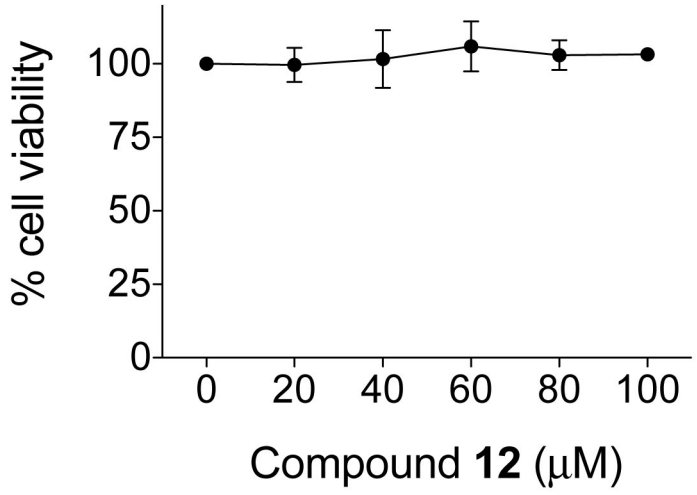
SUPPORTING INFORMATION

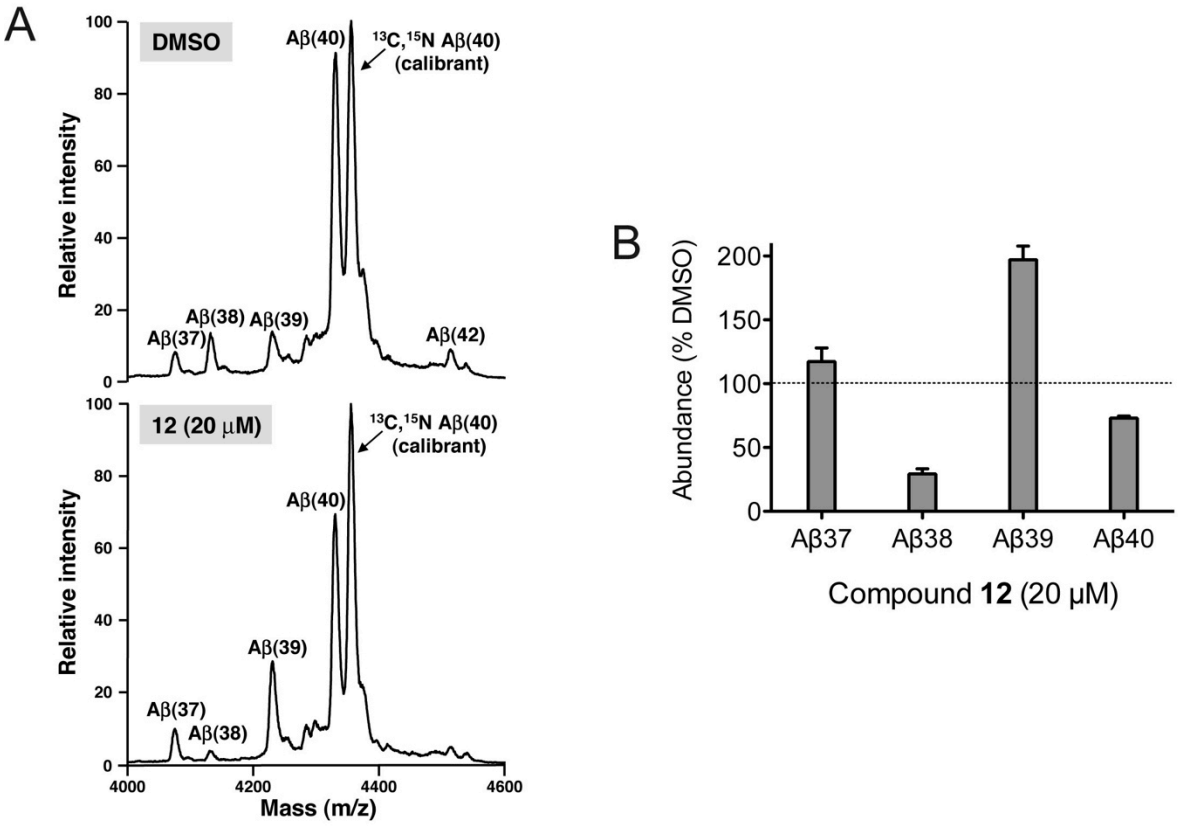
Identification of novel-type γ-secretase modulators by text-based virtual screening

Heiko Zettl[†], Julia Ness[§], Volker Hähnke[†], Dirk Beher[‡], Thorsten Jumpertz[§], Arman Saric[‡], Karlheinz Baumann[#], Claus U. Pietrzik[¶], Bruno Bulic[Δ], Gisbert Schneider[†*], and Sascha Weggen[§*]

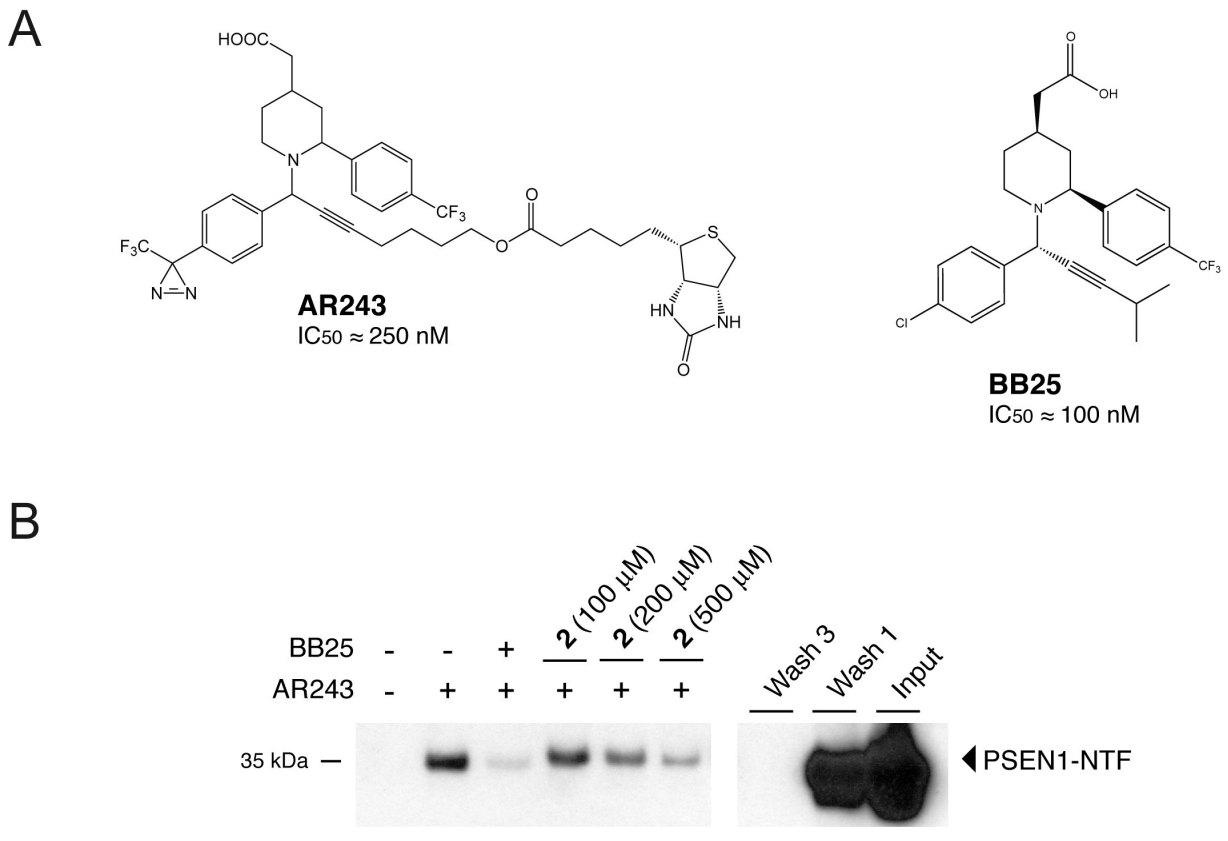
This material is available free of charge via the Internet



Supplementary Figure 1. No cytotoxicity was observed after treatment of CHO-APP/PSEN1 cells with compound **12** up to 100 μM.



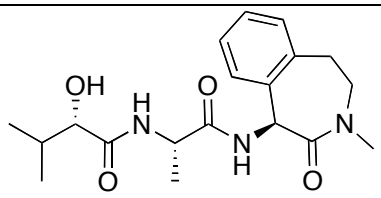
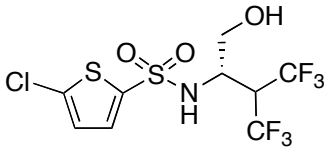
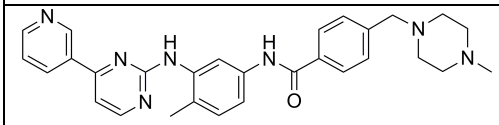
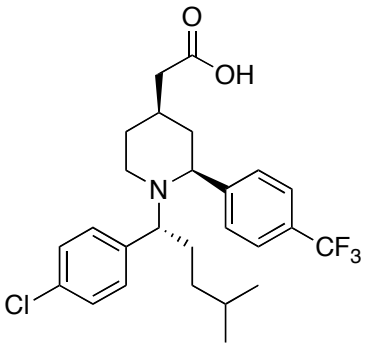
Supplementary Figure 2. (A) IP-MS spectra of Aβ peptide species secreted by CHO-APP/PSEN1 cells after treatment with 20 μM of compound **12**. IP-MS analysis demonstrated a reduction in signals corresponding to Aβ1-42 and Aβ1-38 and a concomitant increase in Aβ1-37 and Aβ1-39. (B) Quantification of the IP-MS results from two independent experiments. Changes after compound treatment are expressed relative to DMSO vehicle control. At this high concentration of **12** approximately 5-fold above the IC_{50} for Aβ42 inhibition, around 27% reduction in Aβ1–40 levels was also observed.

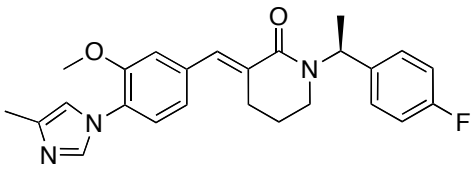


Supplementary Figure 3. (A) Chemical structure of the bioactive acidic GSM photo-probe AR243 and the parental competitor compound BB25. The GSM photo-probe contained a diazirine photo-reactive group and a biotin moiety for affinity purification. In a cell-based assay, this photo-probe has been demonstrated to lower Aβ42 levels with an IC_{50} of 290 nM (*6*). (B) The hydroxypyridin-2-one derivative **2** competed binding of the GSM photo-probe to its molecular target PSEN1. Cellular membranes were prepared from neuronal N2a-ANPP cells and incubated with 0.5 µM of the photo-probe or DMSO vehicle. After UV irradiation, putative target proteins were affinity purified using streptavidin-functionalized beads. Western blotting of the purified material demonstrated that the GSM photo-probe AR243 bound the N-terminal fragment of PSEN1 (PSEN1-NTF) with a MW of approximately 35 kDa. Incubation of membranes with DMSO vehicle did not result in a Western blotting signal. Adding an excess of the parent compound BB25 (100 µM) during incubation efficiently reduced binding of the photo-probe to the PSEN1-NTF. Similarly, co-incubation of the photo-probe with increasing concentrations of compound **2**

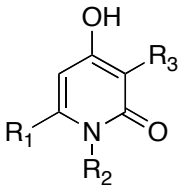
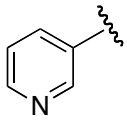
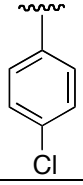
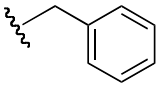
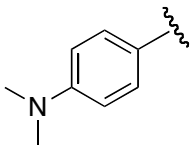
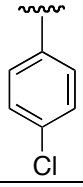
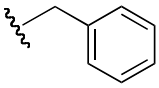
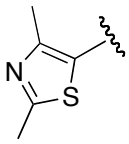
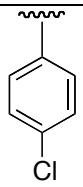
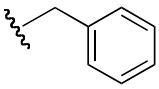
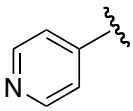
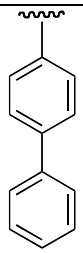
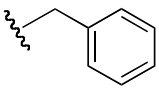
reduced the labeling signal in a dose-dependent fashion. Material that was bound non-specifically to the streptavidin beads is visible in the supernatant of the first washing steps but is completely removed after the third wash. Input represents 0.02% (PSEN1-NTF) of the total membrane material.

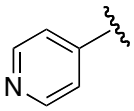
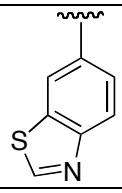
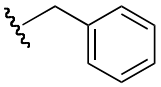
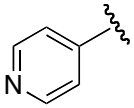
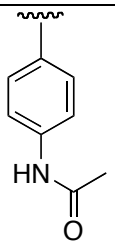
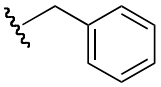
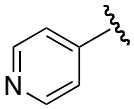
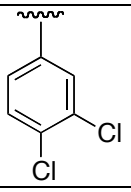
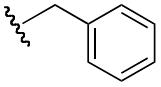
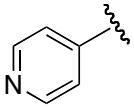
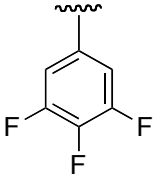
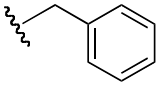
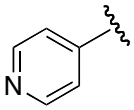
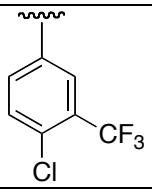
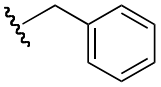
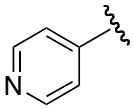
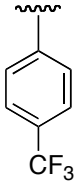
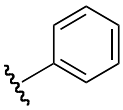
Supplementary Table 1. Representative pharmacological profiles of γ-secretase inhibitors (GSIs) and modulators (GSMs).

Compound	IC_{50}				Ref.
	Aβ38	**Aβ40**	**Aβ42**	**NOTCH**	
 Semagacestat (GSI)	12 nM	12 nM	11 nM	14 nM	(*1*)
 Begacestat (NOTCH-sparing GSI)	n/a	15 nM	12 nM	209 nM	(*2*)
 Imatinib (Nucleotide Binding Site-Inh.)	≈ 10 μM	≈ 10 μM	≈ 10 μM	→	(*3*)
 GSM-1 (acidic GSM)	↑	→	180 nM	→	(*4*)

 E-2012 (non-acidic GSM)	↑	n/a	53 nM	→	(*5*)

Supplementary Table 2. Synthesis and purity of inactive compounds.

Com-pound				**Synthesis**		HPLC-MS	
	R_1	**R_2**	**R_3**	Conditions	Yield	purity	ESI+ [m/z]
3				μW, 235°C, 3min	30%	95.6%	389
4				μW, 235°C, 3min	32%	89.1%	431
5				μW, 235°C, 3min	63%	98.3%	423
6				μW, 235°C, 15min	51%	99%	431

7				μW, 235°C, 3min	49%	100%	412
8				μW, 235°C, 3min	43%	99%	431
9				μW, 235°C, 3min	38%	97.8%	424
11				μW, 235°C, 3min	49%	95.5%	409
13				μW, 200°C, 3min	32%	91.5%	457
14				μW, 200°C, 3min	7%	91.6%	409

SUPPLEMENTARY METHODS

Compounds and Chemistry: The structures of active compounds **10** and **12** were confirmed by ^{1}H-NMR, ^{13}C-NMR, and high-resolution mass spectrometry (HR-MS, ESI+). Purity was determined by HPLC analysis (detection at 254nm, Shimadzu LC-MS2020 system). Inactive compounds were verified by HPLC-MS analysis (Shimadzu LC-MS2020 system, see Supplementary Table 2). Commercial chemicals and solvents were of reagent grade and used without further purification. ^{1}H and ^{13}C NMR spectra were measured in DMSO-*d*6 on a Bruker Av400 spectrometer. Chemical shifts are reported in parts per million (ppm) using tetramethylsilane (TMS) as internal standard. Mass spectra were obtained on a Bruker Daltonics maXis spectrometer measuring in the positive-ion mode (ESI-MS system).

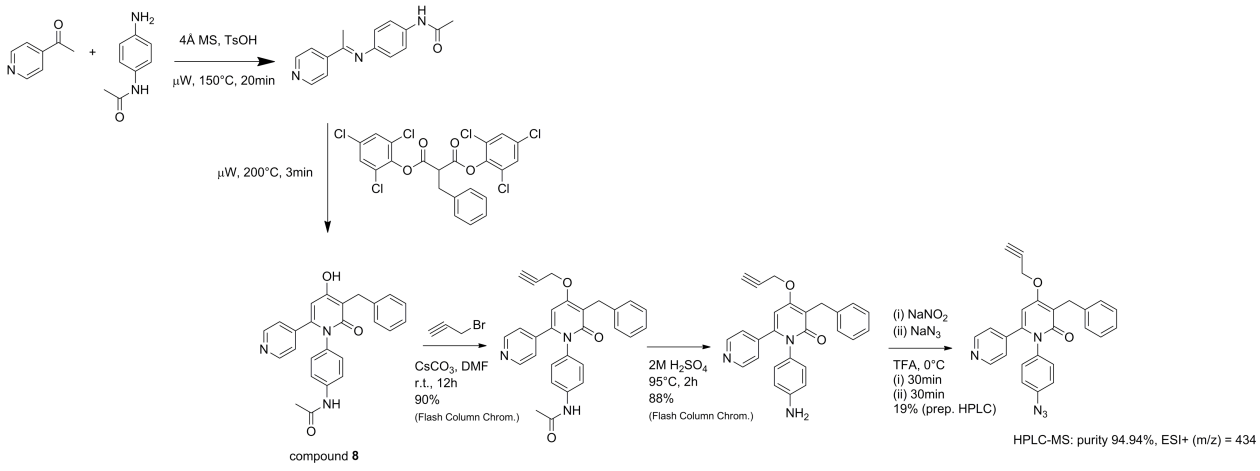
Synthesis of 5-benzyl-4-hydroxy-1-(4-(trifluoromethyl)phenyl)-[2,4'-bipyridin]-6(1*H*)-one (compound 10). First, the imine *N*-(1-(pyridin-4-yl)ethylidene)-4-(trifluoromethyl)aniline was prepared by condensation of 4-acetylpyridine and 4-(trifluoromethyl)aniline under microwave irradiation (step (i): 10 min, 150°C, 4Å molecular sieves, catalytic amounts of TsOH; purification by flash column chromatography using n-hexane/EtOAc as mobile phase; reaction control by HPLC-MS: m/z = 265 (ESI+)). Bis(2,4,6-trichlorophenyl) 2-benzylmalonate was prepared as described in literature (step (iii)). Finally, the imine was heated with the malonic ester under microwave conditions (3 min, 200°C) and the product was recrystallized by dissolving the reaction mixture in ACN. Total yield was 39%

^{1}H-NMR (400.13 MHz, DMSO-*d*6) 3.78 (s, 2H, C*H*$_2$), 6.12 (s, 1H, Pyr-5*H*), 7.14 (t, 1H, Ar-*H*), 7.20 – 7.26 (m, 4H, Ar-*H*), 7.32 (d, 2H, Ar-*H*), 7.41 (d, 2H, Ar-*H*), 7.64 (d, 2H, Ar-*H*), 8.42 (dd, 2H, Ar-*H*) 10.92 (br s, 1H, O*H*). ^{13}C-NMR (100.61 MHz, DMSO-*d*6) 29.00, 102.08, 109.94, 123.36, 125.40 125.44 125.52 (*C*F$_3$), 127.95, 128.55, 130.73, 140.90, 142.31, 142.53, 143.86, 149.40, 161.84, 163.21. HPLC-MS (H$_2$O + 0.05% HCOOH/MeOH + 0.5% HCOOH, RP18, 254nm, ESI+): compound purity 97.53% (area normalization), m/z = 423 [M+H$^+$]. HR-MS (ESI+) calc. m/z = 423.1315, meas. m/z = 423.1319.

Synthesis of 5-benzyl-1-(2-chloro-4-(trifluoromethyl)phenyl)-4-hydroxy-[2,4'-bipyridin]-6(1*H*)-one (compound 12). First, the imine 2-chloro-*N*-(1-(pyridin-4-yl)ethylidene)-4-(trifluoromethyl)aniline was prepared by condensation of 4-acetylpyridine and 2-chloro-4-(trifluoromethyl)aniline under

microwave irradiation (step (i): 10 min, 150°C, 4Å molecular sieves, catalytic amounts of TsOH; purification by flash column chromatography using n-hexane/EtOAc as mobile phase; reaction control by HPLC-MS: m/z = 298 (ESI+)). Bis(2,4,6-trichlorophenyl) 2-benzylmalonate was prepared as described in literature (step (iii)). Finally, the imine was heated with the malonic ester under microwave conditions (3 min, 200°C) and the product was purified by preparative HPLC (CAN + 0.05% HCOOH/ H_2O + 0.5% HCOOH). Total yield was 7%

^{1}H-NMR (400.13 MHz, DMSO-*d*6) 3.77 (q, 2H, CH_2), 6.16 (s, 1H, Pyr-5*H*), 7.13 (t, 1H, Ar-*H*), 7.20 – 7.26 (m, 6H, Ar-*H*), 7.69 (dd, 1H, Ar-*H*), 7.75 (d, 1H, Ar-*H*), 7.92 (s, 1H, Ar-*H*), 8.47 (dd, 2H, Ar-*H*) 11.00 (br s, 1H, O*H*). ^{13}C-NMR (100.61 MHz, DMSO-*d*6) 28.86, 102.00, 111.34, 122.96, 124.53, 126.49 126.53 126.59 (*C*F_3), 126.65, 127.95, 128.36, 133.46, 140.79, 141.41, 142.53, 143.34, 149.50, 161.90, 163.33. HPLC-MS (H_2O + 0.05% HCOOH/MeOH + 0.5%HCOOH, RP18, 254nm, ESI+): compound purity 98.57% (area normalization), m/z = 457 [M+H^+]. HR-MS (ESI+) calc. m/z = 457.0925, meas. m/z = 457.0923.



Supplementary Scheme 1: Synthesis of compound **15**

Determination of γ-secretase modulator activity: To characterize the GSM activity of novel analogs, their effects on the generation of Aβ40, Aβ42, and Aβ38 peptides were determined in a previously described cell-based ELISA assay with modifications (*7*). In brief, CHO cells stably overexpressing wild type human amyloid precursor protein and wild type human presenilin-1 (CHO-APP/PSEN1 cells, (*5*)) were maintained in DMEM supplemented with 10% FBS, and treated in 96-well plates for 24h with increasing concentrations of respective compounds or DMSO vehicle. Culture media were collected and analyzed by ELISA as follows: monoclonal antibody IC16 raised against amino acids 1-15 of the Aβ sequence was used as a capture antibody. To distinguish different Aβ species, C-terminal antibodies specific for Aβ40, Aβ42 and Aβ38 labeled with horseradish peroxidase (HRP) using the Pierce EZ-Link™ Plus Activated Peroxidase kit (Thermo Fisher Scientific) were used for detection. In some experiments, a novel Aβ38-specific antibody (PZ1.11) with increased sensitivity was used to allow detection of Aβ38 levels below the baseline of untreated CHO-APP/PSEN1 cells. Full characterization of this antibody will be published elsewhere. 96-well high-binding microtiter plates were coated overnight at 4°C with capture antibody IC16 diluted 1:250 in PBS, pH 7.2. Capture antibody was removed and conditioned media samples (10 µl for detection of Aβ40, 100 µl for Aβ42, 50-70 µl for Aβ38) or standard peptides were loaded. HRP-coupled detection antibodies diluted in assay buffer (PBS containing 0.05 % Tween-20, 1 % BSA) were added to each well and incubated overnight at 4°C. Plates were washed 3 times with PBS containing 0.05 % Tween-20. 50 µl TMB ultra substrate (Thermo Fisher Scientific) was added, incubated for 1-3 min at RT, and the reaction was stopped by adding 50 µl 2 M H_2SO_4. Absorbance at 450 nm was recorded with a Paradigm™ microplate reader (Beckman-Coulter). Synthetic Aβ40, Aβ42 and Aβ38 peptides (Bachem AG) were used to generate standard curves. These Aβ peptides were solubilized in DMSO and stored frozen at -80°C. Immediately before use, peptides were diluted in culture media to 250-3000 pg/ml. Triplicate measurements from each drug concentration were averaged and normalized to DMSO control condition. For calculation of IC_{50} values, cells were treated with 7-8 increasing concentrations of each compound, and a non-linear curve-fit with variable slope model was

applied. Statistics were performed using GraphPad Prism Version 5 (GraphPad Software).

Analysis of APP processing. CHO cells with stable overexpression of wild type human APP and wild type human PSEN1 (CHO-APP/PSEN1 cells (*8*)) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1mM sodium pyruvate and 100 units/ml penicillin/streptomycin (Invitrogen), and treated for 24 h with increasing concentrations of compounds or DMSO vehicle. Crude cell lysates were prepared using NP40 buffer (50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8), total protein was separated on 10% Bis-Tris gels, and levels of full-length APP (holo-APP) and APP-CTFs were analyzed by Western blotting using polyclonal antibody CT-15 raised against the C-terminal 15 amino acids of APP (*5*). To measure secreted metabolites of APP, conditioned cell culture media from the same cells were separated on 12% Bis-Tris gels. Levels of the soluble ectodomain APPs-α and Aβ peptides were determined by Western blotting with the monoclonal antibody IC16 raised against amino acids 1-15 of the Aβ sequence (*7*). Levels of the soluble ectodomain APPs-β were measured by Western blotting with a polyclonal antibody from IBL International. The γ-secretase inhibitor DAPT and the β-secretase inhibitor IV were purchased from Sigma and Merck KGaA.

NOTCH reporter assay. The NOTCH reporter assay was performed as described (*9*). Subconfluent CHO cells were transiently transfected in 96-well plates with plasmid pcDNA3-Notch-ΔE-GVP encoding truncated NOTCH fused to a Gal4 DNA-binding/VP16 transactivation domain and the MH100 reporter plasmid encoding firefly luciferase under the UAS promotor (50 ng each) using GeneJuice transfection reagent (Merck Chemicals Ltd.). Co-transfection of the MH100 reporter with empty pcDNA3 plasmid served as a negative control. 5ng of plasmid pRL-TK encoding renilla luciferase was added to the plasmid mix to control for transfection efficiency. 24h after the transfection, cells were treated for an additional 24h with increasing concentrations of the compounds **2** and **12**, γ-secretase inhibitor DAPT (5 μM) or DMSO vehicle. The cells were then extracted and firefly and renilla luciferase activities were quantified using the Dual Glo™ Luciferase Assay System (Promega) and a Paradigm™ microplate reader. Normalisation for transfection efficiency and cell growth was achieved by dividing the firefly luciferase

values by the corresponding renilla luciferase values. Percent activation of the reporter was calculated by normalization of triplicate measurements from each condition to the DMSO control values. Results from three independent experiments were analyzed by one-way ANOVA with Dunnett's post tests using GraphPad Prism. ***, $p<0.001$.

Cytotoxicity assay. CHO-APP/PSEN1 cells were seeded at low density in 96-well plates (4000 cells/well) and cultured for 24 h. The cells were then treated in duplicates with increasing concentrations (0 - 100 μM) of the respective compounds or DMSO as vehicle for additional 24 h. Cell viability was assessed using the alamarBlue® reagent (Invitrogen). 20 μl alamarBlue® was added to cells cultured in 200 μl medium and incubated overnight. Absorbance was measured with a Paradigm™ microplate reader at 570 nm, using 600 nm as the reference wavelength. Percent viability of vehicle control was calculated from two independent experiments.

Photo-crosslinking and competition experiments. For photo-affinity labeling studies, membranes were prepared from N2a cells stably transfected with human APP harboring the "Swedish" mutation and all four subunits of the γ-secretase complex: human PSEN1, Nicastrin, APH-1a and PEN-2 (N2a-ANPP cells) as described (*6*). Cells were washed and harvested in PBS and incubated with hypotonic buffer (10 mM Tris pH 7.4, 1x protease inhibitor mix) for 10 min on ice. To prepare cellular membranes, cells were passed 10 times through a 30G needle and centrifuged at 800 x g, 10 min, 4°C. The post-nuclear supernatant was centrifuged at 18.000 x g, 45 min, 4°C. The resulting membrane pellet was washed once with MES buffer (50 mM MES, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, pH 6.0), centrifuged at 18.000 x g, 45 min, 4°C, and again dissolved in MES buffer. Prior to the addition of compounds, membrane preparations were incubated with streptavidin magnetic beads to deplete endogenously biotinylated proteins. Membranes were then incubated with 0.5 μM of the acidic GSM photo-probe AR243 or DMSO vehicle for 1 h at 4°C with gentle shaking. In competition experiments, the photo-probe was co-incubated with 100 μM of the parental acidic compound BB25 or with increasing concentrations of compound **2** (100-500 uM). Samples were UV-irradiated on ice for 30 min at 365 nm and for 30 min at 302 nm. After the photo-crosslinking, to ensure exposure of the bound biotin-tagged GSM, 1x RIPA

buffer was added to unfold/denature proteins. Samples were then incubated overnight with streptavidin-functionalized magnetic beads (Invitrogen) at 4°C with gentle shaking, and the streptavidin beads were washed 3 times for 30 min with 1x RIPA buffer. Bound material was eluted with Laemmli buffer and incubation at 65°C for 10 min with intermittent vortexing. The eluted material was separated on 12% Bis-Tris gels and analyzed by Western blotting with monoclonal antibody PSN2 against a synthetic peptide corresponding to amino acids 31-56 of human PSEN1 (a kind gift of Dr. Hiroshi Mori).

SUPPLEMENTARY REFERENCES

1. Mitani, Y., Yarimizu, J., Saita, K., Uchino, H., Akashiba, H., Shitaka, Y., Ni, K., and Matsuoka, N. (2012) Differential Effects between gamma-Secretase Inhibitors and Modulators on Cognitive Function in Amyloid Precursor Protein-Transgenic and Nontransgenic Mice, *J Neurosci 32*, 2037-2050.
2. Mayer, S. C., Kreft, A. F., Harrison, B., Abou-Gharbia, M., Antane, M., Aschmies, S., Atchison, K., Chlenov, M., Cole, D. C., Comery, T., Diamantidis, G., Ellingboe, J., Fan, K., Galante, R., Gonzales, C., Ho, D. M., Hoke, M. E., Hu, Y., Huryn, D., Jain, U., Jin, M., Kremer, K., Kubrak, D., Lin, M., Lu, P., Magolda, R., Martone, R., Moore, W., Oganesian, A., Pangalos, M. N., Porte, A., Reinhart, P., Resnick, L., Riddell, D. R., Sonnenberg-Reines, J., Stock, J. R., Sun, S. C., Wagner, E., Wang, T., Woller, K., Xu, Z., Zaleska, M. M., Zeldis, J., Zhang, M., Zhou, H., and Jacobsen, J. S. (2008) Discovery of Begacestat, a Notch-1-Sparing gamma-Secretase Inhibitor for the Treatment of Alzheimer's Disease, *J Med Chem 51*, 7348-7351.
3. Netzer, W. J., Dou, F., Cai, D., Veach, D., Jean, S., Li, Y., Bornmann, W. G., Clarkson, B., Xu, H., and Greengard, P. (2003) Gleevec inhibits beta-amyloid production but not Notch cleavage, *Proc Natl Acad Sci U S A 100*, 12444-12449.
4. Kretner, B., Fukumori, A., Gutsmiedl, A., Page, R. M., Luebbers, T., Galley, G., Baumann, K., Haass, C., and Steiner, H. (2011) Attenuated A{beta}42 responses to low potency {gamma}-secretase modulators can be overcome for many pathogenic presenilin mutants by second-generation compounds, *J Biol Chem 286*, 15240-15251.
5. Hahn, S., Bruning, T., Ness, J., Czirr, E., Baches, S., Gijsen, H., Korth, C., Pietrzik, C. U., Bulic, B., and Weggen, S. (2011) Presenilin-1 but not amyloid precursor protein mutations present in mouse models of Alzheimer's disease attenuate the response of cultured cells to gamma-secretase modulators regardless of their potency and structure, *J Neurochem 116*, 385-395.
6. Jumpertz, T., Rennhack, A., Ness, J., Baches, S., Pietrzik, C. U., Bulic, B., and Weggen, S. (2012) Presenilin is the molecular target of acidic gamma-secretase modulators in living cells, *PloS one 7*, e30484.
7. Hieke, M., Ness, J., Steri, R., Dittrich, M., Greiner, C., Werz, O., Baumann, K., Schubert-Zsilavecz, M., Weggen, S., and Zettl, H. (2010) Design, synthesis, and biological evaluation of a novel class of gamma-secretase modulators with PPARgamma activity, *J Med Chem 53*, 4691-4700.
8. Leuchtenberger, S., Kummer, M. P., Kukar, T., Czirr, E., Teusch, N., Sagi, S. A., Berdeaux, R., Pietrzik, C. U., Ladd, T. B., Golde, T. E., Koo, E. H., and Weggen, S. (2006) Inhibitors of Rho-kinase modulate amyloid-beta (Abeta) secretion but lack selectivity for Abeta42, *J Neurochem 96*, 355-365.
9. Karlstrom, H., Bergman, A., Lendahl, U., Naslund, J., and Lundkvist, J. (2002) A sensitive and quantitative assay for measuring cleavage of presenilin substrates, *J Biol Chem 277*, 6763-6766.