Inhibition of Acetylcholinesterase, β-Amyloid Aggregation, and NMDA Receptors in Alzheimer's Disease: A Promising Direction for the Multi-target-Directed Ligands Gold Rush

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Abstract: Alzheimer's disease (AD) is a multifactorial syndrome with several target proteins contributing to its etiology. To confront AD, an innovative strategy is to design single chemical entities able to simultaneously modulate more than one target. Here, we present compounds that inhibit acetylcholinesterase and NMDA receptor activity. Furthermore, these compounds inhibit AChE-induced Aβ aggregation and display antioxidant properties, emerging as lead candidates for treating AD.

Alzheimer's disease (AD[a]) is a multifactorial syndrome with a combination of aging, genetic, and environmental factors triggering the pathological decline. AD is initiated by a cascade of molecular events creating dysfunctions in different neurotransmitter systems, with a major involvement of the cholinergic system, causing the cognitive and neuropsychiatric impairment that characterizes the disease. To date, the only molecules developed and marketed to specifically treat AD are acetylcholinesterase inhibitors (AChEIs), valuable in restoring cholinergic dysfunction, and the NMDA receptor (NMDAR) antagonist memantine (**1**, see Table 1), limiting glutamate excitoxicity.[1] Despite considerable scientific progress, current therapeutic approaches for AD treatment offer only limited and transient benefits to patients. Therefore, in response to the molecular complexity of AD, a new strategy has recently emerged aimed at simultaneously targeting multiple pathological processes involved in the neurodegenerative cascade.

So far, multiple targeting has been pursued in the clinical setting through the polypharmaceutical approach, that is, a combination of therapeutic agents that act independently on different etiological targets. This strategy has already proven to be successful in the treatment of similarly complex diseases, such as cancer, HIV, and hypertension.[2,3] Thus, associations of AChEIs with compounds targeting other pathogenetic factors of AD offer the prospect of additional benefits, as revealed by the large number of patented combinations that has overcome, in recent years, that of single drug entities.[4]

Different clinical trials have shown that combination of AChEIs with **1** is safe and produces enhanced therapeutic effects over AChEI monotherapy.[5,6] Although a recent investigation pointed out that coadministration of donepezil markedly potentiates the neurotoxicity of **1** in rats,[7] the rationale for combination of drugs affecting the cholinergic and glutamatergic systems remains persuasive.[8]

However, a pharmaceutical combination of several drug molecules raises many challenges, not least of which are the associated complexities encountered when combining drug entities that have potentially different degrees of bioavailability, pharmacokinetics, metabolism, and toxicity.[9]

In light of this, an alternative strategy, based on the assumption that a single compound may be able to hit multiple targets, is now emerging,[9,10] leading to the shift from single- to multi-target-directed ligands (MTDLs)[11] that are more adequate to face the complexity of the disease. Herein, it is our aim to combine, in the same molecule, the cholinergic activity through acetylcholinesterase (AChE) inhibition offering a symptomatic relief, with the neuroprotective action of NMDAR antagonism.

Excitotoxic (glutamate-related) neuronal cell injury and death are thought to contribute to AD and occur in part because of the overactivation of NMDARs, leading to an excessive Ca^{2+} influx through the receptor's associated ion channel. Moreover, oxidative stress and increased intracellular Ca^{2+} generated in response to β-amyloid (Aβ) have been reported to enhance glutamate mediated neurotoxicity in vitro, with additional experiments suggesting that Aβ can increase NMDA responses and therefore excitotoxicity. It is becoming evident that a close relationship may occur between glutamate excitotoxicity, oxidative stress, and Aβ formation.[12]

With these concepts in mind, we focused our attention on carvedilol (**2**), a vasodilating β-blocker and antioxidant approved for treatment of mild to moderate hypertension, which is endowed with a neuroprotective efficacy related to its modulatory action at NMDARs as low-affinity antagonist.[13] We selected its carbazole moiety in which the antioxidant properties reside. Moreover, since substituted carbazoles are efficient inhibitors of Aβ fibril formation,[14] this pharmacophore emerges as an intriguing building block in the search of new rationally designed MTDLs to confront AD.

In order to add to multiple carbazole activities an effective AChE inhibition, we selected the chloro-substituted tetrahydroacridine moiety of 6-chlorotacrine (**3**) that has already proven successful in affording lipocrine (**4**, 5-[1,2]dithiolan-3-ylpentanoic acid [3-(6-chloro-1,2,3,4-tetrahydroacridin-9-ylamino)-propyl]amide), a promising MTDL lead for new anti-Alzheimer drugs.[15] The lack of cytotoxicity of **4**, as well as the recently reported neuroprotection elicited by bis(7)-tacrine (*N*,*N*′-bis-(1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine) through a moderate blockade of NMDARs,[16,17] further supported the choice of the tetrahydroacridine pharmacophore for the synthesis of compounds **6**−**9** (Figure 1). **6**−**9** were synthesized by coupling **10** (see Table 1)[15] with the alkylating agents **11**−**14**, obtained from the commercially available 4-hydroxycarbazole

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[a] Abbreviations: Aβ, β-amyloid; AChE, acetylcholinesterase; AChEIs, acetylcholinesterase inhibitors; AD, Alzheimer's disease; BChE, butyrylcholinesterase; MTDLs, multi-target-directed ligands; NMDAR, NMDA receptor; PAS, peripheral anionic site.

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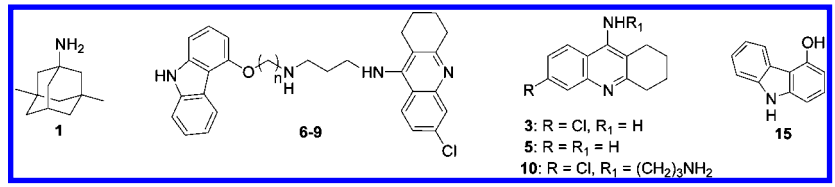
Table 1. Inhibition of AChE and BChE Activities, AChE-Mediated and Self-Induced Aβ Aggregation, and NMDAR Antagonism by **6**−**9** and Reference Compounds **1**, **3**, **5**, **10**, and **15**



		IC$_{50}$ (nM)[a]		% inhibition of Aβ aggregation		IC$_{50}$ (μM)[d]
compd	*n*	AChE	BChE	AChE-induced[b]	self-induced[c]	NR1/NR2A (−100 mV)
1						9.52 ± 2.28
3		8.32[e]	916[e]	8.5[e]		
5		424 ± 21	45.8 ± 3.0	7	<5	
6 (carbacrine)	3	2.15 ± 0.49	296 ± 32	57.7 ± 6.1	36.0 ± 2.3	0.74 ± 0.19
7	4	1.65 ± 0.23	211 ± 29	61.0 ± 7.0	29.7 ± 4.9	30.3 ± 7.0
8	5	1.54 ± 0.13	189 ± 8	63.2 ± 4.9	25.5 ± 5.0	18.2 ± 7.2
9	6	2.57 ± 0.24	137 ± 9	61.7 ± 1.4	23.7 ± 5.4	13.0 ± 4.3
10		21.5 ± 0.8	2580 ± 60	25.2 ± 4.9	22.0 ± 0.5	
15					13.1 ± 2.0	

[a] Human recombinant AChE and BChE from human serum were used. IC$_{50}$ values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements, each performed in triplicate. [b] Inhibition of AChE-induced Aβ(1−40). The concentration of the tested inhibitor and Aβ(1−40) was 100 and 230 μM, respectively, whereas the Aβ(1−40)/AChE ratio was equal to 100/1. [c] Inhibition of self-induced Aβ(1−42) aggregation (50 μM) produced by the tested compound at 10 μM. [d] The concentration of inhibitor required to cause 50% of maximum inhibition of NR1/NR2A mediated current evoked by 100 μM NMDA plus 10 μM Gly at a holding potential of −100 mV. IC$_{50}$ values were estimated from concentration−inhibition curves using 4−14 oocytes for each curve. [e] Data taken from ref 18.

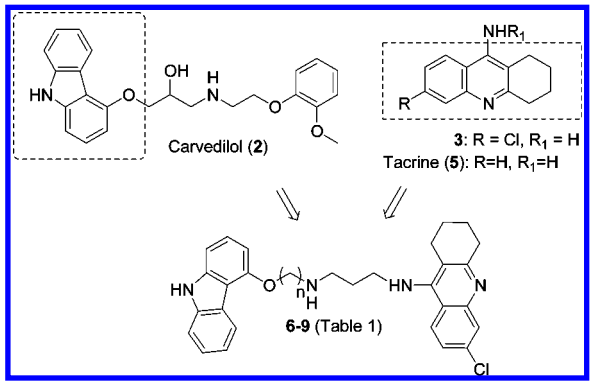
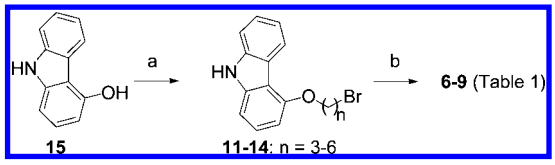


Figure 1. Design Strategy for Compounds **6**−**9**.

Scheme 1. Synthesis of **6**−**9**[a]



[a] Reagents and conditions: (a) CH_3CN, KOH, $Br(CH_2)_nBr$ (n = 3−6), KI, room temp, 24 h; (b) DMF, K_2CO_3, KI, **10**, N_2, 80 °C, 4 h.

(**15**) and the appropriate dibromo derivatives, according to Scheme 1 (see also Supporting Information, SI).

Initially, to determine the potential interest of **6**−**9** as MTDLs for the treatment of AD, their AChE inhibitory activity was determined on human recombinant AChE. Furthermore, the butyrylcholinesterase (BChE) inhibitory activity of **6**−**9** was also evaluated. All the designed compounds were effective AChEIs in the nanomolar range, being more potent than tacrine (**5**) and its 6-chloro derivative **3**,[18] while a homogeneous but significantly lower affinity profile was obtained for BChE inhibition. This selectivity profile might be advantageous in terms of toxicity, since it has been postulated that some severe side effects of AChEIs such as **5** might be attributed to their poor selectivity.[19]

Pure competitive AChEIs are mainly endowed with symptomatic effects; therefore, in the search for effective therapeutics for AD, the mechanism of action of compounds **6**−**9** was also investigated. In fact, the concomitant inhibition of the AChE peripheral anionic site (PAS), which is supposedly associated with the aggregation of Aβ,[20] may turn AChEIs into potential disease modifying agents. Lineweaver−Burk plots obtained at increasing concentrations of substrate and inhibitor showed that all the selected compounds were endowed with a mixed type inhibition; i.e., they were able to interact with the catalytic site and PAS (see SI). Therefore, to confirm the effective AChE binding mode of **6**−**9**, docking studies were performed on **6** and **9**, the shortest and the longest analogue, respectively.

Docking simulations were carried out with the software GOLD,[21] and outcomes were rationalized by means of the clustering algorithm AClAP.[22,23] In Figure 2, the binding modes of **6** and **9** are reported. It can be seen that both compounds could favorably interact with the catalytic site and the PAS of AChE. In particular, the tetrahydroacridine and the carbazole moieties could interact with Trp86 of the catalytic pocket and Trp286 of the PAS, respectively. The protonation of the tetrahydroacridine nucleus reduces the electron density of its central aromatic ring, with a beneficial effect on the interaction with the electron-rich indole ring of the Trp86 side chain. Indeed, the three-methylene spacer of **6** was long enough to allow a proper interaction between **6** and both sites of the enzyme (magenta in Figure 2). For binding of both inhibitors, a pivotal role was played by the protonated secondary nitrogen, which established H-bonds and electrostatic interactions with Tyr124 and Asp74, respectively. Moreover, even if to a lesser extent, the oxygen atoms of Tyr337 and Tyr341 side chains also contributed to interactions with the protonated nitrogen atom by making a kind of "electrostatic cage" (Tyr124, Asp74, Tyr337, and Tyr341 side chains), where a positive charge could be trapped. Notably, all these residues were shown to be able to interact with organic and inorganic cations during the AChE−ligand recognition and interaction phases.[24,25] A further comment is required on the binding mode of **6**. Although the pose reported in Figure 2 was the most populated one (see SI), poses where the carbazole and the tetrahydroacridine moieties

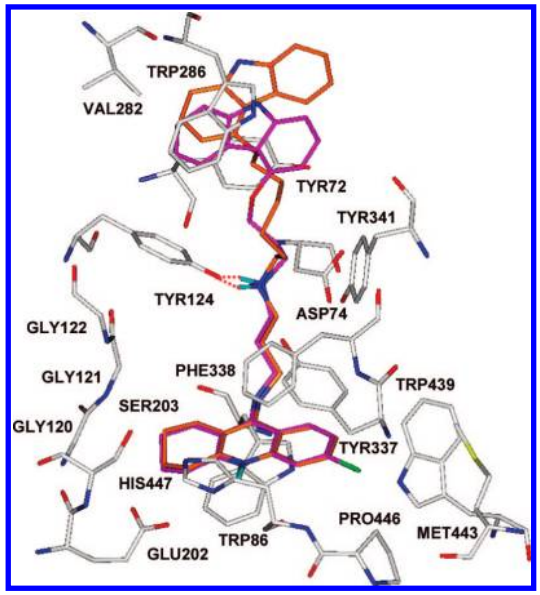


Figure 2. Binding mode of **6** and **9** (carbon atoms in magenta and orange, respectively) at the human AChE gorge. Both molecules are able to properly contact both sites of the enzyme. The protonated tetrahydroacridine and the carbazole moieties establish $\pi-\pi$ stacking with Trp86 and Trp286, respectively. The secondary protonated nitrogen atom is trapped in a kind of "electrostatic cage" formed by Tyr124, Asp74, Tyr337, and Tyr341. In particular, Asp74 and Tyr124 establish electrostatic and H-bond interactions with both inhibitors. The H-bonds are depicted as a dashed red line. The chlorine atom (green) of both inhibitors interacts with a hydrophobic pocket formed by Trp439, Met 443, Pro446, and part of the Tyr337 side chain.

interacting with Trp86 and Trp286, respectively, were also identified. In such poses, the protonated nitrogen was favorably interacting with the residues of the "electrostatic cage" while $\pi-\pi$ interactions were identified between the tetrahydroacridine moiety and Trp286 and between the carbazole moiety and Trp86 (see Figure 2 of SI).

A similar behavior was not observed for **9** because of the asymmetry of the methylene chain (three and six carbon atoms long) with respect to the protonated nitrogen. This made the pose of Figure 2 by far more energetically favorable than poses in which the carbazole and tetrahydroacridine moieties interacted with Trp86 and Trp286, respectively. Indeed, the binding mode where the carbazole moiety interacted with Trp86 and tetrahydroacridine moiety with Trp286 was also prevented by steric hindrance that the six-methylene chain encountered at the narrow inner part of the enzyme gorge. Moreover, the chlorine atom of both inhibitors could establish favorable interactions with a hydrophobic pocket formed by Trp439, Met 443, Pro446, and part of the Tyr337 side chain, thus further supporting the binding mode reported in Figure 2. Notably, such interactions were shown to be responsible for anchoring the chlorine atom in the crystal structure of TcAChE in complex with huprine X, a hybrid compound between chlorotacrine and huperzine, showing high inhibitory activity toward the enzyme.[26]

On the basis of these promising results, the ability of **6**−**9** to inhibit the proaggregating action of AChE toward Aβ(1−40) was assessed through a thioflavin T-based fluorimetric assay.[27] Interestingly, all the synthesized compounds presented a good inhibitory potency on AChE-induced Aβ aggregation, which, like the AChE inhibitory profile, was not influenced by the chain length separating the pharmacophoric functions (Table 1).

In view of the antiaggregating action of substituted carbazoles,[14] the ability of **6**−**9** to reduce Aβ(1−42) self-aggregation was also studied using the two building blocks **10** and **15** as reference compounds.[28] Data in Table 1 show that **6**−**9** at 10 μM inhibited Aβ(1−42) self-aggregation in a range from 36% to 24%, revealing a slight trend of increased efficacy with the reduction of the chain length. As a matter of fact, increasing the methylene chain length reduced the inhibition of Aβ(1−42) self-aggregation. Indeed, **9** behaved similarly to synthons **10** and **15** (Table 1).

In parallel, to verify the capability of **6**−**9** as NMDAR antagonists to join the potential neuroprotective effect to the cholinergic action, they were studied at recombinant NMDARs. These are heteromeric assemblies composed of three different subunits, NR1, NR2, and occasionally NR3, most of them probably comprising two NR1 and two NR2 (NR2A-D) subunits.[29] In particular, the activity profile of **6**−**9** was evaluated at Ca^{2+}-permeable NR1/NR2A NMDARs expressed in *Xenopus laevis* oocytes, using **1** as the reference compound. **1** is a well tolerated NMDAR antagonist that preferentially blocks excessive NMDAR activity without disrupting normal neuronal function.[30,31] With oocytes voltage-clamped at −100 mV, **6**−**9** were coapplied with NMDA (100 μM plus 10 μM Gly) revealing that all of them presented an NMDAR antagonistic effect comparable to that of **1**, with **6** being even more efficacious (Table 1). On the basis of these results, to further explore the mode of action of **6**, its activity was also tested at more positive holding potentials, revealing a voltage-dependent behavior (results not shown). Interestingly, the voltage-dependence of **6** suggests that, as with **1**, it could act as an uncompetitive open-channel blocker.

The close relationships occurring between glutamate excitotoxicity, oxidative stress, and Aβ formation prompted us to select the more interesting carbazole-containing compound to be further studied as an antioxidant agent. In particular, the ability of **6** and the reference compound **15** to counteract the formation of reactive oxygen species (ROS) was assayed in human neuronal-like cells (SH-SY5Y) after treatment with *tert*-butyl hydroperoxide, a compound used to induce oxidative damage.[32] A range of concentrations of tested compounds that did not affect neuronal viability (0.03−30 μM) were used. Interestingly, **6** was able to protect neuronal cells against ROS formation evoked by oxidative stress with a significant, albeit lower (IC_{50} = 23 μM), efficacy with respect to the parent compound **15** (IC_{50} = 0.07 μM). However, **6** showed an antioxidant activity higher than that of trolox (IC_{50} = 49.55 μM), an estsablished antioxidant compound.

In conclusion, in the present study, the MTDL approach allowed us to rationally design **6**−**9**, characterized by a so-far unique multimodal profile. In particular, **6** (carbacrine) was able (i) to inhibit AChE activity in the nanomolar range, (ii) to block in vitro Aβ self-aggregation and aggregation mediated by AChE, (iii) to antagonize NMDARs, and (iv) to reduce oxidative stress. These results represent a first step toward the discovery of MTDLs with potent and appropriately balanced molecular affinities[33] to confront AD neurodegeneration. Clearly, proof of the concept will involve an investigation of the neuroprotectant profile of **6** in vivo. In this regard, even if no specific pharmacokinetic study has been conducted so far, it is encouraging that **6** is not significantly larger or more complex than the parent compound bis(7)-tacrine, which already showed oral acivity in vivo.[34]

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Supporting Information Available: Experimental details for biology, chemistry, and modeling and elemental analysis results of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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