subsequent cleavage of C99 by γ-secretase (GS) affords AICD (APP intracellular domain) and various Aβ species that differ in the length of the C-terminus, among these $A\beta_{42}$. If APP is first cleaved by α-secretase, which competes with β-secretase for APP, the generation of Aβ is precluded. Other shorter forms of Aβ (e.g., $A\beta_{40}$) are also produced in the processing of APP by β-secretase and γ-secretase; however, these shorter forms are less prone to aggregation and are less neurotoxic when aggregated. Interestingly, computational energetics of cleavage of C99 by GS predicts that generation of $A\beta_{40}$ is favored over generation of $A\beta_{42}$, which is consistent with what is experimentally observed.[12] The intraneuronal neurofibrillary tangles of AD were subsequently shown to consist of hyperphosphorylated aggregates of the microtubule-associated protein tau. However, it was unclear if β-amyloid plaques and neuofibrillary tangles were causative for AD or merely epiphenomena. The breakthroughs came in the 1990s from the study of AD genetics of early onset familial AD (FAD) in which mutations in genes for APP or the GS components presenilin-1 (PS-1) and presenilin-2 (PS-2) were causative for FAD. No comparable mutations in AD patients were found in the τ protein, its precursor, or enzymes involved in its biosynthesis. In addition, tau pathology was found to be downstream of β-amyloid pathology. The weight of this evidence led to the initial β-amyloid hypothesis of AD proposed by Hardy and Higgens, which postulates that Aβ peptide aggregates into β-amyloid plaques that lead to neuronal cell death and the clinical symptoms of AD such as

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[a] Abbreviations: AD, Alzheimer's disease; ADAS-Cog, Alzheimer disease assessment scale-cognitive; AICD, amyloid precursor protein intracellular domain; Aph-1, anterior pharynx-1; APP, amyloid precursor protein; BACE, β-amyloid converting enzyme; βAPPs, β-secretase cleaved amyloid precursor protein soluble; AUC, area under the curve; CFC, contextual fear conditioning; CHO, Chinese hamster ovary; CNS, central nervous system; CSF, cerebral spinal fluid; CTF, C-terminal fragment; CYP, cytochrome p450; DMAAD, disease-modifying anti-Alzheimer's drug; FAD, familial Alzheimer's disease; GI, gastrointestinal; GS, γ-secretase; GSI, γ-secretase inhibitor; GSM, γ-secretase modulator; HEK, human embryonic kidney; HTS, high throughput screening; LOAD, late onset Alzheimer's disease; MED, minimum effective dose; NICD, Notch intracellular domain; NRSEs, Notch-related side effects; NTF, N-terminal fragment; PDAPP, platelet-derived growth factor-driven human amyloid precursor protein; Pen-2, presenilin enhancer-2; PK, pharmacokinetic; PS, presenilin; PS-1, presenilin-1; PS-2, presenilin-2; q.d., once daily; RIP, regulated-intramembrane-proteolysis; ROCS, rapid overlay of chemical structures; SAR, structure−activity relationship; SMR, molar refractivity; SPP, signal peptide peptidase; TMD, transmembrane domain.

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severe cognitive impairment manifesting itself by pathological memory loss.[13] Because most FAD is indistinguishable, except for the age of onset and hereditary component, from late onset AD (LOAD), which comprises the majority of AD cases, it is assumed that the β-amyloid hypothesis applies to both forms of AD.[14,15]

Although the β-amyloid hypothesis served as a starting point for the rational design of DMAADs, it was unable to explain why the β-amyloid plaque burden did not correlate with the degree of cognitive impairment in AD. The possible solution to this conundrum has appeared with the recent identification of soluble Aβ oligomers as synaptotoxic species in AD rather than the insoluble β-amyloid plaques.[16] Synaptic loss is the best pathologic correlate of cognitive decline in AD.[17] Synaptic dysfunction is an early event in AD and appears to predate neuronal loss by decades. This led to the evolution of the β-amyloid hypothesis of AD into the Aβ oligomer hypothesis of AD, the current dominant hypothesis to explain AD pathogenesis (for recent reviews, see Selkoe,[18] Viola,[19] and Pimplikar[20]). Currently, the role of β-amyloid plaques in AD pathogenesis is unclear. The insoluble β-amyloid plaques may be the body's attempt to convert the toxic soluble Aβ oligomers into a less toxic species. Alternatively, the insoluble β-amyloid plaques may trigger chronic inflammatory responses that contribute to AD pathophysiology.

Rational Design of DMAADs: Targeting Aβ Oligomer Formation and Clearance

The proposed central role of Aβ oligomers in AD suggests that targeting the following processes may result in a DMAAD: (1) inhibiting the synthesis of Aβ from APP by blocking either β-secretase or GS (Figure 1), (2) inhibiting the aggregation of Aβ into toxic oligomers, or (3) enhancing clearance of Aβ oligomers. A previous seminal Perspective in this journal by Wolfe in 2001[21] has reviewed secretases as potential AD targets, and the current review will highlight recent advances in the identification of γ-secretase inhibitors (GSIs) as potential AD therapeutics. An attempt was made to mainly cover GSIs reported in various scientific journals rather than in patent literature because several extensive reviews are available of the GSI patent literature.[22,23] The reader is also referred to recent reviews on β-secretase inhibitors,[24,25] Aβ aggregation inhibitors,[26,27] and enhancers of Aβ clearance.[28]

Recent Progress in Elucidating the Nature of γ-Secretase

GS is an unusual aspartyl protease that cleaves its substrates within the transmembrane region in a process termed regulated-intramembrane-proteolysis (RIP).[29−32] This enzyme was shown to consist of four protein components: presenilin (PS) 1 or 2 (which contains the catalytic domain), nicastrin (which may serve to dock substrates), Aph-1 (anterior pharynx-1), and Pen-2 (presenilin enhancer-2) in a 1:1:1:1 ratio (Figure 2; for recent reviews on GS, see Dries[33] and Wolfe[34,35] and Tolia[36]). PS-1 and PS-2 are 476- and 448-amino acid polytopic membrane proteins consisting of nine TMDs (transmembrane domains) and are endoproteolytically cleaved into a ~30 kDa N-terminal fragment and a ~20 kDa C-terminal fragment, each of which contains an active site aspartate. The N-terminal catalytically active site of PS is embedded in a conserved YD motif, whereas the C-terminal active site domain contains the equally conserved GXGD motif. The cleavage occurs in the large cytoplasmic loop between TMD6 and TMD7 within a short hydrophobic domain that is believed to dive into the membrane. This endoproteolysis is thought to be an autoproteolytic event, although this has not been formally proven. Nicastrin is a 709-amino acid type 1 membrane glycoprotein with a large ectodomain that may act as a GS substrate receptor.

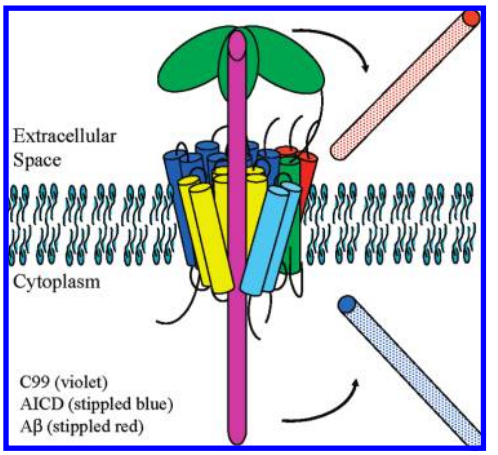


Figure 2. Processing of C99 by the γ-secretase enzyme: structure of the γ-secretase enzyme complex (nicastrin = green, Aph-1 = blue, Pen-2 = red, PS N-terminal = yellow, PS C-terminal = light blue).

It is hypothesized that the free N-terminus of GS substrates first binds to the ectodomain of nicastrin, which may facilitate its interaction with the docking site on PS which is followed by relocation to the active site on PS where it is cleaved. Pen-2 at ~10 kDa is the smallest component of GS and is thought to be required for the stabilization of the PS fragments in the GS complex. Aph-1 is a seven-TMD 20 kDa protein whose function in GS is currently unclear. It has been found that Aph-1 can exist as two splice variants: Aph-1a and Aph-1b.[37,38] Thus, several different GS complexes are possible depending on whether they contain PS-1 or PS-2 and whether they contain Aph-1a or Aph-1b. It has been proposed that nicastrin and Aph-1 form an initial complex that sequentially adds PS and Pen-2. Yet, nicastrin was recently shown to be dispensable for GS activity in the presence of certain PS mutants.[39] The first low resolution structures of GS have been recently obtained and reveal a hydrophilic active site buried in the protein interior, sequestered from the hydrophobic environment of the lipid bilayer.[40,41] This location may explain how hydrolysis can occur within the hydrophobic environment of the membrane. A recent higher resolution structure of GS at 12 Å has further revealed a globular structure that possesses a number of cavities that are open to either the extracellular space or the cytosol as well as an almost continuous surface groove at the membrane region that could be a substrate entry site.[42,43]

Partial, Selective Inhibition of γ-Secretase: A Major Goal for an AD Therapeutic Agent

Subsequent to the discovery of the role of GS in Aβ biosynthesis, it was discovered that this enzyme also cleaves many other protein substrates within their transmembrane regions (for recent reviews, see Beel,[44] Lleo,[45] and Hemming[46]). Some of these cleavages may be merely removing membrane stubs left from prior extramembrane cleavages by other proteases; yet, at least in one case, GS cleavage of Notch, the proteolysis, leads to the release of a smaller cytosolic fragment NICD (Notch intracellular domain)

important in signal transduction pathways.[47] GSIs that are not Notch-sparing have been shown to have untoward effects on GI (gastrointestinal) and T cells (e.g., see Hadland,[48] Milano,[49] Searfoss,[50] Wong,[51] and Wu[52]). However, some blockade of Notch processing by GS is tolerated without affecting Notch signaling and the Bristol-Myers Squibb group has shown that ~15-fold Notch sparing in a GSI is sufficient to avoid Notch-related side effects (NRSEs).[53] The question then becomes how much Aβ inhibition is needed to see a therapeutic effect. Wyeth recently reported that in the contextual fear conditioning (CFC) model of AD employing Tg2576 mice, ~30% inhibition of Aβ synthesis is sufficient to reverse cognitive impairment.[54] Therefore, in theory, a 15-fold Notch-sparing GSI with ~30% inhibition of Aβ synthesis may reverse cognitive impairment in the clinic without NRSEs. Toward this end, a useful parameter is the minimum effective dose (MED) for Aβ reduction in vivo. It is hoped that a therapeutic window can be established between Aβ lowering and NRSEs because GS is only partially inhibited. As a precedent, it is noted that partial enzyme inhibition has been successfully employed before in the clinic with the statins for cholesterol lowering.[55]

Classification of GSIs

GSIs can be classified into three general types based on where they bind to GS: (1) active-site-binding GSIs that mimic the transition state of C99 cleavage by GS, (2) substrate docking-site-binding GSIs, and (3) alternative binding site GSIs that can be further subdivided into carboxamide- and arylsufonamide-containing GSIs.[56] GSIs mimicking the transition state of C99 cleavage by GS have been identified by several groups. Subsequent work with photoaffinity probes based upon these active-site-binding GSIs have identified either PS-1 or PS-2 as the location of the binding site. Prior to occupying the active site of GS, C99 binds to a docking site on GS. GSIs based upon the partial sequence of C99 have been shown to inhibit Aβ synthesis presumably by occupying the docking site that prevents C99 from binding to GS. In addition, from GS binding displacement studies, it appears there also exists alternative binding sites on GS that are distinct from the active site and docking site and to which a large number of GSIs bind.

Recent Advances in Active-Site-Binding GSIs

The design of active-site-binding GSIs was expedited by the knowledge that GS is an aspartyl protease inhibitor and by the large body of work directed toward active-site-binding inhibitors of HIV protease, another aspartyl protease.[57] These efforts led to first generation active-site-binding GSIs **1** (L-685,458) from the Merck group[58] and **2** from Wolfe's group[59] (Figure 3). These first generation compounds proved to be useful biological tools. Use of tritiated **1** in adult rat brain revealed that binding sites in the adult brain were differentially distributed across regions and laminas, with heavy binding localized to the olfactory glomeruli, hippocampal CA3, and cerebellar molecular layer and moderate binding in the cerebral cortex, amygdala, and selected subcortical regions.[60] All of these regions showed labeling for PS-1 N-terminal fragments (NTFs). A distinct correlation of dense binding sites with abundant presence of PS1-NTFs was verified in hippocampal mossy fiber terminals and olfactory bulb glomeruli, suggestive of a rich expression of GS in the synapses at these locations that are characteristic of dynamic plasticity.

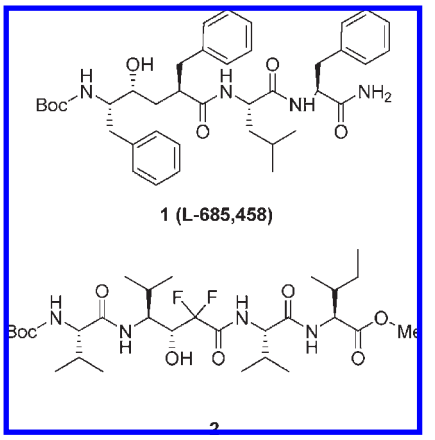


Figure 3. First generation active-site-binding GSIs.

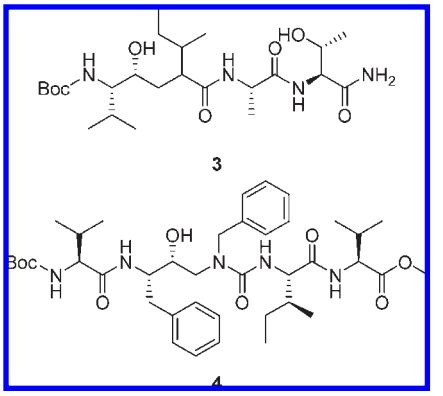


Figure 4. Second generation active-site-binding GSIs.

In another study with **1** in rats, it was found that post-training blockade of GS activity led to enhanced long-term memory in two hippocampus-dependent tasks.[61]

Wolfe's group has sought to improve upon **1** by replacing the chiral carbon atom in P1′ with an achiral nitrogen.[62] The resulting series of (hydroxyethyl) urea peptidomimetics not only produced potent GSIs (e.g., **4**, Figure 4, $A\beta_{total}$ $IC_{50} = 70$ nM) but also revealed a remarkable lack of clear specificity in the S2−S4′ pockets. Further work on this series employing D-amino acids afforded GSIs that had comparable potency to their L-amino acids counterparts when incubated for extended times.[63] These results are consistent with the loose sequence specificity required by GS.

Merck has sought to improve upon **1** by synthesizing hydroxyethylene-containing analogues that incorporate the peptide sequences of C99 cleaved by GS.[64] Surprisingly, these analogues (e.g., **3**) were inactive in blocking Aβ formation at concentrations up to 10 μM. This suggests that the factors governing substrate-based design of GSIs are more complicated than first thought.

Employing high throughput screening (HTS), the Bristol-Myers Squibb group identified the peptidomimetic lead **5** (Figure 5, $A\beta_{total}$ $IC_{50} = 5000$ nM in hAPPH4 neuroglioma cells), which contains the hydroxyethylene moiety found in other active-site GSIs.[65] Extensive structure−activity relationship (SAR) efforts on **5** significantly improved GSI potency, leading to **6** ($A\beta_{total}$ $IC_{50} = 160$ nM). Although active-site-binding GSIs such as **1** are potent GSIs and have been useful tools, they do not possess any

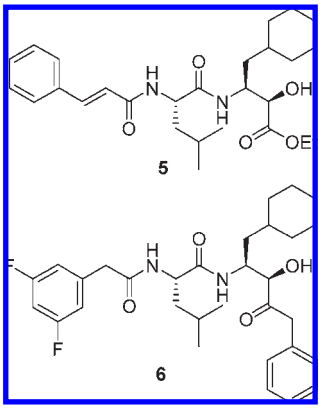


Figure 5. Bristol-Myers Squibb active-site-binding GSIs.

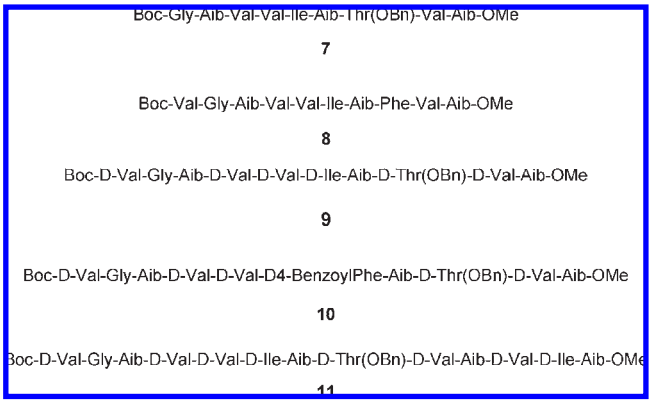


Figure 6. Substrate docking-site-based GSIs.

significant Notch-sparing selectivity[66] and no compound from this class of GSI has entered AD clinical trials.

Recent Advances in Substrate Docking-Site-Based GSIs

The identification of a substrate docking site on the outer surface of GS that is occupied before the active site on the interior of GS has led to the design of a novel class of GSIs, the so-called substrate docking-site-based GSIs.[56] Wolfe's group has designed first generation helical peptides (e.g., **7** and **8**; Figure 6) by incorporating the helix-inducing residue α-aminoisobutyric acid (Aib) that mimic the C-99 substrate conformation and inhibit Aβ production in the low micromolar range in APP-transfected CHO cells.[67] Surprisingly, the helical peptides containing D-amino acids were equally or more potent than their L-amino acid counterparts. The D-peptide **9** was also found to block Aβ production in a cell-free GS assay (Aβ_{40} $IC_{50} \approx 100$ nM) without affecting β-secretase. The helical conformation of these peptides was found to be crucial for GSI activity because analogues with disrupted helicity lost substantial potency. Further modifications led to the potent GSI **10** (Aβ_{40} IC_{50} = 10 nM in cell-free assays and 70 nM in cell-based assays), which, when converted into a photoaffinity probe, was shown to interfere with the interaction of APP with PS-1 in intact cells.[67,68] Importantly, this compound did not compete with active-site directed GSIs for binding to PS-1. Second generation compounds with improved potency (e.g., **11**, Aβ_{40} IC_{50} = 140 pM in cell-free assays) were obtained by extending the length of the helical region.[69] Unfortunately, **10** also potently blocks Notch processing by GS and, like the active site-directed GSIs, no compound from this class of GSIs has entered AD clinical trials.

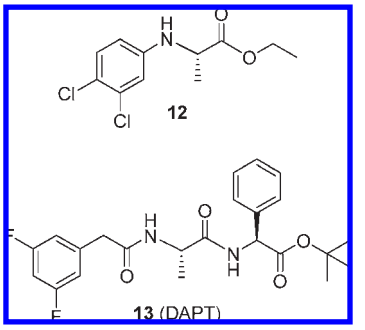


Figure 7. Lilly first generation alternative binding site carboxamide GSIs.

Recent Advances in Alternative Binding-Site-Based GSIs: Carboxamide-Containing GSIs

Because of the absence of a high resolution structure of GS which precluded a structure-based design approach, the majority of efforts to identify GSIs have utilized HTS to identify viable leads. Using this method, the Lilly group in collaboration with Elan identified the GSI lead **12** (Figure 7, Aβ_{total} IC_{50} = 900 nM in HEK cells overexpressing $hAPP_{751}$).[70] Extensive SAR efforts on this lead afforded **13** (DAPT), which not only had greatly improved GSI potency (Aβ_{total} IC_{50} = 20 nM) but also was the first orally active GSI (ED_{50} = 100 mg/kg po for lowering total brain Aβ in platelet-derived growth factor driven hAPP (PDAPP) mice). Further work on DAP-BpB, a biotinylated benzophenone photoaffinity probe derived from **13**, revealed that these compounds bind to the C-terminal fragment of PS-1 in GS.[71] The binding of DAP-BpB to GS could be blocked by the active-site-based GSI **1** or the substrate docking-site-based GSI **8** but only at high concentrations. This suggests that the binding site for **13** is distinct from, but overlaps with, both the catalytic site and substrate docking sites or alternatively that **1** and **8** may slightly alter the conformation of the binding site for **13**. Although **13** has subsequently become an important and widely used tool both in vitro and in vivo, its modest in vivo potency needed to be improved in order to identify a clinical candidate.

Dipeptide amino alcohols related to **13** were, in general, less potent (e.g., **14**, Figure 8, Aβ_{total} IC_{50} = 130 nM).[72] Further elaboration of **13** provided the more potent GSI **15** (LY-411,575, Aβ_{40} IC_{50} = 30 pM), which also had greatly improved in vivo efficacy (ED_{50} < 1 mg/kg po for lowering total brain Aβ in PDAPP mice).[73] Unfortunately, **15** possesses no Notch-sparing selectivity[66] and NRSEs on intestinal goblet cells and T cells were observed when mice were treated with **15** for 15 days.[51] Nevertheless, further studies with **15** revealed that a therapeutic window could be obtained by adjusting the dose.[74] Additional optimization of the **15** scaffold produced the clinical GSI **16** (LY-450,139 or semagacestat, Aβ_{40} IC_{50} = 15 nM).[75] This compound has in vivo efficacy in PDAPP mice comparable to **15** and has progressed into phase III trials despite the lack of Notch-sparing selectivity.[76] Although **16** was able to inhibit plasma Aβ production in the clinic, it was unable to lower CSF Aβ levels at the 4−6 h time points despite achieving dose-related concentrations of **16** in the CSF.[75,76]

The Hoffman-LaRoche group has used **15** as a starting point for the design of novel GSIs.[77] Scaffold modifications of **15** led to the potent malonamide GSI **17** (Figure 9, Aβ_{40} IC_{50} = 2.0 nM in a cell-free GS assay derived from human embryonic kidney (HEK) cells) that suffered from low

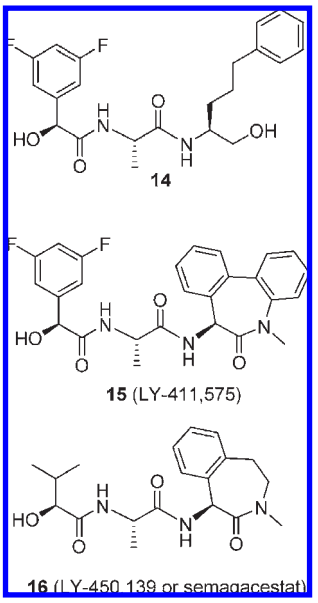


Figure 8. Lilly second generation alternative binding site carboxamide GSIs.

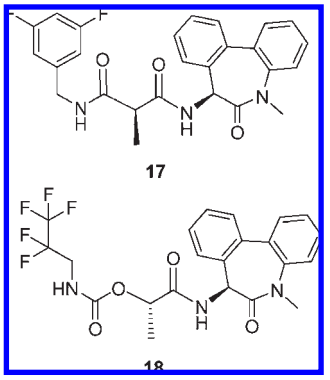


Figure 9. Hoffman-LaRoche alternative binding site carboxamide GSIs.

metabolic stability. Replacement of the metabolically labile difluorobenzyl group with the pentafluoropropyl group and changing the malonamide into its carbamate analogue afforded **18** ($A\beta_{40}$ IC_{50} = 8.6 nM in a cell-free GS assay), which demonstrated good pharmacokinetics (PK) (F = 23%) and in vivo GSI efficacy (MED = 3 mg/kg, po) in the $APP_{swe}/PS\text{-}2_{FAD}$ mouse model. These compounds potently inhibited Notch processing in an HEK cellular assay employing a Notch-1 construct (e.g., **18**, Notch-1 IC_{50} = 1.7 nM) .

Employing HTS, the Bristol-Myers Squibb group identified **19** (Figure 10) as a lead ($A\beta_{40}$ IC_{50} = 4700 nM in $APP_{164SFAD}$ H4 human neuroglioma cells).[78] Extensive SAR work provided the potent GSI **20** ($A\beta_{40}$ IC_{50} = 1 nM). When this compound was administered orally at 200 μmol/kg to Tg2576 mice, only 15% reduction in $A\beta$ was seen in the CNS. This problem was overcome by conversion of the tertiary amide group into a diazepinone ring which afforded **21** ($A\beta_{40}$ IC_{50} = 5 nM).[79] When Tg2576 mice were dosed with **21** at 200 μmol/kg, a 43% reduction in brain $A\beta$ was observed. Surprisingly, total brain exposure of **20** and **21** is similar and it was proposed that the difference in efficacy observed was due to better exposure at the GS target, perhaps reflecting a difference in brain protein binding of these compounds. Subsequent extensive SAR work on this novel scaffold led to the constrained

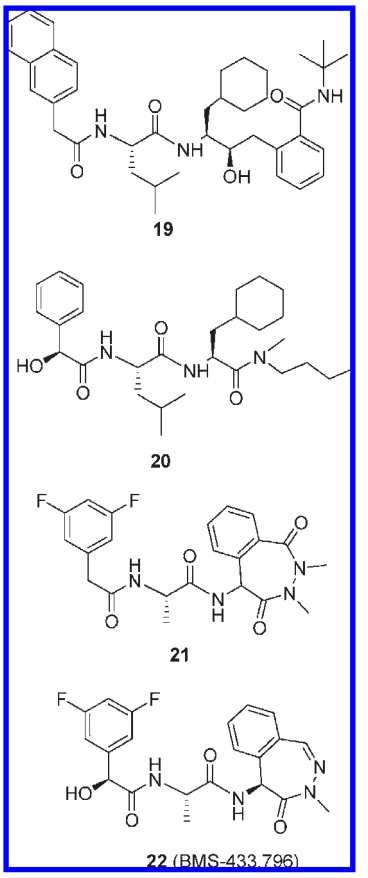


Figure 10. Bristol-Myers Squibb alternative binding site carboxamide GSIs.

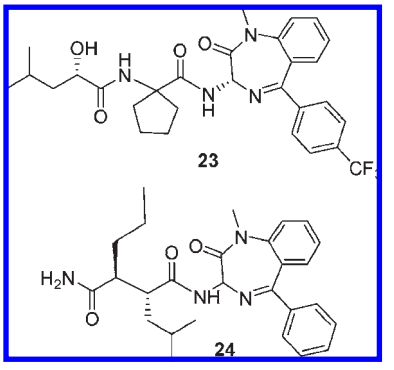


Figure 11. Additional Bristol-Myers Squibb alternative binding site carboxamide GSIs.

diazepine **22** (BMS-433,796, $A\beta_{40}$ IC_{50} = 0.3 nM),[80] which displayed vastly improved in vivo efficacy (ED_{50} for lowering brain $A\beta$ = 4 μmol/kg in Tg2576 mice, po). The structural similarity of **22** to the nonselective GSIs **15** and **16** is noted, and it is not surprising that **22** does not show any Notch-sparing selectivity and that NRSEs (intestinal goblet cell hyperplasia) were observed when Tg2576 mice were dosed chronically with this compound.

In related work,[81] the Bristol-Myers Squibb group prepared benzoazepinone-derived cyclic malonamides and aminoamides that not only were potent GSIs (e.g., **23**, Figure 11, $A\beta_{40}$ IC_{50} = 6 nM) but also exhibited excellent oral pharmacokinetic properties in beagle dogs (F = 84%, $T_{1/2}$ = 6.5 h, C_{max} = 2975 nM). The benzodiazepine malonamide **23** did not show any Notch-sparing selectivity in NIH 3T3 cells

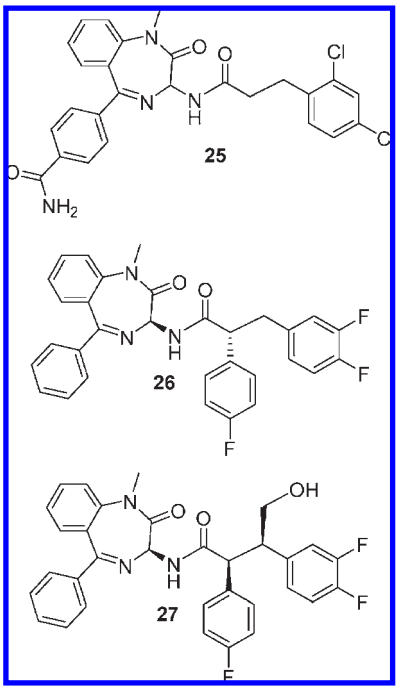


Figure 12. Merck alternative binding site carboxamide GSIs.

containing the NotchΔE construct. A related radiolabled benzodiazepine malonamide **24** was found to be a useful probe to investigate GSI binding sites in rodent brain.[82] When PS-1 knockout mice were used, a correlation was found between the density of GSI binding and the level of PS-1 gene expression. In adult rat brain, the binding sites are predominantly present in the forebrain (mostly in the olfactory bulb, cerebral cortex, and hippocampal formation) and cerebellum. A follow-up study of **24** using human brain tissue found a similar labeling pattern and no difference in labeling between control human brain and AD brain.[83] This is in contrast to β-secretase, where an increase in protein levels was found in AD.[84,85]

Using HTS in a whole cell assay (SHSY5Y cells), the Merck group identified the potent benzodiazepine lead **25** (Figure 12, $A\beta_{40}$ $IC_{50} = 33$ nM).[86] Removal of the C-4 carboxamide to improve membrane permeability led to a loss in potency that could be recovered by elaboration of the hydrocinnamate side chain to analogues such as **26** ($A\beta_{40}$ $IC_{50} = 4$ nM). A QSAR analysis[87] of this series of GSIs revealed that inhibitory activity increases in parallel with increasing lipophilicity and is sensitive to small changes in molar refractivity (SMR). Further optimization of this series led to the picomolar GSI **27** ($A\beta_{40}$ $IC_{50} = 60$ pM).[88] The N(1)-H analogue of **27** could be radiolabeled by N-methylation to yield a radioligand useful for GS binding studies. Unfortunately, no Notch-sparing selectivity data for **27** were provided.

The Bristol-Myers Squibb group also identified a different HTS lead **28** (Figure 13, SR973, $A\beta_{total}$ $IC_{50} = 200$ nM in $hAPP_{695}CHO$ N9 cells).[89] Optimization of this lead afforded inhibitors with < 10 nM cellular potency for inhibition of $A\beta$ production. Selected compounds from this series were profiled in vivo in the beagle dog. The caprolactam succinamide **29** ($A\beta_{total}$ $IC_{50} = 17$ nM in HEK 293 cells) had moderate bioavailability (25%). Once again, this compound did not show any significant Notch-sparing selectivity. Nevertheless, this series led to the development of a photoaffinity probe that in subsequent experiments proved that these GSIs also bind to PS.

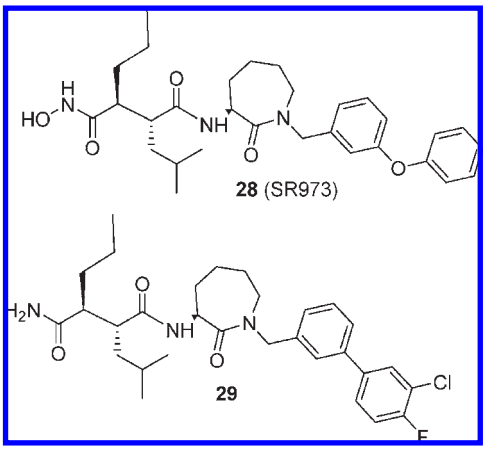


Figure 13. Caprolactam Bristol-Myers Squibb alternative binding site carboxamide GSIs.

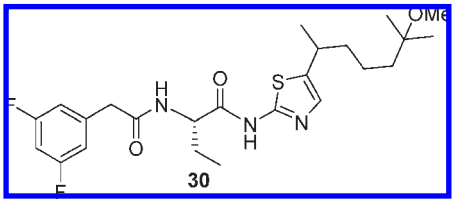


Figure 14. Pfizer alternative binding site carboxamide GSI.

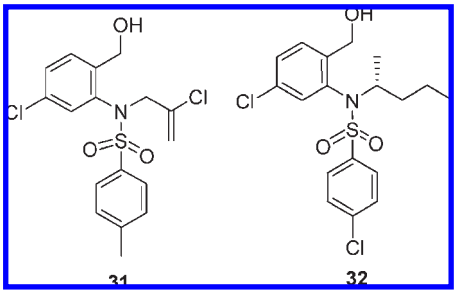


Figure 15. Bristol-Myers Squibb first generation alternative binding site sulfonamide GSIs.

The Pfizer group has prepared a series of potent dipeptide GSIs[90] that contain a substituted aminothiazole at the C-terminus. Lipophilic substitution on C-5 of the thiazole ring led to dramatic increases in potency (e.g., **30**, Figure 14, $A\beta_{total}$ $IC_{50} = 80$ pM in $hAPP_{swe}H4$ cells). The Notch-sparing selectivity of this new series has not been reported.

Recent Advances in Alternative Binding-Site-Based GSIs: Arylsulfonamide-Containing GSIs and Related Compounds

Independent of Amgen's early work on arylsulfonamides,[91] the Bristol-Myers Squibb group had identified lead arylsulfonamides from an HTS screening campaign.[92] Employing a cellular assay ($hAPP_{swe}H4$ cells), they identified **31** (Figure 15) as the initial arylsulfonamide lead ($A\beta_{40}$ $IC_{50} = 850$ nM in $hAPP_{swe}H4$ cells). Significant enhancement in GSI potency (~10-fold) resulted from branching with an α-methyl group at the point of attachment to the sulfonamide nitrogen with the *R* isomer being the eutomer. It was also found that the sulfonamide aryl group was quite sensitive to structural modification with the 4-chlorophenyl group appearing to be the optimal substituent (e.g., **32**, $A\beta_{40}$ $IC_{50} = 5$ nM in

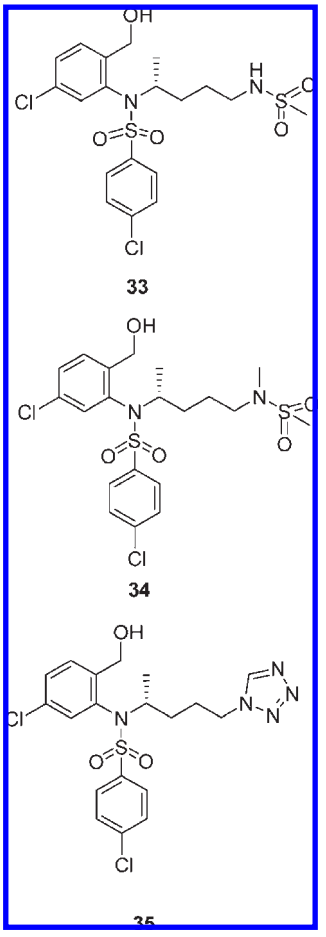


Figure 16. Additional Bristol-Myers Squibb alternative binding site sulfonamide GSIs.

hAPP$_{swe}$H4 cells). When **32** was orally administered to Tg2576 mice at 500 μmol/kg, a 25% reduction in brain Aβ was observed.

The modest reduction in brain Aβ despite achieving robust brain concentrations of 14 μM with **32** suggested that more potent GSIs were required to improve in vivo efficacy. This was achieved by synthesizing various nitrogen-appended analogues of **32**.[93] Synthesis of the methanesulfonamide **33** (Figure 16) afforded the first picomolar inhibitor in this series (Aβ_{40} IC$_{50}$ = 230 pM in hAPP$_{swe}$H4 cells). When the related *N*-methyl analogue **34** (Aβ_{40} IC$_{50}$ = 320 pM in hAPP$_{swe}$H4 cells) was orally administered to Tg2576 mice at 200 μmol/kg, a 27% reduction in brain Aβ was observed. This represents a similar reduction in brain Aβ at less than one-half the dose of **32**; however, the brain/plasma ratio of **34** was poor (0.06). Replacement of the methylsulfonamide with a tetrazole afforded the analogue **35** that not only retained GSI potency (Aβ_{40} IC$_{50}$ = 510 pM in hAPP$_{swe}$H4 cells) but also had improved in vivo efficacy (41% reduction in brain Aβ in Tg2576 at 200 μmol/kg). This compound showed a greatly improved brain/plasma ratio (0.21).

In order to further improve the brain/plasma ratio and absolute brain levels of these GSIs, additional work was carried out on replacing the tetrazole ring in **35** with various carbamates and removing the benzyl alcohol functionality.[94] This effort resulted in **36** (Figure 17, Aβ_{40} IC$_{50}$ = 270 pM in hAPP$_{swe}$H4 cells), which showed improvements in both in vivo efficacy (50% reduction in brain Aβ in Tg2576 at 200 μmol/kg) and brain/plasma ratio (0.42). Final optimization of this series in collaboration with SIBIA Neurosciences produced the Bristol-Myers Squibb clinical GSI **37** (BMS-299,897, APP IC$_{50}$ = 7.1 nM in hAPP$_{swe}$HEK293 cells), which had ED$_{50}$ = 18 mg/kg in reducing brain Aβ40 in Tg2576 mice.[53] Importantly, this compound not only shows 15-fold Notch-sparing selectivity in vitro but also is devoid of NRSEs in vivo. Photoaffinity probes based upon these arylsulfonamides have been synthesized[95] and found to label the N-terminus of PS-1.[96]

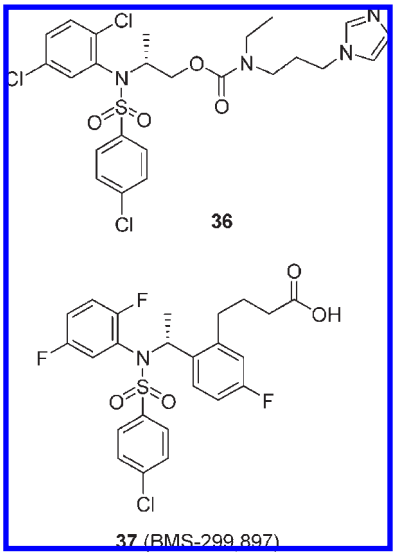


Figure 17. Bristol-Myers Squibb second generation alternative binding site sulfonamide GSIs.

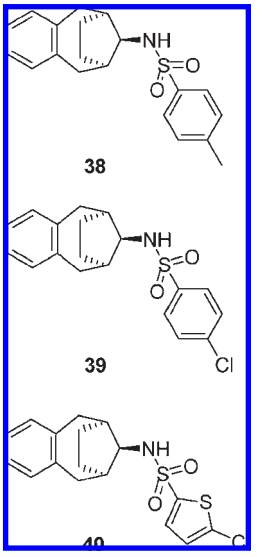


Figure 18. Merck alternative binding site sulfonamide GSIs.

The Merck group has also utilized HTS screening of their compound collection employing a whole cell assay (SHSY5Y cells) to identify the arylsulfonamide lead **38** (Figure 18, Aβ_{40} IC$_{50}$ = 651 nM).[97] Variation of the substituents of the phenyl ring of the sulfonamide led to a modest improvement in potency (e.g., **39**, Aβ_{40} IC$_{50}$ = 467 nM). In contrast, replacement of the phenylsulfonamide by thiophenesulfonamide led to an unexpectedly significant increase in potency (e.g., **40**, Aβ_{40} IC$_{50}$ = 62 nM).

Subsequent elaboration of the aromatic ring of the benzofused bicycle afforded potent glycinamides (e.g., **41**, Figure 19,

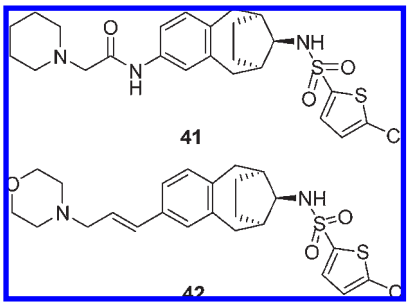


Figure 19. Additional Merck alternative binding site sulfonamide GSIs.

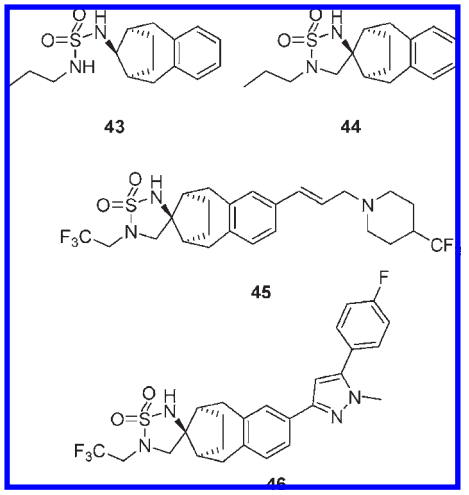


Figure 20. Merck alternative binding site sulfamide GSIs.

$A\beta_{40}$ $IC_{50} = 6$ nM); however, the PK properties of these compounds were poor. Replacement of the amide linkage in **41** with either an ether or olefin linker improved PK properties while retaining GSI potency. The most potent GSI from this work **42** ($A\beta_{40}$ $IC_{50} = 1$ nM) demonstrated excellent brain levels of drug (16.70 μM), a good brain to plasma ratio (~2.11), and promising in vivo efficacy in the APP-YAC mouse model ($A\beta_{40}$ ED_{50}(brain) = 100 mg/kg, po). Further work on this chemotype revealed that the arylsulfonamide group occupied an axial orientation and that the bridgehead position of the bicycle[4.2.1]nonane ring could be substituted with a wide variety of substituents, some of which were shown to improve oral absorption in rats.[98]

The Merck group also explored alternative motifs to the sulfonamide group found in **42**.[99] In their earlier work on the SAR of **38**, the Merck group found that the aryl portion of the arylsulfonamide moiety could be replaced by alkyl side chains. Heteroatoms were subsequently incorporated into these alkyl side chains that eventually led to the sulfamide **43** (Figure 20, $A\beta_{40}$ $IC_{50} = 132$ nM). Synthesis of the conformationally constrained cyclic sulfamide **44** gave a compound with similar GSI potency ($A\beta_{40}$ $IC_{50} = 138$ nM) but with improved PK properties, presumably due to the removal of one sulfamide N−H group (rat systemic exposures: AUC = 0.6 μM h for **43** dosed at 30 mg/kg po and 0.1 μM h for **44** dosed at 1 mg/kg po). Further optimization afforded the picomolar GSI **45** ($A\beta_{40}$ $IC_{50} = 240$ pM), which demonstrated improved efficacy over **42** in the APP-YAC mouse model ($A\beta_{40}$ ED_{50}(brain) = 17 mg/kg, po). Subsequent work provided the analogue **46** which had improved in vitro ($A\beta_{40}$ $IC_{50} = 60$ pM)

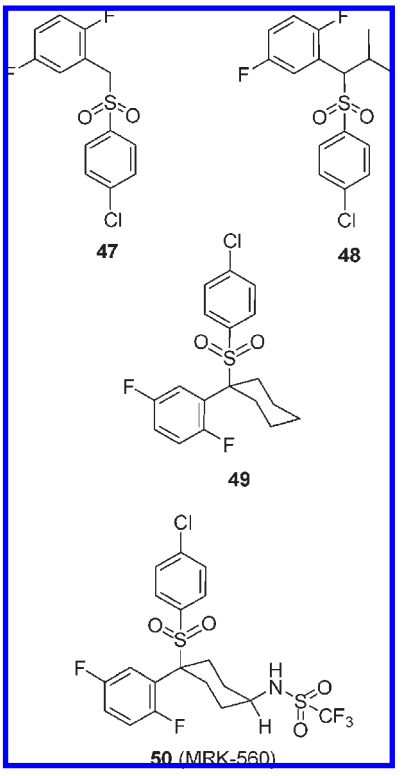


Figure 21. Merck alternative binding site sulfone GSIs.

and in vivo potency ($A\beta_{40}$ ED_{50}(brain) = 1 mg/kg, po in APP-YAC mouse model).[100] Unfortunately, **46** exhibited no Notch-sparing selectivity.

The Merck group has also used the Bristol-Myers Squibb arylsulfonamide scaffold as a starting point for design of novel arylsulfone-containing GSIs in which the sulfonamide nitrogen was replaced by a C−H group.[101] Although **47** (Figure 21), the initial lead derived from this work, was a weak GSI ($A\beta_{40}$ IC_{50} = 4408 nM), its GSI activity could be significantly improved by increasing the size of the lipophilic group at the benzylic position (e.g., **48**, $A\beta_{40}$ IC_{50} = 70 nM) and by preparing more rigid analogues (e.g., **49**, $A\beta_{40}$ IC_{50} = 3 nM). Interestingly, the 2,5-difluoroaryl group in **49** appears to occupy the axial position. Further optimization of this novel series at the 4-position of the cyclohexane ring gave the picomolar GSI **50** (MRK-560, $A\beta_{40}$ IC_{50} = 650 pM), which demonstrated an MED of 1 mg/kg po for reducing brain $A\beta_{40}$ in the APP-YAC mouse model.[102] Although **50** displays no Notch-sparing selectivity in vitro, the Merck group was able to chronically reduce brain $A\beta_{40}$ levels in Tg2576 mice by 43% and amyloid plaque deposits with 3 mg/kg po **50** without observation of NRSEs.[103] This further indicates that an in vivo therapeutic window between GS processing of substrates APP and Notch is possible. Compound **50** was also shown to reduce brain and CSF $A\beta$ levels in rats with ED_{50} values of 6 and 10 mg/kg, respectively.[104] Investigation of the effect of substitution at the 3-position of the cyclohexane ring of this series has subsequently revealed that in most examples the cis isomer is more potent than the trans isomer because of conformational effects.[105] The cis isomer can adopt a conformation in which both the sulfone and the 3-substituent are equatorial that fits their pharmacophore model well. However, in the trans isomer the sulfone no longer dominates the conformation and it is forced into an axial position that does not fit well into the pharmacophore.

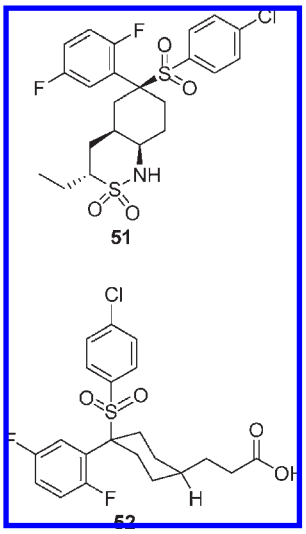


Figure 22. Additional Merck alternative binding site sulfone GSIs.

Although **50** is a potent GSI with promising in vivo efficacy in reducing Aβ levels, this compound shows a relatively short half-life in rodents (e.g., F = 31%, $T_{1/2}$ = 1.1 h in rats) and a high turnover in rat liver microsomes (67% remaining after 20 min). By fusing sulfone, sulfonamide, and sulfamides onto the 3,4 position of the cyclohexane of **50**, the Merck group was able to obtain compounds such as **51** (Figure 22) with improved metabolic stability in rat that retained picomolar in vitro GSI activity (93% remaining after 20 min, Aβ_{40} IC_{50} = 60 pM) and demonstrated good in vivo reduction of Aβ in the APP-YAC mouse model (Aβ_{40} ED_{50}(brain) = 2.8 mg/kg, po).[106] As with the previous Merck sulfones, no Notch-sparing selectivity was demonstrated for these compounds. Subsequently, the structure of one of Merck's clinical GSIs (**52**) was published and is derived from their arylsulfone work.[107]

The Wyeth group has utilized two approaches in identifying GSI leads: (1) molecular modeling and (2) HTS screening. Like the Merck group, Wyeth also used the Bristol-Myers Squibb arylsulfonamide scaffold as a starting point for design of novel arylsulfone-containing GSIs; however, a different approach was taken.[108] Using the ROCS (rapid overlay of chemical structures) program that identifies molecules that have a similar three-dimensional shape, the Wyeth group analyzed the Bristol-Myers Squibb arylsulfonamide **53**[109] (Aβ_{40} IC_{50} = 100 nM in hAPP$_{swe}$CHO cells). After conformational searching and energy minimization of **53** by employing Macromodel, the ROCS query gave 500 hits with shape similar to that of **53** using an in-house compound database. Analysis of these 500 hits was guided by three structural features thought to be important for GSIs: two hydrophobes about 4 Å apart and a hydrogen bond acceptor about 6−8 Å from the hydrophobes (Figure 23). Further analysis of one of these hits, **54**, which was not active in their GSI assays, led Wyeth to replace the hydroxamic acid in this compound with a phenyl ring which would satisfy the first requirement of the Wyeth GSI pharmacophore model. Second, the Wyeth group replaced the alkene in **54** (Figure 24) with a carbamate which added the requisite hydrogen bond acceptor. These two modifications afforded compound **55** which overlapped well while maintaining the pharmacophore orientation and had promising GSI activity (Aβ_{40} IC_{50} = 3500 nM in hAPP$_{swe}$-CHO cells). Unfortunately, in subsequent SAR efforts the

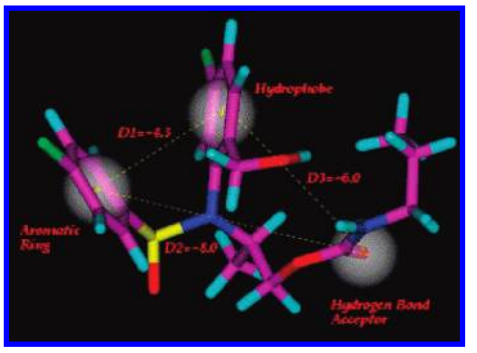


Figure 23. Wyeth GSI pharmacophore overlaid on lowest energy conformation of **53**. Reprinted with permission from *Bioorganic & Medicinal Chemistry Letters*.[110] Copyright 2005 Elsevier.

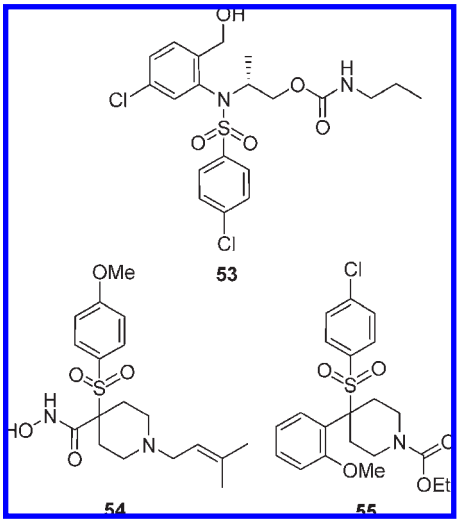


Figure 24. Alternative binding site GSIs and related compounds.

Wyeth group was unable to significantly improve the GSI potency of this lead.

The Wyeth group found a more successful approach, in a collaboration with ArQule, by utilizing HTS screening of their combined compound collections employing a whole cell assay (hAPP$_{swe}$CHO cells)[111] which provided two related HTS hits **56** (Figure 25, Aβ_{40} IC_{50} = 5449 nM) and **57** (Aβ_{40} IC_{50} = 2214 nM). Systematic SAR on these leads to improve GSI potency revealed a narrow tolerance for structural variation. Extending the length of the side chain to afford **58** did significantly improve GSI potency (Aβ_{40} IC_{50} = 294 nM). Compound **58** also exhibited significant Notch-sparing selectivity (13.9-fold). Unexpectedly, when the 4-chlorophenyl ring in **58** was replaced by a 5-chlorothiophene moiety to afford **59**, a large increase in GSI potency (Aβ_{40} IC_{50} = 25 nM) was observed with retention of Notch-sparing selectivity.[112,113] Interestingly, the Merck group has subsequently also observed a large increase (7.5×) in GSI potency when the 4-chlorophenyl ring is replaced by a 5-chlorothiophene moiety in their series of arylsulfonamides.[97] The reasons for this increase in GSI potency are not clear. With the potent GSI **59** in hand, the Wyeth group profiled this compound in vivo in the Tg2576 mouse model and was gratified to observe a 25% reduction of brain Aβ_{40} and Aβ_{42} at 100 mg/kg po, which was comparable to that obtained with the benchmark GSI **13**.[113] To improve the in vivo efficacy of **59**, the Wyeth group sought to elucidate the properties of this molecule that could limit its

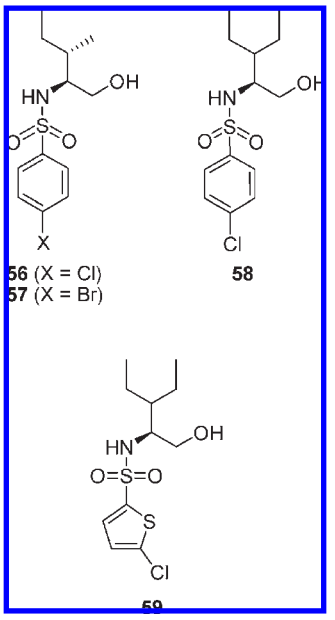


Figure 25. Wyeth first generation alternative binding site sulfonamide GSIs.

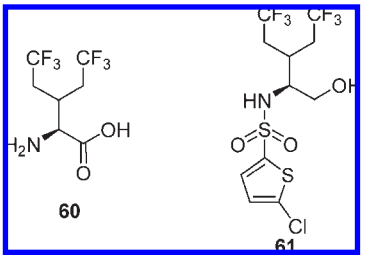


Figure 26. Wyeth second generation alternative binding site sulfonamide GSI and related synthon.

oral in vivo activity. Compound **59** was found to have excellent solubility (6.7 mg/mL in Phosal PG 50/Tween-80/water in a ratio of 10:2:88), good permeability (CACO2: A-B, $P_{app} = 50 \times 10^6$ cm/s; B-A, $P_{app} = 42 \times 10^6$ cm/s), and a favorable brain plasma ratio (2.0), ruling out these properties as limiting the in vivo activity. However, the in vitro metabolic stability of **59** was low in microsomes [$t_{1/2}$ = 1 min (rat), 2 min (mouse), 8 min (human)], indicating that the rapid metabolism may be a key factor in limiting the in vivo efficacy.

To improve the metabolic stability of **59**, the major sites of metabolism were identified as glucuronidation of the primary hydroxyl group and oxidation of the terminal methyl groups of the side chain. Blocking glucuronidation in this series led to an unacceptable loss of GSI potency; however, oxidation of the terminal methyl groups could be blocked, with retention of GSI potency, by replacing the terminal methyl groups with CF_3 groups. This necessitated developing a synthesis of the novel α-amino acid **60** (Figure 26), which was subsequently converted to the desired target **61**.[114] This compound demonstrated improved metabolic stability [$t_{1/2}$ = 8 min (rat), 24 min (mouse), 8 min (human)] while retaining GSI potency ($A\beta_{40}$ IC_{50} = 16 nM) and Notch-sparing selectivity (15-fold). When Tg2576 mice were dosed with **61**, 5 mg/kg po, both brain $A\beta_{40}$ and $A\beta_{42}$ were reduced by 27% and 22%, respectively.

Although the Wyeth group had improved the in vivo efficacy of this series with **61** by addressing metabolic stability, the group still needed to address the short in vitro metabolic

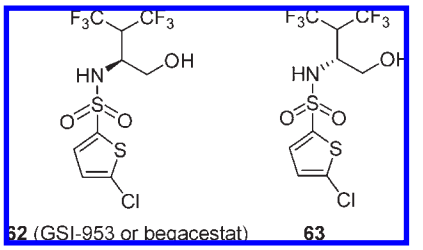


Figure 27. Wyeth clinical GSI and enantiomer.

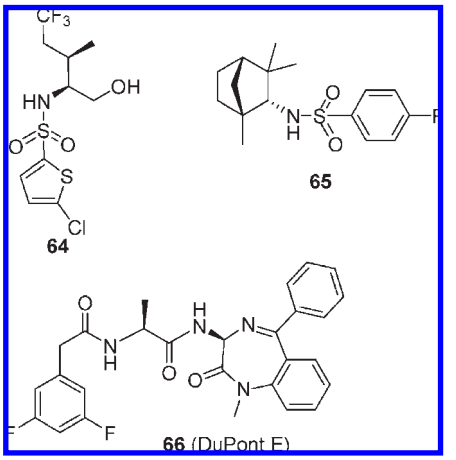


Figure 28. Miscellaneous GSIs.

stability of this compound in human microsomes. They hypothesized that by removing the two methylene groups in the side chain of **61**, they could improve the metabolic stability by eliminating potential sites of metabolism. When the target compound **62** (Figure 27, GSI-953 or begacestat) was prepared, they discovered that, in fact, in vitro metabolic stability in human microsomes was greatly improved [$t_{1/2}$ = 3 min (rat), 48 min (mouse), >90 min (human)]. This compound also retained potent GSI activity ($A\beta_{40}$ IC_{50} = 15 nM) and achieved targeted Notch-sparing selectivity (14-fold). When Tg2576 mice were dosed with **62** at 5 mg/kg po, both brain $A\beta_{40}$ and $A\beta_{42}$ were reduced by 37% and 25% at 4 h, respectively. In the CFC behavioral model developed by the Wyeth group,[54] **62** reversed cognitive deficits at 10 mg/kg po whereas its enantiomer **63**, which is much less active as a GSI ($A\beta_{40}$ IC_{50} > 30 000 nM), was inactive in this model.[115] On the basis of its favorable pharmacological profile and lack of NRSEs in several animal models, **62** was selected for clinical evaluation in the treatment of AD.

It is interesting to compare the results of the SAR leading to **62** with the structural features present in the Bristol-Myers Squibb GSIs **37** and **53**. In contrast to **37** and **53**, there is a requirement in the Wyeth series of GSIs, represented by **62**, for *S* absolute configuration at the 2-amino alcohol-derived chiral center, a free hydroxyl group, a large β-branched side chain, and an unalkylated sulfonamide nitrogen. Considering the structural differences between the Wyeth arylsulfonamides and the Bristol-Myers Squibb arylsulfonamides, it was of interest to determine if these compounds compete for the same binding site on GS. Toward this end the Wyeth group prepared the GSI **64** (Figure 28, $A\beta_{40}$ IC_{50} = 100 nM) and introduced two tritium atoms on the primary hydroxyl carbon by standard methodology.[116] Using a GS cell-free preparation derived from membranes isolated from human neuroblastoma SY5Y cells, they were able to show that not

Table 1. Correlation of GS Binding with Aβ_{40} Inhibition[117]

	IC_{50} (nM)	
compd	GS binding	Aβ_{40}
62	8	15
63	> 10000	> 30000
13	29	28
15	1	0.4
16	38	26
66	1	2.7
1	22	281
53	57	47
65	966	2634

only **53** but also a wide variety of structurally diverse GSIs (**13**, **15**, **16**, **62**, **65**, **66** (DuPont E)) could competitively displace tritiated **64** from GS (Table 1).[117] This is consistent with these GSIs competing for the same binding site on GS. However, displacement of tritiated **64** from GS by the active site directed GSI **1** was incomplete even at high (saturating) levels of compound. The binding affinities to GS in this assay correlated with GSI potencies in Wyeth's cellular assay ($hAPP_{swe}CHO$ cells) except for the active site directed GSI **1**.

The Schering-Plough group also utilized HTS screening of a cell-free GS assay to identify an arylsulfonamide lead **67** (Figure 29, Aβ_{40} IC_{50} = 2500 nM in GS derived from C99 transfected 293 cells),[118] which was elaborated into novel potent GSIs such as **68** (Aβ_{40} IC_{50} = 39 nM). Unfortunately, these tetrahydroquinoline sulfonamides had poor PK properties. Further SAR in this series led to the identification of a simplified piperidine core bearing cis C-2 and C-6 substituents with good potency (e.g., **69** Aβ_{40} IC_{50} = 11 nM).[119] Interestingly, the C-2 and C-6 substituents were proposed to be in a diaxial conformation of the chairlike piperidine ring based upon analysis of the vicinal proton−proton *J* coupling constants and NOEs of the resulting MTPA esters of the primary alcohol derived from **69**. Replacement of the piperidine ring with a pyrrolidine ring in this series led to a ~10-fold decrease in GSI potency.[120] Poor PK was also seen with **69** when TgCRND8 mice were dosed with this compound.[121] It was hypothesized that the poor PK was due to the rapid in vivo hydrolysis of the carbamate linker. Therefore, groups were introduced adjacent to the carbamate in order to provide steric hindrance toward hydrolysis. This eventually led to the potent GSI **70** (Aβ_{40} IC_{50} = 2.4 nM) which exhibited >75% reduction of plasma Aβ when TgCRND8 mice were dosed orally with this compound at 10 mg/kg. Reduction of the levels of both Aβ_{40} and Aβ_{42} in the cortex of the brain of these animals tracked well with the effect in the plasma.

Further testing of **70** and related analogues in this series revealed that these compounds are potent inhibitors of cytochrome P_{450} (Cyp) 3A4 (for **70**, IC_{50} = 60 nM).[122] Interaction with Cyp3A4 has been linked to lipophilicity (cLogP = 6.05 for **70**) and the presence of basic amines; therefore, the strategy was to prepare less lipophilic analogues containing less basic amine groups. This effort afforded **71** (Figure 30, Aβ_{40} IC_{50} = 6.9 nM) that not only had reduced Cyp3A4 inhibition (IC_{50} > 2 μM) but also led to near complete reduction of plasma Aβ levels (and presumed reduction in brain Aβ levels based on high brain penetration) when administered to TgCRND8 mice at 30 mg/kg po. Interestingly, linking the 6-ethyl and 2-cyclopropyl side chains in this series to form conformationally restricted analogues retained GSI potency and provided support that the diaxial conformation of the C-2 and C-6 substituents is the active conforma-

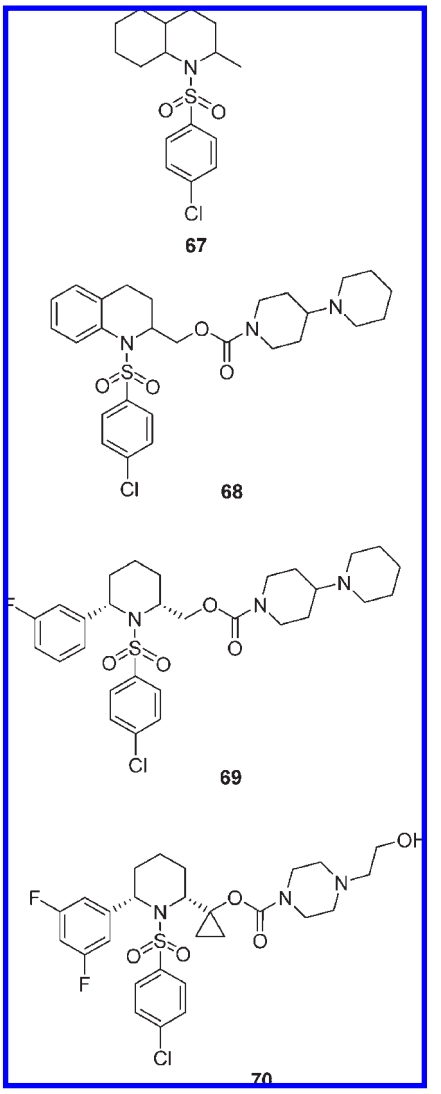


Figure 29. Schering-Plough alternative binding site sulfonamide GSIs.

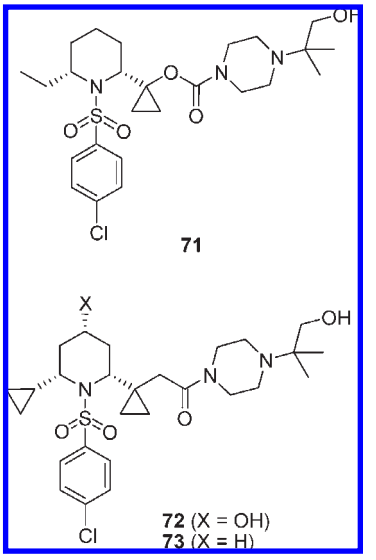


Figure 30. Additional Schering-Plough alternative binding site sulfonamide GSIs.

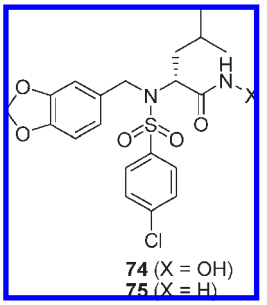


Figure 31. Hoffman-LaRoche first generation alternative binding site sulfonamide GSIs.

tion. Further efforts to reduce Cyp3A4 inhibition by introducing a polar group on the 4-position of the piperidine ring led to compounds such as **72** which had GSI activity comparable to that of its 4-unsubstituted counterpart **73** ($A\beta_{40}$ IC_{50} = 8.1 and 9.9 nM, respectively) with a greatly improved Cyp3A4 profile (IC_{50} = 11 and 1.5 μM, respectively).[123] Subsequently it was found that replacing the carbamate group in this series with either an amide group or a heterocycle retains in vivo efficacy (e.g., **73** caused 80% reduction of plasma $A\beta_{40}$ in CRND8 mice when dosed 30 mg/kg po).[124] No data related to Schering-Plough GSIs concerning the extent of Notch sparing selectivity have been published.

The Hoffman-LaRoche group identified **74** (Figure 31) as an initial screening hit ($A\beta_{40}$ IC_{50} = 2600 nM in hAPP HEK cells).[125] Conversion of the hydroxamic group in **74** to a primary amide group afforded the nanomolar GSI **75** ($A\beta_{40}$ IC_{50} = 8 nM). Unfortunately, **75** had poor solubility (<1 μg/mL), poor permeability, and high microsomal clearance (282 (μL/min)/mg protein in mouse) that needed to be improved.

Synthesis of ring constrained analogues such as lactam **76** (Figure 32) did lower microsomal clearance (39 (μL/min)/mg protein in mouse) while retaining GSI potency ($A\beta_{40}$ IC_{50} = 20 nM). Difluoro substitution on the lactam ring affording the potent GSI **77** ($A\beta_{40}$ IC_{50} = 4 nM) further lowered microsomal clearance (2.2 (μL/min)/mg protein in mouse). When PS-2/APP Tg mice were treated with **77** at 20 mg/kg (3 times per day) po or ip, no reduction of brain Aβ was observed. This was attributed to the possible low brain penetration of **77** due to the presence of two amide groups in its structure. Removal of one of the amide groups in **77** led to the potent GSI **78** ($A\beta_{40}$ IC_{50} = 2 nM) that displayed MED = 20 mg/kg po for brain Aβ reduction in PS-2/APP Tg mice. Further work is in progress to optimize this series; yet, again, the Notch-sparing selectivity of these compounds is unknown.

Finally, the Elan group has recently disclosed the structure of a novel, potent ($A\beta_{40}$ IC_{50} = 2 nM), Notch-sparing (13.4-fold) arylsulfonamide GSI **79** (Figure 33, ELN-475, 516)[126−129] that was able to reduce brain Aβ levels in both wild type (MED < 30 mg/kg) and Tg mice when given orally. Unlike most in vitro Notch-sparing assays, the Notch and APP processing assays were run simultaneously in a single cell and NICD was measured directly. In vivo, no NRSEs were observed with **79** in a 7-day repeat dose mouse model up to 1000 mg/kg b.i.d.[127]

Clinical Trials of GSIs in AD

A number of GSIs have entered clinical trials for the treatment of AD: **16**, **37**, **52**, **62**, **80** (Figure 34, BMS-708,163),[130] **81** (MK-0752,[131] structure not disclosed), and

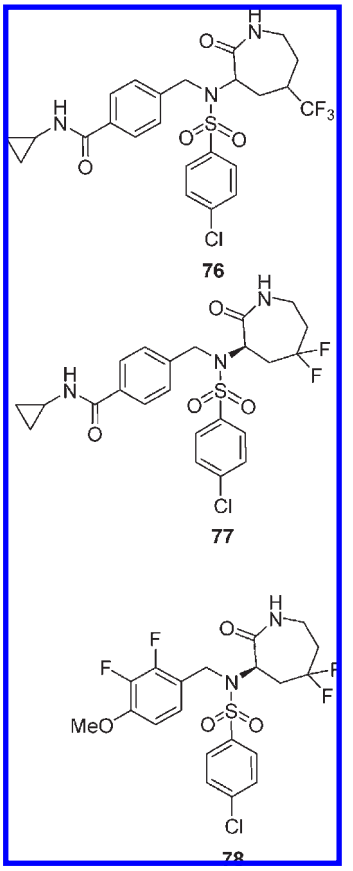


Figure 32. Hoffman-LaRoche second generation alternative binding site sulfonamide GSIs.

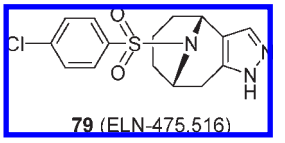


Figure 33. Elan alternative binding site sulfonamide GSI.

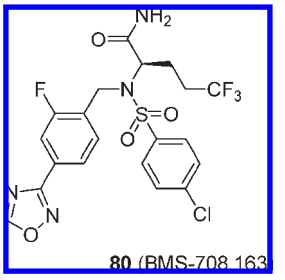


Figure 34. Bristol-Myers Squibb second generation clinical GSI.

ELN-006[132] (structure not disclosed). The most advanced GSI is **16**, which has entered phase III trials despite the lack of Notch-sparing selectivity.[76] A 14-day phase 1 study of 5−50 mg doses of **16** ($t_{1/2}$ = 2.5 h) in healthy volunteers revealed that plasma Aβ concentrations decreased in a dose−dependent manner over a 6 h period following drug administration, with a maximum decrease of ~40%.[133] Increasing the dose of **16** to 140 mg in healthy volunteers led to a 73% reduction in plasma Aβ but no reduction in CSF Aβ.[75] In a related 6 week study in AD patients[134] **16** was well tolerated at 40 mg q.d. and demonstrated a 38% reduction in plasma Aβ. In this study

again, despite the fact that subsequent clinical studies employing radiolabled leucine have shown a reduction by **16** on newly synthesized Aβ in the CSF, no reduction of CSF Aβ was observed.[135] In a phase II safety trial in AD patients,[76] **16** was well tolerated at doses up to 140 mg/d for 14 weeks, which led to a 65% reduction in plasma Aβ. Again, no significant reduction of CSF Aβ was observed nor improvement in the ADAS-Cog (Alzheimer disease assessment scale-cognitive) score that measures cognitive improvement.

Although the Merck GSI **52** has entered clinical trials for AD, few details are available on these studies.[107] The Merck GSI **81** ($A\beta_{40}$ $IC_{50} \approx 50$ nM, $T_{1/2} = 20$ h) was able to lower both plasma and CSF Aβ when administered to healthy volunteers at single doses greater than 300 mg.[136,137] A mean CSF Aβ reduction of $>35\%$ was observed for doses greater than 500 mg. It is noted in this study that drug concentrations in CSF were similar to those of estimated free drug in plasma; however, a slight time shift was noted in CSF drug concentrations relative to plasma concentrations ($\sim$ 2 h later for CSF T_{max}). In addition, the time course of the CSF Aβ lowering appeared to lag the time course of both the plasma and CSF drug concentrations. Note that whether Merck GSI **52** is in fact **81** is currently unknown.

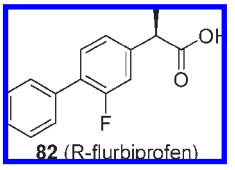
In healthy subjects, the Wyeth GSI **62** was able to lower plasma Aβ by 40% when given at a single oral dose of 600 mg to healthy young volunteers.[138] Interestingly, in this study the clearance of **62** was $\sim$40% lower in healthy, elderly subjects. CSF concentrations of **62** were 10-fold lower relative to plasma concentrations, and no reduction of CSF Aβ was observed. A translational medicine study was subsequently performed on **62**, comparing PK/PD biomarker relationships in Tg2576 mice and humans.[139] It was found that a 10 mg/kg dose of **62** in Tg2576 mice produces different exposures and effects on Aβ in the plasma (AUC = 5951 ng·h/mL and 9−20% lowering of Aβ), brain (AUC = 9338 ng·h/mL and 22−33% lowering of Aβ), and CSF (AUC = 350 ng·h/mL and no lowering of Aβ). The exposures and effects on Aβ in the plasma (AUC = 2334 ng·h/mL and a mean 28% lowering of Aβ) and CSF (AUC = 240 ng·h/mL and no lowering of Aβ) obtained in the clinic with a 450 mg dose of **62** in AD subjects were correlated with the Tg2576 results and suggested that the exposure in human brain would lead to AUC = 2400 ng·h/mL, which should produce brain Aβ lowering similar to what was observed in plasma. The apparent disconnect between CSF and brain Aβ lowering in Tg2576 mice may be explained by the lower drug levels observed in CSF compared to brain.

Like **81**, the Bristol-Myers Squibb GSI **37** has entered clinical trials for AD but no details have been published on these studies. This compound was subsequently terminated because the PK profile was not adequate, and this limited the ability to achieve target drug exposures.[140] A second generation Bristol-Myers Squibb GSI **80**[130] has recently entered clinical trials for the treatment of AD.[141] This compound was a potent GSI ($A\beta_{40}$ IC_{50} = 300 pM in $hAPP_{swe}H4$ cells) with a large reported Notch-sparing selectivity (193-fold). Consequently, **80** was able to chronically lower brain Aβ in both rats and dogs without NRSEs. In clinical trials, this compound was well tolerated up to 400 mg as a single dose and up to 150 mg following 28-day multiple dosing. A single 200 mg dose of **80** ($T_{1/2}$ = 40 h) in healthy, young subjects led to decreases in both plasma and CSF Aβ (50% and 40%, respectively). Dosing healthy, young volunteers for 28 days resulted in reductions in CSF Aβ without NRSEs.

Table 2. Clinical Lowering of Aβ by GSIs

compd	dose (mg)	% plasma Aβ reduction	% CSF Aβ reduction
16[75,135]	140	73 at 6 h	52 at 12 h[a]
81[137]	300		29 at 12 h
81[150]	450	46 at 4 h	
62[139]	450	28 at 2 h	NS[b] at 1−24 h
80[141]	200	50 at 4 h	40 at 12 h

[a] Newly synthesized Aβ. [b] NS = not significant.



82 (R-flurbiprofen)

Figure 35. Myriad clinical GSM.

Perspective and Future Directions

Since Wolfe's Perspective on secretase inhibitors as AD targets appeared in this journal in 2001,[21] numerous advances have been made in the field. The components of GS have been identified, and low resolution structures of this enzyme complex have been obtained. Numerous GSI leads have been identified by HTS screening, and subsequent optimization efforts have afforded several clinical candidates. The issue of NRSEs of GSIs has been addressed either by adjusting the dose of nonselective GSIs such as **15** or by identifying Notch-sparing GSIs such as **37**, **62**, and **80**. A recent report has demonstrated that Notch-based GI toxicity of GSIs could be abrogated in mice by coadministration of glucocorticoids.[142] It remains to be seen whether this combination therapy is as safe in man as it is in mice. Initial clinical trials with GSIs have demonstrated that lowering of plasma and CSF Aβ is possible without significant side effects (Table 2). A new class of drugs targeting GS has recently emerged, the so-called GS modulators (GSMs), which selectively lower $A\beta_{42}$ rather than total Aβ without affecting Notch-processing.[143−145] Two of these GSMs have entered clinical trials: **82** (Figure 35, *R*-flurbiprofen) from Myriad and E-2012 (structure not disclosed) from Eisai. At least in the case of **82**, the drug interacts with APP rather than with GS.[146] However, the Merck group has shown that **82** and the GSM sulindac sulfide could noncompetitively block binding of an active-site directed GSI to GS, implying that these GSMs also interact with GS.[147] An additional level of complexity recently uncovered for the processing of APP by GS is that APP binds to GS as a dimer and must partially unwind for GS processing.[148]

The first BACE inhibitor CTS21166 **83** (structure not disclosed) from CoMentis has also entered clinical trials[149] and has been shown to reduce plasma Aβ by 80% when given iv at a 150 mg dose. More advanced AD clinical trials will be required to determine if GSIs and BACE inhibitors lead to disease-modifying effects on cognition and memory. Nevertheless, the identification of second generation of GSIs such as **62**, **79**, and **80** with improved Aβ-lowering potency, Notch-sparing selectivity, and PK is a cause for optimism that NRSEs may be able to be avoided in AD clinical trials of GSIs.

Despite this impressive progress, much work remains to be done. The physiological roles of APP, Aβ, and AICD need to be clarified as does the possible role of AICD signaling in AD.[151] Recent evidence suggests that APP may function as a receptor for TAG1[152] and may be involved in neurogenesis.

In addition, APP and DR6 were recently identified as components of a neuronal self-destruction pathway and suggest that an extracellular fragment of APP, acting via DR6 and caspase 6, may contribute to AD.[153] The non-GS roles of PS also need to be elucidated. Recent evidence suggests that PS is involved in calcium channel function that is not affected by GSIs.[154,155] A high resolution structure of GS would be helpful in understanding how the four components of GS fit together and interact and may lead to the design of GSIs targeting other GS components such as nicastrin, Aph-1, or Pen-2. In lieu of this, more indirect methods will need to be employed. A recent example of this is the use chemical cross-linking agents to analyze GS which revealed that the PS-1 CTF subunit interacts with Aph-1a.[156] It would also be useful to identify the specific amino acid sequences on PS responsible for binding the different classes of GSIs. A recent investigation toward this goal has identified specific regions of PS to which GSIs from different classes bind.[157] The sequence of intermediates in the cleavage of C99 by GS to afford $A\beta_{40}$ and $A\beta_{42}$ needs to be further explored because there is evidence for both successive and nonsuccessive cleavage models. For example, recent work by Zhao et al. suggests that C99 is first cleaved to $A\beta_{49}$ (and AICD) by GS and further processing by GS affords $A\beta_{46}$ which is proposed to lead to both $A\beta_{40}$ and $A\beta_{42}$[158,159] In contrast, Ihara et al. have presented evidence for a nonsequential process in which processing of $A\beta_{46}$ by GS affords $A\beta_{43}$ or $A\beta_{40}$.[160] It should also be noted that the steps involved in the processing of C99 by GS have been shown to vary in sensitivity across different GSI inhibitor classes,[160] and more work is needed to clarify this.

The pharmacology of different complexes of GS (e.g., GS-containing PS-1 versus GS-containing PS-2) may vary, and this needs to be further investigated.[161] Recent work by the Elan group has shown that GSIs exhibit different activities toward PS-1-containing GS versus PS-2-containing GS.[162] Interestingly, the Schering-Plough group recently presented evidence that PS-1 selective GSIs such as **50** show less in vivo NRSEs in mice than PS-1 nonselective GSIs such as **15**.[163] In addition, the PS-1 selectivity and PK parameters of GSIs may vary across species, which may contribute to a difference in tolerability of GSIs. De Strooper's group[164] has proposed that Aph-1a-containing GS is responsible for Notch processing while Aph-1b-containing GS is responsible for APP processing. This needs to be investigated further and may provide the key to the design of improved Notch-sparing GSIs. Toward this end, human GS has been recently purified and characterized.[165] Although the structural features of GSIs that lead to potent inhibition of $A\beta$ production are fairly well understood, the structural features of GSIs that lead to inhibition of Notch processing and Notch-sparing selectivity are not well understood at present and will require more extensive SAR work. GSMs have emerged as an exciting area that may also provide a Notch-sparing drug; however, the potency and brain penetration of these drugs need to be improved over the low potency ($A\beta_{42}$ $IC_{50} \approx 250$ μM)[143] and poor brain levels of **82** which failed to reach its clinical end points in a phase III trial.[166] Although many substrates for GS have been identified besides APP, Notch remains the most relevant physiologically.[44] One caveat is that Notch-sparing drugs may cause other toxicities by inhibition of cleavage of other GS substrates (e.g., ErbB4, *N*-cadherin). Another area in which GSI selectivity will need to be addressed is in the inhibition of signal peptide peptidase (SPP), which is an intramembrane aspartyl protease with homology to PS.[167,168]

A confounding observation in vivo both in animals and in man is the observation of a rebound in $A\beta$ plasma levels after the initial reduction of plasma $A\beta$ with GSIs, a phenomenon that was not seen with the BACE inhibitor **83**.[149] This rebound in $A\beta$ levels was also seen in rat brains dosed with **37**[169] but not in dogs brains dosed with **16**.[170] Interestingly, the rebound in $A\beta$ levels seen in rat brains dosed with **37** correlated with partial GS occupancy,[169] implying that high GS occupancy will be required to avoid the brain $A\beta$ rebound, at least in rats. This phenomenon needs to be investigated further in multiple species. Another potential pitfall in the development of GSIs is the difference in GSI potency in cells expressing wild type versus FAD mutant APP.[169] The generality and magnitude of this potency difference needs to be investigated further in other series of GSIs and in other cell types because FAD mutant APP is frequently used for the selection and validation of GSIs. A recent publication from the Novartis group using transgenic mice containing the Swedish APP mutation did not show a difference in potency compared to wild type mice with the GSI **15**.[171]

Finally, GSIs may have other uses beyond AD. For example, because excessive Notch signaling is involved in certain cancers, a Notch-selective GSI may be useful to treat these cancer subtypes.[172] In fact, the GSI **81** has entered clinical trials for the treatment of leukemia and breast cancer.[136,150] Notch-sparing GSIs may be useful to treat glaucoma based upon the proposed role of $A\beta$ in apoptosis of retinal ganglion.[173] Further identification of Notch-selective and Notch-sparing GSIs will allow the identification of other diseases that may be amenable to treatment with GSIs. Identification of GSIs that are selective for other GS substrates besides APP and Notch may also expand the potential therapeutic uses of GSIs.

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Robert Martone received his B.S. in Biology from the State University of New York at Albany and conducted graduate studies in Anatomy and Neurobiology at the University of Vermont. He studied blood−brain barrier biology and familial

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Alexander Porte received his B.S. in Chemistry from Calvin College in Grand Rapids, MI, in 1992. He attended graduate school at Texas A&M University and obtained his Ph.D. in 1998 under the supervision of Prof. Kevin Burgess. He joined ArQule in Boston, MA, in 1998 and subsequently moved to Wyeth. Currently, he is a Senior Research Scientist II at Wyeth Research in Princeton, NJ, working on Alzheimer's programs including β- and γ-secretase.

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