Benzofuran-Based Hybrid Compounds for the Inhibition of Cholinesterase Activity, β Amyloid Aggregation, and Aβ Neurotoxicity

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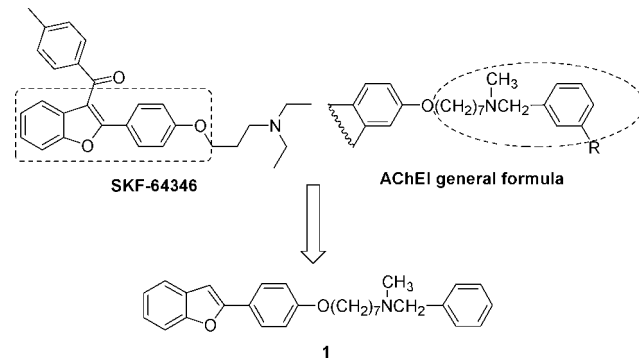
Abstract: The complex etiology of Alzheimer's disease (AD) prompts scientists to develop multitarget strategies to combat causes and symptoms. We therefore designed, synthesized, and tested new hybrid molecules linking a benzofuran ring to a *N*-methyl-*N*-benzylamine through a heptyloxy chain, affording a series of potential multifunctional drugs for AD. The cholinesterase inhibitory activity was extended to the inhibition of Aβ fibril formation for **1**, **3**, and **5**. Compound **3** showed an additional neuroprotective effect.

Alzheimer's disease (AD[a]) is a progressive neurodegenerative brain disorder that affects millions among the aging population worldwide and is going to affect millions more in the next 20 years. AD is characterized by progressive memory loss and severe cognitive decline associated with a degeneration of cholinergic neurons in many areas of the CNS and with a dramatic reduction of the neurotransmitters levels, among which ACh is the most important one.[1] One of the major neuropathological findings in AD is an abnormal extracellular accumulation of β-amyloid peptide (Aβ), the main component of the senile plaques that could be responsible for the onset of the disease.[2] Aβ is a proteolytic fragment derived from the amyloid precursor protein (APP), a transmembrane glycoprotein that is usually processed by the enzyme α-secretase to generate in physiological conditions small and soluble peptides. In AD affected brain a second pathway, known as "amyloidogenic pathway", takes place involving the sequential action of β-secretase followed by γ-secretase to generate two predominant Aβ peptides, either 40 or 42 amino acids in length,[3] that are able to aggregate into fibrils via soluble oligomers resulting, as generally accepted, in neuronal toxicity. Consequently, many drug discovery approaches are targeting the slowing and/or blocking of the amyloid polymerization process.[4,5]

Current treatment of AD focuses on symptomatic aspects of the pathology and is based on drugs increasing cholinergic neurotransmission by inhibiting acetylcholinesterase (AChE), like donepezil, rivastigmine, or galantamine. In recent times, the role of butyrylcholinesterase (BuChE) inhibition in AD has received increasing attention from the medicinal chemistry and the clinical points of view.[6,7] Several lines of evidence indicate that BuChE might be a co-regulator of the activity of the neurotransmitter ACh.[8] Remarkably, cortical levels of BuChE show a significant increase in AD. In this respect, the research efforts are focused on the development of selective inhibitors that are necessary to clearly evaluate the role of the enzyme and the therapeutic feasibility of its inhibition.[9,10]

Chart 1. Design of Target Molecule **1**



Because of the complexity of AD and the involvement of different enzymes in its progression, the modulation of a single protein might not be sufficient to produce the desired efficacy. Thus, researchers are now turning to the design of structures that should be able to simultaneously interact with different targets.[11–14] With this new paradigm, two compounds binding with very high selectivity to their respective targets are used as the starting points and their structural elements are combined to incorporate activity at both targets into a single molecule.

Our research group has been involved for many years in the development of AChE and BuChE inhibitors as potential drugs for AD.[15–17] Since compounds containing a benzofuran moiety have been identified as inhibitors of Aβ fibrils formation,[18] we report here a new hybrid molecule (**1**) based on the frameworks of our AChE/BuChE inhibitors and of SKF-64346 (Chart 1). The AChEI general formula (Chart 1) shows *N*-methyl-*N*-benzylamine linked to a heteroaryl moiety through an heptyloxy chain that allows the molecule to extend from the active anionic site to the PAS, located at the bottom and at the top of the gorge, respectively. In the design of the new molecule, the heteroaryl moiety was substituted with the benzofuran ring contained in SKF-64346, to introduce the inhibitory activity toward Aβ fibril formation. With following acylation of this newly introduced moiety (as in SKF-64346) a new series of potential multi-target-directed compounds for AD was synthesized (**2**−**8**) whose structures are reported in Table 1.

According to Scheme 1, **1** was synthesized starting from 4-benzofuran-2-yl-phenol[19] and 1,7-dibromoheptane in the presence of $K_2CO_3$to afford the bromoheptane derivative **9**, which was then condensed with *N*-methyl-*N*-benzylamine to give the final compound **1**. Friedel−Craft acylation of **1**, using tin(IV) chloride and the selected acylchloride in dichloromethane, gave the final compounds **2**−**8**.

The inhibitory activities of the newly synthesized compounds against both cholinesterases were studied using the method of Ellman[20] to determine the rate of acetylthiocholine or butyrylthiocholine hydrolysis in the presence of the inhibitor and are

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[a] Abbreviations: AD, Alzheimer's disease; ACh, acetylcholine; Aβ, β-amyloid peptide; APP, amyloid precursor protein; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; AChEI, acetylcholinesterase inhibitors.

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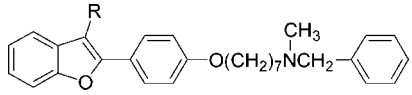
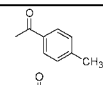
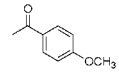
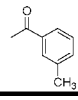
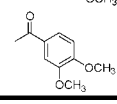
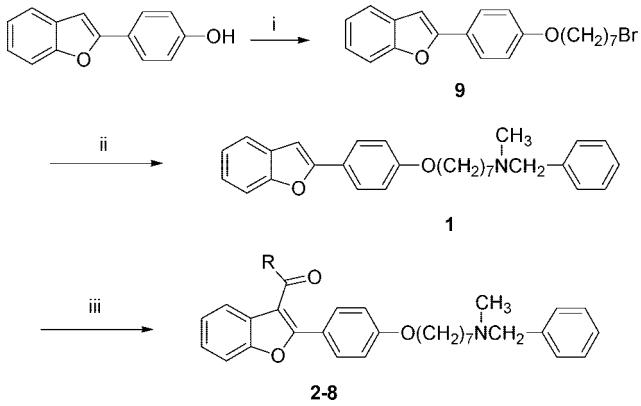
Table 1. Structures of the Studied Compounds



Compound	R	Compound	R
1	H	5	
2		6	
3		7	
4		8	

Scheme 1. Synthesis of the Studied Compounds[a]



[a] Reagents: (i) $Br(CH_2)_7Br$, K_2CO_3; (ii) *N*-methyl-*N*-benzylamine; (iii) RCOCl, $SnCl_4$.

Table 2. AChE, BuChE, Aβ Fibril Formation Inhibitory Activities and Aβ_{25-35} Peptide Neurotoxicity, Expressed as IC_{50}

	$IC_{50} \pm$ SEM (μM)[a]		IC_{50} (μM)[b]	IC_{50} (μM)[c]
compd	AChE	BuChE	Aβ_{25-35}	Aβ_{25-35}
1	32.6 ± 11.9	0.28 ± 0.02	7.0	nd[d]
2	17.4 ± 1.0	1.83 ± 0.12		
3	40.7 ± 3.5	38.1 ± 2.2	12.5	5.6
4	127 ± 42	40.9 ± 1.1		
5	10.5 ± 1.3	1.82 ± 0.09	13.0	
6	127 ± 10	21.7 ± 1.6		
7	281 ± 95	89.1 ± 1.6		
8	177 ± 43	74.1 ± 0.2		
rivastigmine	3.03 ± 0.21	0.30 ± 0.01		

[a] Human recombinant AChE and BuChE were used. IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two indipendent measurements, each performed in triplicate (SEM = standard error of the mean). [b] IC_{50} values defined as the concentrations of inhibitor to inhibit the formation of Aβ_{25-35} fibrils to 50% of the control value (reference compound: curcumin $IC_{50} = 10.0$ μ M). [c] IC_{50} values represent the concentration of compound resulting in 50% inhibition of Aβ_{25-35} peptide toxicity in human neuronal SH-SY5Y cells. The neuronal viability was determined by the MTT assay (as described in Supporting Information) after 3 h of incubation with 10 μM Aβ_{25-35} peptide in the presence or absence of different concentrations of the compounds (1−30 μM). The values are the mean ± SD of at least two independent experiments. [d] nd: IC_{50} not determined because less than 50% inhibition was observed at the highest tested concentration (30 μM).

reported in Table 2, expressed as IC_{50} values. The inhibitory activity of Aβ fibril formation was studied with an original in vitro assay that uses UV−vis measurements and electron microscopy[21] (Figure 1). The Aβ_{25-35} amino acid peptide, which preserves the properties of neurotoxicity and aggregation, was used.[22,23] For the compounds exhibiting an inhibitory activity at least equal to that of curcumin (compound reported to have antiamyloidogenic properties[24]), IC_{50} values were calculated as reported in Table 2. The neuroprotective effects of the most interesting Aβ antiaggregating compounds were also determined against the Aβ_{25-35} peptide induced toxicity in human neuronal-like SH-SY5Y cells using a colorimetric MTT assay[25,26] (Table 2 and Figure 3).

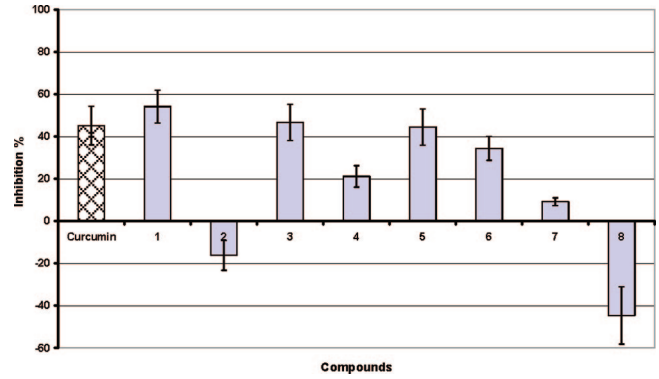


Figure 1. Compounds **1**−**8** Aβ_{25-35} fibril inhibition compared to that of curcumin. The mean ± SD values from three independent experiments are shown.

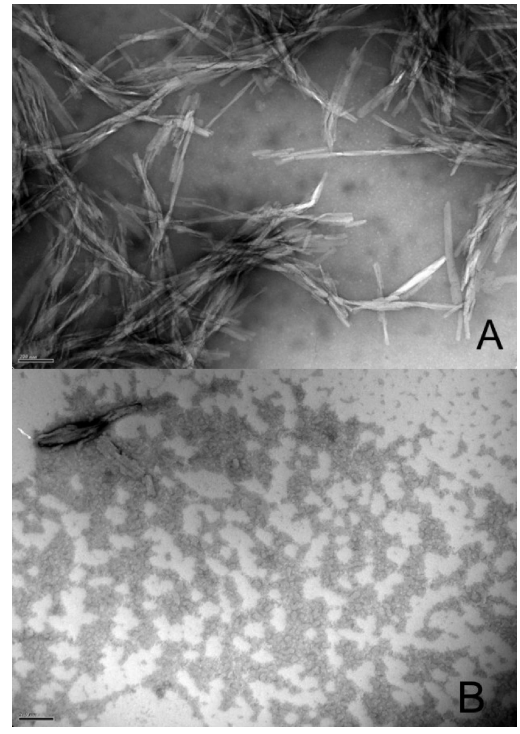


Figure 2. Electron micrographs of fibrils of Aβ_{25-35} alone (A) and in presence of the inhibitor **1** after the end of inhibitory activity measurements (B). The bar represents 200 nm.

The anticholinesterase activity of the new molecules was generally similar to that of rivastigmine, showing a better activity toward BuChE than AChE. In detail, **2** and **5** showed the best AChE inhibitory activity of the series (17.4 and 10.5 μM, respectively), still keeping a fairly good BuChE inhibition (1.83 and 1.82 μM, respectively), probably as a consequence of a higher affinity. There are differences in terms of topology between AChE and BuChE, since in this latter enzyme Lys286 and Val288 line the gorge, compared to the large Phe of the

corresponding residues of AChE, and this could allow bulky compounds to better fit inside the gorge of BuChE and to stabilize its occupancy probably by means of hydrophobic interactions. In particular, **1** was found to possess a good BuChE inhibitory activity (0.28 μM), about 100-fold higher than its activity toward AChE. Several lines of evidence indicate that BuChE might be a co-regulator of the activity of the neurotransmitter ACh[8] and that it might be important to inhibit this enzyme in the treatment of AD. Several drugs that proved to be selective BuChE inhibitors have been evaluated; for example, rivastigmine showed clinical efficacy without remarkable side effects.[27] It is intriguing that specific BuChE inhibitors not only improve cognition, presumably through an increase in acetylcholine concentration, but also reduce levels of APP, which is the source of Aβ peptide, the main component of plaques in AD. The effect of these compounds on APP seems to be independent of their ability to inhibit BuChE enzymatic activity, and it has been suggested that it involves interactions with interleukin-1, a proinflammatory molecule that has also been implicated in the pathogenesis of AD.[8]

Regarding Aβ fibril inhibition, **1** showed a higher potency compared to that of the standard curcumin (IC_{50} = 7 and 10 μM, respectively) and the highest one among the series. Acylation of **1** with acetylchloride led to **2**, whose activity was surprisingly proaggregatory, whereas acylation of **1** with benzoyl chloride retained the activity (**3**, IC_{50} = 12.5 μM), suggesting that the introduction of an aryl moiety in position 3 of the benzofuran ring was tolerated. Modifications of **3** by the introduction of a methyl group in the meta or para position on the aryl group led respectively to **4** and **5** and resulted in a lower activity for **4**, whereas **5** retained the potency of the unsubstituted **3**. In the opposite direction, the introduction of a methoxy group in the meta position (**6**) resulted in a slightly lower but still remarkable activity, though moving the methoxy group from the meta to the para position (**7**) was considerably detrimental for the activity. Moreover, the acylation of **1** with 3,4-dimethoxybenzoyl chloride led to **8**, which surprisingly proved to have remarkable proaggregating activity.

The assembly of Aβ aggregates, derived from Aβ oligomers, into fibrils is toxic to neurons. The formation of Aβ is related to protein misfolding, since the conformational transition to β-sheet leads to a faster formation of aggregates.[28] Thus, compounds that are able to slow or block the amyloid polymerization process could be considered potential drugs for inhibition of AD progression. During incubation of Aβ in the presence of **1**, only small bulk aggregates were visible and no characteristic Aβ fibrils were observed in the electron micrographs (Figure 2).

Interestingly, **3** also showed a marked neuroprotective effect against Aβ_{25-35} peptide induced neurotoxicity (Table 2). As reported in Figure 3, treatment of SH-SY5Y cells with **3** at 10 and 30 μM significantly reduced the neuronal viability loss evoked by Aβ_{25-35} peptide, in a dose-dependent manner, with a maximum of inhibition of 63%. Since the same trend was not observed with **1**, these results prove that the modification of **1** by introduction of an aryl moiety in the benzofuran ring is crucial for the observed neuroprotective effects. Aβ_{25-35} peptide contains a number of hydrophobic residues (i.e., Ile31, Ile32, and Met35) that are critical for neurotoxicity and aggregation processes.[23,29] In this regard, recent studies have suggested that unaggregated Aβ_{25-35} and Aβ_{31-35} peptides could initiate a cascade of events leading to neurotoxicity solely after their internalization within the neuronal cells.[30] Neuroprotective effects of **3** against Aβ_{25-35} toxicity could be ascribed to its

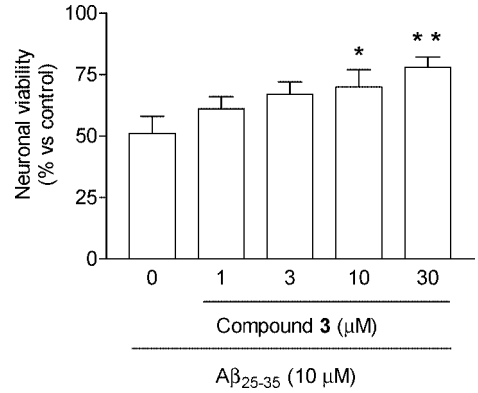


Figure 3. Compound **3** protects human neuronal SH-SY5Y cells from Aβ_{25-35} peptide induced toxicity. The neuronal viability was determined by the MTT assay (as described in Supporting Information) after 3 h of incubation with 10 μM Aβ_{25-35} peptide in the presence or absence of various concentrations of **3**. The results are expressed as percentage of control cells. Values are reported as the mean ± SD of three independent experiments: (*) $p < 0.05$, (**) $p < 0.01$ vs untreated sample, ANOVA with Dunnett post hoc test.

hydrophobic properties (compare **3** with **1**: 8.00 vs 6.80 log *P*) and its ability to block the interaction of Aβ_{25-35} with the lipid bilayer of the neuronal plasma membrane. It is therefore reasonable to predict that there are adequate opportunities for functionally important hydrophobic interactions between **3** and the Aβ_{25-35} peptide. However, these preliminary results could encourage further studies to elucidate the neuroprotective mechanisms of **3**.

In conclusion, because of the multifactorial nature of AD, molecules that modulate the activity of a single protein target are unable to significantly modify the progression of the disease. Following this new paradigm, here we have reported a new series of hybrid molecules based on the frameworks of our AChE/BuChE inhibitors and of SKF-64346. Promising hits proved to be **1**, with very good inhibitory activity of BuChE and Aβ aggregation, and **3**, which turned out to inhibit AChE/BuChE enzymes and showed remarkable inhibition of Aβ aggregation and Aβ neurotoxicity. This multi-target-directed compound could be considered as a new lead for further optimization.

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Supporting Information Available: Details of syntheses and biological evaluation of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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