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Curcumin-Derived Pyrazoles and Isoxazoles: Swiss Army Knives or Blunt Tools for Alzheimer's Disease?

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Curcumin binds to the amyloid β peptide (Aβ) and inhibits or modulates amyloid precursor protein (APP) metabolism. Therefore, curcumin-derived isoxazoles and pyrazoles were synthesized to minimize the metal chelation properties of curcumin. The decreased rotational freedom and absence of stereoisomers was predicted to enhance affinity toward $A\beta_{42}$ aggregates. Accordingly, replacement of the 1,3-dicarbonyl moiety with isosteric heterocycles turned curcumin analogue isoxazoles and pyrazoles into potent ligands of fibrillar $A\beta_{42}$ aggregates. Additionally, several compounds are potent inhibitors of tau protein aggregation and depolymerized tau protein aggregates at low micromolar concentrations.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by a gradual loss of cognitive function and increasing behavioral abnormalities.[1] The pathological hallmarks of AD are extracellular deposits of aggregated amyloid β (Aβ) peptide and intracellular neurofibrillary tangles (NFTs). The major proteinaceous components of NFTs are abnormal filaments, termed either straight filaments (SFs) or paired helical filaments (PHFs). The core protein of these filaments is the microtubule-associated protein tau. Aβ is generated by sequential proteolytic processing of Aβ precursor protein (APP) by β-secretase and γ-secretase. β-Secretase has been identified as the membrane-bound aspartyl protease BACE (β-site APP-cleaving enzyme). β-Secretase cleavage leaves a C-terminal fragment of 99 amino acids in the membrane (C99). This fragment is a substrate for γ-secretase. γ-Secretase catalyzes the final cleavage, which occurs in the transmembrane domain (TMD), thus liberating Aβ from the membrane into the extracellular space and the APP intracellular domain (AICD) into the cytosol. γ-Secretase is an intramembranous protease complex that requires the correct assembly of at least five components: presenilin-1 (PS-1), nicastrin, presenilin enhancer-2 (Pen-2), anterior pharynx defective-1 (Aph-1), and the substrate.[2–4] PS-1, the catalytic subunit of the γ-secretase complex, harbors two aspartate residues in transmembrane domains 6 and 7 that define the active site which closely resembles those of other recently identified aspartic proteases.[5] Cleavage by γ-secretase occurs with little specificity and generates Aβ peptides with variable C termini. The longer form of Aβ, $A\beta_{42}$, is highly fibrillogenic and is believed to trigger the amyloid cascade, a pathological series of neurotoxic events that eventually lead to neurodegeneration and finally AD.

In addition to Aβ pathology, the disruption of tau protein functions plays a key role in this neurotoxic cascade. Normal tau binds to microtubules and facilitates microtubule assembly and stability. Tau phosphorylation is regulated by the delicate balance between multiple kinases (such as GSK-3β and CDK5) and phosphatases (such as PP-1 and PP-2A); hyperphosphorylation is the net result of an imbalance between kinase and phosphatase activities, which normally tightly regulate its phosphorylation. Hyperphosphorylation of tau leads to its detachment from microtubules and subsequent disassembly of microtubules. Moreover, enhanced levels of microtubule-bound tau can inhibit motor proteins.[6] Both of these lead to impaired axonal transport, compromising neuronal and synaptic function.

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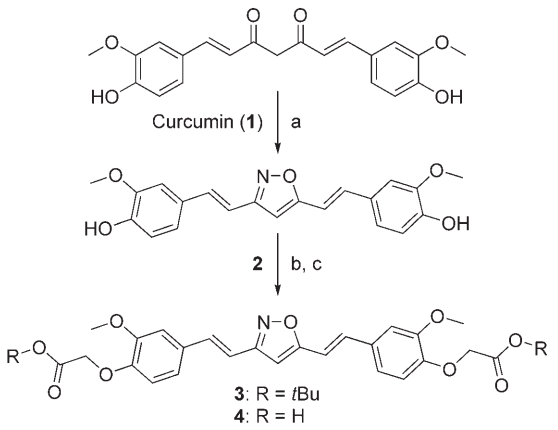
There is currently no approved causal treatment for AD. Current advances related to AD treatment include antioxidant therapy,[7,8] acetyl cholinesterase (AChE) inhibitors,[9,10] nicotinic and muscarinic agonists,[11,12] nerve growth factor (NGF),[13] lipophilic compounds that can trigger the neurotrophic factor signaling pathway,[14,15] anti-inflammatory drugs,[15] compounds that impede Aβ formation and deposition,[16] and drugs that attenuate Aβ toxicity.[17]

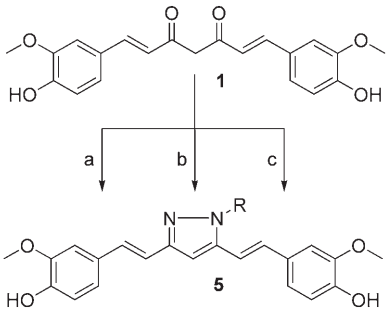
Curcumin is a major constituent of the yellow spice turmeric[18] and is derived from the plant *Curcuma longa* Linn. Turmeric is used in traditional diet and as an herbal medicine in India. Curcumin has potent anti-inflammatory[19,20] and antioxidant activities.[21,22] It can decrease oxidative damage, inflammation, amyloid accumulation, and is under development as a chemotherapeutic agent.[23] Curcumin is much stronger than vitamin E as a free-radical scavenger,[24] protects the brain from lipid peroxidation, and scavenges nitric oxide (NO)-based radicals.[25] Curcumin inhibits amyloid aggregation ($IC_{50} = 0.9\ \mu M$) in vitro and disaggregates fibrillar $A\beta_{40}$ ($IC_{50} = 1\ \mu M$). It binds to small Aβ oligomers and blocks aggregation and fibril formation in vitro and in vivo.[26] On the other hand, an alternative mechanism may be the enhanced decrease in Aβ aggregation due to the metal chelation properties of curcumin; curcumin binds to redox-active metal ions such as iron and copper, and such complexes may cause a net protective effect through decreased Aβ aggregation and toxicity or impaired metal induction of NF-κB.[27]

Recently we reported that curcumin (**1**)-derived pyrazoles and isoxazoles (**2**–**5 d**, **5 f**,**g**) inhibit or modulate APP metabolism by interfering with γ-secretase activity.[28] The keto–enol tautomerism and *E*/*Z* enol stereoisomerism of curcumin result in dynamic equilibria of isomers, which hinder the evaluation of the structure–activity relationships (SARs) of substituted curcumins. We therefore anticipated that replacement of the 1,3-dicarbonyl moiety by isosteric heterocycles, isoxazole and pyrazole, would lock the accessible conformation into defined arrangements and would decrease pleiotropic effects derived from rotational freedom and equilibria of stereoisomers. Replacement of the 1,3-dicarbonyl moiety by isosteric isoxazoles and pyrazoles provided potent γ-secretase inhibitors.[28] Herein we report our studies on their inhibitory effect toward tau aggregation and their affinity to polymeric $A\beta_{42}$ aggregates, and compare them with the previously reported results on Aβ secretion.



Scheme 1. Reagents and conditions: a) $NH_2OH{\cdot}HCl$, py, EtOH, reflux, 18 h, 80%; b) $ClCH_2CO_2tBu$, K_2CO_3, Me_2O, reflux, 14 h, 90%; c) TFA (20%) in CH_2Cl_2, room temperature, 4 h, 95%.

Pyrazole derivatives of curcumin were synthesized as reported previously (Scheme 2).[28] Curcumin was treated with hydrazine hydrate in acetic acid at ambient temperature for 8 h to



Compd	Method	R	Yield [%]
5a	a	H	65
5b	b	benzyl	75
5c	c	phenyl	63
5d	c	4-nitrophenyl	35
5e	c	6-chloropyridazin-3-yl	54
5f	c	4-tolyl	68
5g	c	4-isopropylphenyl	59
5h	b	methyl	45
5i	c	hydroxyethyl	50
5j	c	3-nitrophenyl	30
5k	c	3,5-dichlorophenyl	43

Scheme 2. a) $NH_2NH_2{\cdot}H_2O$, AcOH, room temperature, 8 h; b) $RNHNH_2$, MeOH, NEt_3, room temperature, 72 h; c) $RNHNH_2$, TFA, toluene, reflux, 18–24 h.

Results and Discussion

The isoxazole derivatives of curcumin were prepared as depicted in Scheme 1 as reported previously. Curcumin was converted into its corresponding isoxazole **2** with hydroxyamine hydrochloride and pyridine in ethanol at reflux for 18 h.[28] The hydroxy groups of isoxazole **2** were alkylated with 2-chloro-*tert*-butyl acetate and anhydrous K_2CO_3 at 60–70 °C for 12 h to give the bisalkylated compound **3**. The diacid **4** was released by treatment with 20% trifluoroacetic acid in dichloromethane for 4 h.

give the pale-yellow pyrazole **5 a**. The *N*-benzyl pyrazole **5 b** and *N*-methyl pyrazole **5 h** were obtained from the corresponding hydrazine sulfate or hydrazine dihydrochloride. Curcumin was converted into the *N*-phenyl, phenyl, and pyridazin-3-yl pyrazoles (**5 c**–**k**) by treatment with the corresponding hydrazines or hydrazine hydrochlorides and catalytic trifluoroacetic acid in toluene at reflux for 18–24 h.

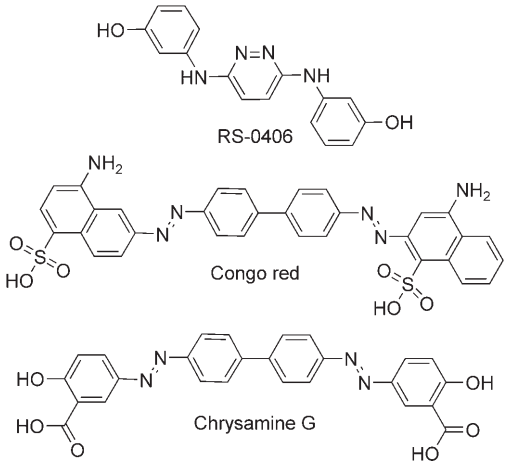
The potent γ-secretase inhibitor **2**[28] was found to display affinity for polymeric $A\beta_{42}$. This stimulated a systematic evaluation in five assays (Table 1). The impact on γ-secretase activity

Table 1. Activity of curcumin derivatives on the secretion of tau, $A\beta_{42}$, and $A\beta$.

Compd	Code	Tau aggregation IC_{50} [μM]	Tau depolymerization DC_{50} [μM]	$A\beta_{42}$ affinity assay IC_{50} [nM][a]	$A\beta$ secretion IC_{50} [μM]			Cell toxicity [μM][b]
					$A\beta_{38}$	$A\beta_{40}$	$A\beta_{42}$	
1	Curcumin	>200	>200	107±48	>20	>20	>20	>20
2	BSc3596	>200	>200	138±95	6.1±0.5	7.4±0.3	6.8±0.4	>20
3	BSc3597	>200	>200	>1000	>40	>40	>40	>40
4	BSc3598	>200	>200	>1000	>40	14.1±0.9	>40	>40
5a	BSc3732	34	17	476±67	5.2±1.4	9.7±0.9	8.7±0.8	>20
5b	BSc3733	35	73	>1000	7.7±0.1	11.5±0.2	15.3±2.1	>20
5c	BSc3734	103	73	>1000	6.3±1.3	11.3±0.4	14.2±1.0	>20
5d	BSc3736	1.2	1.1	>1000	3.5±1.2	6.2±1.0	3.5±0.9	>40
5e	BSc3735	54	46	>1000	3.4±0.2	5.4±0.1	4.8±0.02	>10
5f	BSc3738	35	>200	>1000	6.0±2.0	8.7±0.1	5.0±0.1	>20
5g	BSc3737	18	190	>1000	4.7±0.2	6.9±0.9	4.4±0.7	>20
5h	BSc3748	13	8	NT[c]	6.3±1.3	10.4±0.8	9.0±1.3	>10
5i	BSc3749	69	65	NT	13.6±0.3	17.8±0.2	17.3±0.01	>20
5j	BSc3751	5	4	NT	8.1±0.6	15.1±0.2	14.2±0.1	>20
5k	BSc3753	10	>200	NT	3.3±1.3	6.3±1.1	3.5±1.4	>10

[a] The IC_{50} value refers to the displacement of the reference compound [^{125}I]IMPY from $A\beta_{42}$ fibrils; typical error limits for tau aggregation are apparent from Figure 1. [b] Cell toxicity assay A. [c] Not tested.

may be due to affinity for the precursor APP or C99. However, this affinity is striking, because these monomeric peptides differ in their secondary structures from $A\beta_{42}$ aggregates. Therefore, all novel compounds (**5e**, **5h**–**k**) were tested for their inhibitory activity toward Aβ secretion in a cell-based assay. Indeed, they turned out to be effective Aβ secretion inhibitors and diminished cleavage at the $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ sites to varying extents (see Table 1). The most potent inhibitors of Aβ secretion were compounds **5d** and **5e**. In accordance with previous results, the addition of an aryl ring to the *N*-aryl pyrazoles (compounds **5c**–**g**, **5j**–**k**) increased the inhibition of Aβ secretion twofold. Variation of the aryl substituents resulted in subtle differences; *para* positioning of electron-withdrawing groups (in **5d**–**e**) was superior to 4-alkyl substitution (in **5f**–**g**). The 4-nitro derivative was more active than the 3-nitro analogue (see **5d** and **5j**). However, the 3,5-dichloro substitution is equipotent to the 4-nitro derivative. The introduction of an electron-donating group (**5h**–**i**) on the pyrazole group decreased Aβ secretion inhibition to a similar extent.



Congo red, chrysamine G, and RS-0406 are known inhibitors of Aβ aggregation, and inhibit $A\beta_{42}$ fibrillogenesis and oligomer formation. Curcumin shows similarity to these three compounds, binds to plaques, prevents oligomer formation, and recognizes secondary structure in fibrillar and oligomeric Aβ.[26] In our search for selective ligands for Aβ aggregates and depolymerizing agents for tau aggregates, we tested the curcumin-derived isoxazoles **2**–**4** and pyrazoles **5a**–**k** for their affinity toward fibrillar $A\beta_{42}$ aggregates and their ability to inhibit the formation of PHFs. Compounds **2** and **5a** turned out to be potent ligands for fibrillar $A\beta_{42}$; they displayed nanomolar affinity in the radioligand binding assay against the reference compound [^{125}I]IMPY (Table 1). Replacement of the 1,3-dicarbonyl moiety in curcumin by an isosteric isoxazole (compound **2**) retained the affinity. The isosteric curcumin pyrazole **5a** displayed fourfold decreased activity. N-arylation or N-alkylation of the pyrazoles **5b**–**k** decreased affinity toward fibrillar $A\beta_{42}$; these N-substituted derivatives did not displace the reference ligand at concentrations up to 1 μM. Small substituents on the pyrazole (compounds **5h**–**i**) or the isoxazole (compound **2**) may result in androgen receptor antagonism or inhibit cell proliferation, and thus their SAR was not pursued further.[29,30]

Several inhibitors of Aβ aggregation are known to inhibit tau aggregation as well. Therefore, we tested the same set of compounds on tau aggregation and PHF depolymerization in a cell-free assay. Curcumin neither inhibited nor depolymerized tau protein aggregates. Similar results were observed with the curcumin isoxazole **2** and the isoxazole derivatives **3**–**4**. However, curcumin-derived pyrazoles turned out to be potent inhibitors of tau aggregation and depolymerized tau aggregates (Table 1). The most active 4-nitrophenyl- (**5d**) and 3-nitrophenyl- (**5j**) substituted curcumin pyrazoles displayed tau aggregation inhibition at low micromolar concentrations (Table 1 and Figure 1). The introduction of an electron-withdrawing group on the aryl ring of the *N*-aryl pyrazoles increased the inhibition of tau aggregation (compounds **5d**, **5j**) 2–100-fold relative to

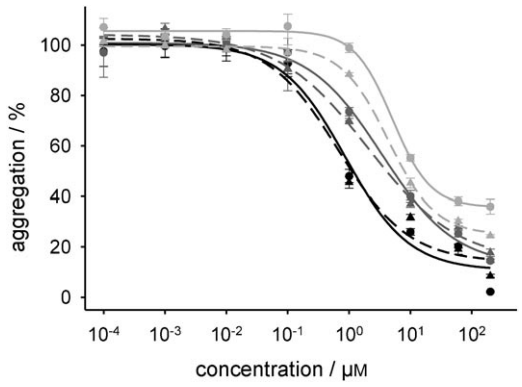


Figure 1. Tau aggregation inhibitors of the curcumin class. The figure shows the results of the most active compounds **5h** (gray), **5j** (dark gray), and **5d** (black) for the inhibition of tau aggregation (circles, solid lines) and depolymerization of preformed tau aggregates (triangles, dashed lines) in the thioflavin-S assay. For the test, 10 μM of the protein K19 was used in a dilution series of each compound of 200, 60, and 1 μM down to 100 pM.

the parent *N*-phenyl curcumin pyrazole. Remarkably, even the introduction of an electron-donating group increased the inhibition of tau aggregation (compounds **5f**–**g**) by two- to fivefold relative to the parent *N*-aryl curcumin pyrazole. Introduction of a methyl group (in **5h**) improved the inhibitory activity threefold, but introduction of a 2-hydroxyethyl group (in **5i**) decreased the inhibitory activity twofold relative to the parent curcumin pyrazole **5a**. A similar SAR was observed with respect to disaggregation of tau protein. The introduction of a nitro group on the aryl ring of the *N*-aryl pyrazoles increased the tau depolymerization activity (compounds **5d**, **5k**) 18–70-fold relative to the parent *N*-phenyl curcumin pyrazole. Introduction of a methyl group (in **5h**) gave a twofold improvement PHF depolymerization. However, the introduction of a 2-hydroxymethyl group (in **5i**) decreased activity by a similar magnitude in comparison with the parent curcumin pyrazole **5a**.

We tested the same set of compounds for their ability to inhibit the aspartic protease BACE, which is associated with AD, to rule out upstream cleavage inhibition. None of these compounds, including curcumin, displayed inhibition in the cell-free assay (data not shown). However, curcumin and most of the curcumin derivatives share a dark yellow–brown color, which led to staining and color quenching in the radioligand binding assay (RLBA). The fluorescence resonance energy transfer (FRET) assay indicated a certain degree of apparent activation by some compounds, but this might be due to the redox properties of the phenols. The apparent inhibition in the RLBA in the presence of detergent (Tween 20) most likely reflected poor compound solubility and micelle formation in the assay.

Additionally, the curcumin isoxazoles **2**–**4** and curcumin pyrazoles **5a**–**g** were tested for their potential to inhibit PrP^{Sc} formation by the scanning for intensely fluorescent target (SIFT) assay to validate the selectivity for protein aggregation.[31] None of the compounds including curcumin exhibited prion aggregate inhibitory activity (data not shown) in the SIFT assay at 100 μM concentration.

The most active compound toward tau inhibition is **5d**, which features a notorious nitro substituent, a frequent cause for cellular toxicity. Compound **5a** is characterized by high activity in all assays, but it was reported to be an androgen receptor antagonist and a potent inhibitor of cell proliferation; it was therefore omitted from further investigations.[29,30] Thus, compounds **5d** and the tau-inactive isoxazole **2** were analyzed further for their potential to alter Aβ production in primary telencephalic chicken neuronal cultures. The Aβ sequence of the chicken is identical to the human sequence.[32] Therefore, in contrast to rodent primary neurons, detection of endogenous Aβ peptides in chicken neurons is feasible with available human-specific Aβ antibodies. Furthermore, primary chicken neurons were shown to secrete qualitatively and quantitatively similar Aβ species to those in human cerebrospinal fluid.[32] Thus, chicken neurons are an attractive physiological culture system to assess the effects of small molecules on APP processing and Aβ production. Primary chicken neurons were treated for 24 h with 10–25 μM curcumin derivatives **2** or **5d**, or DMSO vehicle. Total Aβ levels in conditioned culture media were then quantified by Western blotting with monoclonal antibody 26D6 against the N terminus of the human Aβ sequence. In agreement with the results in H4 neuroglioma cells, compound **2** decreased Aβ levels in a dose-dependent fashion by 50–70% relative to the DMSO control conditions (Figure 2A and B). Compound **5d** had no significant effect, but a strong trend to decrease Aβ levels was observed at 25 μM. Evaluation of cytotoxicity did not reveal toxic effects of the compounds in this concentration range, suggesting that the reductions in Aβ production were not caused by nonspecific cell damage. However, detection of cellular APP protein levels in corresponding cell lysates showed that compound **5d** induced dramatic decreases in APP expression (data not shown). This indicates that the observed reductions in total Aβ secretion after treatment with **5d** were most likely secondary to reductions in APP protein levels. In contrast, compound **2** did not change overall APP levels but induced a slight increase in the more mature, highly glycosylated forms of APP (Figure 2C).

Replacement of the 1,3-dicarbonyl in curcumin by the isosteric heterocycles isoxazole and pyrazole, resulted in potent γ-secretase inhibitors, potent ligands for fibrillar $A\beta_{42}$ aggregates, tau aggregation inhibitors, and depolymerizing agents for tau aggregates. Compound **5d** displayed good γ-secretase inhibition in the Aβ liquid-phase electrochemiluminescence assay and inhibited tau aggregation but lacked affinity to fibrillar $A\beta_{42}$ aggregates. Curcumin–isoxazole **2** was found to be a potent inhibitor of γ-secretase and has affinity for $A\beta_{42}$ aggregates, but no significant activity toward tau aggregates. Shifting the nitro substituent on the *N*-aryl moiety from the *para* to the *meta* position resulted in three- to sixfold decreased activity on amyloid formation and tau aggregation (see **5d** and **5j**). The 6-chloropyridazin-3-yl derivative **5e** is approximately equipotent to the 3,5-dichlorinated **5k** on Aβ formation, but fivefold less active toward tau aggregation. Only compound **5a** displayed good activity in all three assays: it displayed affinity

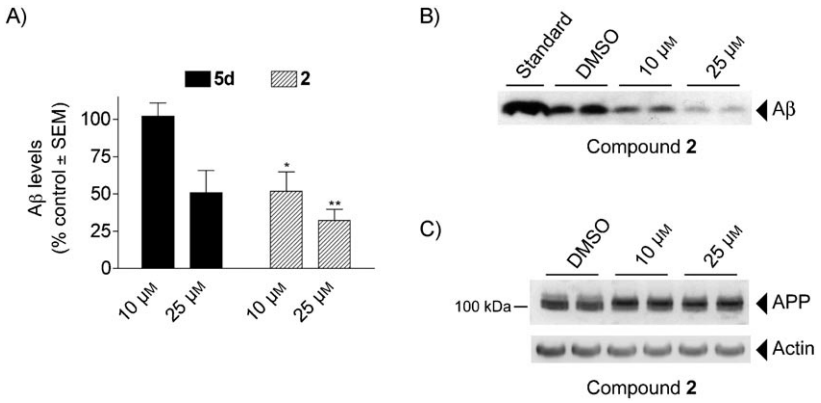


Figure 2. A) Modulation of endogenous Aβ production in primary chicken neurons by curcumin derivatives. Primary neurons were treated for 24 h with the indicated concentrations of curcumin derivatives or DMSO vehicle. Total Aβ levels in conditioned media were analyzed by combined immunoprecipitation/Western blotting with antibody 26D6 directed against the N terminus of human Aβ. Compound **2** decreased total Aβ levels by 50–70% at 10–25 μM in a dose-dependent manner. With derivative **5d**, a strong trend to decrease Aβ levels was observed at the higher 25 μM concentration. ($n=3$); one-way ANOVA, $^{**}p<0.01$, $^{*}p<0.05$, Dunnett's post tests. B) A representative Western blot of the experiments quantified in part A for compound **2**. Standard $A\beta_{1-40}$ peptides were separated on the same gel for identification of secreted Aβ in culture media. C) Cellular APP expression in primary neurons after treatment with curcumin derivative **2**. Corresponding cell lysates from cultures treated with **2** for 24 h were analyzed with antibody CT-15 against the C terminus of APP. Overall APP levels were unchanged, whereas an increase in APP maturation (upper band) was observed. Re-probing of Western blot membranes with anti-actin antibody confirmed equal protein loading.

for $A\beta_{42}$ aggregates, and inhibited tau aggregates and γ-secretase in the low nanomolar and micromolar concentration range.

The impact of acetic acids or phenols on the activity of γ-secretase inhibitors has been reported previously for three different scaffolds: isoxazole–curcumins, carbazoles, and diarylsulfonamides,[28,33,34,35] and several polyphenols have been identified in tau aggregation assays.[36] The free phenol crucially contributes to the simultaneous inhibition of γ-secretase, tau aggregation, and the affinity for $A\beta_{42}$ aggregates. The alkylation of the OH group resulted in the loss of activity. This effect is not due to impaired solubility, as three equally soluble alkylaminated derivatives (structures and data not shown) do not inhibit γ-secretase. The multifunctional compounds **2** and **5d** were further evaluated for Aβ production in primary telencephalic chicken neuronal cultures. However, **5d** decreased APP expression dramatically, and the observed decreases in total Aβ secretion were most likely due to decreases in APP protein levels. Interestingly, compound **2** decreased Aβ levels but did not change overall APP levels.

In conclusion, curcumin-derived isoxazoles and pyrazoles inhibit Aβ secretion, bind to or inhibit the formation of fibrillar $A\beta_{42}$ and tau aggregates. The enhancement in potency in comparison with curcumin is 10–100-fold. It is apparent from these data that curcumin-derived heterocycles, isoxazole and pyrazoles have multiple targets in AD. Several curcumin derivatives (such as **5a**) displayed good inhibition of γ-secretase activity, tau aggregation, and/or affinity to fibrillar $A\beta_{42}$ aggregates and thus may effectively serve as 'Swiss army knives' for AD research. However, a significant decrease in APP levels in primary telencephalic chicken neuronal cultures by compound **5d** makes it a risky candidate for further evaluation in animals. Currently, this compound must be regarded as 'blunt tool'. The multifunctional curcumin–isoxazole **2** displayed interesting properties as an Aβ-modulating agent in primary neuronal cultures. Further studies, such as the rescue of tau-aggregation-derived toxicity in the N2a cell model,[37] are required to elucidate the potential of **2** and the exact mechanism underlying its Aβ-decreasing activity.

Experimental Section

General comments: ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AC 300 spectrometer at 300 and 75 MHz, respectively. Chemical shifts (Δ in ppm) are reported downfield from Me_4Si. Mass spectrometry was performed with a Bruker-Franzen Esquire LC mass spectrometer. Flash column chromatography was carried out with Merck silica gel 60 (15–40 μm). Thin-layer chromatography (TLC) was carried out with aluminum sheets precoated with silica gel 60 F_{254} (0.2 mm, Merck). Chromatographic spots were visualized by UV light and/or spraying with an ethanol solution of ninhydrin followed by heating. All commercial chemicals were used without further purification.

4,4′-(1*E*,1′*E*)-2,2′-(Isoxazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxyphenol) (2): Hydroxylamine hydrochloride (500 mg, 6.78 mmol) and pyridine (0.55 mL, 6.78 mmol) were added to a stirred solution of curcumin (500 mg, 1.36 mmol) in ethanol (10 mL) and held at reflux for 48 h. After completion of reaction (TLC), the mixture was cooled to ambient temperature, and the solvent was evaporated in vacuo to obtain the crude product. It was purified by column chromatography (EtOAc/hexanes, 3:7) to afford the title compound as a yellow solid (258 mg, 55%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.17$ (d, $J=16.2$ Hz, 1H), 7.06 (d, $J=16.2$ Hz, 1H), 6.96–6.90 (m, 4H), 6.77–6.71 (m, 4H), 6.42 (s, 1H), 3.82 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=170.2$, 163.7, 152.5, 152.1, 149.3, 149.2, 137.9, 136.6, 130.0, 129.8, 122.8, 122.6, 120.5, 120.1, 117.1, 116.7, 115.8, 115.5, 98.8, 56.9 ppm; MS (EI): $m/z=365.2$ ($[M]^+$, 100%), 336.2 (64%), 229.1 (40%), 177.1 (28%), 145 (26%).

***tert*-Butyl-2,2′-(4,4′-(1*E*,1′*E*)-2,2′-(isoxazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy)diacetate (3)**: Anhydrous K_2CO_3 (170 mg, 1.23 mmol) was added to a stirred solution of 4,4′-(1*E*,1′*E*)-2,2′-(isoxazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxyphenol) (**2**) (150 mg, 0.41 mmol) in acetone (10 mL) and stirred at ambient temperature for 30 min. *tert*-Butyl chloroacetate (70 μL, 0.49 mmol) was added and heated at 60–70 °C for 12 h. The reaction mixture was cooled to room temperature and filtered. The residue was washed with acetone (3×). The combined organic extracts were evaporated in vacuo and purified by crystallization (EtOAc/hexanes) to yield the title compound as a lemon-colored solid (230 mg, 95%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.06$–6.94 (m,

6H), 6.79–6.72 (m, 4H), 6.36 (s, 1H), 4.54 (s, 4H), 4.06 (s, 3H), 4.03 (s, 3H), 1.41 ppm (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 168.3, 167.6, 162.0, 161.2, 149.6, 148.5, 148.2, 135.2, 134.5, 130.1, 129.7, 120.6, 120.4, 114.6, 113.4, 111.5, 109.9, 97.9, 82.4, 66.4, 66.3, 56.0, 55.9, 28.0 ppm; MS (ESI): m/z = 616.3 ([M+Na]$^+$).

***tert*-Butyl-2,2′-(4,4′-(1*E*,1′*E*)-2,2′-(isoxazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy)diacetate (4)**: TFA (40 μL) was added to *tert*-butyl-2,2′-(4,4′-(1*E*,1′*E*)-2,2′-(isoxazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxy-4,1-phenylene))bis-(oxy)diacetate (**3**) (100 mg, 0.17 mmol) in CH_2Cl_2 (2 mL) at 0 °C and stirred at ambient temperature for 6 h. After completion of reaction (TLC), the solvent was evaporated in vacuo to afford the crude acid, which was purified by acid–base treatment to obtain the title compound as a lemon-colored solid (73 mg, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.36–7.28 (m, 4H), 7.19–7.09 (m, 4H), 6.09–6.84 (m, 3H), 4.70 (s, 2H), 4.69 (s, 2H), 3.84 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 169.9, 168.1, 162.5, 162.1, 149.0, 148.2, 148.0, 136.0, 134.3, 129.2, 128.9, 124.5, 124.1, 121.1, 120.7, 113.8, 113.9, 111.5, 110.1, 98.3, 64.8, 55.6 ppm; MS (ESI): m/z = 482.2 ([M+H]$^+$), 504.2 ([M+Na]$^+$).

4-((1*E*,1′*E*)-2-(5-(4-hydroxy-3-methoxystyryl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5a): Hydrazine hydrate (5 equiv) was added to a stirred suspension of curcumin (200 mg, 0.54 mmol) in CH_3COOH (10 mL) and stirred at room temperature for 48 h. The reaction mixture was then poured on ice, and the precipitate was filtered and dried to afford the crude product, which was purified by column chromatography (EtOAc/hexanes, 3:7) to obtain the title compound (88 mg, 45%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.04–6.94 (m, 6H), 6.85–6.54 (m, 4H), 6.54 (s, 1H), 4.63 (br s, 2H), 3.87 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 151.8, 151.4, 148.3, 148.2, 137.9, 136.6, 130.0, 129.8, 124.9, 124.4, 122.8, 122.6, 117.1, 116.7, 113.8, 110.9, 98.8, 56.9 ppm; MS (ESI): m/z = 365.2 ([M−H]$^+$).

General methods for the synthesis of N-substituted curcumin pyrazole derivatives:

Method A: Hydrazine hydrochloride or sulfate (5 equiv) and Et_3N (5 equiv) were added to a stirred solution of curcumin (1 equiv) in MeOH followed by AcOH (cat.), and the resulting mixture was stirred at room temperature for 48 h. The reaction was monitored by TLC. The reaction mixture was then evaporated in vacuo to yield the crude product, which was further purified by flash column chromatography (EtOAc/hexanes, 5:5) to afford the desired product.

Method B: Hydrazine or hydrazine hydrochloride (2 equiv) and TFA (cat.) were added to the suspension of curcumin (1 equiv) in toluene and held at reflux for 24–48 h. The reaction was monitored by TLC. The reaction mixture was then cooled to ambient temperature, and the solvent was evaporated in vacuo to afford the crude product. The crude product was purified by column chromatography (EtOAc/hexanes, 5:5) to obtain the desired curcumin pyrazoles.

4-((1*E*,1*E*′)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-benzyl-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5b): The title compound was synthesized by method A. Yield: 185 mg, 75%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.36–7.32 (m, 3H), 7.20 (dd, J = 9.0, J = 1.5 Hz, 2H), 7.08–6.96 (m, 4H), 7.08–6.96 (m, 5H), 6.70 (s, 2H), 5.76 (br s, 2H), 5.44 (s, 2H), 3.92 (s, 3H), 3.91 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 155.3, 152.3, 146.7, 146.3, 140.6, 137.1, 132.5, 129.9, 129.0, 128.8, 126.6, 120.6, 115.6, 115.3, 113.5, 113.2, 108.5, 55.9, 55.8, 55.3 ppm; MS (ESI): m/z = 453.2 ([M−H]$^+$), 455.3 ([M+H]$^+$).

4-((1*E*,1′*E*)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-phenyl-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5c): The title compound was synthesized by method B. Yield: 150 mg, 63%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.48–7.40 (m, 3H), 7.36–7.31 (m, 2H), 7.08–6.98 (m, 2H), 6.95–6.83 (m, 6H), 6.80–6.61 (m, 3H), 5.57 (br s, 2H), 3.88 (s, 3H), 3.84 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 154.7, 151.5, 151.3, 146.7, 146.2, 142.5, 139.5, 132.9, 130.5, 129.7, 129.0, 127.8, 125.2, 124.8, 120.7, 120.3, 120.1, 114.7, 113.4, 108.7, 107.9, 100.4, 55.9, 55.8 ppm; MS (ESI): m/z = 441.3 ([M+H]$^+$)

4-((1*E*,1′*E*)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-(4-nitrophenyl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5d): The title compound was synthesized by method B. Yield: 92 mg, 35%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.30 (br s, 1H), 9.19 (s, 1H), 8.41 (dd, J = 9.0, J = 1.5 Hz, 2H), 7.81 (dd, J = 9.0, J = 3.0 Hz, 2H), 7.29–7.14 (m, 5H), 7.07–6.90 (m, 4H), 6.80–6.76 (m, 2H), 3.83 (s, 3H), 3.79 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 159.7, 152.4, 147.8, 147.6, 147.0, 145.4, 145.1, 133.9, 132.0, 128.0, 127.5, 126.3, 125.0, 124.3, 120.7, 120.4, 120.1, 115.6, 115.5, 111.8, 110.7, 109.7, 55.7, 55.5 ppm; MS (ESI): m/z = 486.3 ([M+H]$^+$).

4-((1*E*,1′*E*)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-(6-chloropyridazin-3-yl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5e): The title compound was synthesized by method B. Yield: 139 mg, 54%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.28 (br s, 1H), 9.19 (br s, 1H), 7.88 (d, J = 9.0 Hz, 1H), 7.32 (d, J = 15.0 Hz, 1H), 7.20–7.08 (m, 6H), 7.03–6.97 (m, 3H), 6.80–6.76 (m, 2H), 3.83 (s, 3H), 3.82 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 159.7, 154.3, 151.8, 147.8, 147.4, 143.3, 141.9, 132.7, 132.2, 129.9, 128.0, 127.8, 123.8, 120.5, 120.4, 116.6, 115.6, 115.5, 110.1, 109.7, 101.6, 55.6, 55.5 ppm; MS (ESI): m/z = 475.2 ([M−H]$^+$).

4-((1*E*,1′*E*)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-*p*-tolyl-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5f): The title compound was synthesized by method B. Yield: 155 mg, 68%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.34 (d, J = 6.0 Hz, 2H), 7.22 (d, J = 9.0 Hz, 2H), 7.04–6.91 (m, 6H), 6.88–6.62 (m, 5H), 3.85 (s, 3H), 3.84 (s, 3H), 2.36 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 154.9, 151.7, 146.7, 146.2, 142.4, 137.3, 156.6, 132.1, 131.9, 130.3, 129.8, 129.1, 124.1, 123.8, 120.7, 120.1, 119.8, 114.7, 113.6, 108.9, 107.9, 100.2, 55.9, 55.8, 21.1 ppm; MS (ESI): m/z = 455.3 ([M+H]$^+$).

4,4′-(1*E*,1′*E*)-2,2′-(1-(4-Isopropylphenyl)-1*H*-pyrazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxyphenol) (5g): The title compound was synthesized by method B. Yield: 150 mg, 59%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.37 (dd, J = 9.0, 1.5 Hz, 2H), 7.27 (d, J = 9.0 Hz, 2H), 7.05–6.89 (m, 6H), 6.84–6.80 (m, 3H), 6.72–6.55 (m, 2H), 5.63 (br s, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 2.99–2.84 (m, 1H), 1.23 ppm (d, J = 6.0 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 156.4, 151.7, 148.7, 146.7, 146.2, 142.4, 137.3, 132.1, 131.3, 129.5, 129.1, 127.1, 125.1, 120.7, 120.4, 118.4, 118.1, 114.7, 113.6, 108.9, 107.9, 100.2, 55.9, 55.8, 33.8, 23.9 ppm; MS (ESI): m/z = 486.3 ([M+H]$^+$).

4-((1*E*,1*E*′)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-methyl-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5g): The title compound was synthesized by method A. Yield: 40 mg, 45%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.02–6.88 (m, 5H), 6.84–6.58 (m, 6H), 3.90 (s, 3H), 3.87 (s, 3H), 3.62 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 153.8, 151.6, 151.5, 149.1, 147.1, 134.2, 133.2, 132.4, 128.6, 124.5, 124.1, 119.2, 119.0, 115.2, 114.9, 113.2, 112.8, 106.1, 59.6, 59.5, 40.0 ppm; MS (ESI): m/z = 377.2 ([M−H]$^+$), 379.3 ([M+H]$^+$).

4-((1*E*,1*E*′)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-(2-hydroxyethyl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5i): The title compound was synthesized by method B. Yield: 88 mg, 40%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.19–6.90 (m, 7H), 6.84–6.74 (m, 4H), 4.30 (t, J = 6.0 Hz, 2H), 3.91 (s, 3H), 3.90 (s, 3H), 3.65 ppm (t, J = 6.0 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 151.8, 149.3, 149.2, 147.9, 145.2,

131.7, 131.5, 130.2, 121.9, 121.2, 118.3, 114.4, 113.9, 110.8, 110.4, 108.3, 62.3, 56.5, 56.4, 52.2 ppm; MS (ESI): $m/z = 407.1$ ($[M-H]^+$), 409.3 ($[M+H]^+$).

4-((1*E*,1′*E*)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-(3-nitrophenyl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5 j): The title compound was synthesized by method B. Yield: 68 mg, 23%; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.41$–8.39 (m, 1H), 8.28–8.24 (m, 1H), 8.14–7.98 (m, 1H), 7.76–7.69 (m, 1H), 7.18–7.07 (m, 3H), 6.99–6.87 (m, 5H), 6.84–6.78 (m, 2H), 6.66 (d, $J = 16.2$ Hz, 1H), 3.89 (s, 3H), 3.84 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 156.3$, 152.3, 151.6, 150.1, 146.3, 146.1, 142.1, 138.4, 133.8, 132.8, 130.4, 130.3, 126.1, 124.6, 124.5, 122.0, 121.6, 120.4, 116.6, 116.2, 115.1, 113.4, 112.9, 105.3, 59.5, 59.4 ppm; MS (ESI): $m/z = 484.1$ ($[M-H]^+$), 486.3 ($[M+H]^+$).

4-((1*E*,1′*E*)-2-(5-(4-Hydroxy-3-methoxystyryl)-1-(3,5-dichlorophenyl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (5 k): The title compound was synthesized by method B. Yield: 120 mg, 45%; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.49$–7.38 (m, 3H), 7.16–7.05 (m, 3H), 6.99–6.70 (m, 7H), 6.64 (d, $J = 15.0$ Hz, 1H), 3.89 (s, 3H), 3.86 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 155.2$, 153.9, 148.7, 148.0, 144.6, 142.3, 136.8, 133.5, 129.6, 129.0, 124.8, 122.1, 117.9, 117.7, 113.0, 110.7, 110.4, 107.6, 56.9, 56.4 ppm; MS (ESI): $m/z = 509.2$ ($[M]^+$)

Analysis of endogenous Aβ production and APP expression in primary telencephalic chicken neurons: Fertilized chicken eggs were obtained from Charles River Laboratories (Wilmington, MA, USA). Telencephalic cortices were dissected from 8-day-old chicken embryos, and neurons were prepared and cultured according to previously described procedures.[32] Experiments were carried out after 5 days of cultivation in neurobasal medium supplemented with B27 supplement, L-glutamine (2 mM), and penicillin/streptomycin (100 U mL^{-1}, Invitrogen, Carlsbad, CA, USA). Primary chicken neurons were treated in 12-well plates for 24 h with curcumin derivatives **2**, **5 d** (10–25 μM), or DMSO vehicle. Cell viability was assessed morphologically and quantified using the CytoTox-ONE homogeneous membrane integrity assay (Promega GmbH, Mannheim, Germany) according to the manufacturer's protocol (cell toxicity assay B). For analysis of endogenous Aβ production, conditioned cell culture media (400 μL) were mixed with 1× complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), and subjected to immunoprecipitation with monoclonal antibody 26D6 recognizing amino acid residues 1–12 of the human Aβ sequence[38] and magnetic M-280 sheep anti-mouse IgG Dynabeads (Invitrogen). Immunoprecipitated material was resolved on 4–12% Bis-Tris gels (Invitrogen), and Aβ peptides were detected by Western blotting with antibody 26D6. Standard $Aβ_{1–40}$ peptides (Sigma–Aldrich, Munich, Germany) were separated on the same gel for identification of Aβ in media. For quantitative analysis, chemiluminescence was recorded with a LAS 3000 ECL camera system and quantified with the densitometry software AIDA (Fuji Photo Film GmbH, Düsseldorf, Germany). Duplicate measurements from each drug concentration were averaged and normalized to DMSO control conditions. These experiments were repeated three times, and results were analyzed by one-way ANOVA with Dunnett's post tests. Statistics were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). For analysis of cellular APP expression, cells were lysed in NP40 buffer (1% NP40, 50 mM Tris, pH 8.0, 150 mM NaCl). Equal volumes of cell lysates were separated on 4–12% Bis-Tris gels and analyzed by Western blotting with polyclonal antibody CT-15 against the C-terminal 15 amino acid residues of human APP.[38] Western blotting membranes were re-probed with monoclonal anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to confirm equal protein loading.

Aβ liquid-phase electrochemiluminescence (LPECL) assay: To evaluate the compounds for their potency in inhibiting and modulating γ-secretase activity, we used the LPECL assay to measure Aβ isoforms.[33] 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. Antibodies were labeled with TAG electrochemiluminescent label according to the manufacturer's protocol (Bioveris). Labeled antibodies were purified from unincorporated label using a PD-10 column (Pharmacia) and stored in phosphate-buffered saline (PBS) containing 0.1% NaN_3 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 per 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. Compound-containing medium (100 μL) 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, 1% Tween 20, pH 7.4). Conditioned culture medium (50 μL) from the plated cells was incubated with beads (50 μL) and each labeled antibody (25 μL; 6E10-bio and BAP29-TAG for detection of $Aβ_{1–38}$, 6E10-bio and BAP24-tag for detection of $Aβ_{40}$, 6E10-bio and BAP24-TAG for detection of $Aβ_{42}$; 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 $Aβ_{38}$, $Aβ_{40}$ and $Aβ_{42}$ peptides (Bachem) were used to generate standard curves. These Aβ peptides were solubilized in DMSO at a concentration of 1 mg mL^{-1}, aliquoted and stored frozen at −80 °C. Immediately before use, they were diluted in culture medium to 16–2000 pg mL^{-1}. Electrochemiluminescence was quantified using an M-Series M8 analyzer (Bioveris). IC_{50}/EC_{50} values were calculated using GraphPad Prism v.4 software.

Cell toxicity assay A: Cell viability was measured in the corresponding cells after removal of the media for the Aβ assays by a colorimetric cell proliferation assay (CellTiter 96TM AQ assay, Promega) using the bioreduction of MTS (Owen's reagent) to formazan according to the manufacturer's instructions as reported previously.[34]

Radioligand binding competition assay: To investigate compounds for their ability to bind $Aβ_{42}$ fibrils, we used a radioligand binding competition assay against the reference [^{125}I]IMPY as reported previously.[39] The competition assays employed a fixed concentration of $Aβ_{42}$ (10 μg mL^{-1}), PBS, 0.1% BSA, tracer ([^{125}I]IMPY, 0.1 nM) and varying concentrations of the ligand (0.5 nM–1000 nM). The mixtures were incubated for 3 h at 20 °C and filtered through a Whatman GF/B filter.

Thioflavin-S assay: To monitor the aggregation of tau into PHFs and the disassembly of preformed PHFs in vitro, a thioflavin-S fluorescence assay was performed. The chemicals, proteins, and methods used are described elsewhere.[36,40]

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