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Novel and Potent Tacrine-Related Hetero- and Homobivalent Ligands for Acetylcholinesterase and Butyrylcholinesterase

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Abstract—Based upon synthetic and biochemical results, a novel and potent tacrine analogue and heterobivalent analogues of tacrine, were designed. The role played by the amino groups of homo- and heterobivalent ligands in the interaction with the peripheral and catalytic sites of AChE and BuChE were investigated. The syntheses of these materials together with the results of AChE/BuChE inhibition assays are detailed.

The role of acetylcholine (ACh) in the regulation of cognitive functions in both normal and pathological conditions has been widely reviewed.[1] Neurodegenerative diseases involving impairment of cognitive functions, such as dementia of the Alzheimer's type (AD), are characterized by a loss of basal forebrain neurons and reduced cortical and hippocampal levels of ACh. Since one of the major deficits in AD patients involves the cholinergic system, the use of reversible inhibitors of acetylcholinesterase (AChE) is considered to be one viable and attractive therapeutic approach to the disease. In the past decade, tetrahydroaminoacridine (THA) **1** has become the subject of intense clinical studies and has been marketed for use in the long-term palliative treatment of AD since 1993. Tacrine is a weak reversible inhibitor of AChE, and an even stronger inhibitor of butyrylcholinesterases (BuChE). Unfortunately, the severe liver toxicity associated with this drug as well as side-effects stemming from its low target specificity (competing blockade of K^+ ion channels, M1 and M2 receptor antagonism, inhibition of the neuronal monoamine uptake processes) conspire to limit its widespread applicability.[2] Recently,[3–5] the synthesis of several homobivalent and heterobivalent tacrine-based AChE inhibitors was reported, whose increased inhibitory potency and target specificity was the result of the simultaneous binding of the units to the active and peripheral anionic sites of AChE. Of consequence, tacrine-related homo- and heterobivalent ligands could be promising drug candidates for the treatment of AD. The aim of this study was to produce novel tacrine analogues (**2** and **3**) for pharmacological evaluation against AChE, and molecular probes to contribute to the elucidation of the nature of the interactions with the AChE/BuChE binding sites. Among the new tacrine analogues identified, we report herein the synthesis of a representative compound of the new series: 9-amino-6,8-dichloro-1,2,3,4-tetrahydroacridine (**2**), a tacrine analogue characterized by enhanced inhibition potency, probably due to a better interaction at the catalytic site of AChE. Based on tacrine and the potent and selective analogue **2**, several new homo- and heterobivalent ligands were synthesized (**3b–j**) (Fig. 1), characterized by a different length of the tether, and tested on fetal bovine serum (FBS) AChE and equine (Eq) BuChE (92 and 88% homology with human AChE and BuChE, respectively).

Compound **2** was synthesized starting from 6,8-dichloro-9-hydroxy-1,2,3,4-tetrahydroacridine **4**, in turn obtained, via thermal ring closure, from the suitable dichloroaniline and 2-ethoxycarbonylcyclohexanone in refluxing diphenylether. Treatment of **4** with phosphorous oxychloride led to the corresponding chloro derivative, which by refluxing with sodium azide in 80% EtOH was converted to the corresponding 9-azido

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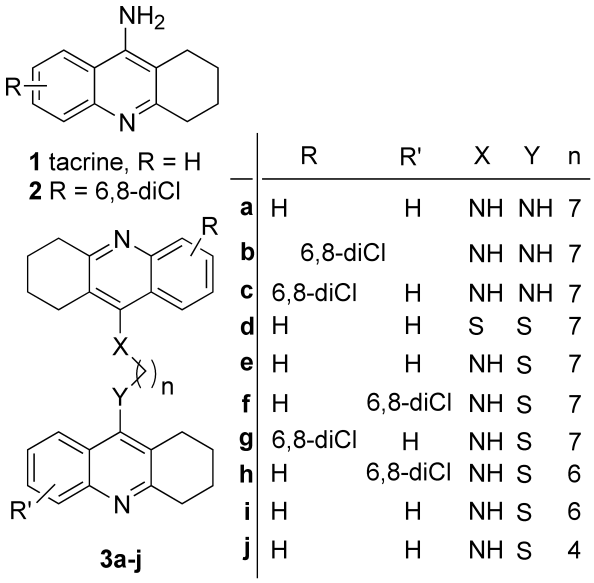
derivative **5**. Reduction with $LiAlH_4$ in Et_2O gave the desired compound **2** in good overall yield (Scheme 1). The tacrine homobivalent ligand **3a** described earlier by Carlier[3] was resynthesized and tested in the same experimental conditions, while the bivalent ligands **3b–j** were prepared using the two straightforward routes shown in Scheme 2.

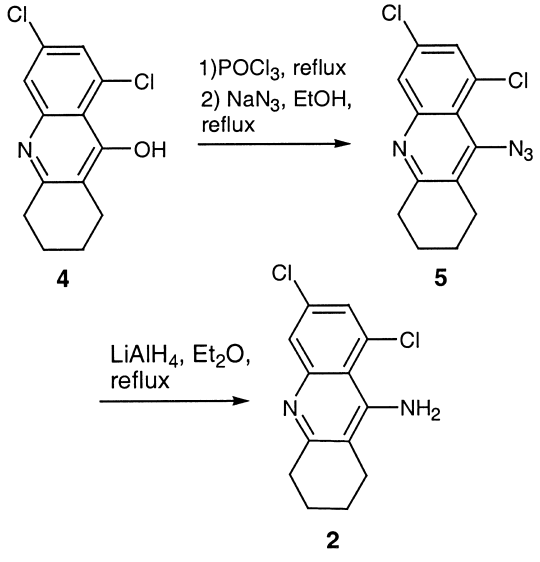


	R	R'	X	Y	n
a	H	H	NH	NH	7
b	6,8-diCl		NH	NH	7
c	6,8-diCl	H	NH	NH	7
d	H	H	S	S	7
e	H	H	NH	S	7
f	H	6,8-diCl	NH	S	7
g	6,8-diCl	H	NH	S	7
h	H	6,8-diCl	NH	S	6
i	H	H	NH	S	6
j	H	H	NH	S	4

Figure 1.

The first synthetic pathway provided the bivalent ligands **3b** and **3c**. Reaction of 9-chloro-1,2,3,4-tetrahydroacridine **7** with 1,7-diaminoheptane in refluxing 1-pentanol[3] afforded the intermediate **8**, which, after reaction with **7** or **9** gave the analogues **3b**,**c**.

Compounds **3d–j** were synthesized starting from **2** or tacrine, by reaction with the appropriate dibromoalkyl tether[6] to give the intermediates **10a–d**; from these latter intermediates, by reaction with **11a** or **11b** the desired compounds **3d–j** were obtained.[7]



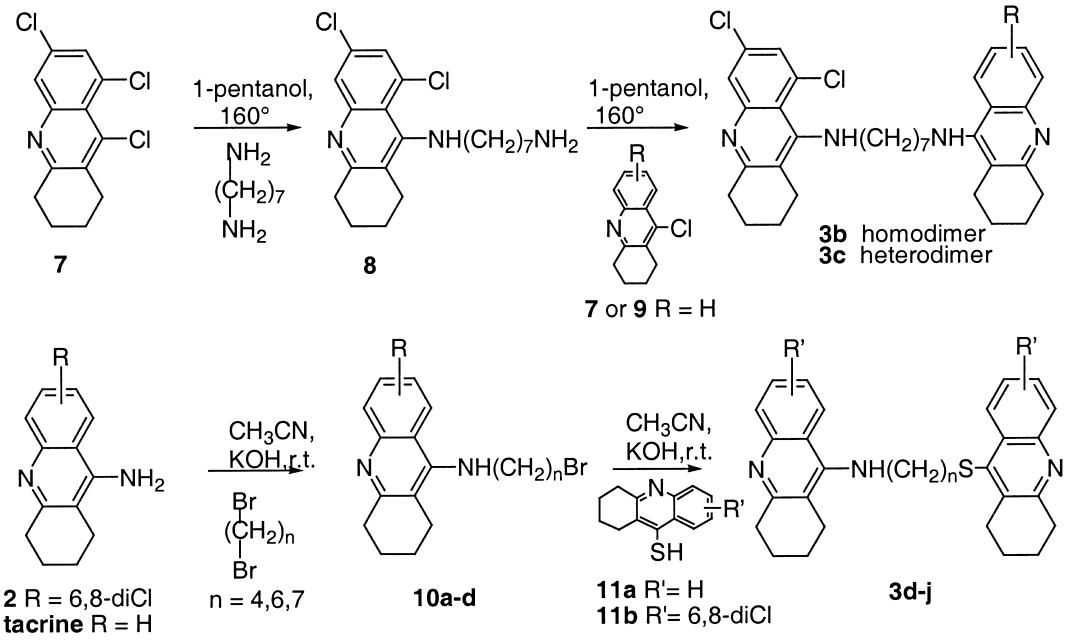
Scheme 1.

Measurement of AChE/BuChE Activity

The biological activity of the new homo- and heterobivalent analogues of THA (THA-An) were evaluated using purified FBS AChE and Eq BuChE. AChE and BuChE activities were measured in 50 mM sodium phosphate, pH 8.0, at 25 °C as described[8] using acetylthiocholine (ATC) and butyrylthiocholine (BTC) as substrates, respectively. Inhibition of enzyme activity was measured in 50 mM sodium phosphate, pH 8.0, over a substrate concentration range of 0.01–30 mM and at least six inhibitor concentrations to determine the components of competitive and non-competitive inhibition. Plots of initial velocities versus substrate concentrations at a series of inhibitor concentrations were analyzed by non-linear least squares methods to determine the values of K_m (Michaelis–Menten constant) and V_{max} (maximal velocity). Non-linear regression analysis of the plots of V_{max}/K_m values versus THA-An concentrations were used for the determination of K_i values.[9] The values of the inhibition constants for the inhibition of FBS AChE and Eq BuChE by the various inhibitors are reported in Table 1.

Discussion

As shown in Table 1, compound **2** showed potent anti-AChE activity, much higher than that of tacrine, with an improved selectivity. This analogue was found to be 8-fold less potent as an inhibitor of BuChE than tacrine itself, indicating that the BuChE catalytic site does not tolerate the substitutions at positions 6 and 8. Previously other chloro substituted tacrines have been reported and compound **2** appears to be more potent than the monosubstituted 6- and 8-chlorotacrine,[10,11] suggesting that the two chlorine atoms of **2** could work in a complementary manner. Accordingly, the 6,8-dichlorotetrahydroacridine system of **2** and the tetrahydroacridine system of tacrine were chosen to develop homo- and heterobivalent probes to elucidate the nature of the interactions of these inhibitors with the active sites of AChE and of BuChE. The first question addressed was whether by synthesizing heterobivalent ligands we could get information on a hypothetical binding mode of these compounds with respect to the two AChE binding sites. Thus, choosing a 7-methylene tether (**3a–g**) (the 7-methylene tether was already found to be optimal for dual-site binding to AChE[3]), starting from **2** we obtained the homobivalent ligand **3b**, to be compared to **3a**. It is noteworthy that **3a** showed a potent affinity for Eq BuChE, similar to that for FBS (or rat brain[3]) AChE. The different affinity found for BuChEs ($K_i = 2$ nM for Eq BuChE and $IC_{50} = 149$ nM for rat serum BuChE[3]) is probably due to significant differences between the amino acids present at the lip of the gorge of the two BuChEs, found by sequence alignments. Indeed, a Trp residue, responsible for cation–π



Scheme 2.

Table 1. Dissociation constants for the inhibition of FBS AChE and Eq BChE by analogues of tacrine

Inhibitor	Tether length $(CH_2)_n$	AChE[a] K_i (nM)	BuChE[a] K_i (nM)
1 tacrine		40	7
2		1	50
3a tac-homo	7	1.3	2
3b homo	7	150	>20,000
3c hetero	7	6	180
3d homo	7	>10,000	380
3e hetero	7	340	35
3f hetero	7	290	80
3g hetero	7	41	250
3h hetero	6	120	45
3i hetero	6	100	30
3j hetero	4	650	40

[a]K_i is mean ± standard deviation; standard errors were all within 10% of the mean.

interaction in the anionic peripheral site of AChEs, is replaced by an Ala, Val and Lys residue in human, equine and rat BuChE, respectively. The presence of a positively charged amino acid at the lip of the gorge of BuChE could explain the lower affinity of **3a** for this enzyme. On the other hand, the presence of chlorine substituents on both units of Tac-homodimer (**3b**) is less tolerated by Eq BuChE than by FBS AChE active site (**3b** vs **3a**), while the replacement of one dichloro substituted unit with a tacrine unit (**3c**) improved the potency of AChE inhibition, **3c** being slightly less potent than **3a** at AChE, and, by consequence, more selective with respect to BuChE (**3c** vs **3a**). Comparing **3a–c** activities with those of **1** and **2**, we propose that the heterobivalent ligand **3c** binds to AChE by placing the most potent unit (9-amino-6,8-dichlorotetrahydroacridine) at the catalytic site. The second question addressed was the elucidation of the roles that cation–π and hydrophobic interactions play in the binding of these inhibitors to AChE and BuChE. Consequently, a subset of heterobivalent ligands was synthesized (**3d–g**). In accordance to Carlier hypothesis,[3] compound **3d** was found inactive. In contrast, **3g**, in which the protonable amino group of the tacrine unit has been replaced by a sulfur, showed significant AChE inhibition potency (**3g** vs **3a**), likely with the 9-mercaptotetrahydroacridine moiety directed to the peripheral site and the potent 9-amino-6,8-dichlorotetrahydroacridine unit to the catalytic site. Despite the presence of an anionic peripheral site in AChE, the lack of the protonatable function only slightly lowered the potency (**3g** vs **3c**). These data suggest the critical role played also by hydrophobic interactions at AChE active sites. Furthermore, initially Pang,[5] and later Carlier[3] and co-workers stated that their data were consistent with the absence of a peripheral site on the BuChE family of enzymes, since the bivalent ligands they synthesized[3,5] showed low affinity for rat serum BuChE. In contrast, in the case of Eq the BuChE, the affinity found for the homobivalent inhibitor **3a** and the heterobivalent ligands we developed (**3e**,**f**) could be consistent with the presence of a 'peripheral site' also on this enzyme. This results highlighted the different complementary effect played by hydrophobicity and cation–π interactions at the active sites of BuChEs and AChEs (**3f**,**g** vs **3e**). By comparing the data of compounds **1**–**3g** toward Eq BuChE, it is reasonable to propose that the heterobivalent ligands bind predominantly to BuChE by facing the tacrine–NH unit to the catalytic site, while the acridine–S unit is directed to the lip of the gorge. This hypothesis is supported by multiple sequence alignment studies, demonstrating the high degree of identity in the amino acid composition of AChE and BuChE catalytic sites, with the exception of the substitution of a Pro residue in the AChEs with a Met residue in BuChEs. In contrast, many aromatic residues in the gorge and peripheral site of AChEs are replaced with aliphatic residues in BuChEs. Accordingly, in the

case of Eq BuChE the lack of a protonatable amino group on one unit of the ligand could be partially compensated by a high degree of hydrophobicity for binding with high affinity the peripheral site (**3e**,**f** vs **3a**). On the other hand, in contrast to FBS AChE, the catalytic site of Eq BuChE does not tolerate the chlorine substituents of **3g**, but they could be better accommodated at the peripheral site (**3e** > **3f** vs **3g**). Consequently, the position of the 6,8-dichloro substituents on the bivalent ligands, together with the replacement of an amino group with a non-protonatable atom, play a key role for AChE/BuChE selectivity. Furthermore, another aspect that has been investigated is the dependence of BuChE affinity on the tether length of the new compounds. As shown in Table 1, **3h** and **3i** proved to be slightly more potent than **3f** and **3e** on both enzymes while reduction of the spacer length to four methylenes (**3j**) greatly reduced the affinity for AChE but not for BuChE. In fact, **3j** is a new selective ligand of Eq BuChE. Since 6 of 14 aromatic residues in the active site gorge of FBS AChE are replaced with aliphatic amino acids in Eq BuChE,[13] the consequent lack of specific cation–π interactions at the lip of the gorge, and the larger dimension of Eq BuChE active site gorge, may allow hydrophobic forces to drive the accommodation of inhibitors characterized also by a shorter tether. These results further support the earlier observations made by Saxena et al. that the amino acids lining the gorge as well as the dimensions of the active site gorge determine the binding of these inhibitors to AChE and BuChE.[14]

Conclusions

A series of new tacrine analogues and tacrine-related hetero- and homobivalent ligands containing protonatable and non-protonatable functions were described. Among the tacrine analogues described so far,[10–12] compound **2** could be considered one of the most potent tacrine-based AChE inhibitors. The subset of novel bivalent ligands **3b–j** led us to identify structural properties responsible for AChE/BuChE selectivity. Furthermore, compounds **3e–j** allowed us to: (i) evaluate the capability of the Eq BuChE gorge to accommodate tacrine-related bivalent ligands, with high affinity; (ii) hypothesize a possible binding mode of these compounds with respect to both sites of FBS AChE and Eq BuChE; and (iii) elucidate the complementary role played by hydrophobic and cation–π interactions for high affinity binding to both enzymes. In summary we identified novel and selective AChE and BuChE inhibitors. The latter can represent useful pharmacological tools to study the physiological role of BuChEs.

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