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Piperidine Acetic Acid Based γ-Secretase Modulators Directly Bind to Presenilin-1

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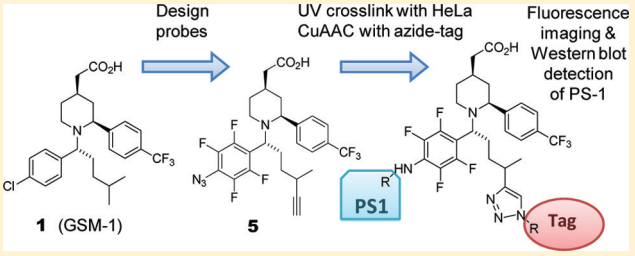
Supporting Information

ABSTRACT: Aβ42 is believed to play a causative role in Alzheimer's disease (AD) pathogenesis. γ-Secretase modulators (GSMs) are actively being pursued as potential AD therapeutics because they selectively alter the cleavage site of the amyloid precursor protein (APP) to reduce the formation of Aβ42. However, the binding partner of acid based GSMs was unresolved until now. We have developed clickable photoaffinity probes based on piperidine acetic acid GSM-1 and identified PS1 as the target within the γ-secretase complex. Furthermore, we provide evidence that allosteric interaction of GSMs with PS1 results in a conformational change in the active site of the γ-secretase complex leading to the observed modulation of γ-secretase activity.



KEYWORDS: *Alzheimer's disease, presenilin, γ-secretase modulator, GSM-1, click chemistry, photoaffinity labeling*

INTRODUCTION

γ-Secretase has emerged as an appealing drug target for Alzheimer's disease (AD) due to its central role in the generation of Aβ peptides, which are widely believed to play a causative role in the initiation of the neuropathogenesis of AD.[1] γ-Secretase produces Aβ40 and Aβ42 peptides during the cleavage of the amyloid precursor protein (APP). However, γ-secretase also cleaves an array of other substrates[2] including Notch proteins, which are key molecules that regulate neuronal development[3] and other physiological processes.[4] This wide spectrum of γ-secretase substrates has made the development of γ-secretase inhibitor based therapies a formidable challenge, exemplified by the recently failed phase III clinical trial of Semagacestat, a nonselective γ-secretase inhibitor (GSI), partially due to Notch-mediated skin tumor progression.[5,6] Aβ42 is generally considered to be a more pathogenic species, since it is highly prone to aggregation and becomes the more predominantly deposited species in brains of AD subjects.[7,8] Therefore, there is considerable interest in finding selective Aβ42-lowering therapies that do not inhibit key γ-secretase-dependent signaling pathways. The γ-secretase modulators (GSMs) exhibit such characteristics[9,10] and can be generally divided into two categories: NSAID derived carboxylic acid analogues[11] and non-NSAID imidazole compounds.[12]

Multiple mechanisms of action of GSMs have been proposed including direct and selective binding of GSMs to the APP substrate within the Aβ region.[13,14] However, other studies indicate that these GSMs do not specifically interact with the APP substrate, but instead bind to the γ-secretase complex, which is composed of presenilin, nicastrin, Aph1, and Pen2.[15−17] A recent study suggested that these acid GSMs interact with the binding site within the γ-secretase complex when the substrate docking site is occupied.[18] These studies have been limited by the weak potency and/or poor solubility of the probe compounds used. Clearly, further investigation is required in order to gain a better understanding of the specific target(s) that these GSMs interact with to facilitate the development of potent and selective Aβ-lowering therapies for AD. In recent years, several next generation GSMs with improved Aβ42 lowering potency have been reported as exemplified by **1** (GSM-1) and **2** (Figure 1),[9,17] offering an unprecedented opportunity to elucidate the mechanism of action of GSMs.

Photoaffinity labeling is a powerful method to covalently capture the protein targets of small molecules[19] and has been

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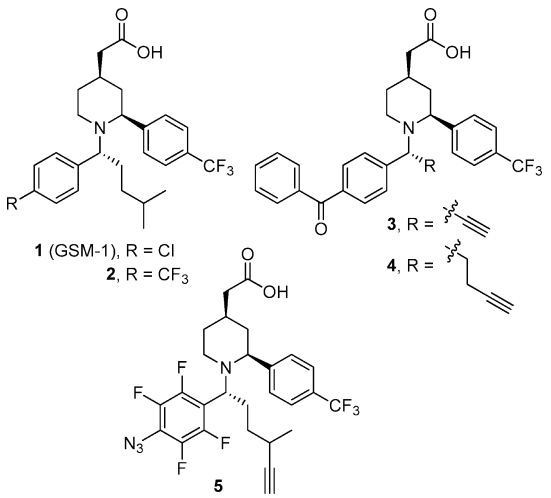

Figure 1. Structure of piperidine acetic acid GSMs **1** and **2** and clickable photoaffinity probe analogues **3**–**5** designed to label target proteins upon UV-irradiation. The labeled proteins are then reacted with an azide-linked reporter tag for further characterization.

successfully applied to characterize the binding of γ-secretase inhibitors.[20−23] However, in many cases, incorporation of a bulky biotin group can influence the biological activity of the probes by reducing the potency or limiting permeability into cells. Therefore, we aimed to develop clickable photoaffinity probes for our studies. This approach has proven to be an effective way to profile and identify small molecule interactions with enzymes and other target proteins.[24−31]

We have designed and synthesized clickable photoaffinity probe analogues **3**–**5** by substituting the chlorophenyl group of **1** with a benzophenone or perfluorophenyl azide photoreactive group and incorporating an alkyne into the alkyl side-chain for click chemistry conjugation of azide-reporter tags (see Schemes S1–S3 in the Supporting Information). We determined their potencies in cell-free HeLa membrane assays using recombinant APP and Notch1 substrates[32,33] and found that the potency was improved by extending the length of the alkyl substituent (**3** to **4** in Table 1). Incorporation of either the

Table 1. In Vitro Inhibitory Potency (IC50) of 1–5

	IC50 (nM)[a]		
compd	Aβ42	Aβ40	Notch
1	120	1010	>5000
2	170	880	>5000
3	1010	2500	>5000
4	300	1700	>5000
5	310	1520	>5000

[a]Average of at least three independent assays.

benzophenone or perfluorophenyl azide photoactivatable moiety led to similar potency and selectivity (**4** and **5** in Table 1). The potency of the resulting clickable photoprobes was reduced by only 2- to 3-fold when compared to the parent compounds (**1** and **2**). We then utilized both **4** and **5** for photoaffinity labeling experiments.

Initially, we determined whether these compounds are active in our newly developed reconstitution system with recombinant presenilin-1 (PS1), the catalytic subunit of γ-secretase.[34] PS1ΔE9 is a PS1 mutant which lacks the exon 9 loop and therefore does not require endoproteolysis for activity. This unique feature allows for the preparation of PS1ΔE9 proteoliposomes with γ-secretase activity in the absence of any of the other components or coactivators of the γ-secretase complex (Figure 2a).[34] First, we found that **1** is capable of

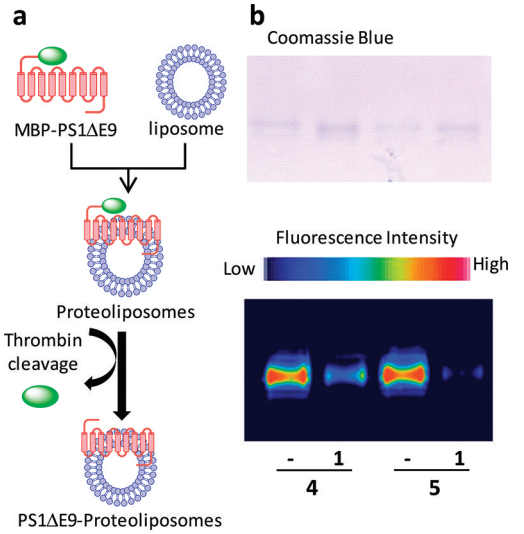

Figure 2. (a) Illustration of the preparation of PS1ΔE9 proteoliposomes for γ-secretase activity assay or photolabeling. (b) PS1ΔE9 proteoliposomes were labeled with **4** or **5** in the presence or absence of 10 μM **1**, followed by click chemistry with TAMRA-azide, in-gel fluorescence, and Coomassie blue staining.

inhibiting the activity of PS1ΔE9 proteoliposomes with an IC50 of 610 and 3300 nM for Aβ42 and Aβ40, respectively, suggesting that these acid GSMs could bind to PS1 for their modulatory activity. Second, we performed photoaffinity labeling studies with **4** and **5** followed by click chemistry with TAMRA-azide. We found that both **4** and **5** at 200 nM robustly label PS1ΔE9 proteoliposomes. Moreover, the labeling was blocked by **1** at 10 μM (Figure 2b), indicating that both probes specifically and directly interact with PS1ΔE9 in the reconstituted proteoliposome system.

Next, we examined whether both probes can interact with native PS1 within the γ-secretase complex isolated from HeLa cell membranes. Compound **4** or **5** was incubated with HeLa membranes and then UV irradiated to cross-link it to nearby proteins, followed by click chemistry with biotin-azide. Biotinylated proteins were captured with streptavidin beads and analyzed by Western blot with anti-PS1 antibodies (Figure 3a). Compound **5** was found to have a more robust labeling than **4**, but both labeled PS-1 NTF. Again, the labeling of PS1-NTF was blocked by the excess of **1** and **2**, indicating the specific binding of the probe to PS1 in cell membranes, which corroborated with the PS1ΔE9 proteoliposome studies. We were not able to detect specific labeling of other γ-secretase components such as Aph1, Pen2, and nicastrin. Together, these findings demonstrate that these particular acid GSMs can directly bind to PS1 in both reconstituted PS1 and native forms of the γ-secretase complex. Finally, we carried out similar studies in which **5** was incubated with HeLa membranes and UV irradiated followed by click chemistry with TAMRA-azide (Figure 3b). We identified a predominant band specifically labeled at 30 kDa, the apparent molecular weight of PS1-NTF. In addition, high molecular weight bands (>75 kDa) were

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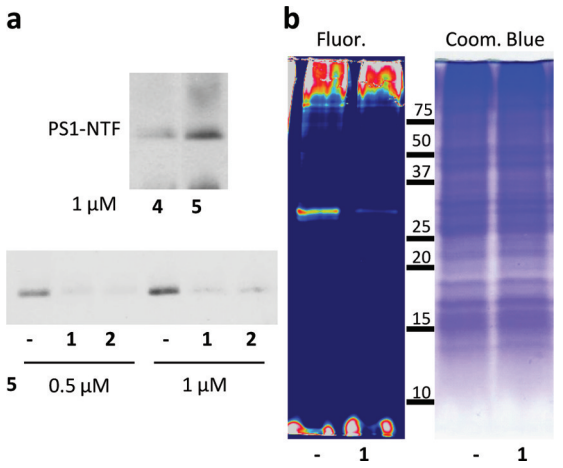

Figure 3. (a) HeLa membranes were labeled with **4** or **5** (top panel) or labeled with **5** in the presence or absence of 50 μM **1** or **2** (bottom panel), followed by click chemistry with biotin-azide, streptavidin pull down, and Western blot analysis with PS1-NTF antibody. (b) HeLa membranes were labeled with 150 nM **5** in the presence or absence of 10 μM **1**, followed by click chemistry with TAMRA-azide, in gel fluorescence, and Coomassie blue gel staining.

observed; however, they were not competed by an excess of **1**, suggesting that they may represent nonspecific or aggregated species.

Since signal peptide peptidase (SPP) is a PS1 type of aspartyl protease,[35] we wanted to determine if acid GSMs also bind SPP. It was found that **5** specifically labels both the SPP monomer and dimer/multimer forms (Figure S1, Supporting Information). Since SPP is a single subunit protease, this finding further supports that acid GSMs directly bind to PS1. However, the SPP monomer did not appear as a dominant band in Figure 3b. It is possible that SPP dimer and multimer labeling is hidden within the strong uncompeted labeling of aggregated proteins above 75 kDa. Clearly, interaction of acid GSMs with SPP and other potential higher molecular weight off-targets needs to be further investigated. Nevertheless, our studies indicate that GSM1 directly binds to PS1 within the γ-secretase complex to modulate γ-secretase activity.

Furthermore, we hypothesized that GSMs modulate γ-secretase activity by allosterically changing the shape of the active site of PS1. To probe the shape of the active site of γ-secretase, our lab has developed a series of photoreactive probes based on the core structure of the active site directed peptidomimetic GSI, L458.[32] A benzophenone group was incorporated into the P2, P1, P1′, or P3′ position of L458 and the corresponding compounds are referred to as L646, GY4, JC8, or L505. Each of these inhibitors interacts with and labels the S2, S1, S1′, and S3′ subsites of the γ-secretase complex active site, respectively (Figure S2, Supporting Information). We found that at 4 μM **1** had little to no effect on the labeling of the S2, S1′, and S3′ subsites as observed with L646, JC8, and L505 labeling. However, we did find that **1** is capable of enhancing the labeling of the S1 subpocket with the GY4-P1 probe (Figure 4a). Moreover, this enhancement is concentration-dependent (Figure 4b), and observed with both **1** and **2** (data not shown). Collectively, these findings lead to a proposed mechanism in which the interaction of acid GSMs with PS1 allosterically changes the active site of γ-secretase, as illustrated for GY-4 labeling in Figure 4c, and ultimately leads to an altered Aβ profile, that is, selective lowering of Aβ42.

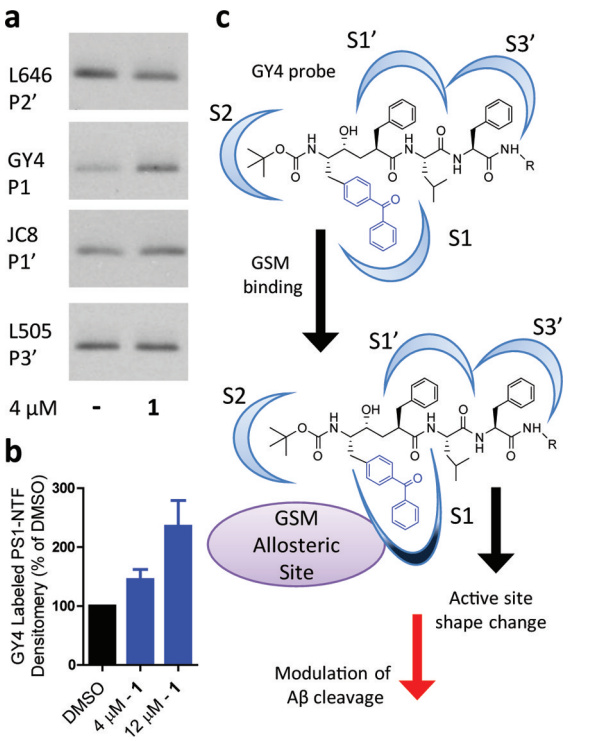

Figure 4. (a) HeLa membranes were labeled with 20 nM of GSI photoprobe L646, GY4, JC8, or L505 in the presence or absence of 4 μM **1**, followed by streptavidin pull down and Western blot analysis with PS1-NTF antibody. (b) Densitometry analysis from PS1-NTF labeling of GY4 with coincubation of **1** at 4 or 12 μM. (c) Proposed model for the mechanism of action of acid GSMs. GSM binding to PS1 allosterically influences the S1 subsite within the active site, leading to an alteration of γ-secretase specificity and an observed increase in GY4 labeling. R = biotin linker.

In conclusion, we have synthesized and characterized clickable photoaffinity probes of GSM-1 that target PS1. These studies provide direct evidence that acid GSMs bind to PS1, which is consistent with previous kinetic and biochemical analyses showing that acid GSMs allosterically modulate γ-secretase activity and specificity.[16,36,37] Moreover, we demonstrated that GSMs are capable of interacting with PS1 in the absence of substrate and cause a conformational change in the active site. Our studies also suggest that PS1 contains the allosteric site for γ-secretase modulation. Finally, our work builds a foundation for further elucidating the mechanism of acid GSMs and other classes of GSMs, and offers a molecular basis for developing more effective Alzheimer's disease therapeutics.

METHODS

Synthesis of GSM Compounds. The syntheses of compounds **1**–**5** are described in the Supporting Information.

General Materials and Methods of Biology. HeLa cells were purchased from BioVest, and membrane was prepared as described previously.[20] Biotin-azide was purchased from Invitrogen. Streptavidin plus UltraLink resin was purchased from Pierce. In-gel fluorescence scans were obtained using the Typhoon Trio Variable Mode Imager instrument (GE Healthcare). The antibody against PS1-NTF was kindly provided by Dr. Min-Tain Lai (Merck Research Laboratories). The antibody against SPP was generated by immunizing rabbits with a peptide epitope from the N-terminal region of SPP. AlphaLISA detection reagents for γ-secretase activity assays were purchased from Perkin-Elmer.

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In Vitro γ-Secretase Activity Assay for IC50s. γ-Secretase activity assays were performed similarly to a method described previously.[21] Biotinylated recombinant APP substrate, Sb4 (1 μM), or Notch1 substrate, N1-Sb1 (0.4 μM), was incubated with 40 μg/mL HeLa membranes in 0.25% CHAPSO for 2 h in the presence or absence of GSM compounds. The amount of cleavage product generated was then determined using a detection mixture with cleavage specific antibodies for Aβ42 (10-G3), Aβ40 (G2-10), or Notch1 intracelluar domain (SM320) in combination with AlphaLISA Protein A (for Aβ42 and NICD) or AlphaLISA anti-mouse (for Aβ40) acceptor beads and streptavidin coated donor beads (Perkin-Elmer). Equal volumes of reaction mixtures were added to detection mixtures in a 384 well plate and incubated at room temperature overnight, and then the AlphaLisa signal was read using the EnVision multilabel plate reader (Perkin-Elmer).

Photolabeling of PS1ΔE9 Proteoliposomes with Clickable GSMs Followed by in-Gel Fluorescence. The photoreactive probes **4** and **5** (200 nM) were incubated with 25 μg of PS1ΔE9 proteoliposomes, which were prepared as described previously,[34] and 0.25% CHAPSO for 1 h at 37 °C in the presence or absence of 10 μM **1** in 250 μL of PBS followed by UV irradiation at 350 nm for 30 min. Cross-linked proteins were labeled with tetramethyl rhodamine using Cu catalyzed azide alkyne cycloaddition (CuAAC) click chemistry with 1 mM $CuSO_4$, 1 mM TCEP, 0.1 mM TBTA, and 80 μM TAMRA-azide, in PBS with 5% *t*-butanol, 2% DMSO, and shaking for 1 h at 25 °C. Labeled proteins were then precipitated with 1 mL of cold acetone at −20 °C for 30 min and washed once with 500 μL of cold acetone. Precipitated proteins were centrifuged at 15 000*g* for 10 min, the acetone was removed, and the protein pellet was air-dried for 10 min. The protein pellets were resolubilized in 50 μL of PBS buffer with 1% SDS, and 5 μL of sample was loaded on to an SDS-PAGE gel for protein band separation and then scanned for fluorescent bands. The same gel was then stained with Coomassie blue to compare the total amount of protein loaded in each sample.

Photolabeling of HeLa Membranes with Clickable GSMs Followed by Western Blot Analysis. The photoreactive probe **5** (0.5 or 1 μM) was incubated with 800 μg of HeLa cell membranes for 1 h at 37 °C in the presence or absence of 50 μM **1** or **2** in 1 mL volume of PBS followed by UV irradiation at 350 nm for 30 min to cross-link the probe to nearby proteins. The samples were then ultracentrifuged at 90 000*g* to reduce the volume to 225 μL, and the pellets were resuspended with PBS buffer by homogenization. Proteins were labeled with biotin by using CuAAC click chemistry with 1 mM $CuSO_4$, 1 mM TCEP, 0.1 mM TBTA, and 100 μM biotin-azide, in PBS with 5% *t*-butanol, 2% DMSO, and shaking for 1 h at 25 °C. The samples were then ultracentrifuged at 90 000*g* to remove click chemistry reagents, and the pellets were resuspended by homogenization and solubilized in 750 μL of RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% deoxycholate), followed by centrifugation at 12k rpm to remove particulate matter. The supernatant was decanted and added to 20 μL of streptavidin ultralink resin slurry and then incubated overnight at 4 °C. The streptavidin resin was washed four times by centrifugation at 0.5*g* with 500 μL of RIPA buffer. Biotinylated proteins were eluted by boiling with 30 μL of 2× SDS sample buffer for 10 min at 95 °C. Then 25 μL of the eluent was loaded on to an SDS-PAGE gel for protein band separation and then transferred to a PVDF membrane and blotted for PS1-NTF, PS1-CTF, nicastrin, Pen2, Aph1a, or SPP.

Photolabeling of HeLa Membranes with Clickable GSMs Followed by in-Gel Fluorescence. The photoreactive GSM probe **5** (150 nM) was incubated with 300 μg of HeLa cell membranes for 1 h at 37 °C in the presence or absence of 10 μM **2** in 1 mL volume of PBS followed by UV irradiation at 350 nm for 30 min to cross-link the probe to nearby proteins. The samples were then ultracentrifuged at 90000*g* to reduce the volume to 225 μL and the pellets were resuspended with 1% SDS. Proteins were labeled with tetramethyl rhodamine (TAMRA) using CuAAC click chemistry with 1 mM $CuSO_4$, 1 mM TCEP, 0.1 mM TBTA, and 100 μM TAMRA-azide, in PBS with 5% *t*-butanol, 2% DMSO, and shaking for 1 h at 25 °C. Labeled proteins were then precipitated with 1 mL of cold acetone at −20 °C for 30 min and washed once with 500 μL of cold acetone. Precipitated proteins were centrifuged at 15 000*g* for 10 min and the acetone was removed, and the protein pellet was air-dried for 10 min. The protein pellets were resolubilized in 100 μL of PBS buffer with 1% SDS, and 10 μL of sample was loaded on to an SDS-PAGE gel for protein band separation and then scanned for fluorescent bands. The same gel was then stained with Coomassie blue to compare the total amount of protein loaded in each sample.

Photolabeling of HeLa Membranes with Biotinylated GSIs. Experiments were performed as described previously.[32,38] Briefly, prepared HeLa membranes (400 μg) were incubated with 20 nM of GSI probes (L646, GY4, JC8, or L505) and 4 or 12 μM of GSM compound **1** or **2** in 1 mL vol PBS in a 24-well microplate. After UV irradiation at 350 nm for 30 min, labeled membranes were RIPA solubilized and pulled-down with ultralink streptavidin resin overnight at 4 °C; bound proteins were eluted and separated via SDS-PAGE and analyzed by Western Blot using PS1-NTF antibody. The chemical syntheses for L646 and L505,[20] GY4,[39] and JC8[40] have been described previously.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Chemical synthesis and characterization of GSMs and supplementary figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

C.J.C., K.R.B., D.S.J., and Y.M.L. designed experiments and prepared the manuscript. B.A.F. and C.S. synthesized compounds. C.J.C. performed photolabeling studies. D.M.C. developed activity assays. S.V.C., N.G., and K.A. carried out assays. N.P. gave scientific input.

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■ ABBREVIATIONS

AD, Alzheimer's disease; APP, amyloid precursor protein; GSM, γ-secretase modulator; GSI, γ-secretase inhibitor; NSAID, nonsteroidal anti-inflammatory drug; PS1, presenilin 1; PS1-NTF, PS1 N-terminal fragment; SPP, signal peptide peptidase; TAMRA, tetramethyl rhodamine

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