Letters

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Discovery of γ-Secretase Modulators with a Novel Activity Profile by Text-Based Virtual Screening

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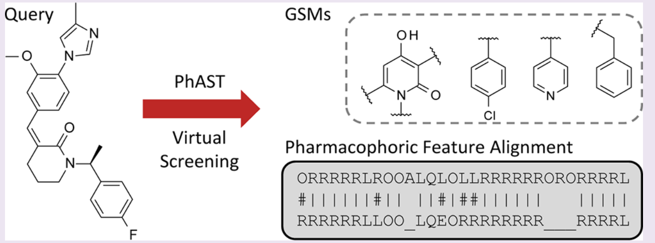
Supporting Information

ABSTRACT: We present an integrated approach to identify and optimize a novel class of γ-secretase modulators (GSMs) with a unique pharmacological profile. Our strategy included (i) virtual screening through application of a recently developed protocol (PhAST), (ii) synthetic chemistry to discover structure−activity relationships, and (iii) detailed *in vitro* pharmacological characterization. GSMs are promising agents for treatment or prevention of Alzheimer's disease. They modulate the γ-secretase product spectrum (*i.e.*, amyloid-β (Aβ) peptides of different length) and induce a shift from toxic Aβ42 to shorter Aβ species such as Aβ38 with no or minimal effect on the overall rate of γ-secretase cleavage. We describe the identification of a series of 4-hydroxypyridin-2-one derivatives, which display a novel type of γ-secretase modulation with equipotent inhibition of Aβ42 and Aβ38 peptide species.



Integrated drug discovery approaches linking computational chemistry, structural compound optimization, and subsequent biological characterization are promising strategies to promote lead identification and optimization. We have recently reported the development of PhAST, a novel virtual screening tool for rapid ligand-based hit and lead finding.[1,2] Herein, we present its successful application to identify novel γ-secretase modulators (GSMs), as candidates for the treatment or prevention of Alzheimer's disease.[3]

γ-Secretase, a membrane-bound aspartic protease, is the terminal enzyme in the proteolytic cleavage of amyloid precursor protein (APP) and is responsible for the release of toxic Aβ42 peptides.[4] γ-Secretase generates various Aβ species with abundant peptides in cell supernatants ranging from Aβ1-37 to Aβ1-43. However, Aβ42 is particularly prone to form soluble oligomers, which are neurotoxic and believed to have a crucial disease-initiating role in the pathogenesis of Alzheimer's disease.[4]

Several classes of small molecules that reduce Aβ generation by interfering with γ-secretase activity have been discovered (for a comprehensive overview see Supplementary Table 1). The biological effects of these compound classes can be categorized by (i) the way they affect γ-secretase activity and Aβ generation (inhibition *vs* modulation) and (ii) whether they display a degree of selectivity for APP over the NOTCH receptor, another important physiological substrate of γ-secretase.[3,5] γ-Secretase inhibitors (GSIs) function as protease inhibitors by reducing the overall activity of γ-secretase and the production of all species of Aβ peptides.[5] However, GSIs (*e.g.*, semagacestat) were shown to cause mechanism-based toxicity in both preclinical animal studies and clinical trials, largely attributable to inhibition of NOTCH processing and signaling.[5] More recently, GSIs with selectivity toward APP have been advanced. These NOTCH-sparing GSIs include compounds with structural similarity to earlier sulfonamide-based GSIs (*e.g.*, begacestat), as well as certain kinase inhibitors such as imatinib (Gleevec).[5−7] Still, it remains to be demonstrated that the degree of selectivity seen *in vitro* is sufficient to avoid toxicity in humans.[8]

Intriguingly, GSMs have been identified that selectively lower Aβ42 generation.[9] Concomitantly, GSMs increase shorter Aβ species such as Aβ38, suggesting that compensating changes in the Aβ42/Aβ38 ratio might be mechanistically linked.[9,10] Besides the originally described GSMs in the class of nonsteroidal anti-inflammatory drugs and a second generation of structurally related acidic GSMs with nanomolar potency

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Table 1. GSM Activity of Compounds 1−15

Compound	R1	R2	R3	γ-secretase modulation: Aβ42 Inhibition (IC_{50} ± SEM)	γ-secretase modulation: Aβ38 Inhibition (IC_{50} ± SEM)
	OH, R3, R1, N, O, R2				
1		Cl		inverse modulation 5-10μM	
2	N	Cl		17±2 μM	18±2 μM
3	N	Cl		inactive	
4	N	Cl		weak inverse modulation >10μM	
5	N, S	Cl		inactive	
6	N	S, N		inactive	
7	N	HN, O		inactive	
8	N	Cl, Cl		inactive	

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Table 1. continued

Compound	R1 (OH, R3, R1, N, O, R2)	R2	R3	γ-secretase modulation	
	R1	**R2**	**R3**	Aβ42 Inhibition (IC_{50} ± SEM)	Aβ38 Inhibition (IC_{50} ± SEM)
9	N	F, F, F		inactive	
10	N	CF_3		21±6 μM	22±6 μM
11	N	CF_3, Cl		weak inverse modulation >30μM	
12	N	Cl, CF_3		9±2 μM	8±2 μM
13	N	CF_3		inactive	
14	N	Cl, CF_3		not determined due to poor solubility	
15	O, N, O, N, N_3			inactive	

(*e.g.*, GSM-1), structurally different non-acidic GSMs (*e.g.*, E-2012) have been described.[3] Notably, these non-acidic GSMs display less selectivity for A*β*42 and also reduce A*β*40.[11] In addition, inverse GSMs that increase A*β*42 and lower A*β*38 production have been described.[3] GSMs do not reduce the total amount of A*β* production, indicating no or negligible effects on the overall turnover rate of *γ*-secretase. Consequently, selectivity of GSMs can be extensive with some compounds offering a >1000-fold selectivity window between A*β*42 and NOTCH inhibition.[12]

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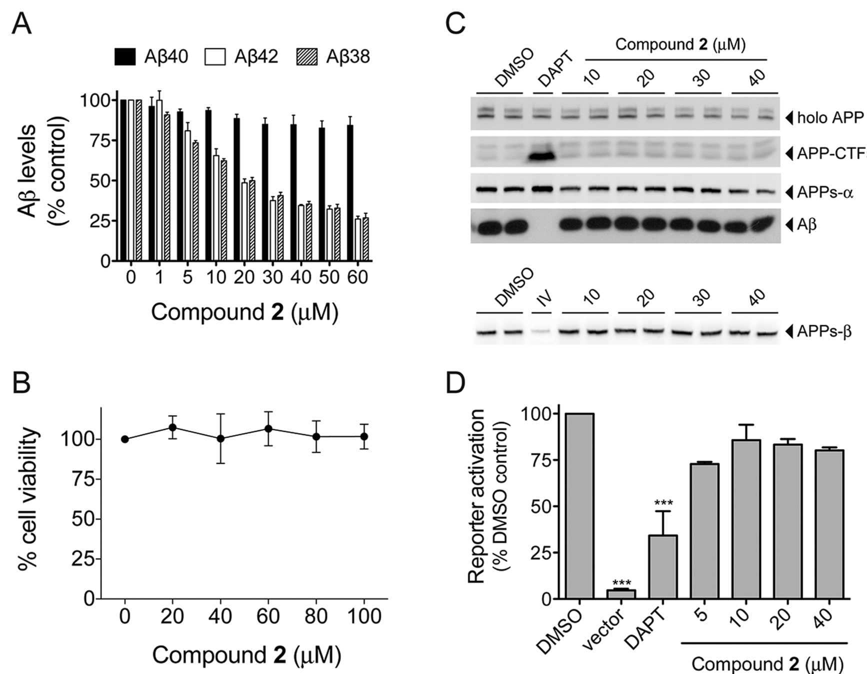

Figure 1. (A) Unique GSM profile of the PhAST hit compound **2**. CHO cells with stable overexpression of wild type APP and PSEN1 (CHO-APP/PSEN1 cells) were treated with compound **2**, and Aβ40, Aβ42, and Aβ38 levels in conditioned media were analyzed by sandwich ELISA. Compound **2** reduced Aβ42 and Aβ38 levels in a dose-dependent fashion with equal potency. Results represent averages of 3 independent experiments performed in triplicates. (B) Compound **2** did not cause any toxicity in CHO-APP/PSEN1 cells up to 100 μM. (C) The upper panel shows the analysis of APP processing after treatment of CHO-APP/PSEN1 cells with compound **2** or the γ-secretase inhibitior DAPT (5 μM). As expected, DAPT abolished Aβ production and induced strong accumulation of APP C-terminal fragments (APP-CTFs). In contrast, compound **2** did not affect APP expression, secretion of the soluble ectodomain APPs-α, or total Aβ levels. Consistent with **2** being a genuine GSM, the compound did not cause accumulation of APP-CTFs. The two bands represent APP-CTF species generated either by α-secretase (lower band) or β-secretase (upper band) cleavage of APP. In the lower panel, CHO-APP/PSEN1 cells were treated with compound **2** or the β-secretase inhibitior IV (1 μM), and the levels of the soluble ectodomain APPs-β were analyzed with an antibody that recognizes the C-terminal neo-epitope generated by β-secretase cleavage. Compound **2** did not affect APPs-β levels, while β-secretase inhibitor IV strongly reduced APPs-β levels. (D) Potential effects of compound **2** on NOTCH processing were analyzed using an established reporter assay. DAPT treatment or replacing the plasmid encoding NOTCH with empty vector caused a significant decrease in reporter activity. In contrast, treatment with compound **2** did not reduce reporter activity. $n = 3$, one-way ANOVA, *** $p < 0.001$ Dunnett's post tests.

Using PhAST virtual screening, we have discovered a class of small molecules with a novel mode of γ-secretase modulation. These compounds reduce both Aβ42 and Aβ38 levels and qualify as genuine GSMs as the reduction of longer Aβ42 species is accompanied by increased production of shorter Aβ peptides and preservation of NOTCH processing.

We used the non-acidic GSM E-2012 (see Table 1) as template for ligand-based virtual screening with PhAST (please refer to the Methods section for a brief description of PhAST). Four compounds ranked 12th, 13th, 20th, and 77th were selected from the resulting list by visual inspection for pharmacological characterization. Among these, the 4-hydroxypyridin-2-one derivative **2** (ranked 13th) displayed GSM activity in a previously described cell-based assay.[13] Treatment of CHO cells with stable overexpression of wild type human APP and presenilin-1 (PSEN1, CHO-APP/PSEN1 cells) with compound **2** caused a dose-dependent reduction in Aβ42 levels (IC_{50} = 17 ± 2 μM) with minor effects on Aβ40 levels (Figure 1A). Surprisingly, this hit also dose-dependently lowered Aβ38 levels (IC_{50} = 18 ± 2 μM). Treatment of CHO-APP/PSEN1 cells with compound **2** did not result in any cytotoxicity up to 100 μM (Figure 1B). This unusual profile of γ-secretase modulation with equipotent reduction of Aβ42 and Aβ38 levels has not been previously reported. Therefore, we performed additional assays to test whether compound **2** showed the hallmark characteristics of a GSM. First, we determined its effects on the proteolytic processing of APP in comparison to the established GSI DAPT in CHO-APP/PSEN1 cells.[5] Compound **2** did not affect APP holo-protein expression in cell lysates or the levels of the soluble APP-ectodomains APPs-α and APPs-β in cell supernatants (Figure 1C). Consistent with the ELISA results for Aβ40, the total amount of Aβ peptides was not reduced by compound **2**, whereas DAPT at 5 μM completely abolished cellular Aβ production (Figure 1C). Importantly, compound **2** did not induce accumulation of APP C-terminal fragments (APP-CTFs), which are the proximal substrates for γ-secretase. In contrast, DAPT treatment substantially increased APP-CTF levels, indicating strongly reduced γ-secretase activity (Figure 1C). Next, we determined NOTCH signaling using an established reporter assay for NOTCH-1.[13] DAPT treatment significantly reduced NOTCH-1 reporter activity compared to vehicle control. In contrast, treatment with compound **2** did not impair NOTCH-1 signaling (Figure 1D). Finally, to examine whether the Aβ42/Aβ38 reductions induced by compound **2** were accompanied by opposite changes in other Aβ species not detectable by our ELISA assay, we chose to analyze the full spectrum of secreted peptides by immunoprecipitation mass spectrometry (IP-MS). Clearly detectable peptides in the mass spectra were Aβ1-37, 1-38, 1-39, 1-40, and 1-42 (Figure 2A). Confirming the ELISA results, treatment of CHO-APP/PSEN1 cells with 20 μM of compound **2** reduced the peak heights corresponding to Aβ1-

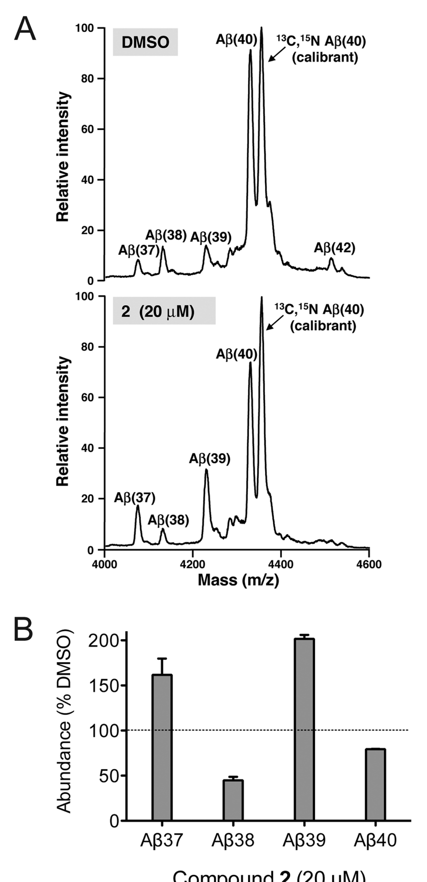

Figure 2. (A) Immunoprecipitation mass spectrometry (IP-MS) spectra of Aβ peptide species secreted by CHO-APP/PSEN1 cells after treatment with compound **2**. IP-MS analysis demonstrated a reduction in peak heights corresponding to Aβ1-42 and Aβ1-38 as compared to DMSO vehicle, confirming the sandwich ELISA results. In addition, signals corresponding to Aβ1-37 and Aβ1-39 were elevated, showing that levels of these Aβ species were increased by compound **2**. (B) Quantification of the IP-MS results from two independent experiments. Changes after compound treatment are expressed relative to DMSO vehicle control. Aβ1-42 levels could not be accurately quantified due to the small signal size commonly observed with this peptide species.[16]

42 and Aβ1-38. Concomitantly, peaks corresponding to Aβ1-39 and Aβ1-37 were elevated indicating that compound **2** increased levels of these Aβ species. In these experiments, a slight decrease of around 20% in the signal corresponding to Aβ1-40 was observed, which was less pronounced when measured by ELISA. While this could be attributed to experimental variation due to the less quantitative nature of IP-MS, we cannot fully exclude the possibility that our C-terminal specific ELISA antibody for Aβ40 might have minor affinity to Aβ39. In this case, slight reductions in Aβ40 levels could be obscured in the ELISA assay by the concomitant increase in Aβ39 peptides. Taken together, these results demonstrate NOTCH sparing, lack of APP-CTF accumulation, and compensatory changes in shorter Aβ species with no effect on total Aβ production and strongly suggest that compound **2** is a genuine GSM with a novel profile of Aβ modulation.

Surprisingly, analogue **1** (ranked 12th), in which the pyridin-4-yl substituent in 6-position of the central 4-hydroxypyridin-2-one is replaced by phenyl, was completely inactive. This first SAR result indicated an essential role of the pyridin-nitrogen in compound **2**. Encouraged by this initial finding, we decided to elucidate the SAR of γ-secretase affecting 4-hydroxypyridine-2-ones and developed a synthetic strategy toward structural analogues. Synthesis (Scheme 1) started with the condensation of commercially available acetophenone analogues and aromatic primary amines. Desired imines were obtained by microwave irradiation. For the final cyclization step (ii), the imines were heated with activated malonic ester derivatives, a reaction that was also performed under microwave irradiation. Therefore, 2-benzyl (and 2-phenyl) malonic acid was esterified with 2,4,6-trichlorophenol to obtain so-called "magic malonates" (step (iii); synthesis was not successful with malonic acid ethyl esters).[14] Our SAR study included 13 derivatives and was focused on the terminal aromatic residues in the 1-, 3-, and 6-positions of the central 4-hyrdoxypyridin-2-one (Table 1). First, we varied the pyridin-4-yl residue in the 6-position. As the SAR of the ordered compounds **1** and **2** suggested the presence of the pyridin-nitrogen as an essential structural requirement, we maintained the nitrogen atom and varied its position as in analogues **3** (3-pyridin), **4** (4-dimethylaminophenyl), and **5** (2,4-dimethylthiazol). These slight structural changes either caused a complete loss of activity (**3**, **5**) or led to inverse GSM activity (**4**). Next, we focused on the 4-chlorophenyl residue in the 1-position. This moiety was initially replaced by larger

Scheme 1. Synthesis of 4-Hydroxypyridin-2-ones[a]

[a]Reagents and conditions: (i) TsOH, 4 Å molecular sieves, 160 °C, 10−60 min; (ii) μW, 200−230 °C, 1.5−3 min; (iii) $POCl_3$, reflux, 2 h.

residues such as benzothiazol (**6**) and 4-acetanilide (**7**). However, these residues were not tolerated and resulted in inactive compounds. On the basis of these results, we decided to prepare the structurally closer analogues **8** (3,4-dichlorophenyl), **9** (3,4,5-trifluorophenyl), and **10** (4-trifluorophenyl). Intriguingly, the addition of one chlorine in 3-position (**8**) caused a complete loss of activity as well as the 3,4,5-trifluorophenyl residue. Only replacement of the initial chlorine with a trifluoromethyl group (**10**) yielded an active GSM (IC_{50} = 21 ± 6 μM). We further explored this finding by preparation of the 4-chloro-3-(trifluoromethyl)phenyl-substituted analogue **11** and the 2-chloro-4-(trifluoromethyl)phenyl-substituted analogue **12**. Again, an additional substituent in 3-position was detrimental (but caused inverse GSM activity, **11**), whereas the additional chlorine in 2-position combined with the 4-trifluoromethyl-substituent led to the bioactive compound **12** with slightly improved activity (IC_{50} = 9 ± 2 μM).

Compound **12** was the most active GSM in this study, and further analysis confirmed that it modulates γ-secretase activity with the same characteristic profile as the initial PhAST hit **2**. ELISA analysis of secreted Aβ species demonstrated equipotent inhibition of both Aβ42 and Aβ38 species (Table 1). Compound **12** did not cause any cytotoxicity up to 100 μM (Supplementary Figure 1). Similar to the results obtained for compound **2**, IP-MS analysis of secreted Aβ species demonstrated reductions in Aβ1-42 and Aβ1-38 and elevation of Aβ1-39 and Aβ1-37 levels (Supplementary Figure 2). To further explore the SAR of the 3-benzyl group of this scaffold, we replaced it by a shorter 3-phenyl residue and prepared the analogues of the most active compounds **10** and **12**. The 4-trifluoromethylphenyl-substituted analogue **13** did not show any GSM activity, while the pharmacological characterization of the 2-chloro-4-trifluoromethylphenyl-substituted analogue **14** was not possible due to poor aqueous solubility.

Finally, a photoprobe containing an azide as UV-labile functional group and an alkine tag for click reaction-mediated biotin conjugation was synthesized (**15**), which did not show GSM activity. However, we recently demonstrated binding of an acidic GSM photoprobe to PSEN, the catalytic subunit of the γ-secretase complex.[15] In competition experiments, compound **2** was able to displace the acidic GSM photoprobe from its target PSEN (Supplementary Figure 3). This suggested that the binding site of 4-hydroxypyridin-2-one based GSMs within the γ-secretase complex might overlap either physically or allosterically with the molecular target of other GSM classes.

The unique way of Aβ modulation by compounds **2** and **12** has implications for current models to explain the molecular mechanism of GSMs. Following cleavage of APP by β-secretase, which generates the N-terminus of the Aβ peptides, γ-secretase cleaves at multiple sites within the APP transmembrane domain, and various Aβ peptide species have been identified in cell supernatants (Aβ33, 34, 37, 38, 39, 40, 42, 43) and lysates (Aβ45, 46, 48, 49). According to the sequential cleavage model of Aβ generation, γ-secretase cleavage takes place sequentially every 3−4 amino acids along the α-helical surface of the substrate APP, thereby converting longer Aβ peptides into shorter species. This model is consistent with the idea that Aβ42 might be the direct precursor of Aβ38, and that GSMs act by promoting the turnover of Aβ42 to shorter species such as Aβ38.[10] However, it was previously shown that overexpression of PSEN mutants associated with familial forms of Alzheimer's disease rendered cells resistant to the Aβ42-lowering activity of GSMs, while GSMs were able to elevate Aβ38 levels in this cellular system, arguing that Aβ42 and Aβ38 peptides were generated independently by γ-secretase.[11,16,17] Likewise, the identification of GSMs that reduce both Aβ42 and Aβ38 generation and predominantly increase Aβ1-39 does not readily conform to the sequential cleavage model of Aβ generation. Alternatively, the 4-hydroxypyridin-2-one derivatives might promote turnover of Aβ42 to Aβ38 and, in a second step, rapid conversion of Aβ38 to shorter species ≤ Aβ35. However, in the IP-MS spectra, we observed only one peak corresponding to an Aβ peptide shorter than Aβ1-37, and the abundance of this species (Aβ1-34) was not affected by compounds **2** and **12** (data not shown).

In conclusion, we have demonstrated the strength of an integrated approach using computational virtual screening, medicinal chemistry, and *in vitro* pharmacology for rapid hit identification and optimization. Considering the patent literature of the past 5 years it is apparent that beyond the NSAID-like acidic GSMs (*e.g.*, GSM-1) and the non-acidic heteroaryl-type GSMs (*e.g.*, E-2012) no novel pharmacophores have emerged.[18] It is fair to assume, therefore, that the hit rates for the identification of GSMs in conventional high-throughput campaigns are extremely low and that most pharmaceutical companies have rather approached this field by knowledge-based design. With the discovery of 4-hydroxypyridin-2-one derivatives characterized by equipotent Aβ42 and Aβ38 inhibition, we were able to add a new class to the non-acidic GSMs, which appear to dominate ongoing drug development efforts.[3] This corroborates not only the effectiveness of the ligand-based PhAST screening protocol in finding novel chemical entities but also its ability to discover active compounds with slightly distinct pharmacological properties. The identified 4-hydroxypyridin-2-one derivatives displayed an extremely steep SAR, and minor structural changes in almost every part of the molecule led to either inactive compounds or inverse GSMs. Nevertheless, we were able to improve the activity of the initially identified PhAST hit **2**, yielding the low micromolar active, drug-like compound **12** with potential for further investigation and development as a drug candidate for Alzheimer's disease.

■ METHODS

Pharmacophore Alignment Search Tool (PhAST). PhAST[1,2] is a text-based approach to virtual screening. It encodes molecules as strings, which describe patterns of pharmacophoric molecular features. The conversion of molecules (with suppressed hydrogen atoms) into string representations follows three steps: (i) A pharmacophoric feature is assigned to each atom. Each feature type has an associated symbol. (ii) Graph canonization is performed utilizing Minimum Volume Embedding[19] in combination with a Diffusion Kernel[20] (diffusion parameter β = 0.4) yielding a set of invariant atom indices. (iii) Symbols are concatenated as defined by invariant atom indices in ascending order. Sequences are compared by global pairwise sequence alignment. The alignment score is interpreted as indicator of similarity, with higher scores identifying higher similarity.

Screening Library. The screening compound pool combined the vendor catalogues of Specs (v01/2010, Specs, Delft, The Netherlands) and Asinex Gold and Platinum (v11/2008, Asinex, Moscow, Russia). Protonation states were assigned using the "wash" function of the Molecular Operating Environment (v2009.10 The Chemical Computing Group Inc., Montreal, QC, Canada) with the following settings: protonation of strong bases, deprotonation of strong acids.

Compounds and Chemistry. Compounds **1** and **2** were purchased from Specs (www.specs.net, catalogue numbers AE-406/41057207, AE-406/41056370). Synthesis was performed using an adapted protocol (Scheme 1) first published by Kafka and Kappe.[21]

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Imines were prepared by microwave irradiation under standard conditions. "Magic malonates" were synthesized in a reaction of the respective malonic acid, phosphorus oxychloride, and 2,4,6-trichlorophenol as previously described.[14] The cyclization reaction to the final 4-hydroxypyridin-2-ones was carried out under microwave irradiation without any solvent. Temperature and reaction time were varied depending on the substituents of the imines.

Immunoprecipitation Mass Spectrometry (IP-MS). CHO-APP/PSEN1 cells were treated for 24 h with 20 μM concentration of compounds **2** or **12** or DMSO vehicle. Three micrograms of 4G8 and 6E10 monoclonal antibodies and 60 ng of isotopically labeled ^{13}C,^{15}N-Aβ1-40 (Anaspec) internal standard for peak intensity and mass calibration were added to 1.5 mL of conditioned media and incubated for 3 h at RT. Immunocomplexes were captured by incubation with anti-IgG coated magnetic beads (Dynal) overnight at 4 °C followed by repeated washing of the beads according to Beher *et al.*[22] Bound peptides were eluted in 4 μL of α-cyano-4-hydrocinnamic acid matrix solution (50% MeCN, 0.1%TFA), and 2 μL was dried on a target plate. The spectra were collected on a Voyager-DE PRO Workstation (Applied Biosystems) MALDI-TOF mass spectrometer in linear positive ion mode averaging 5 times 100 shots for each sample. For data analysis the individual peak intensities were normalized to the internal calibrant by calculating the ratio of peptide versus calibrant peak intensity. Changes after compound treatment were expressed relative to the individual ratios obtained in DMSO vehicle controls.

Analysis of γ-Secretase Modulator Activity, APP Processing, NOTCH Processing, and Cytotoxicity. GSM activity, APP processing, NOTCH processing, and cytotoxicity were determined in previously described cell-based assays (see Supporting Information and refs13 and 23).

Photocross-Linking and Competition Experiments. Photoaffinity labeling studies were performed as described (see Supporting Information and ref 15).

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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