Multi-Target-Directed Drug Design Strategy: From a Dual Binding Site Acetylcholinesterase Inhibitor to a Trifunctional Compound against Alzheimer's Disease

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Received September 27, 2007

Abstract: A design strategy to convert a dual-binding site AChE inhibitor into triple functional compounds with promising in vitro profile against multifactorial syndromes, such as Alzheimer's disease, is proposed. The lead compound bis(7)-tacrine (**2**) was properly modified to confer to the new molecules the ability of chelating metals, involved in the neurodegenerative process. The multifunctional compounds show activity against human AChE, are able to inhibit the AChE-induced amyloid-β aggregation, and chelate metals, such as iron and copper.

Alzheimer's disease (AD[a]) is a multifaceted neurodegenerative disorder characterized at a molecular level by protein misfolding and aggregation, oxidative stress, mitochondrial abnormalities, and neuroinflammatory processes.[1] Unfortunately, this complex molecular pathogenesis has halted the development of effective therapeutic tools to combat the disease progression.[2] Historically, the relation between the observed cholinergic dysfunction and AD severity provided a rationale for the therapeutic use of acetylcholinesterase inhibitors (AChEIs), such as tacrine (**1**) (Figure 1). Notwithstanding an accepted clinical practice,[3,4] the effectiveness of AChEIs has been questioned, since, although beneficial in improving cognitive and behavioral symptoms, they do not delay or prevent the neurodegeneration.[3,4] Conversely, the multifactorial nature of AD and the current lack of an accepted unitary theory on its etiology lend credence that a more successful approach will be a single compound able to interact with several molecular targets involved in the neurotoxic cascade. As a consequence, drug discovery in AD is gradually moving from the development of molecules able to modulate the biological function of a single target to the "multi-target-directed ligands" (MTDLs).[5–7] MTDLs are, in principle, effective in treating complex diseases because of their ability to interact with multiple targets supposed to be responsible for the pathogenesis. With the advent of high-throughput screening assays, which allow efficient profiling of a large number of compounds against large panels of targets, the multifunctional nature of many existing drugs and leads has been verified and probably should be expected for the majority of the therapeutic agents.[8] The plethora of such examples coming from the recent literature might raise the perception that MTDLs cannot be rationally designed but rather discovered accidentally by screening approach.[8] Indeed, a growing number of compounds have been specifically designed to exhibit polypharmacology.[8] The most common strategy to rationally design MTDLs has been mainly based on linking through a spacer two properly selected pharmacophores endowed with activity against two different molecular targets. If it is reasonable for a compound to hit two targets, converting a dual-acting ligand into a triple ligand is not an easy task. In fact, designing a triple MTDL by conjugating three distinct pharmacophores might be disadvantageous from a pharmacokinetic point of view, since it might lead to high molecular weight compounds, with an intrinsic lower probability of optimal druglikeness.[9,10]

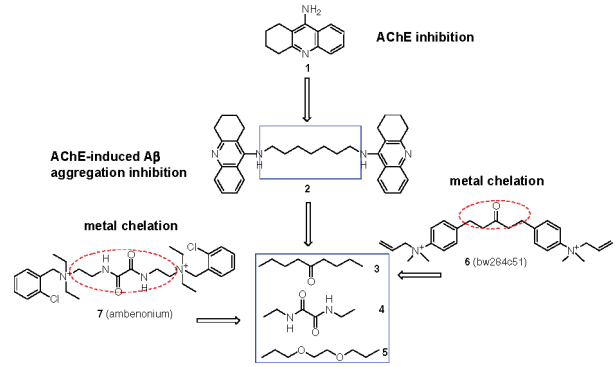

Figure 1. Design strategy for compounds **3**−**5**.

In a search of new rationally designed MTDLs having multiple activities against AD, we started from "dual binding site" AChEIs, i.e., molecules able to simultaneously interact with the catalytic site and the peripheral anionic site (PAS) of the enzyme. Such inhibitors are of particular interest as disease-modifying agents, having the potential of restoring the cholinergic deficit by blocking acetylcholinesterase (AChE) catalytic activity and at the same time of interfering with amyloid-β (Aβ) deposition and aggregation by PAS interaction.[11]

In designing such AChEIs, the bivalent ligand strategy already developed in the field of opioid research[12] has been particularly successful because of the peculiar architecture of the enzyme, with the two target sites at the top and the bottom of a gorge.[13–16] In the first documented application, two tacrine units were bound together by a polymethylene spacer of optimized length to contact both the catalytic and peripheral sites, resulting in the bivalent ligand **2**, with a marked enhanced affinity toward AChE over the monomeric **1**.[17] Original computational analysis[17] and subsequent crystallographic studies[18] have unequivocally demonstrated that **2** is able to interact with the catalytic site and PAS of the enzyme. Conversely, its ability to block PAS and consequently to retard Aβ assembly had never been experimentally proved. The availability of a suitable test for the determination of the Aβ(1–40) aggregation mediated by AChE[19] allowed us to have the proof of concept that such a dual-binding inhibitor was also able to decrease the AChE-induced Aβ aggregation. Indeed, **2** inhibited the AChE-induced Aβ aggregation with an IC_{50} of 41.7 $\pm$ 3.5 μM, only slightly higher than that of propidium (12.6 $\pm$ 0.5 μM),[19] one of the most potent reference compounds commonly used for this assay. In light of this new result and in the search of new MTDLs, we reasoned to expand the role of the spacer of tacrine-based inhibitors through the synthesis of different bis-ligands, in which the 14

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[a]Abbreviations: Aβ, amyloid-β; AChE, acetylcholinesterase; AChEI, acetylcholinesterase inhibitor; AD, Alzheimer's disease; BChE, butyrylcholinesterase; MD, molecular dynamics; MTDL, multi-target-directed ligand; PAS, peripheral anionic site.

10.1021/jm701225u CCC: $37.00
Published on Web 11/30/2007

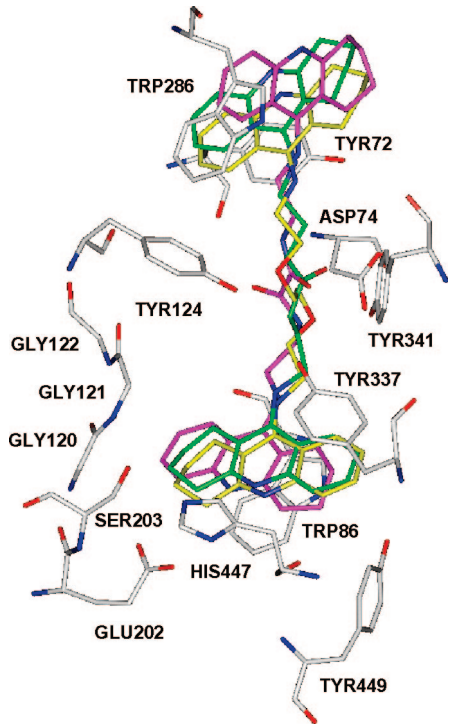
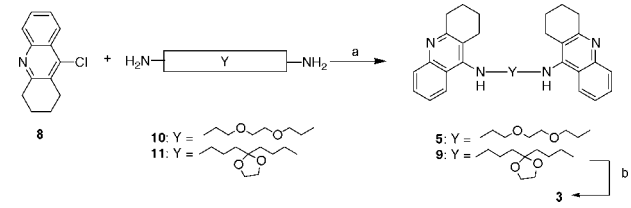


Figure 2. Binding modes of **3**, **4**, and **5** (green, magenta, and yellow, respectively) at the human AChE gorge. The new molecules are able to properly contact both sites of the enzyme. The two tacrine moieties establish $\pi-\pi$ stacking with Trp86 and Tyr337 and with Trp286 and Tyr72, while the spacer does not seem to be detrimental to the interaction with the enzyme. Conversely, some interactions could be identified for **3** and **4** with Tyr341 or Tyr124, although they were not strong enough to increase the AChE inhibitory potency of the new molecules.

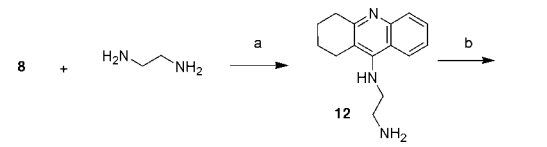
Å distance spanning the two enzymatic sites is featured by properly designed scaffolds. To rationally convert the bivalent ligand **2** into a triple MTDL against AD, we focused on the spacer as the carrier of a third biological activity. In this regard, accumulating evidence indicates that dyshomeostasis of biometals (Fe, Cu, Zn) in the brain and their interactions with APP and Aβ may contribute to AD pathology, and thus metal chelation represents another rational therapeutic approach for interdicting AD pathogenesis.[20–22] We therefore scanned the spacers of several dual-binding AChEIs to identify those with functional groups bearing potential metal-chelating properties. The spacers of **6** (bw284c51)[23] and ambenonium (**7**) were selected because these inhibitors carry carbonyl and oxalamide functions, likely endowed with the desired property, and because their dual-binding mode has been confirmed through X-ray diffraction[23] and molecular modeling studies,[24] respectively. Therefore, the new tacrine dimers **3** and **4** were designed, whereas, following the same basic idea, in **5** the saturated alkylene chain of **2** was substituted with a poly(ethylene glycol) chain of similar length (see Figure 1 for design rationale). To confirm that substituting the aliphatic spacer of **2** with those of **3**−**5** would have not negatively affected the ability of the new compounds to interact with AChE, docking simulations were carried out using GOLD[25] and AClAP[26] as computational tools. As shown in Figure 2, **3**−**5** were still able to interact with both sites of AChE, and the new spacers were not detrimental for a proper positioning of the ligands at the AChE gorge. Therefore, they might have the potential to function as new MTDLs against AD because they might bear the metal-attenuating ability with the capacity to modulate AChE and Aβ aggregation. In principle, additional activity has been conferred to the dual-binding inhibitors without significantly increasing the ligand size and altering their main structural features for binding to the original target (AChE).

Scheme 1[a]



[a] Reagents and conditions: (a) PhOH, NaI, 180 °C, 2 h; (b) HCl, MeOH, reflux, 2 h.

Scheme 2[a]



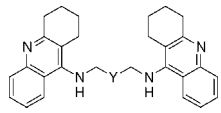
[a] Reagents and conditions: (a) PhOH, NaI, 180 °C, 2 h; (b) diethyl oxalate, $CHCl_3$, room temp, 24 h.

The synthetic pathway to **3**−**5** is illustrated in Schemes 1 and 2. Although initial reports for the synthesis of bis-tacrines began with tacrine itself, a more reliable method was further developed involving reaction of bis-amination of 9-chlorotetrahydroacridine **8** by proper diamines in phenol.[27] Following this latter route, the commercially available diamine **10** or **11**[28,29] were chosen as key intermediates for the synthesis of **5** and **9**. Deprotection of **9** in acidic medium afforded final compound **3**. In the case of the bis-tacrine **4**, a modified procedure was followed: monosubstitution of chloride **8** with an excess of 1,2-diaminoethane furnished **12**, which in turn, after condensation with diethyloxalate, gave **4**.

Initially, to determine the potential interest of **3**−**5** as MTDLs for the treatment of AD, they were assayed for their human AChE inhibitory activity in comparison with prototypes **1** and **2**. Furthermore, **3**−**5** butyrylcholinesterase (BChE) inhibitory activity was also evaluated and expressed as IC_{50}. The use of inhibitors of AChE-induced Aβ oligomerization has been recognized as an effective pharmacotherapy for AD.[15,17] Therefore, the ability of bis-tacrines **3**–**5** to inhibit the proaggregating action of AChE toward Aβ(1–40) was assessed through a thioflavin T-based fluorometric assay.[19] Finally, the ability of **3**−**5** to act as mild chelating agents was evaluated through spectroscopic analyses, in comparison with **2**.

All the designed dimers were potent inhibitors of human AChE, being dramatically more potent than **1**. The increased AChE inhibitory activity of **3** and **5** vs BChE reversed their selectivity profile in comparison to **1**. This aspect might be advantageous in terms of toxicity profile. In fact, it has been postulated that some severe side effects of **1**, including hepatotoxicity, might be attributed to its poor selectivity.[30] Moreover, the high potency displayed, in the nanomolar range, is consistent with the simultaneous binding at active and peripheral sites, as shown by docking simulations (Figure 2). However, if replacement of the heptylene spacer with the selected ones reduced only slightly the AChE binding affinity (compare **3** and **4** vs **2**), this is not the case of **5**, which was 24-fold less potent than **2**. Although all the derivatives could contact both sites of AChE and also establish favorable interactions with some midgorge residues,[30] the increased polarity of **3**−**5** with respect to **2** could account for a major energy penalty during their desolvation,[31]

Table 1. Inhibition of AChE and BChE Activities and AChE-Mediated Aβ Aggregation by Bis-tacrines **2–5** in comparison with prototype **1**



compd	Y	IC$_{50}$ ± SEM (nM)[a] AChE	IC$_{50}$ ± SEM (nM)[a] BChE	% Aβ aggregation inhibition[b]
1		424 ± 21	45.8 ± 3.0	<7
2	$(CH_2)_5$	0.81 ± 0.09	5.66 ± 0.15	68.0 ± 3.5
3	$(CH_2)_2CO(CH_2)_2$	1.83 ± 0.08	11.3 ± 1.0	76.1 ± 1.5
4	$CH_2NHCOCONHCH_2$	6.65 ± 0.47	5.44 ± 0.12	53.7 ± 9.4
5	$CH_2O(CH2)_2OCH_2$	19.7 ± 2.3	35.1 ± 1.9	61.8 ± 1.9

[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.

which in the cases of **3** and **4** could partially be compensated by H-bonding (Figure 2).

In light of our earlier results on **2** (see above), we next tested **3**−**5** for their ability to modulate the proaggregatory action of PAS. The data of Table 1 clearly showed that all the bis-tacrines were effective in inhibiting AChE-induced Aβ fibrillogenesis. In particular, 100 μM inhibitors inhibited Aβ aggregation from 53% to 76%, **3** being the most potent and slightly more potent than **2** (76% vs 68%). It appears that the effective inhibitor concentration in the aggregation assay is much higher than the IC$_{50}$ values vs the enzyme. Nevertheless, as was pointed out elsewhere,[19] if "normalized" on the basis of the different amounts of enzyme used in the Ellman's and aggregation assays, the ratio [inhibitor]/[AChE] is in the same range in both assays. Consequently, it seems plausible that similar amounts of inhibitor can simultaneously carry out the anticholinesterase and antiaggregating actions.

Finally, electronic spectra of **3**−**5** in methanol changed in the presence of Fe^{2+} and Cu^{2+} ions. In Figure 3a, UV–vis spectra of **3** at increasing Cu^{2+} concentrations are shown as an example. The increase in absorbance, which could be better estimated by an inspection of differential spectra (Figure 3b), indicated that there was an interaction between Cu^{2+} and **3**. With the molar ratio method, the stoichiometry of metal(II)−ligand complexes could be determined as 1:1. A similar behavior was also observed for **4** and **5** (Table 2), while no spectral change was observed for **2**, indicating the specificity of interaction of metal ions with the spacer. Since spectral changes were dependent on both the relative concentration of metal/ligand and saturability, it was possible to use UV–vis spectroscopy to measure complex stability, as recently described for curcumin.[32] Scatchard analysis[33,34] afforded the formations constants (K_f), collected in Table 2, with the free energy of the chelation reactions (see Supporting Information for more details). As already reported for curcumin, the interaction of bis-tacrines-metals could take place at micromolar concentration, which is compatible with ion concentrations in the AD brain. This suggests a potential physiological relevance of the metal-chelating properties of **3**−**5**.[32] Affinities are also comparable to that for desferrioxamine,[32] which was effective in a clinical trial for AD. Thus, from these preliminary results, one can suggest that **3**−**5**, but **2**, might also act against AD by a chelation mechanism.

In conclusion, **3**−**5** are bis-tacrine derivatives that maintain a potent AChE-inhibiting activity (nanomolar range), are able

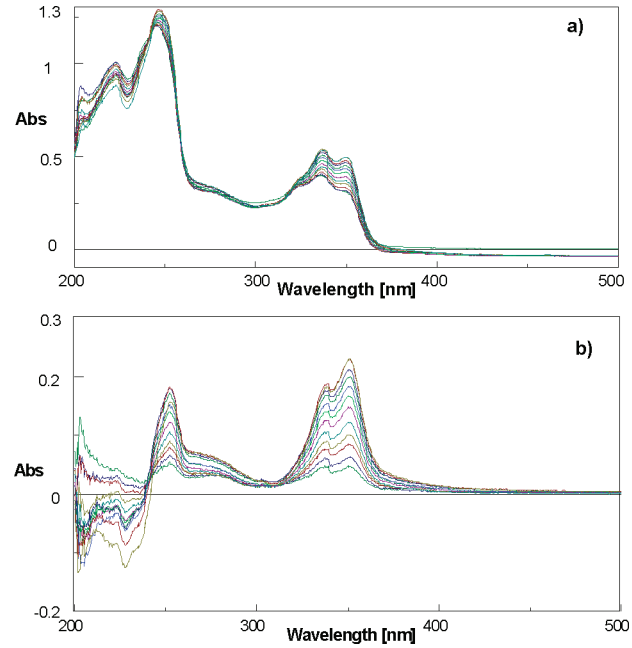


Figure 3. UV–vis (200–500 nm) absorption spectra of **3** (3.33×10^{-5} M) in methanol after addition of ascending amounts of $CuCl_2$ ((1–40) $\times$ 10^{-6} M) (a) and differential spectra due to **3**−Cu^{2+} complex formation obtained by numerical subtraction from the above spectra of those of $CuCl_2$ and **3** at the corresponding concentrations (b).

Table 2. Stability constants (K_f), Gibbs Energy of Formation ($\Delta G°_f$) and Number of Binding Sites (n) (Molar Ratio Ligand/Metal) As Obtained from Scatchard Analysis of Spectroscopic Data for Binding of Cu^{2+} and Fe^{2+} to Test Compounds **2–5**[a]

	Cu^{2+}			Fe^{2+}		
compd	log K_f (M^{-1})	n	$\Delta G°_f$ (kcal/mol)	log K_f (M^{-1})	n	$\Delta G°_f$ (kcal/mol)
3	5.07	1.0	−6.9	4.30	1.1	−5.9
4	5.94	1.0	−8.1	4.40	1.2	−6.0
5	5.55	1.1	−7.6	4.64	1.1	−6.3
curcumin	~5.2[b]	0.5	−7.1	~5.1[b]	0.5	−7.0

[a] Values obtained for curcumin with analogous technique are quoted from the literature[32] for comparison purposes. [b] Calculated from ref 32 as the reciprocal of the average between K_{d1} and K_{d2} for binding of the two curcumin molecules per metal ion.

to reverse AChE-induced amyloid fibrillogenesis, and at the same time have the additional property of acting as metal chelators. More importantly, the new MTDLs are not significantly larger or more complex than parent compound **2**, which already showed oral activity in vivo.[33] All these points demonstrate the efficiency of the proposed MTDL design strategy and underline the so-far unique trifunctional nature of bis-tacrines **3–5**, which could therefore be promising lead candidates as AD therapeutics. However, an accurate investigation of the pharmacokinetic and toxicological profile is mandatory before assessing their real therapeutic value. We believe that the development of MTDLs might be an innovative avenue for the treatment of complex neurodegenerative diseases like AD.[34]

Acknowledgment. We thank Rosalba Scalise and Luisa Ceccarini for their technical assistance. This research was supported by grant from MUR (FIRB RBNE03FH5Y) and the University of Bologna.

Supporting Information Available: Experimental details on biology, chemistry, spectroscopy, and modeling and elemental

analysis data for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM701225U