

Soluble γ -Secretase Modulators Selectively Inhibit the Production of the 42-Amino Acid Amyloid β Peptide Variant and Augment the Production of Multiple Carboxy-Truncated Amyloid β Species

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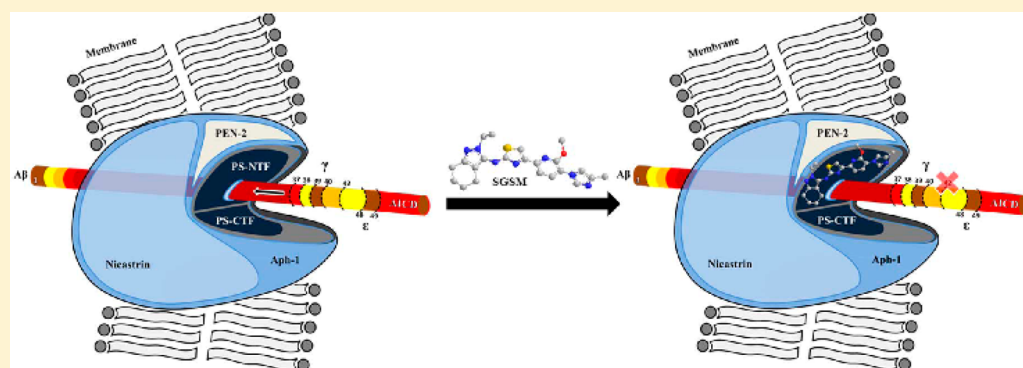
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ABSTRACT: Alzheimer's disease (AD) is characterized pathologically by an abundance of extracellular neuritic plaques composed primarily of the 42-amino acid amyloid β peptide variant ($A\beta_{42}$). In the majority of familial AD (FAD) cases, e.g., those harboring mutations in presenilin 1 (PS1), there is a relative increase in the levels of $A\beta_{42}$ compared to the levels of $A\beta_{40}$. We previously reported the characterization of a series of aminothiazole-bridged aromates termed aryl aminothiazole γ -secretase modulators or AGSMs [Kounnas, M. Z., et al. (2010) *Neuron* 67, 769–780] and showed their potential for use in the treatment of FAD [Wagner, S. L., et al. (2012) *Arch. Neurol.* 69, 1255–1258]. Here we describe a series of GSMs with physicochemical properties improved compared to those of AGSMs. Specific heterocycle replacements of the phenyl rings in AGSMs provided potent molecules with improved aqueous solubilities. A number of these soluble γ -secretase modulators (SGSMs) potently lowered $A\beta_{42}$ levels without inhibiting proteolysis of Notch or causing accumulation of amyloid precursor protein carboxy-terminal fragments, even at concentrations approximately 1000-fold greater than their IC_{50} values for reducing $A\beta_{42}$ levels. The effects of one potent SGSM on $A\beta$ peptide production were verified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, showing enhanced production of a number of carboxy-truncated $A\beta$ species. This SGSM also inhibited $A\beta_{42}$ peptide production in a highly purified reconstituted γ -secretase *in vitro* assay system and retained the ability to modulate γ -secretase-mediated proteolysis in a stably transfected cell culture model overexpressing a human PS1 mutation validating the potential for use in FAD.

Alzheimer's disease (AD) is characterized pathologically by abundant neuritic plaques and neurofibrillary tangles in several brain regions, especially those important for cognition.¹ AD may become an even greater health and economic burden.² Currently, only palliative treatments that provide temporary benefit with no effect on disease progression exist. Potential disease-modifying therapeutic approaches for AD involving both immunological and inhibitory strategies have focused on reducing the levels of all $A\beta$ peptide variants and have

demonstrated little if any efficacy or significant side effects.^{3–7} However, in AD, neuritic plaques are composed primarily of the $A\beta_{42}$ peptide variant,⁸ and the most consistent biochemical phenotype of the more than 200 different familial AD or FAD-linked mutations is an increased $A\beta_{42}/A\beta_{40}$ ratio.⁹ This

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finding raises the possibility that more selectively attenuating A β 42 levels relative to the shorter A β peptide variants (i.e., A β 40, A β 38, and A β 37) may prove to be safer and effective.¹⁰ All A β peptides, including the pathogenic A β 42, are ultimately generated by γ -secretase-mediated proteolysis of APP-CTFs, the β -secretase cleavage product of the amyloid protein precursor (APP).¹¹

γ -Secretase is an enzyme complex composed of four critical subunits: presenilin 1 (PS1) or presenilin 2 (PS2), anterior pharynx defective 1 homologue A (APH-1a or APH-1b), presenilin enhancer 2 (PEN-2), and nicastrin.¹² The γ -secretase complex functions as a unique aspartyl protease that cleaves its substrates within the membrane. It cleaves β -secretase-cleaved APP-CTFs to produce each A β peptide variant. Once released from the membrane, longer A β peptides (e.g., A β 42) oligomerize, ultimately forming insoluble deposits.¹³ One therapeutic approach to AD was focused on lowering the total level of A β peptide production by inhibiting the catalytic activity of γ -secretase. Extensive efforts led to the discovery of many γ -secretase inhibitors (GSIs) that until recently were being developed for clinical use.^{4–7}

A potential liability of GSIs is adverse events resulting from the inhibition of Notch proteolysis,^{6,7} which yields the notch intracellular domain (NICD), a γ -secretase-generated peptide necessary for proper cellular differentiation and the development of key organs. In addition, γ -secretase is now known to hydrolyze a rather large number of type I membrane proteins,¹⁴ including the Notch 1 receptor. Therefore, inhibiting this enzymatic complex, which has been described as the “proteosome of the membrane”,¹⁵ may in fact be detrimental to an aged AD population. Side effects associated with inhibition of γ -secretase-dependent Notch signaling, via inhibiting NICD production, have frequently been observed (both preclinically and clinically) upon repeated exposures to GSIs and were of great concern with respect to the clinical development of GSIs.^{6,7} In all likelihood, as is the case for other age-related degenerative disorders (e.g., cardiovascular disease), successful disease-modifying therapeutic approaches will require long-term administration, beginning early in the disease process, that are without side effects or engender readily manageable ones.

More recently, a safer and more selective approach for modulating A β generation utilized NSAID-like substrate-targeted GSMs (e.g., tarenflurbil) that have been shown to selectively lower levels of A β 42; however, poor potency combined with limited ability to cross the blood–brain barrier resulted in a lack of efficacy in the clinic.^{16–18} We recently discovered and characterized a series of GSMs with potencies >1000-fold improved compared to that of tarenflurbil and with good brain penetrance.¹⁹ These first-generation aryl aminothiazole-containing GSMs (AGSMs) are bridged aromates that appear to bind directly to specific subunits of the γ -secretase complex, elicit a decrease in the level of A β 42 and A β 40 production, and concomitantly increase the level of A β 38 and A β 37 production without measurably affecting γ -secretase-mediated enzymatic processing of other known substrates, such as E-cadherin and Notch.¹⁹ AGSMs were shown to be potent and efficacious *in vivo* in decreasing the levels of A β 42 and A β 40 in both the plasma and brain of APP transgenic mice, and chronic efficacy studies revealed that AGSMs dramatically attenuated AD-like pathology in the Tg2576 APP transgenic mouse model. In addition, the AGSMs, by virtue of their distinct noninhibitory mechanism of action unlike the GSIs, did

not show Notch-related side effects such as intestinal goblet cell hyperplasia.¹⁹ Unfortunately, the poor aqueous solubility of AGSMs (<0.1 μ M at neutral pH) may hinder further preclinical and clinical development.

Herein we describe a structurally and mechanistically related, yet novel, GSM chemotype (termed SGSMs for soluble γ -secretase modulators) with significantly improved physicochemical properties (e.g., aqueous solubility) that may be more suitable for comprehensive preclinical evaluation. A GSM compound, capable of modulating enzyme activity to selectively lower A β 42 levels, without inhibiting the enzymatic machinery or reducing the absolute levels of A β peptides, would likely circumvent many if not all of the major obstacles that attended the development of GSIs. An efficacious and well-tolerated GSM could also reverse the increased A β 42/A β 40 ratio present in FAD-linked mutations.²⁰ The studies described herein demonstrate that SGSMs behave like AGSMs with respect to effects on A β peptide variant production. Both of these methylimidazole-containing aminothiazole-bridged series of GSMs reduced the levels of A β 42, and to a limited degree that of A β 40, while concomitantly increasing the level of production of both A β 38 and A β 37. The SGSMs described here show a dramatic separation between their ability to inhibit the production of A β 42 compared to that of A β 40 and also demonstrate the ability to increase the level of production of A β 34 and A β 33.

We thoroughly characterized these SGSMs with respect to a number of physicochemical properties and potency for lowering A β 42, potency for lowering A β 40, and potency for potentiating production of A β 38. Importantly, none of the SGSMs tested inhibited proteolysis of a truncated Notch protein harboring the γ -secretase ϵ -cleavage sites, even at concentrations 200–1000-fold higher than their IC₅₀ values for decreasing the level of A β 42. The SGSMs tested also showed no effect on levels of APP-CTFs at similar concentrations. Finally, it was also critical to test the ability of the SGSMs to modulate γ -secretase under conditions where the A β 42/A β 40 ratio was increased (i.e., cells overexpressing a mutant PS1), as well as the ability to modulate a highly purified γ -secretase enzyme complex in a fully reconstituted enzymatic assay.

MATERIALS AND METHODS

Reagents and Compounds. All SGSM compounds were designed at the University of California, San Diego, and Massachusetts General Hospital and synthesized at Synchem (Elk Grove Village, IL) and determined to be >95% pure based on liquid chromatography–tandem mass spectrometry and nuclear magnetic resonance analyses. A β peptides were purchased from Bachem (Torrance, CA).

Physicochemical Property Determinations. Kinetic solubility measurements were conducted at Analiza (Cleveland, OH) using chemiluminescent nitrogen detection (CLND) from DMSO stock solutions at pH 6.6 and 7.4 or using ultraviolet (UV) detection in phosphate-buffered saline (PBS, pH 7.4) at Albany Molecular Research, Inc. (AMRI, Albany, NY). ClogP values were calculated using Advanced Chemistry Development Laboratories, Inc. (ACD/Laboratories), software (Toronto, ON).

Stable Cell Lines and Cell-Based Assays for Measuring Extracellular A β Levels. Human SHSY5Y neuroblastoma cells stably overexpressing wild-type human APP751 (SHSY5Y-APP) have been described previously.¹⁹ A β peptide variants in conditioned medium from SHSY5Y-APP cells following

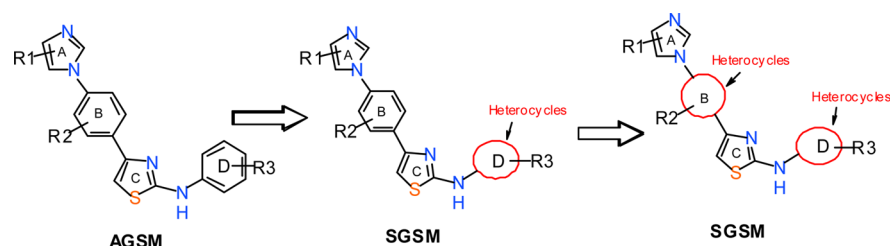


Figure 1. Schematic illustration of the key structural features of AGSMs vs SGSMs. Synthesis of SGSMs was achieved by replacing the phenyl D ring and/or B ring of AGSMs with a variety of different heterocycles.

treatment for 24 h with either vehicle or SGSM were quantitated using sandwich ELISAs for human A β 42, human A β 40, and total human A β as described previously.¹⁹ A β 42, A β 40, and A β 38 peptides from stably transfected CHO-APP-PS1M146L cells treated for 24 h were quantitated in conditioned medium by an ELISA utilizing Meso Scale Sector 6000 multiplex technology (Gaithersburg, MD). Total A β peptide levels were quantitated in conditioned medium by a sandwich ELISA as described previously.¹⁹

Cell-Based Notch Proteolytic Processing Assays.

Human H4 neuroglioma cells stably overexpressing the human APP751 isoform, or H4-APP751 cells, were transfected with the Myc-tagged Notch (N Δ E) construct and then treated with compounds or vehicle (DMSO) at a variety of concentrations by serial dilution for an additional 24 h. Cells were harvested 48 h post-transfection, and cell lysates were prepared and analyzed for levels of NICD by Western blotting using an anti-Myc antibody (1:1000) (Cell Signaling, Danvers, MA). APP and the C99 and C83 APP carboxyl-terminal fragments (APP-CTFs) were probed with the APP carboxyl-terminal antibody, A8717 (Sigma, St. Louis, MO), targeting the 19 carboxyl-terminal amino acids of APP. β -Actin was probed using an anti- β -actin antibody (1:10000) (Sigma). Compound activity was verified by simultaneously analyzing aliquots of conditioned medium for extracellular levels of A β 42 that were quantitated by ELISA kits (Wako, Richmond, VA). HRP-conjugated secondary antibodies (anti-mouse and anti-rabbit) were used at a 1:10000 dilution (Pierce, Rockford, IL).

Mass Spectrometric Analysis of Immunoprecipitated A β Peptides Secreted from Human Neuroblastoma Cells following Treatment with SGSMs. Human SHSY5Y-APP neuroblastoma cells were treated for 24 h with either vehicle (DMSO), compound, 49 or compound 46. Harvested medium was immunoprecipitated using the mAb4G8 antibody (Cell Sciences, Canton, MA), and the immune precipitates were extracted with a trifluoroacetic acid/water/acetonitrile mixture [1/20/20 (v/v/v)] and subjected to matrix-assisted UV laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as described previously.¹⁹

Reconstituted γ -Secretase Assays Using HeLa Cell Membranes and a Tandem Affinity-Purified (TAP) Protein- γ -Secretase Complex. *In vitro* γ -secretase assays capable of detecting the generation of A β 42 and A β 40 from a truncated, biotinylated APP artificial substrate (APP Sb4) were conducted using either γ -secretase-enriched HeLa cell membrane preparations or TAP protein- γ -secretase isolated complexes exactly as described previously.^{19,21} *In vitro* γ -secretase assays capable of detecting NICD from a truncated biotinylated Notch 1 artificial substrate (N1-Sb1) were conducted using both the TAP protein- γ -secretase subunit-

containing complexes and γ -secretase-enriched HeLa cell membranes as described previously.^{19,22}

RESULTS

Heterocycle Substitutions of Phenyl Rings in AGSMs Provide Compounds with a Broad Range of Physico-chemical Properties and Potencies for Inhibiting the Production of A β 42 and A β 40.

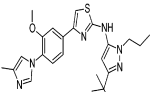
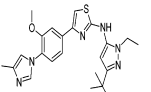
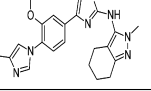
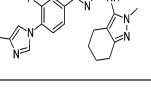
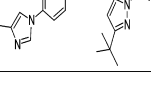
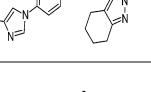
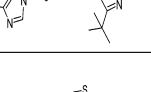
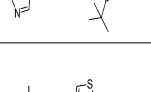
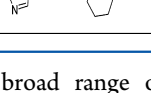
The key structural features of aryl aminothiazole γ -secretase modulators (AGSMs) versus heterocycle-containing or soluble γ -secretase modulators (SGSMs) are schematically illustrated below (Figure 1). Kinetic solubility measurements for a number of aryl aminothiazole AGSMs described previously¹⁹ were below the limits of detection ($<0.1 \mu\text{M}$) regardless of which method of detection was utilized (S. L. Wagner, data not shown). These compounds were focused around diarylimidazoles with an aminothiazole linker (C ring).¹⁹ SGSMs were synthesized utilizing specific heterocycle substitutions replacing the phenyl B rings and D rings of the AGSMs to improve aqueous solubilities while retaining good potencies. Twenty structurally related SGSMs were synthesized and characterized using the assays described and depicted in Table 1 and Figure 2. ClogP values as low as 3.3 and as high as 5.7 were calculated for the 20 SGSM compounds. Kinetic solubility measurements using the chemiluminescent nitrogen detection (CLND) method from DMSO stock solutions were very similar between those performed at pH 6.6 or 7.4. In addition, kinetic solubility measurements for several SGSM compounds were conducted using an ultraviolet (UV) detection method. In the few circumstances where UV detection and CLND methods were compared (compounds 31, 36, and 38), UV detection gave slightly lower estimates of kinetic solubility than did the CLND method (Table 1). In summary, the various B ring and D ring heterocycle substitutions resulted in compounds containing a broad spectrum of kinetic solubilities ranging from 18.4 to $\leq 1.6 \mu\text{M}$ (the lower limit of detection for the UV method). The vast majority had kinetic solubilities (regardless of the method of detection) significantly higher than those of the AGSM series.

Structure-Activity Relationships (SARs) within the B Ring and D Ring of SGSMs. The various B ring and D ring substitutions also led to a wide range of potencies for the inhibition of A β 42 production. These IC₅₀ values were determined with highly reproducible cell-based assays utilizing a human SHSY5Y neuroblastoma cell line stably overexpressing the wild-type human APP751 amino acid isoform (SHSY5Y-APP)¹⁹ and were derived from 10-point concentration response curves (in duplicate at each concentration) using a four-parameter fit nonlinear regression analysis following a 24 h treatment with either compound or vehicle. The assays were required to demonstrate a Z' score of ≥ 0.5 and to have a test-retest reliability with an r^2 of ≥ 0.75 for the 20 SGSM

Table 1. Chemical Structures, Physicochemical Properties, and *in Vitro* Potencies for Novel Soluble γ -Secretase Modulators

Cmpd #	Structure	Clog P	IC ₅₀ for A β ₄₂ inhibition (nM)	IC ₅₀ for A β ₄₀ inhibition (nM)	EC ₅₀ for A β ₃₈ potentiation (nM)	(CLND) Kinetic Solubility (μ M)		(UV) Kinetic Solubility (μ M) pH 7.4
						pH 6.6	pH 7.4	
42		3.83	1275 \pm 92	4125 \pm 1534	2285 \pm 870	5.5	6.3	NA
30		3.3	1051 \pm 141	5815 \pm 5112	376 \pm 149	3.8	3.6	NA
34		3.3	857 \pm 1	1960 \pm 212	3760 \pm 2234	12	11.8	NA
38		3.83	647 \pm 46	1945 \pm 78	320 \pm 125	3.5	3.7	< 1.6
27		4.62	564 \pm 2	4630 \pm 3592	723 \pm 420	2.4	2.3	NA
31		4.61	386 \pm 33	1300 \pm 71	476 \pm 58	7.5	6.2	4.7
39		5.15	347 \pm 83	1115 \pm 7	613 \pm 55	2.3	2.6	NA
28		4.64	296 \pm 11	1242 \pm 492	240 \pm 4	3	3.1	NA
43		5.26	292 \pm 55	1490 \pm 113	1229 \pm 1048	NA	NA	NA
35		5.15	254 \pm 31	830 \pm 31	219 \pm 21	2.7	2.5	NA
29		4.38	223 \pm 1	2010 \pm 382	105 \pm 79	14.8	11.6	5.5

Table 1. continued

Cmpd #	Structure	Clog P	IC ₅₀ for Aβ ₄₂ inhibition (nM)	IC ₅₀ for Aβ ₄₀ inhibition (nM)	EC ₅₀ for Aβ ₃₈ potentiation (nM)	(CLND) Kinetic Solubility (μM)		(UV) Kinetic Solubility (μM) pH 7.4
						pH 6.6	pH 7.4	
45		5.45	185 ± 7	1335 ± 148	241 ± 42	NA	NA	NA
37		4.91	175 ± 8	953 ± 34	196 ± 46	3.2	2.4	NA
33		4.38	165 ± 13	441 ± 13	877 ± 584	18.4	12.1	NA
32		4.64	145 ± 33	657 ± 200	648 ± 319	4.8	4.2	NA
46		5.52	121 ± 9	426 ± 141	131 ± 11	NA	NA	5.5
40		5.17	96 ± 1	350 ± 6	238 ± 40	2.8	2.6	NA
48		5.7	95 ± 2	519 ± 42	149 ± 20	NA	NA	< 1.6
36		5.17	94 ± 3	427 ± 86	245 ± 1	1.8	2.1	3.3
49		4.25	30 ± 6	104 ± 4	305 ± 54	NA	NA	2.7

compounds representing a broad range of potencies (IC₅₀ values for inhibiting the production of Aβ₄₂ ranging from ~1.3 μM to 30 nM).

The structure–activity relationships (SARs) with respect to the heterocycle substitutions in the D ring, for compounds containing a fluorophenyl B ring (compounds 28, 46, 48, and 36), showed that substitution of either an ethyl (36), *n*-propyl (48), or isopropyl (46) group for the methyl group (28) at position 1 of the pyrazole D ring led to a 3-fold increase in potency (compare compound 28 to compounds 36, 46, and

48). These same pyrazole D ring substitutions did not have any effects on potencies for compounds containing the methoxyphenyl B ring (compare compounds 43, 29, 45, and 37).

Briefly, in terms of the B ring SAR, for compounds containing the 3-*tert*-butyl-1-ethylpyrazole D ring (38, 35, 37, and 36), introduction of a fluoro group at position 2 of the phenyl B ring (compare 36 to 35) led to a 2–3-fold increase in potency; introduction of a methoxy group at position 2 of the phenyl B ring led to a <2-fold increase in potency (compare 37 to 35). Alternatively, substituting the phenyl B ring with the 2-

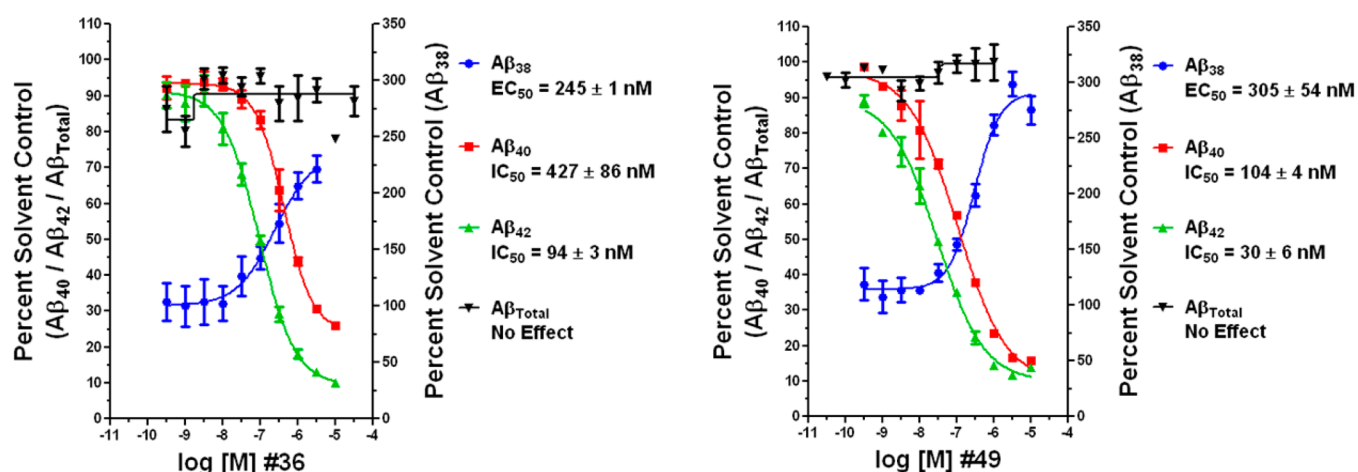


Figure 2. Representative concentration response curves depicting effects of two SGSMs (compounds 36 and 49) on steady state levels of specific A β peptide variants and total A β peptide levels secreted by SHSY5Y-APP cells treated for 24 h with various concentrations of either SGSM 36, SGSM 49, or vehicle (DMSO). Individual A β peptide variants (A β 42, A β 40, and A β 38) and total A β peptide levels were quantitated using MesoScale Sector 6000 multiplex technology and sandwich ELISAs, respectively. IC₅₀ values were derived using four-parameter fit nonlinear regression analyses.

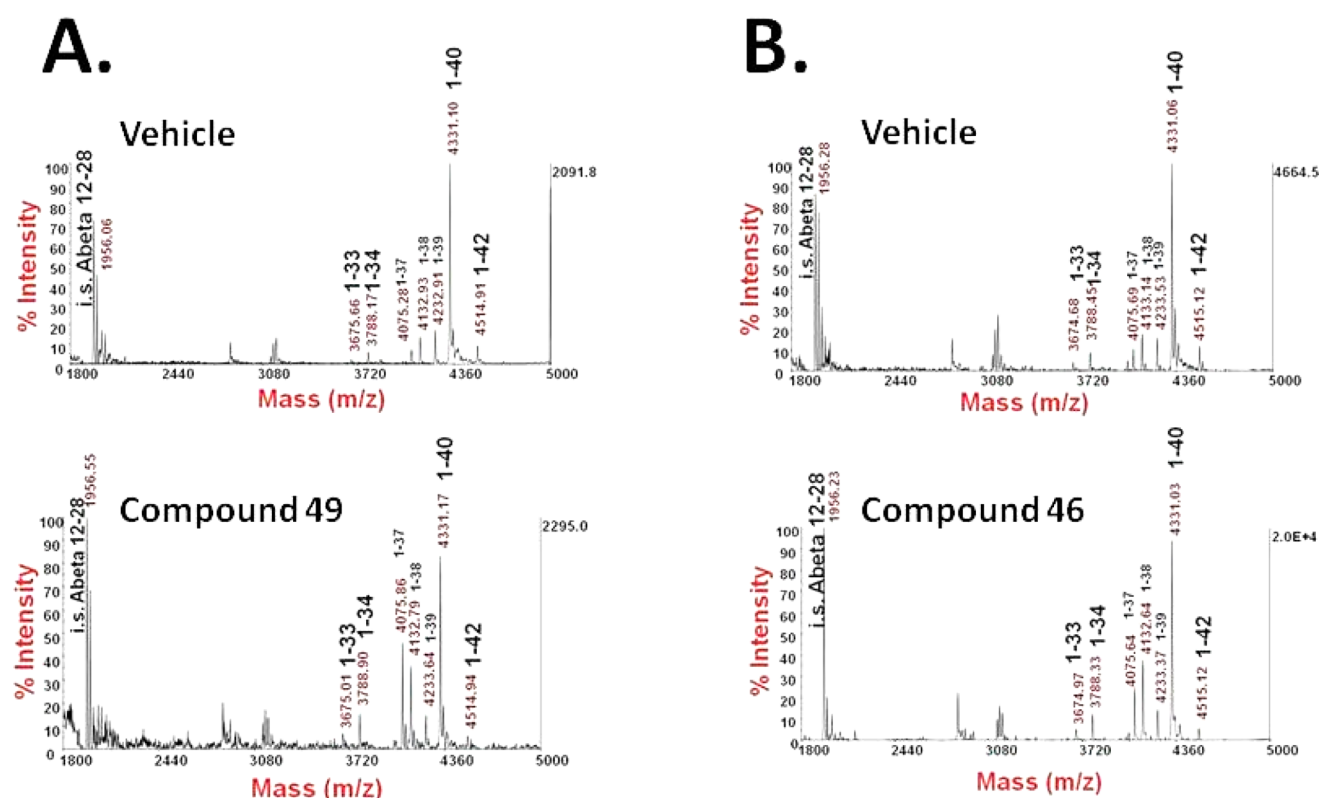


Figure 3. (A) Immune precipitation–MALDI-TOF analysis of A β peptide variants in conditioned medium of SHSY5Y-APP cells following a 24 h treatment with either the DMSO vehicle (top) or SGSM 49 at a concentration of 30 nM (bottom). (B) Immune precipitation–MALDI-TOF analysis of A β peptide variants in conditioned medium of SHSY5Y-APP cells following a 24 h treatment with either the DMSO vehicle (top) or SGSM 46 at a concentration of 150 nM (bottom). The A β 12–28 internal standard (i.s.) was used as the immune precipitation loading control.

pyridyl ring (compare 38 to 35) caused a >2-fold loss of potency. A similar B ring substitution pattern in terms of effects on potency held for compounds containing the 2-ethyl-tetrahydro-indazole D ring (compare compounds 42, 39, and 40; potency for the fluorophenyl 40 > phenyl 39 > pyridyl 42). However, the most striking effect on potency in terms of B ring substitution was achieved by replacing the phenyl B ring (39)

with a 2-methoxy 3-pyridyl B ring (49). This led to a >10-fold increase in potency.

SGSMs Do Not Affect the Levels of Total A β Peptides, Reduce A β 42 Levels Much More Efficiently Than A β 40 Levels, and Increase Levels of A β 38, A β 37, A β 34, and A β 33. Regardless of compound potency, there was a consistent relationship between the IC₅₀ values for A β 42 and A β 40 in the SHSY5Y-APP cell-based assay using ELISAs for A β 42 and

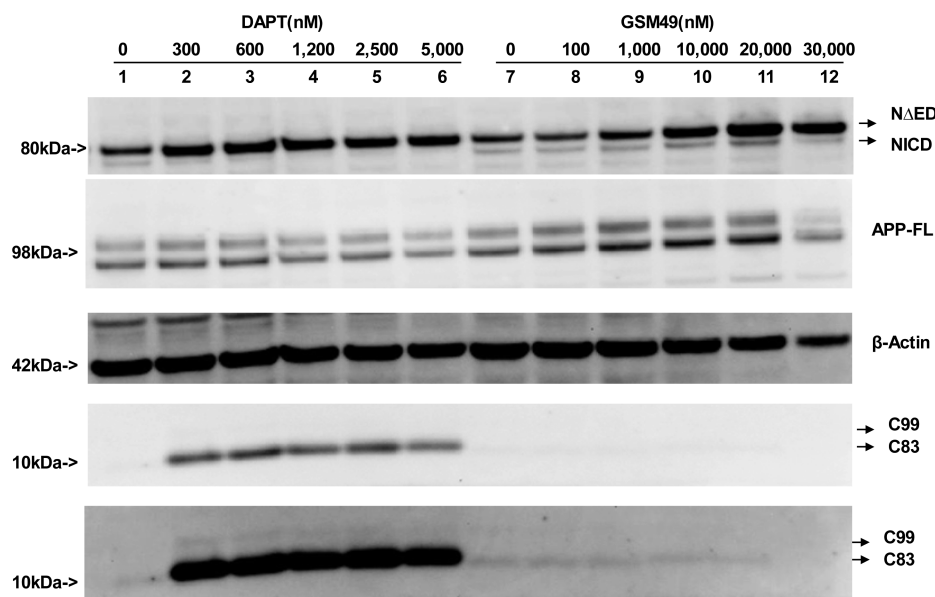


Figure 4. Stable H4 human neuroglioma cells overexpressing human APP751, or H4-APP751 cells, were transfected with the NΔED construct (top panel, lanes 1–12) and then treated with either vehicle (lanes 1 and 7), DAPT (lanes 2–6), or SGSM 49 (lanes 8–12) for an additional 24 h. Cells were harvested 48 h post-transfection, processed, and subjected to Western blotting analysis. The Myc antibody was utilized to assess the NΔED and NICD tagged with Myc at their N-termini (top). APP (second panel from top) and C99 and C83 APP-CTFs (bottom two panels) were probed with the APP carboxyl-terminal antibody (A8717). The bottom panel included a longer exposure to visualize the C99 (APP β -CTFs). β -Actin (middle) was utilized as the loading control.

A β 40¹⁹ or the MesoScale multiplex ELISA used herein. Interestingly, via comparison of the IC₅₀ values for decreasing the level of A β 42 (in Table 1, compounds are listed in order of increasing potency based on IC₅₀ values for decreasing the level of A β 42) and the IC₅₀ values for decreasing the level of A β 40, IC₅₀ values for decreasing the level of A β 42 are significantly more potent (~3–5-fold). This is consistent across a range of IC₅₀ values extending from the least potent compound 42 (A β 42 IC₅₀ = 1275 \pm 92 nM) to the most potent compound 49 (A β 42 IC₅₀ = 30 \pm 6 nM). The IC₅₀ values for decreasing the level of A β 40 for this same series of SGSMs also defined a wide range of potencies extending from the least potent compound 42 (A β 40 IC₅₀ = 4125 \pm 1534 nM) to the most potent compound 49 (A β 40 IC₅₀ = 104 \pm 4 nM). This relationship is well-illustrated for the two most potent SGSMs, compounds 49 and 36 (Figure 2). These data suggest that this series of aminothiazole-bridged heterocycle-containing methyl-imidazoles impact enzyme activity differently with respect to cleavage events leading to A β 42 and A β 40. Remarkably, the discrepancy between A β 42 and A β 40 potencies was not seen with respect to the EC₅₀ values for the potentiation of A β 38 and the relationship between the concentrations of these SGSMs required for decreasing the level of A β 42 and those required for the potentiation of A β 38. Importantly, all of the SGSMs within this series maximally increased the levels of A β 38 approximately 1.5–3-fold. In general, there was more variability with respect to measuring levels of A β 38 in this multiplex ELISA, especially at the lower SGSM concentrations compared to those of A β 42 (see Figure 2). For example, compound 49, the most potent SGSM based on A β 42 inhibition, displayed an EC₅₀ for potentiating A β 38 (305 \pm 54 nM) that was 10-fold higher than its IC₅₀ for inhibiting A β 42 (30 \pm 6 nM). Alternatively, compound 46 potentiated A β 38 with an EC₅₀ (131 \pm 11 nM) that was very similar to its IC₅₀ for inhibiting A β 42 (121 \pm 9 nM).

We used MALDI-TOF to evaluate the effects of these two molecules on A β peptide variant levels at each of their respective IC₅₀ values (for inhibiting A β 42) using the conditioned medium from SHSYSY-APP cells treated for 24 h to comprehensively assess their effects on A β peptide variants at these concentrations (Figure 3). MALDI-TOF analysis, though semiquantitative, showed good agreement with the multiplex ELISAs. As expected, both compounds 49 and 46 substantially lowered A β 42 levels at concentrations near their IC₅₀ values (30 and 150 nM for compounds 49 and 46, respectively). Both compounds also increased the levels of A β 34 and A β 33, and neither had a comparable effect on the level of A β 40 or A β 39 at these concentrations (although compound 49 did lower the levels of A β 40 only slightly at a concentration of 30 nM). Interestingly, at 30 nM, compound 49 significantly increased the level of A β 37 to a greater extent than it did the level of A β 38. This was not the case for compound 46, which when tested near its IC₅₀ increased the level of A β 38 more so than it did that of A β 37. Whether differential effects on other A β peptide variants (e.g., A β 37) can explain differences between the IC₅₀ for A β 42 inhibition and the EC₅₀ for A β 38 potentiation for compounds such as 49 is unknown at this time but does offer an interesting possibility. For example, for compound 49, the EC₅₀ for A β 37 potentiation may be closer to the IC₅₀ for decreasing the level of A β 42 than is the EC₅₀ for the potentiation of A β 38. However, because of the lack of a robust A β 37 ELISA, we could not make this determination at this time. For the nine most potent compounds, in terms of decreasing the level of A β 42 (IC₅₀ values of \leq 200 nM), all enhanced A β 38 generation at EC₅₀ values above their corresponding A β 42 IC₅₀ values (see Table 1); however, a few of these, including compound 46, had EC₅₀ values for A β 38 that were very near their corresponding A β 42 IC₅₀ values. Interestingly, previous studies investigating the equilibrium between A β 42 inhibition and A β 38 potentiation in

cell-based assays overexpressing a number of different FAD-linked PS1 mutations and the effects of NSAID-like carboxylic acid-containing GSMs on decreasing the level of A β 42 versus A β 38 potentiation clearly demonstrated that these two activities are not coupled,²³ yet those compounds described herein with IC₅₀ values for decreasing the level of A β 42 that are the same as their EC₅₀ values for potentiating A β 38 raise the possibility that they could be.

We also assessed the effects of the two most potent SGSMs (compounds **49** and **36**) on total A β peptide levels using a sandwich ELISA that detects total A β peptides provided they contain the first six amino acids of the A β peptide motif (Figure 2). When SHSY5Y-APP cells were treated (for 24 h) with increasing amounts of each compound over a broad range of concentrations (100 pM to 10 μ M), they failed to elicit a change in total A β peptide levels. While levels of A β 42 and A β 40 were reduced in a concentration-dependent manner, A β 38 levels increased to a maximum that was approximately 2.5–3-fold greater than that of vehicle-treated cells in a concentration-dependent manner for these two compounds.

SGSMs Do Not Inhibit γ -Secretase-Mediated Proteolysis of the Notch 1 Receptor or APP-CTFs at the ϵ -Cleavage Sites. It was also critical to assess whether these SGSMs had any effect on γ -secretase-mediated proteolysis of the Notch 1 receptor. We utilized three different assays to fully evaluate this. The first was cell-based and utilized human H4 neuroglioma cells overexpressing both the APP751 isoform and the N Δ E Notch 1 receptor construct. We used the dipeptidic carboxamide GSI, DAPT, as a positive control and treated cells with increasing concentrations of either DAPT, SGSM **49**, or vehicle for 24 h. For the DAPT-treated cells (Figure 4), inhibition of the Notch intracellular domain (NICD) occurred at concentrations as low as 300 nM (the lowest concentration tested). Alternatively, in the SGSM **49**-treated cells (Figure 4), there was no inhibition of NICD generation, even at concentrations as high as 20 μ M, which is almost 1000-fold higher than its IC₅₀ for inhibiting A β 42 production in these very same cells (data not shown). In the SGSM **49**-treated cells, we did observe a slight decrease in the level of NICD generation at a concentration of 30 μ M; however, at this extremely high concentration, we also observed a decrease in holo-APP and β -actin levels that was supported by toxicity measurements at this very high concentration (data not shown). These latter findings suggest a toxic effect rather than inhibition of γ -secretase-mediated ϵ -site proteolysis. In addition, the lack of an effect of SGSM **49** on the accumulation of APP-CTFs (Figure 4) is consistent with the lack of effect on NICD production and further demonstrates the fact that SGSM **49** does not affect the ability of γ -secretase to perform ϵ -site cleavages of either of these substrates.

We confirmed that SGSM **49** did not inhibit γ -secretase-mediated ϵ -site proteolysis using both a cell-free HeLa cell membrane *in vitro* γ -secretase assay and a reconstituted γ -secretase assay with highly purified tandem affinity-purified (TAP) γ -secretase subunits (Figure 5). In both systems (Figure 6), SGSM **49** effectively inhibited cleavage of a truncated, biotinylated APP artificial substrate (Sb4)²¹ at the A β 42 cleavage site with IC₅₀ values in the single-digit nanomolar range (IC₅₀ values of 6 and 7 nM for the HeLa membrane and TAP γ -secretase assays, respectively). SGSM **49** was slightly less efficacious at inhibiting γ -secretase-mediated cleavage at the A β 40 site (IC₅₀ values of 37 and 132 nM for the HeLa membrane and TAP γ -secretase assays, respectively) yet

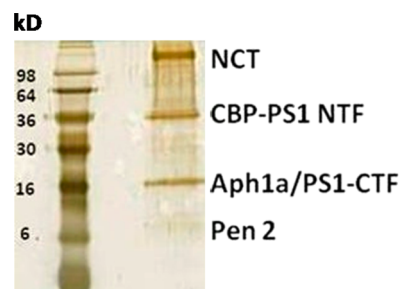


Figure 5. Silver staining of TAP γ -secretase subunit purification: bovine serum albumin (BSA), 500 ng (lane 1); molecular mass markers (lane 2), with masses in kilodaltons given at the left; purified γ -secretase components (lane 3), indicated at the right. Note that PS1 NTF has a higher than expected mass because of the presence of the calmodulin binding peptide tag (CBP). The faint band for Pen 2 is due to its known low affinity for silver stain.¹⁹ Aph1a and PS1-CTF comigrate. Nicastrin (NCT), calmodulin binding peptide-tagged PS1 N-terminal fragment (CBP-PS1 NTF), anterior pharynx defective 1 homologue A (Aph1a), PS1 carboxyl-terminal fragment (PS1-CTF), and presenilin enhancer 2 (Pen 2).

required concentrations that were 200–400 times higher to inhibit cleavage of a truncated, biotinylated Notch 1 receptor artificial substrate (N1-Sb1)²² at the NICD cleavage site by 50% [IC₅₀ values of 2042 and 1423 nM for the cell-free HeLa membranes and TAP γ -secretase assays, respectively (Figure 6)]. Collectively, these studies demonstrate that SGSM **49** is “notch sparing” and apparently unable to affect γ -secretase-mediated proteolysis at the ϵ -sites at concentrations required to affect γ -secretase-mediated proteolysis at γ -sites.

SGSMs Modulate A β Peptide Variant Production in Cells Overexpressing an FAD-Linked Mutant PS1. Finally, we conducted experiments aimed at discerning whether SGSM **49** was capable of modulating A β peptide variant production in a cell-based assay harboring a missense mutation in PS1 (M146L). In these experiments, two different cell lines (CHO-APP wt/PS1 and CHO-APP M146L/PS1) were utilized. Ten-point concentration response curve experiments were conducted exactly like those performed using the human SHSY5Y-APP cell line. In the experiment involving the CHO-APPwt/PS1M146L cells, SGSM **49** was essentially as effective at inhibiting A β 42 (IC₅₀ = 47 \pm 5 nM) and A β 40 (IC₅₀ = 103 \pm 4 nM) and at potentiating A β 38 (EC₅₀ = 499 \pm 203 nM) as the wild-type CHO-APPwt/PS1wt cells (A β 42 IC₅₀ = 47 \pm 17 nM; A β 40 IC₅₀ = 130 \pm 35 nM; A β 38 EC₅₀ = 646 \pm 326 nM) and the human SHSY5Y-APP751 cell line (A β 42 IC₅₀ = 30 \pm 6 nM; A β 40 IC₅₀ = 104 \pm 4 nM; A β 38 EC₅₀ = 305 \pm 54 nM) (Figure 7).

DISCUSSION

Since the recent demise of the GSIs, γ -secretase modulation via GSMs has attracted the majority of the attention in terms of exploiting γ -secretase as a therapeutic target for AD.²⁴ GSMs can be broken down into two basic classes of molecules: (1) NSAID-derived GSMs and (2) non-NSAID-derived GSMs.²⁴ The original series of non-NSAID-derived GSMs were recently shown to be capable of effectively attenuating β -amyloid deposition in a transgenic mouse model of AD following chronic administration.¹⁹ These small molecules are aryl aminothiazole GSMs (AGSMs) and have the general structure schematically depicted in Figure 1. The most potent of these are very lipophilic in character with very poor aqueous

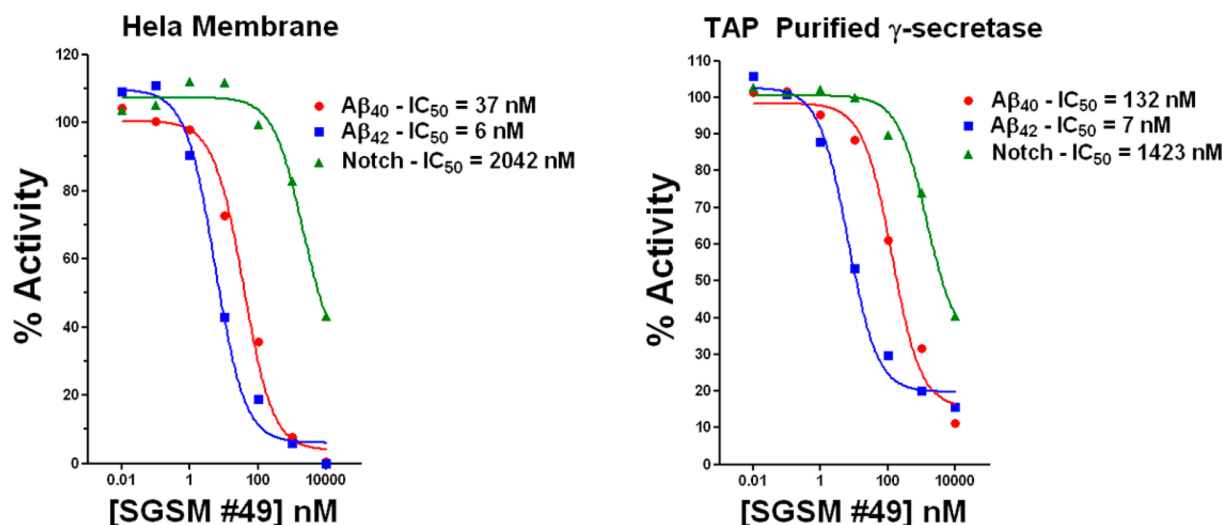


Figure 6. Concentration response curves of SGSM 49 for the inhibition of Aβ₄₂ (blue), Aβ₄₀ (red), and NICD (green) in a HeLa cell membrane (left) or in TAP reconstituted γ-secretase subunits (right) during *in vitro* γ-secretase assays. IC₅₀ values were derived using four-parameter fit nonlinear regression analyses.

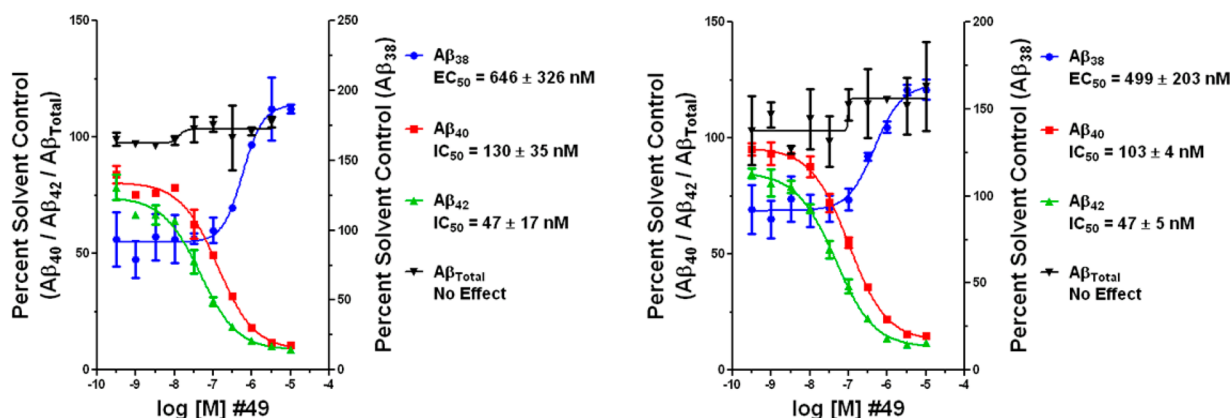


Figure 7. Concentration response curves displaying differential effects of SGSM 49 on steady state levels of specific Aβ peptide variants and total Aβ peptide levels secreted by CHO cells overexpressing human APP751 and either wild-type human PS1 (left) or a mutant human PS1 (M146L) (right) following treatment with either SGSM 49 or vehicle (DMSO). Individual Aβ peptide variants (Aβ₄₂, Aβ₄₀, and Aβ₃₈) and total Aβ peptide levels were quantitated using MesoScale Sector 6000 multiplex technology and a sandwich ELISA, respectively. IC₅₀ values were derived using four-parameter fit nonlinear regression analyses.

solubilities. This particular scaffold has been the basis of a large number of medicinal chemistry investigations that have focused mostly on C ring substitutions.²⁴ We attempted to identify permissive heterocycle substitutions of the phenyl B and D rings in the hope of attaining more aqueously soluble molecules while retaining the excellent potencies of some of those from the original AGSM series.¹⁹ We were able to synthesize and characterize 20 SGSM compounds; nine of these had IC₅₀ values of <200 nM (for inhibiting Aβ₄₂ production) with kinetic solubilities ranging from <1.6 to 18.4 μM. All 20 of these SGSM molecules, regardless of their potency for inhibiting Aβ₄₂, had the same profile with respect to their effects on Aβ peptide variant production. They consistently inhibited Aβ₄₂ with potencies much better than those for inhibition of Aβ₄₀, and they enhanced the production of Aβ₃₈ with potencies that were highly variable. This profile of inhibiting Aβ₄₂ much more effectively than Aβ₄₀ would seem to be ideal for a disease-modifying AD drug.²⁰ This is based on a large number of observations involving both early onset familial AD (EOFAD)^{9,23} and the more common sporadic late-

onset form of AD⁸ that denote the pathogenic character of the Aβ₄₂ peptide variant. In terms of EOFAD resulting from missense mutations in one of three genes (*APP*, *PSEN1*, and *PSEN2*), the vast majority of these genetic mutations cause an increase in the Aβ₄₂/Aβ₄₀ ratio.²⁵ A pivotal study utilizing transgenic mice expressing fusion proteins between the BRI protein and either Aβ₄₂ or Aβ₄₀ demonstrated that Aβ₄₂ was essential for both parenchymal and vascular amyloid deposition.²⁶ This latter study also showed that overexpression of Aβ₄₀ in a well-characterized AD transgenic mouse model (Tg2576) caused a reduction in the extent of amyloid pathology and that overexpression of Aβ₄₂ increased the extent of amyloid pathology in the same model.²⁶ The large disparity between the ability of the SGSMs described here to inhibit Aβ₄₂ versus Aβ₄₀ would seem to be desirable, and one would be expected to be able achieve dosing regimens that would allow selective inhibition of Aβ₄₂ without affecting the levels of Aβ₄₀ and therefore effectively reversing the biochemical phenotype (increased Aβ₄₂/Aβ₄₀ ratio) elicited by the large number of EOFAD-linked mutations.

The most potent of these SGSM compounds, **49**, contained a methylimidazole A ring, a methoxypyridyl B ring, an aminothiazole C ring, and a 2-ethyl-terahydro-indazole D ring. This compound inhibited A β 42 in the SHSYSY-APP cell-based assay with an IC₅₀ of 30 \pm 6 nM, while the IC₅₀ for inhibiting A β 40 was >3-fold higher (IC₅₀ = 104 \pm 4 nM). This compound was also studied in a number of additional assays, including MALDI-TOF mass spectrometry to assess its effects on total A β peptide production, the ability to inhibit Notch 1 receptor proteolysis, APP ϵ -site proteolysis, the ability to modulate γ -secretase in cell-free and purified enzyme assays, and the ability to modulate γ -secretase in cell-based assays harboring a missense mutation in PS1.

As expected, compound **49**, although able to effectively inhibit the production of A β 42 and A β 40 (much less effectively), was able to potentiate the generation of A β 38 yet was unable to affect total A β levels over a broad range of concentrations (100 pM to 10 μ M). MALDI-TOF analysis demonstrated that when SHSYSY-APP cells were treated with 30 nM compound **49** (the IC₅₀ for inhibiting A β 42 in this SHSYSY-APP cell-based assay) there was a decrease in the levels of A β 42; a slight decrease in the levels of A β 40; an increase in the levels of A β 38, A β 34, and A β 33; and a considerable increase in the levels of A β 37. This particular A β variant profile is distinct from that elicited by the NSAID-like carboxylic acid-containing GSMs, such as GSM-1;²⁷ however, it is very similar to the profile generated through treatment of cells overexpressing APP by the original AGSM series,¹⁹ although that series of AGSMs did not demonstrate the ability to increase the levels of the shorter A β variants A β 34 and A β 33. Upon comparison of A β profiles elicited by a large number of different GSMs from multiple chemotypes, it appears that there is considerable diversity, even when examining molecules within the same structural class (e.g., methylimidazole-containing GSMs or phenylacetic acid-containing GSMs).¹⁰

Upon rigorous evaluation of the ability of compound **49** to inhibit γ -secretase-mediated proteolysis of the Notch 1 receptor by implementing cell-based, cell-free, and purified γ -secretase subunit-containing reconstituted enzymatic assays, it is clear that this molecule has a very limited liability in this regard. This notch-sparing feature appears to be a general property of GSMs of all structural classes that is not shared by GSIs.^{7,10,19,28} The cell-based assay utilizing H4 glioma cells overexpressing both APP and the N Δ E truncated Notch 1 receptor construct also demonstrated that compound **49** had no effect on ϵ -site proteolysis of APP, even at concentrations ~1000-fold greater than its IC₅₀ for inhibiting A β 42 production in the H4 glioma cell-based assay system. When compound **49** was tested for the ability to inhibit γ -secretase-mediated proteolysis in both cell-free and reconstituted TAP-purified γ -secretase subunit enzyme assays using artificial substrates for both APP (Sb4) and Notch (N1-Sb1), a similar disparity was observed between the ability of compound **49** to inhibit production of A β 42 versus A β 40 and the ability to inhibit A β 42 versus the ability to inhibit NICD production. Although the IC₅₀ values were lower in these assays than the corresponding IC₅₀ values in the cell-based assay systems, the same general rank order of potencies held (i.e., compound **49** inhibited the production of A β 42 \gg A β 40 \gg NICD).

Compound **49** was also tested for the ability to modulate γ -secretase activity in cell-based assay systems harboring a missense mutation in PS1 (M146L). Previous studies have determined that although cell-based assays overexpressing APP

mutations respond to first-generation NSAID GSMs, cell-based assays overexpressing PS1 mutations for the most part do not.^{27,29} Alternatively, second-generation NSAID-like phenylacetic acid-containing GSMs, such as GSM-1, for the most part are able to modulate A β peptide production in cell-based assays harboring PS1 mutants in a pattern similar to how they affect cell-based assays harboring wild-type PS1 (selectively decreasing A β 42 levels and selectively increasing A β 38 levels).²⁷ However, when tested against the G384A PS1 mutant, GSM-1 effectively lowered A β 42, A β 40, and A β 37 (thus removing the A β 42 selectivity). Other notable exceptions were the PS1 mutants L166P and N135S that were resistant to inhibition of A β 42 by GSM-1; however, GSM-1 was able to cause increases in A β 38 levels in these same PS1 mutant-expressing cell lines.²⁷

Compound **49** was tested against a mutant-harboring cell line (PS1M146L), and it behaved the same as it did in the corresponding wild-type PS1-expressing cell lines with respect to the effects on A β 42 and A β 40; A β 38 levels were increased approximately 1.5-fold following treatment with compound **49**. Previous studies demonstrated that a compound termed “TorreyPines”, a first-generation AGSM with a distinct scaffold similar to that of compound **49**, however, with a potency for inhibiting A β 42 much lower in our hands than that of compound **49**, when tested against a large number of PS1 mutants, including both FAD-linked and synthetic or artificial mutations (P117L, N135I, M146L, L166P, M233V, Y256S, Δ E9, L383W, and G384A), was able to increase A β 38 levels in all of the PS1 mutant-harboring cell lines (as was the case with GSM-1) and inhibited A β 42 production in all except the N135I and L166P PS1 mutant-expressing cell lines.²⁷ Another methylimidazole-containing GSM with a cinnamide linker (as opposed to an aminothiazole C ring) termed “Eisai” behaved like the “TorreyPines” compound in that study.²⁷ However, those carefully performed experiments utilized a cell-based assay system encompassing HEK cells overexpressing the various PS1 mutants as well as human APP harboring a “Swedish” mutation to provide optimal amounts of the β CTF (β -carboxyl-terminal fragment of APP) substrate for γ -secretase,²⁷ and it has been shown that increased levels of β CTF can further augment the A β 42/A β 40 ratio.³⁰ Perhaps cell-based assay systems utilizing human neurons differentiated from iPSC cell lines derived from FAD patient fibroblasts may provide a more accurate assay system for evaluating whether transgenic animals and ultimately patients carrying certain PS1 mutations will respond to a given GSM chemotype. Nonetheless, the previously published studies make a strong case for evaluating these molecules against FAD-linked mutations *in vitro* prior to embarking on *in vivo* studies encompassing these various mutations.

The SGSMs described in these studies (aminothiazole-bridged heterocycles) appear to work through a mechanism similar to that of the AGSM chemotype (aminothiazole-bridged aromates) described previously.¹⁹ This is based on the similarity in A β profiles generated by treatment of cells overexpressing wild-type APP with either of these types of non-NSAID-like GSMs. The only difference was the ability of the SGSMs to cause an increase in the shorter A β 34 and A β 33 variants. It is currently unknown precisely how these types of GSMs (AGSMs and SGSMs) alter the A β peptide profiles. A number of photoaffinity labeling studies,³¹ including “photophore walking”²² utilizing a variety of photoprobes of different GSM chemotypes, competition studies with various GSMs, and the use of clickable probes,^{32,33} suggest that there are multiple

allosteric sites within the catalytic PS1-NTF subunit that can accommodate binding of various GSMs¹⁰ (for review).

A recent model describing the sequential generation of shorter A β peptide variants from longer A β peptides via successive proteolysis or “processivity”³⁴ has been used to describe one potential mechanism of action of GSMs.^{10,35} In this model, the GSMs induce a conformational change in the γ -secretase catalytic center that inhibits A β 42 release and allows for A β 42 to be proteolytically processed to shorter A β peptide variants such as A β 38. The identification of specific tripeptide products resulting from carboxy-terminal trimming of the longer A β peptides clearly provides support for this model,³⁴ yet another model has been proposed, the independent cleavage model,¹⁰ which postulates that GSMs induce a conformational change in the active site of the enzyme (within the PS1-NTF) that alters one of the preferred γ -secretase cleavages (preventing cleavage at A β 42 and augmenting cleavage at, e.g., A β 38). The SGSMs described here appear to be much more effective at inhibiting cleavage at A β 42 than inhibiting cleavage at A β 40. At much higher doses (3–5-fold), inhibition of A β 40 production eventually occurs, along with potentiation of A β 38; however, with several of the SGSMs described here, potentiation of A β 38 occurred at concentrations much lower than those required for inhibition of A β 40.

MALDI-TOF analysis of SHSYSY-APP cells treated with two different types of SGSMs [(1) compound 49, which inhibited A β 42 production with an IC₅₀ nearly 10-fold lower than its EC₅₀ for potentiating A β 38, and (2) compound 46, which inhibited A β 42 production with an IC₅₀ nearly identical to its EC₅₀ for potentiating A β 38 production] revealed that both of these compounds potentiated not only A β 38 but also A β 37, A β 34, and A β 33. Interestingly, compound 49 appeared to potentiate A β 37 to a greater degree than A β 38 upon being tested near its A β 42 IC₅₀ (30 nM); however, this was not the case for compound 46, which actually augmented A β 38 to a greater degree than A β 37 upon being tested at a concentration near its A β 42 IC₅₀ (150 nM). One possible interpretation of these data that could explain the apparent disparity between two compounds like 49 and 46 is that for compounds such as 49 that display large differences between their A β 42 IC₅₀ and their A β 38 EC₅₀ one or more of the shorter peptides (e.g., A β 37, A β 34, and/or A β 33) are actually potentiated at concentrations of SGSMs similar to those required for inhibiting A β 42. Thus, if this proves to be correct, this would support the alternative or independent cleavage model in which cleavage at A β 37, A β 34, and/or A β 33 takes the place of A β 42 cleavage.

Under normal circumstances, A β 40 is by far the most abundant product of γ -secretase (γ -site cleavage product), suggesting it as the preferred γ -site cleavage. With this in mind, another model to consider, especially for most of these SGSMs described here (those that inhibit A β 42 at concentrations much lower than that required for either inhibiting A β 40 or potentiating A β 38, e.g., compound 49), is one in which they bind initially to a high-affinity site (at relatively low concentrations) that prevents cleavage at the A β 42 γ -site and then at another perhaps lower-affinity site (at relatively higher concentrations) that eventually reduces the amount of cleavage at A β 40 and concomitantly promotes cleavage at A β 38, A β 37, A β 34, and A β 33 instead.

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Notes

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ABBREVIATIONS

A β , amyloid β -protein; AD, Alzheimer's disease; AGSMs, aryl aminothiazole γ -secretase modulators; SGSMs, soluble γ -secretase modulators; APP, amyloid precursor protein; APP-CTF, amyloid precursor protein carboxy-terminal fragment A β 42, A β 40, and A β 38, the 42-, 40-, and 38-amino acid A β peptide variants, respectively; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBP, calmodulin binding peptide; TAP, tandem affinity-purified; PS1, presenilin 1; PS1-NTF, presenilin 1 amino-terminal fragment; PS1-CTF, presenilin 1 carboxy-terminal fragment; C83, 83-amino acid APP carboxy-terminal fragment; C99, 99-amino acid APP carboxy-terminal fragment; PS2, presenilin 2; APH1A, anterior pharynx defective 1 homologue A; PEN-2, presenilin enhancer 2; NCT, nicastrin; GSI, γ -secretase inhibitor; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *tert*-butyl ester; CLND, chemiluminescent nitrogen detection; UV, ultraviolet; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide.

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