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N-Substituted carbazolyloxyacetic acids modulate Alzheimer associated γ-secretase

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Abstract—*N*-Sulfonylated and *N*-alkylated carbazolyloxyacetic acids were investigated for the inhibition and modulation of the Alzheimer's disease associated γ-secretase. The introduction of a lipophilic substituent, which may vary from arylsulfone to alkyl, turned 2-carbazolyloxyacetic acids into potent γ-secretase modulators. This resulted in the selective reduction of $A\beta_{42}$ and an increase of the less aggregatory $A\beta_{38}$ fragment by several compounds (e.g., **7d** and **8c**). Introduction of an electron donating group at position 6 and 8 of *N*-substituted carbazolyloxyacetic acids either decreased the activity or inversed modulation. The most active compounds displayed activity on amyloid precursor protein (APP) overexpressing cell lines in the low micromolar range and little or no effect on the γ-secretase cleavage at the ε-site.

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that causes the most common form of dementia, affecting approximately 5% of the population over the age of 65 years in Europe. Short-term memory impairment, disorientation, aphasia and a general cognitive decline are common symptoms early in disease development. As the disease progresses, spatial and motor abilities are affected and the patient becomes bedridden and completely dependent on the caretaker. According to the World Health Organization, an estimated 37 million people worldwide currently have dementia; AD affects about 18 million of them.[1]

Pathological lesions and plaques consisting of the amyloid β-peptide (Aβ) are found in the brains of the AD patients. Aβ is heterogeneously produced by the sequential cleavages of amyloid precursor protein (APP) by the two aspartic proteases: β- and γ-secretase. Cleavage at the Aβ N-terminus is executed by the β-site cleaving enzyme (BACE).[2,3] The following intramembrane cleavage by γ-secretase occurs with little sequence specificity, resulting in Aβ fragments of different length, predominantly $A\beta_{40}$, $A\beta_{42}$ and some $A\beta_{38}$. $A\beta_{42}$ being the most aggregatory. The C-terminal fragments generated by α- and β-secretase cleavage, C83 and C99, are cleaved within their transmembrane domains to produce the p3 peptide. The functional γ-secretase complex requires the correct assembly of at least five components: presenilin-1 (PS1), nicastrin, anterior pharynx defective-1 (Aph-1), presenilin enhancer-2 (Pen-2) and the substrate.[4–10] PS1, a catalytic subunit of the γ-secretase complex, harbours two aspartates in the transmembrane domains 6 and 7 that define a novel active site closely resembling that of other recently identified aspartic proteases.[11–14] PS1 acts as a heterodimer of N- and C-terminal fragments (NTF, CTF) that derive from autoproteolysis. γ-Secretase activity can be controlled by the inhibition of the active site of PS1 or by interference with complex assembly or substrate recognition, the latter resulting in allosteric modulation or inhibition. The allosteric mechanisms are particularly attractive targets for drug development as they may control the ratio of the Aβ

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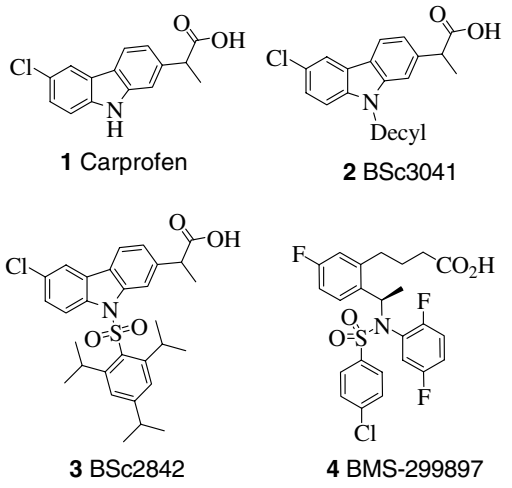
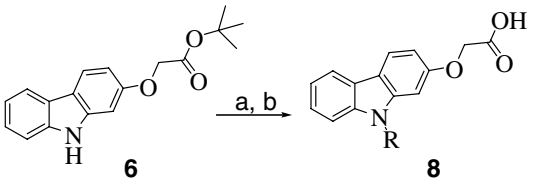
fragments: $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$, while retaining the cleavage of other substrates. Most of the reported and confirmed γ-secretase inhibitors are not substrate specific and inhibit the processing of other γ-secretase substrates equally,[15] for example, Notch and other.[16]

Epidemiological studies indicated an association between nonsteroidal anti-inflammatory drugs (NSAIDs)

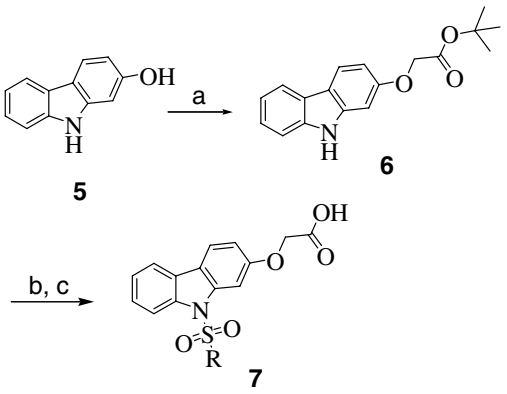


Scheme 1. Carprofen, the most active carprofen derivatives and BMS-299897.



Entry	Compound	R
1	**8a**	Octyl
2	**8b**	Nonyl
3	**8c**	Decyl
4	**8d**	H

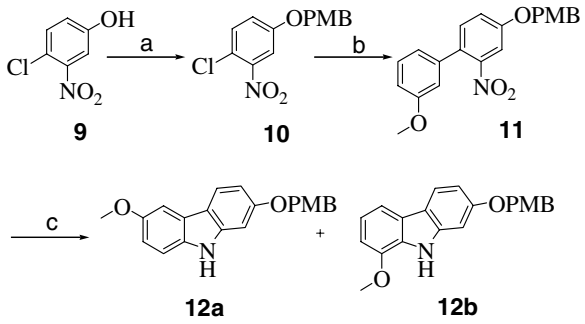
Scheme 3. Synthesis of N-alkylated carbazolyloxyacetic acids and **8d**. Reagents and conditions: (a) KO^{t}Bu, RX, THF, 0 °C rt, 2–8 h, 80–91%; (b) NaOH, MeOH/H_2O (1:1) rt, 2 h, 92–97%.



Entry	Compound	R
1	**7a**	4-methylphenyl
2	**7b**	3,5-bis(trifluoromethyl)phenyl
3	**7c**	4-biphenyl
4	**7d**	2,4,6-tri-*iso*-propylphenyl
5	**7e**	4-*n*-propylphenyl
6	**7f**	Octyl
7	**7g**	(1*S*)-1-(methyl)-7,7-dimethylbicyclo[2.2.1]heptan-2-one
8	**7h**	4-chlorophenyl
9	**7i**	3,5-difluorophenyl
10	**7j**	2-bromophenyl
11	**7k**	3-nitrophenyl

Scheme 2. Synthesis of *N*-sulfonylated carbazolyloxyacetic acid derivatives. Reagents and conditions: (a) $ClCH_2CO_2{}^tBu$, K_2CO_3, acetone, 60–70 °C, 16 h, 100%; (b) NaH, RSO_2Cl, THF, 0 °C rt, 2–8 h, 80–100%; (c) 20% TFA in DCM, 1–4 h, 80–100%.

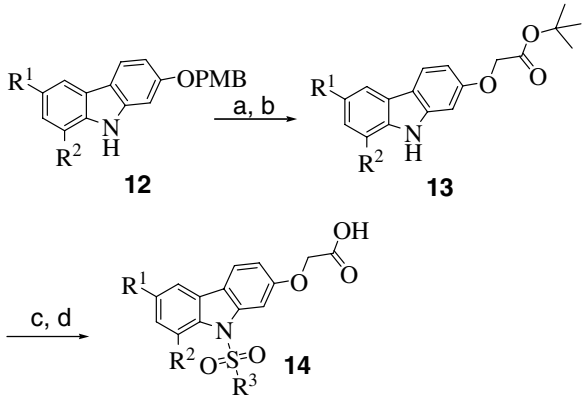
use and a reduced risk for the development of AD.[17–20] Recently, we reported N-sulfonylated and N-alkylated derivatives (**2**, **3**) of carprofen (Scheme 1) that modulate γ-secretase and selectively reduce $A\beta_{42}$.[21,22] This is accompanied by an increase of the less aggregatory $A\beta_{38}$ species. Carprofen **1** is a COX-2 inhibitor (COX = cyclooxygenase) approved for the use in dogs, cows and horses as Imadyl® or Rimadyl®. The selectivity of carprofen versus $COX\text{-}2_{canine}$ and $COX\text{-}1_{canine}$ is greater than 100:1 ($COX\text{-}2_{canine}$ IC_{50}/*R*/*S*-carprofen 102 nM, *R*-carprofen 5.97 μM, *S*-carprofen 37 nM).[23] Original carprofen (as isolated from Rimadyl® tablets 500 mg) was found to be a weak inhibitor of γ-secretase and reduced $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ at high concentrations. Knowing about the relevance of the carboxylic acid and N-substitution of carprofen, we decided to investigate scaffolds not included in the patent applications and publications by Koo et al.[24] or Stock,[25] or Imbimbo[26] and others.[27,28] Carprofen's similarity to the γ-secretase inhibitor BMS-299897 (Scheme 1) prompted us to investigate *N*-sulfonylated and *N*-alkylated 2-hydroxy carbazolyloxyacetic acids. The corresponding sulfonamides **7a**–**k** were readily accessible by straightforward synthesis.



Scheme 4. Synthesis of 6-methoxy and 8-methoxy carbazoles. Reagents and conditions: (a) PMBCl, K_2CO_3, acetone, reflux, 16 h, 97%; (b) $[(Ph_3P)_4Pd]$, 3-methoxybenzene-boronic acid, K_2CO_3, H_2O, toluene, reflux, 18 h, 67% (c) Ph_3P, *o*-dichlorobenzene, reflux, 16 h, 60%.

A series of 2-hydroxy carbazole *N*-sulfonamide derivatives was prepared as outlined in Scheme 2. 2-Hydroxycarbazole **5** was alkylated using anhydrous K_2CO_3 and $ClCH_2CO_2{}^tBu$ in acetone at 60–70 °C to give compound **6**. *N*-Sulfonylation of **6** was carried out using NaH, various sulfonyl chlorides in THF and subsequent *t*-butyl deprotection by 20% trifluoroacetic acid in dichloromethane furnished the desired compound **7**.

N-Alkylated carbazolyloxyacetic acids were prepared to evaluate the contribution of the sulfonamide moiety in



Entry	Compound	R^1	R^2	R^3
1	**14a**	OMe	H	3,5-(bis-trifluoro)phenyl
2	**14b**	OMe	H	4-chlorophenyl
3	**14c**	OMe	H	4-biphenyl
4	**14d**	OMe	H	2-bromophenyl
5	**14e**	H	OMe	4-chlorophenyl
6	**14f**	H	OMe	4-biphenyl

Scheme 5. Synthesis of 6-methoxy and 8-methoxy *N*-sulfonylated carbazolyloxyacetic acids. Reagents and conditions: (a) 10% Pd–C, H_2, MeOH, 60 psi, rt, 12 h, 81%; (b) $ClCH_2CO_2{}^tBu$, K_2CO_3, acetone, reflux, 16–18 h, 82–88%; (c) R^3SO_2Cl, NaH, THF, 0 °C rt, 1–3 h, 55–71%; (d) 20% TFA in DCM, rt, 2 h, 80–94%.

Table 1. Activity report of N-alkylated and N-sulfonylated carbazolyloxyacetic acids

Entry	Compound	Cell toxicity[d](μM)	IC_{50} (μM)		
			$Aβ_{38}$[a]	$Aβ_{40}$	$Aβ_{42}$
1	**7a**	20	13	>40	39
2	**7b**	20	>40	>40	>40
3	**7c**	20	13	40	24
4	**7d**	20	7.8	19	7.5
5	**7e**	>40	ND[b]	>40	38
6	**7f**	>40	ND[b]	>40	37
7	**7g**	40	14	>40	20
8	**7h**	40	32	>40	32
9	**7i**	40	13	>40	>40
10	**7j**	40	>40	>40	>40
11	**7k**	40	16	>40	>40
12	**8a**	>40	24	>40	19
13	**8b**	>40	30	>40	17
14	**8c**	>40	ND[b]	>40	11
15	**8d**	>40	>40	>40	>40
16	**2**	—	5.8	>40	2.9
17	**3**	—	9.3	>20	8.5
18	**4**[c]	—	—	—	0.0071

[a] EC_{50} values are displayed for $Aβ_{38}$.
[b] Maximum effect on $Aβ_{38}$ not observed at the highest concentration tested.
[c] IC_{50} value is displayed for total Aβ.
[d] Significant cellular toxicity observed at this concentration. Viability reduction > 20%.

7g and the most active derivate **7d**, where the sulfonamide is shielded by isopropyl substituents. The *N*-alkylated carbazolyloxyacetic acids were synthesized as shown in Scheme 3. Compound **6** was alkylated using KO^{t}Bu and alkyl halide in dry THF. Subsequent deprotection of *t*-butyl group by base hydrolysis provided desired *N*-alkylated carbazolyloxyacetic acid **8**.

We synthesized the 2-hydroxy-6-methoxy and 2-hydroxy-8-methoxy *N*-sulfonylated carbazolyloxyacetic acids to investigate the impact of an electron donating substituent on the activity. We commenced the synthesis of 6-methoxy and 8-methoxy-2-hydroxy carbazoles from the commercially available 4-chloro-3-nitrophenol **9** as outlined in Scheme 4. The –OH functionality of **9** was protected as a *para*-methoxybenzyl (PMB) ether **10**. Suzuki–Miyaura coupling of **10** with 3-methoxybenzeneboronic acid using the catalyst $[(PPh_3)_4]Pd$ gave biphenyl **11**, which was then cyclized to its corresponding carbazole by refluxing it in *o*-dichlorobenzene with PPh_3. The PPh_3 mediated cyclization resulted in two regioisomers, 6-methoxy carbazole, **12a** and 8-methoxy carbazole, **12b** which were separated by flash column chromatography.

N-Sulfonylated derivatives of 2-hydroxy-6-methoxy and 2-hydroxy-8-methoxy carbazolyloxyacetic acids were synthesized as depicted in Scheme 5. The PMB group of **12** was deprotected by hydrogenation at 60 psi and the free –OH was alkylated using anhydrous K_2CO_3 and $ClCH_2CO_2{}^tBu$ in acetone at 60–70 °C to yield alkylated **13**. *N*-Sulfonylation of **13** was carried out using NaH and R^3SO_2Cl in dry THF and subsequent *t*-butyl group removal by 20% TFA in DCM furnished the desired compound **14**.

Compounds **7a**–**k** and **8a**–**c** turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40 and γ42 sites to a different extent, and particularly reduced the formation of $Aβ_{42}$, while increasing the formation of $Aβ_{38}$ (see Table 1 and Fig. 1).[33] The most potent inhibitors of $Aβ_{42}$ were compounds **7d**, **7g**, and **8c**. Compounds **14a**–**c** showed modulatory activity but required very high concentrations. Compounds **14e** and **14f** showed some indications to be inverse modulators, they increased $Aβ_{42}$ formation and reduced $Aβ_{38}$ formation at very high concentrations (see Fig. 2). The necessary levels for IC_{50}/EC_{50} determination were not reached at 40 μM. The effect of the most potent compounds on γ-secretase cleavage at the ε-site was assessed using an in vitro assay that monitors the de novo generation of β-amyloid precursor protein intracellular domain (AICD).[29,30] The formation of AICD was affected by the compounds to various extent (Fig. 3). However, much higher compound concentrations than those determined to be modulatory were required to inhibit the ε-cleavage. One of the most active *N*-sulfonylated carbazolyloxyacetic acids (**7d)** was selected for evaluation in COX-1 and COX-2 assays to rule out COX-1 or COX-2 mediated effects at the concentrations necessary for γ-secretase modulation. The assays were performed at CEREP (www.cerep.com) using indomethacin as a standard for COX-1 and NS398 for COX-2. The compound **7d** displayed no activity on COX-1 and COX-2 at 10 μM concentration. Only a few compounds within this structural class displayed cell toxicity in H4 cells at the highest concentration tested (40 μM). It is very likely that this toxicity is caused by unique structural properties of single compounds.[34,35]

The introduction of a single lipophilic substituent, which may vary from arylsulfone to alkyl, turns the *N*-substituted carbazolyloxyacetic acids into a potent γ-secretase modulator. Some in silico parameters of these lead candidates are close to the range of drug-like compounds,[31] but the lipophilic substituents cause a dramatic increase of the clog*P*. The carboxylic acid may interfere with uptake, but the total polar surface area of **8c** is just 62.9 Å^2, this compares to COX189 (lumiracoxib, tPSA = 58 Å^2).The more polar compound **7c** displays similar properties (clog*P* = 6.426, tPSA = 107.5 Å^2) as the carboxylic acid BMS-299897 (clog*P* = 5.92, tPSA = 93.4 Å^2).[27] Further increases in chain length will worsen this property, but the determination of the maximum length may provide information on drug localisation or trafficking. We speculate that the lipophilic substituent anchors the *N*-substituted carbazolyloxyacetic acid in the required orientation within the membrane, thus the maximum tolerated length should be similar to natural phospholipids. This hypothesis will be explored in the next series. The desired selective reductions of $Aβ_{42}$ and the accompanying increases of the less aggregatory $Aβ_{38}$ fragment are displayed by several compounds

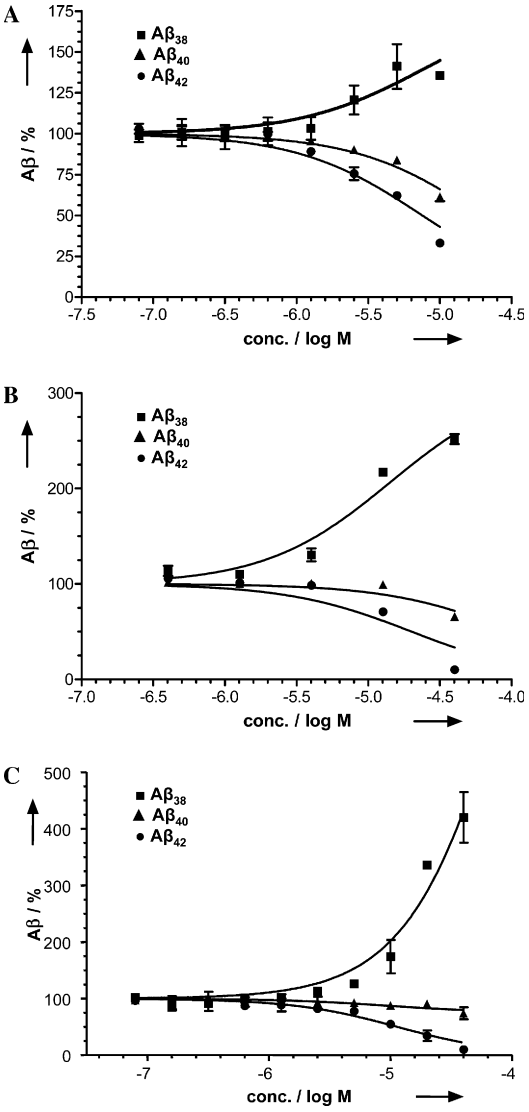


Figure 1. Dose–response curves for the most active N-substituted carbazolyloxyacetic acid derivatives; Aβ (% of control). (A) Compound **7d**. (B) Compound **7g**. (C) Compound **8c**.

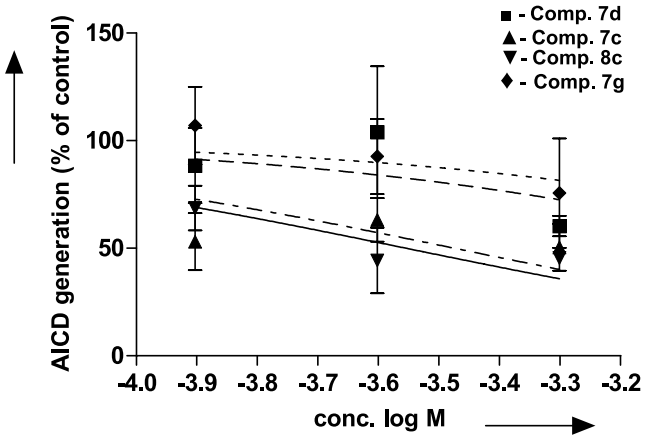


Figure 3. Dose–response curves for the most active *N*-substituted carbazolyloxyacetic acids (**7d**, **7c**, **8c**, and **7g**) on in vitro AICD generation. Results are the average of three independent experiments. Error bars indicate the standard error of the mean.

(e.g., **7d** and **8c**). The introduction of an electron donating substituent at 6- and 8-position of *N*-substituted carbazolyloxyacetic acids either decreased the modulatory activity (**14a**–**14c**) or inversed modulatory activity (**14d**–**14f**). If affected at all, the ε-cleavage of γ-secretase was inhibited at much higher compound concentrations than those determined to be modulatory at the γ-site (Fig. 3). The compounds are thus expected to have little or no impact on γ-secretase-mediated signalling via the AICD or via intracellular domains (ICDs) of other γ-secretase substrates.

The present data qualify the scaffold as a lead structure for γ-secretase modulation, but do not justify extensive investigation of the current compounds in animals. Improvements of potency, solubility and the investigation in neuronal cells are required prior to the investigation of in vivo activity.

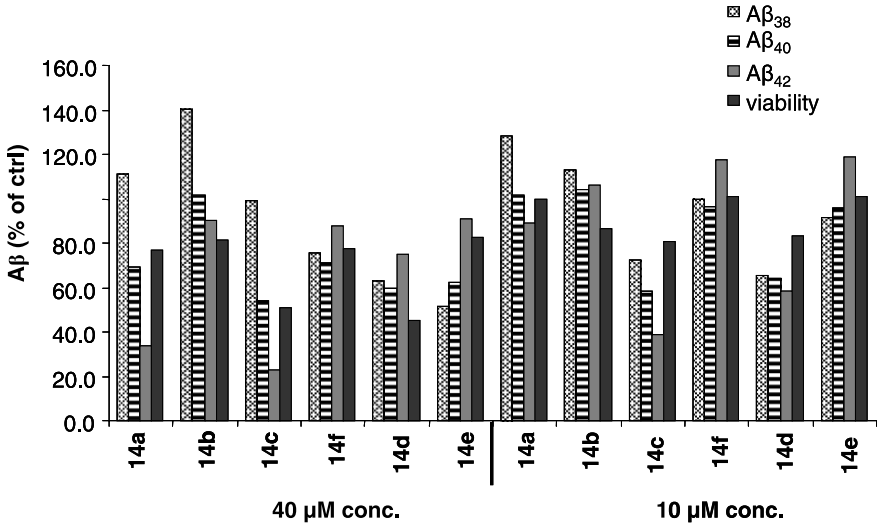


Figure 2. Activity of the compounds **14a**–**14f**.

Acknowledgments

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33. *Aβ liquid phase electrochemiluminescence (LPECL) and AICD assays*. To evaluate the compounds for their potency in modulating γ-secretase activity, we used the LPECL assay to measure Aβ isoforms. APP-overexpressing cell lines were generated by stably transfecting human neuroglioma H4 cells obtained from ATCC (Accession No. CRL-1573 or HTB-148) with human APP695 in vector pcDNA3.1. The biotinylated Aβ specific antibodies 6E10 or 4G8 were used (Signet Laboratories) as capture antibodies. The C-terminal specific Aβ antibodies BAP15, BAP24 and BAP29 were generated as described previously.[32] Antibodies were labelled with TAG electro-chemiluminescent label according to manufacturer's protocol (Bioveris). Labelled antibodies were purified from unincorporated label using a PD-10 column (Pharmacia) and stored in phosphate-buffered saline (PBS) containing 0.1% sodium azide at 4 °C for several weeks or at −80 °C for long-term storage. Cells were plated in 96-well plates (30–60,000 cells/200 μL/well) and allowed to adhere for 2 h. Compounds were dissolved in DMSO (vehicle) and further diluted in cell culture medium to the desired concentration. Hundred microlitres of compound containing media was added to the cells and incubated for 20–24 h at 37 °C. Before use, M-280 paramagnetic beads (Bioveris) were diluted with assay buffer (50 mM Tris, 60 mM NaCl, 0.5% BSA and 1% Tween 20, pH 7.4). Fifty microlitres of conditioned culture medium from the plated cells were incubated with 50 μL of beads and 25 μL of each labelled antibody (6E10-bio and BAP29-TAG for detection of $A\beta_{1\text{-}38}$, 6E10-bio and BAP24-TAG for detection of $A\beta_{40}$, 6E10-bio and BAP15-TAG for detection of $A\beta_{42}$; for detection of total Aβ 4G8-bio and 6E10-TAG were used) in a final volume of 250 μL for 3 h at room temperature with gentle shaking. Synthetic $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ peptides (Bachem) were used to generate standard curves. These Aβ peptides were solubilised in DMSO at a concentration of 1 mg/mL, aliquoted and

stored frozen at −80 °C. Immediately before use, they were diluted in culture media to 16–2000 pg/mL. Electrochemiluminescence was quantified using an M-Series M8 analyzer (Bioveris). IC_{50}/EC_{50} values were calculated using GraphPad Prism ver4 software. EC_{50} values can only be calculated when the dose–response curve had reached a stable plateau at the maximum applied concentration. γ-Secretase activity was monitored by de novo production of AICD (APP intracellular domain) in vitro by the previously reported assay.[30]

34. *Cell toxicity assay*. Cell viability was measured in the corresponding cells after removal of the media for the Aβ assays by a colourimetric cell proliferation assay (CellTiter 96TM AQ assay, Promega) utilizing the bioreduction of MTS (Owen's reagent) to formazan according to the manufacturer's instructions. Briefly, after removal of the supernatant for Aβ assays 80 μL of fresh cell culture medium was added to each well and incubated for 1 h before 20 μL MTS/PES solution was added. The optical density was recorded at 490 nm after 1 h incubation at 37 °C. Cell viability was expressed as % of untreated control.
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