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Letters

Specific Targeting of Acetylcholinesterase and Butyrylcholinesterase Recognition Sites. Rational Design of Novel, Selective, and Highly Potent Cholinesterase Inhibitors

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Abstract: Tacrine-based AChE and BuChE inhibitors were designed by investigating the topology of the active site gorge of the two enzymes. The homobivalent ligands characterized by a nitrogen-bridged atom at the tether level could be considered among the most potent and selective cholinesterase inhibitors described to date. The nitrogen-containing homobivalent ligands **3e**,**g** and the sulfur-containing **3h** validated the hypothesis of extra sites of interaction in the AChE and BuChE active site gorges.

Introduction. 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 acetylcholine (ACh). AD is caused by a progressive and rather specific degeneration of certain neuron populations, with β-amyloid plaques (extracellular deposit), neurofibrillary tangles (intracellular deposit), and synaptic loss being neuropathological hallmarks of the disease. The role of ACh in the regulation of cognitive functions in both normal and pathological conditions has been widely reviewed.[1] In the central nervous system (CNS) the cholinergic system is important in the regulation of memory and learning processes, and there are two cholinesterases (hydrolases) that coexist ubiquitously in humans and contribute to the degradation of the neurotransmitter ACh: acetylcholinesterase (E1.1.1.7; AChE) and butyrylcholinesterase (E1.1.1.8; BuChE).[2] Functionally, both enzymes hydrolyze ACh efficiently but at different rates; BuChE hydrolyzes butyrylcholine (BuCh) at rates faster than ACh, while AChE degrades BuCh much more slowly than ACh. Although highly homologous (50−60%), these enzymes are encoded by different genes and clearly differ in substrate specificity and sensitivity to inhibitors, likely because of a larger void at the BuChE active site gorge and/or structural differences in the catalytic site.[3,4] These differences supported the presence of a specific peripheral anionic site (PAS) at the lip of the gorge of AChE, which is lacking in BuChE.[5−7]

BuChE is widely distributed in the body of vertebrates. It appears in serum, hemopoietic cells, liver, lung, heart, and CNS (neurons, glial cells, and gliomas).[8] Unlike AChE, its physiologic function in normal and diseased humans is still unclear, and recently a role for BuChE in development and neurobiological processes was suggested. In particular, BuChE has been implicated in neurogenesis and regulation of cell proliferation and differentiation, as well as in changes in apoptotic events.[8] The BuChE gene is amplified or abnormally expressed in tumorigenesis[8] and some neuronal disorders, suggesting that BuChE may be involved in cell adhesion phenomena. Amplification of BuChE gene could stimulate tumorigenic cells to proliferate more rapidly, evading regular proliferation control. Furthermore, significant increases in the levels of BuChE and/or AChE in various neurological disorders such as dementias may be indicative of pathological conditions. Thus, the development of selective BuChE inhibitors may be of great interest to clarify the physiological role of this enzyme and to provide novel therapeutics for various diseases.

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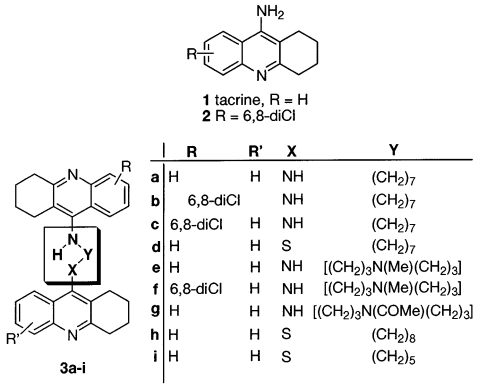
Chart 1. New Tacrine-Based Homo- and Heterodimers

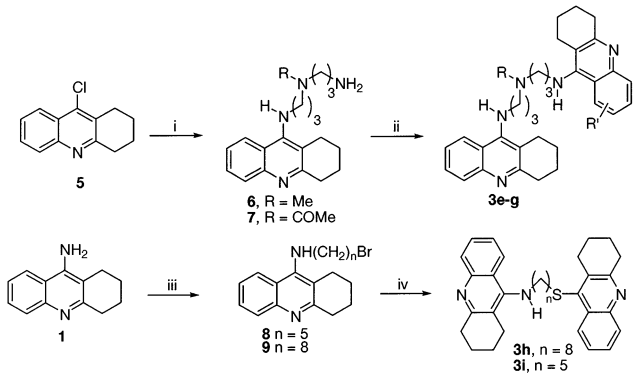


	R	R'	X	Y
a	H	H	NH	$(CH_2)_7$
b	6,8-diCl		NH	$(CH_2)_7$
c	6,8-diCl	H	NH	$(CH_2)_7$
d	H	H	S	$(CH_2)_7$
e	H	H	NH	$[(CH_2)_3N(Me)(CH_2)_3]$
f	6,8-diCl	H	NH	$[(CH_2)_3N(Me)(CH_2)_3]$
g	H	H	NH	$[(CH_2)_3N(COMe)(CH_2)_3]$
h	H	H	S	$(CH_2)_8$
i	H	H	S	$(CH_2)_5$

The use of reversible inhibitors of AChE is considered to be one viable and attractive therapeutic approach to amplify the action of ACh in the brain of AD patients. Tacrine (THA, **1**), a reversible nonselective inhibitor of AChE and an even stronger inhibitor of BuChE, has been marketed for use in the long-term palliative treatment of AD since 1993. The widespread applicability of this drug is limited by its severe liver toxicity as well as side effects stemming from its low target specificity. Since then, other potent AChE inhibitors have been developed, such as huperzine A, E2020, and rivastigmine, with the last two already approved for the treatment of AD. Recently,[7,9−11] the synthesis of several bivalent tacrine-based AChE inhibitors was reported whose increased activity and selectivity were the result of the simultaneous binding of two units to the catalytic and PAS of AChE. These tacrine-related homo- and heterobivalent ligands could be promising drug candidates for the treatment of AD, possessing pharmacological properties superior to those of tacrine. The aim of the present study was to develop, by the investigation of the active site topology of AChE and BuChE, novel tacrine-based homo- and heterobivalent ligands for pharmacological evaluation against AChE and BuChE and to provide molecular probes to get further insights into the AChE/BuChE active site gorges. On the basis of a rational approach, a series of potent tacrine-based homo- and heterobivalent ligands were designed (**3b**−**i**) and tested (Chart 1). The new subset was designed to monitor the existence of extra sites of interaction into the AChE and BuChE active site gorges. Indeed, by use of molecular modeling techniques, a mid-gorge AChE recognition site and a peripheral interaction site in BuChE were hypothesized. The resulting affinity of the newly designed compounds seems to validate these hypotheses. The set of compounds included in this molecular modeling study was synthesized using an already described general procedure.[11] The synthetic pathways to obtain the most representative compounds **3e**−**i** are reported in Scheme 1, while the tacrine homodimer **3a**, described earlier,[6,7] was resynthesized and tested under the same experimental conditions.

Molecular Modeling Studies. Selected conformers of compounds **3e**,**g** and **3h** were docked into both the hAChE X-ray crystal structure (PDB code 1B41) and the homology model of hBuChE (PDB code 1EHO), using a protocol that included molecular mechanic, Monte Carlo, and simulated annealing calculations (Supporting Information). Molecular modeling studies

Scheme 1[a]



[a] Reagents: (i) pentanol, *N*,*N*-bis(3-aminopropyl)acetamide, 160 °C, 12 h; (ii) 9-chloro-1,2,3,4-tetrahydroacridine or 6,8,9-trichloro-1,2,3,4-tetrahydroacridine, pentanol, 160 °C, 12 h; (iii) KOH, $Br(CH_2)_nBr$, CH_3CN, room temp, 6 h; (iv) 1,2,3,4-tetrahydro-9-mercaptoacridine, KOH, CH_3CN, room temp, 2 h.

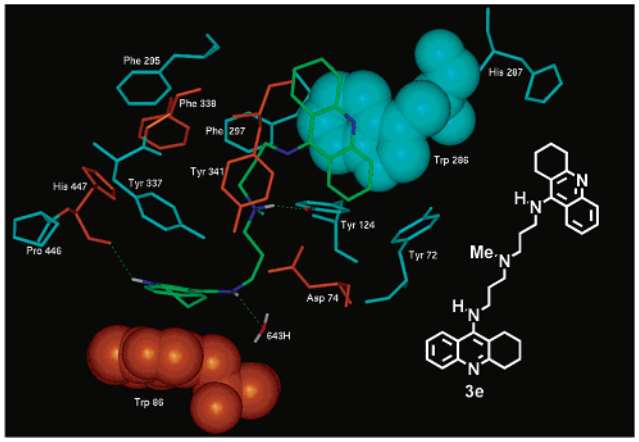


Figure 1. Compound **3e** (colored by atom type) docked into the active site of hAChE. Amino acids conserved in hBuChE are orange, those replaced in hBuChE are cyan. van der Waals volumes of W86 and W286 are displayed. Hydrogen bonds are highlighted by green dashed lines. Hydrogens and water molecules are omitted for clarity with the exception of those involved in hydrogen bonds.

were performed using human enzymes, the final pharmacological target for the development of new drugs, although our pharmacological tests used fetal bovine serum (FBS) AChE and equine (Eq) BuChE. Nevertheless, a comparison of their amino acid sequences with those of the corresponding human enzymes revealed an overall identity of 84% and 88%, respectively. No differences were found in the amino acid composition of the active sites of FBS and hAChE, while only two differences were detected between human and Eq BuChE (Figure 2).

Results and Discussion. Previously,[11] we reported and discussed the ability of compounds **3a** and **3d** to bind to Eq BuChE with good affinity (Table 1) and that AChE/BuChE selectivity could be modulated by exploiting the different amino acid compositions of the active sites of the two enzymes. Indeed, the molecular modeling study herein described suggested that the AChE/BuChE selectivity profiles of compounds **2, 3b**, and **3c**, may be related to the only relevant difference in the catalytic sites of the two enzymes: the substitution of P446 in hAChE with the bulkier M437 in hBuChE (Figures 1 and 2), which reduces the space to optimally

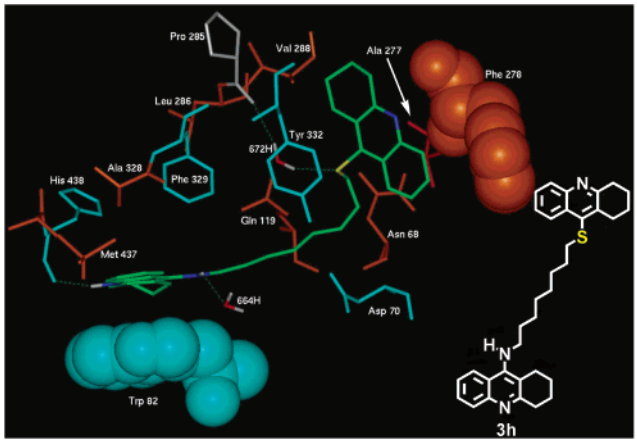


Figure 2. Compound **3h** (colored by atom type) docked into the active site of hBuChE. Conserved amino acids are cyan. Those replaced in hAChE are orange. P285, replaced in Eq BuChE by L313, is white. A277, replaced in hAChE by W286 and in Eq BuChE by V305, is red. van der Waals volumes of W82 and F278 are displayed. Hydrogen bonds are highlighted by green dashed lines. Hydrogens and water molecules are omitted for clarity with the exception of those involved in hydrogen bonds.

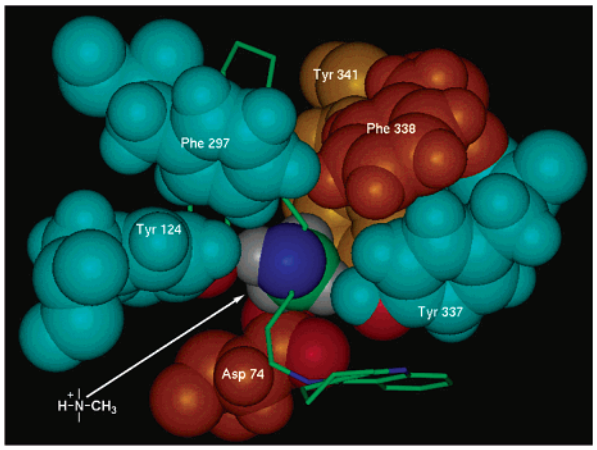


Figure 3. Compound **3e** (colored by atom type) docked into the active site of hAChE. Its interaction with the proposed mid-gorge recognition site is shown. The interactions between the protonatable amine function in the tether and amino acids in the gorge are displayed using their van der Waals volumes. The rest of the inhibitor is displayed as capped sticks, and its hydrogens are omitted for clarity. Cyan residues are aliphatic in hBuChE.

Table 1. Dissociation Constants for the Inhibition of Fetal Bovine Serum AChE and Equine BuChE by Analogues of Tacrine

compound	tether length $(CH_2)_n$	AChE K_i [a] (nM)	BuChE K_i [a] (nM)
1, tacrine		40	7
2		1	50
3a, THA-homo	7	1.3	2
3b	7	150	>20000
3c	7	6	180
3d	7	340	35
3e	7	0.06	6
3f	7	3.7	11
3g	7	1500	2
3h	8	250	0.4
3i	5	210	10
E2020		2.9	640
ethopropazine[b]		173200	20[c]
huprine X[d]		0.026[e]	120[c]

[a] K_i is the mean of at least three determinations. Standard errors were all within 10% of the mean. [b] Reference 4. [c] hBuChE. [d] Reference 13. [e] hAChE.

accommodate the chlorine atoms at C-6 and C-8 of compounds **2**, **3b**, and **3c** in hBuChE. On the other hand, replacement of the amine group of a tacrine unit by a sulfur atom provided compounds with increased selectivity toward BuChE (**3a** vs **3d**),[11] which can be explained by the fact that W286 in hAChE, responsible for cation−π interaction at the PAS, is replaced by an aliphatic residue A277 in hBuChE (Figures 1 and 2).

These results provided the rational for an in-depth molecular modeling analysis to find new and specific sites of interaction in AChE and BuChE active sites that can drive the design of new, potent and selective inhibitors. Inspection of X-ray structures of AChEs from various sources, alone or in complex with inhibitors, revealed that the catalytic site is located at the bottom of a narrow gorge whose striking feature is the presence of 14 aromatic residues highly conserved through different species. The comparison of the amino acid composition of hBuChE (no three-dimensional structure is available to date for BuChEs) with that of hAChE evidenced the same positioning of W86 responsible for the binding of the quaternary ammonium group of the substrate, and the catalytic triad S203, E202, and H447 (hAChE numbering). On the other hand, 6 of the 14 aromatic residues (Y72, Y124, W286, F295, F297 and Y337) in the active site gorge of hAChE are replaced by aliphatic residues (N68, Q119, A277, L286, V288, and A328) in hBuChE.[2,4]

On the basis of sequence analysis and molecular modeling studies, we synthesized homo- and heterobivalent ligands bearing different functional groups on the tether (**3e**−**g**), designed to interact with specific residues in the active site gorge. For example, to obtain more potent and selective inhibitors of hAChE, the central methylene unit of the alkyl tether of **3a** was replaced by a protonatable amine function (**3e**) capable of providing additional specific interactions with aromatic residues in the active site gorge of hAChE (K_{i3a} = 1.3 nM vs K_{i3e} = 60 pM). This is supported by the comprehensive docking study of **3e** in the active site gorge of hAChE, shown in Figure 1. Interaction points with the enzyme include (i) a hydrogen bond between the carbonyl oxygen of H447 and the protonated tetrahydroacridine nitrogen atom, (ii) a hydrogen bond between the amino group at C-9 of the tricyclic system and a water molecule (643H), (iii) two π−π stacking interactions involving the tetrahydroaminoacridine moieties of **3e** and W86 (catalytic site) and W286 (PAS), (iv) a hydrogen bond between the protonated amino nitrogen in the alkyl tether and the hydroxyl group of Y124, and (v) cation−π interactions with a number of aromatic side chains present in the hAChE gorge (Figure 3). The specific interactions with the newly discovered mid-gorge recognition site in the hAChE active site gorge are likely responsible for the picomolar binding affinity of **3e** (three-point interaction, K_i = 60 pM) and its AChE/BuChE affinity ratio of 0.01, with an AChE inhibitory activity 650-fold higher than that of tacrine (**1**). This increase in activity is much higher than that reported by Carlier[7,9] for the homobivalent ligand **3a** (two-point interaction) (under our experimental conditions: **1**, K_i = 40 nM vs **3a**, K_i = 1.3 nM; Table 1).

When a THA unit of **3e** bears two chlorine atoms at C6 and C8 (**3f**), the decreased binding affinity could be due to the inability of different pharmacophore points of **3f** to assume the correct orientation in the AChE active site. On the other hand, docking studies with **3g**, characterized by a bulkier, neutral amide group in the alkyl tether, indicated that this compound could be better accommodated in the larger hBuChE gorge (data not shown). Accordingly, **3g** showed a BuChE affinity of 2 nM and an AChE/BuChE affinity ratio of 750 (Table 1). It is noteworthy that **3e** was found to be more active than E2020 (Table 1), and a comparison of their binding mode is shown in Figure 4 (Supporting Information). To design new selective inhibitors of hBuChE, we also explored the possibility of varying the tether length of **3d**, whose mercaptotetrahydroacridine moiety was found to confer a certain degree of selectivity toward BuChE.[11] While the 7-methylene tether represents a strict requirement for high affinity binding to AChE, the presence of the aromatic residue F278 in hBuChE, located at the rim of the gorge next to A277 (A277 is replaced by W286 located at the PAS of hAChE), suggested that a longer alkyl spacer (8-methylenes, **3h**) could provide a selective BuChE inhibitor, optimally positioning the *S*-tetrahydroacridine moiety to establish a $\pi-\pi$ interaction with F278 (Figure 2). Accordingly, **3h** was found to be a potent BuChE inhibitor and, although less selective than ethopropazine, a prototype for the generation of highly selective BuChE bivalent ligands (K_i = 0.4 nM; AChE/BuChE affinity ratio of 625).

Figure 2 shows the results of our molecular modeling studies with **3h** docked into the active site of hBuChE. The tacrine unit is placed in the catalytic site, reproducing a binding mode similar to that described for compound **3e** in the hAChE catalytic site. In addition, the mercaptotetrahydroacridine moiety, placed at the rim of the gorge, gives a face-to-edge $\pi-\pi$ interaction with F278 and a hydrogen bond between the sulfur atom and a water molecule (672H) (Figure 2). The presence of a specific site of interaction at the rim of BuChE gorge is also supported by the binding affinities of compounds **3i**, **3d**, and **3h** (Table 1), which are characterized by 5-, 7-, and 8-methylene tethers, respectively. Indeed, BuChE affinities of compounds **3d** and **3i** are comparable and could be due to the possible accommodation of the *S*-tetrahydroacridine moiety in the large BuChE gorge. In contrast, the subnanomolar affinity of **3h** is consistent with achievement of a new binding interaction with F278 and supports the presence of a peripheral interaction site in BuChE.

Conclusions. In summary, we disclosed the rational design of novel and potent tacrine-based selective inhibitors of AChE and BuChE. Their biological data confirmed the existence of a peripheral site of interaction in the active site gorge of BuChE and led to the definition of an unprecedentedly described mid-gorge interaction site in the active gorge of AChE. Together with the recently described phenantridinium-based heterobivalent ligand[12] and huprine X,[13] compound **3e** could be considered one of the most potent and selective tacrine-based AChE inhibitors, while the novel, potent, and highly selective bivalent BuChE inhibitors **3g**,**h** may be considered useful pharmacological tools to investigate the physiological role of BuChE and may represent lead structures to generate inhibitors of tumorigenesis. Starting from our molecular modeling approach and the new leads, these studies may also pave the way for future rational design of selective AChE inhibitors as novel therapeutics for neurodegenerative diseases.

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Supporting Information Available: Experimental details for the new compounds **2** and **3b**−**i** (chemistry, molecular modeling, and pharmacology) and Figure 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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