Acetylcholinesterase Noncovalent Inhibitors Based on a Polyamine Backbone for Potential Use against Alzheimer's Disease

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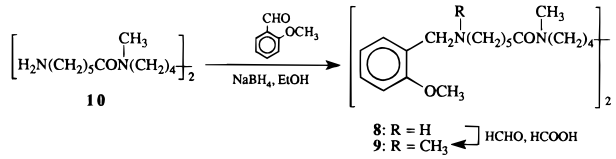
Dementia is the most common psychiatric disorder in elderly patients, and Alzheimer's disease[1] (AD) is the most common cause. Much effort is devoted to elucidating the relationships among the hallmarks of the disease: amyloid plaques, neurofibrillary tangles, and degeneration of the basal forebrain cholinergic neurons.[2] Despite the evidence that there is a profound and consistent loss of cholinergic transmission in AD, pharmacotherapy and practical medicine are still waiting for the possible advantages arising from enhancing the cholinergic system in some way.[3] To date, only some inhibitors of acetylcholinesterase (AChE), such as tacrine and donepezil, resulted to be useful drugs in alleviating the symptoms of AD.[4] On the other hand, the existence of multiple muscarinic receptor subtypes in the central nervous system (CNS) has stimulated the search for new drugs which target only one receptor while not affecting others. It has been advanced that drugs which antagonize selectively presynaptic muscarinic M_2 autoreceptors may also be useful in AD as they will facilitate ACh release.[5]

AChE is the enzyme involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses in the central and peripheral nervous systems. Inhibitors of AChE activity promote an increase in the concentration and the duration of action of synaptic ACh thus causing an enhancement of the cholinergic transmission through the activation of the synaptic nicotinic and muscarinic receptors. It has been demonstrated that AChE could play a key role during an early step in the development of the senile plaques, as revealed by the finding that AChE accelerates β-amyloid peptide (βA) deposition.[6] This peculiar feature of AChE was not affected by active site inhibitors, such as edrophonium, but it was affected by peripheral anionic binding site ligands, such as decamethonium and propidium. Interestingly enough, butyrylcholinesterase (BChE), an enzyme that lacks the peripheral anionic binding site, did not affect amyloid formation. This finding suggests clearly that the catalytic site of AChE does not participate in the interaction of the enzyme with βA, whereas it is possible that the peripheral binding site of AChE may be involved in amyloid formation.[6]

The aim of this study was to produce novel ligands based on a polyamine backbone having affinity for both AChE active and peripheral binding sites and for muscarinic M_2 receptors as well. Inhibition of AChE activity would potentiate the remaining cholinergic transmission in affected brain regions, while inhibition of muscarinic M_2 autoreceptors would have an additive effect by facilitating the release of ACh in the synapse. Furthermore, inhibition of the peripheral binding site would prevent the aggregation of βA induced by AChE. The starting point was the observation that benextramine, a tetraamine disulfide developed as an irreversible α-adrenoreceptor antagonist,[7] displayed also a significant affinity for cardiac muscarinic M_2 receptors and potentiated the effect of ACh on the frog rectus muscle as well.[8,9] To verify whether the latter finding might result from an inhibition of AChE activity, we tested benextramine on human erythrocyte AChE. Since benextramine turned out to be a reversible inhibitor of AChE, we thought that it might represent a new lead for the design of ligands displaying affinity for AChE and muscarinic M_2 receptors thus fulfilling our research goal.

Scheme 1



The role of the 2-methoxybenzyl group of **1** in AChE inhibition was verified by investigating the corresponding unsubstituted tetraamine disulfide **2**. In addition, the contribution, if any, of the disulfide bridge on affinity was studied by assaying the carbon analogue **4**. Since **4** was as active as benextramine, it was apparent that the disulfide bridge is not essential for AChE inhibition. Next, our attention was focused on the chain length separating the two inner nitrogens of tetraamine **4**. Although it turned out that optimum inhibition of AChE activity is associated with a seven-carbon chain, we chose tetraamine **6** (methoctramine) as a lead since it displayed optimum affinity for muscarinic M_2 receptors.[10] Since we had already demonstrated that a diamine diamide backbone can retain significant affinity for muscarinic M_2 receptors,[11] diamine diamides **7**−**9** were designed such as to improve lipophilicity versus methoctramine, while retaining or hopefully improving affinity for AChE.

To ascertain the possibility for the polyamines under study to bind at both the active and the peripheral AChE binding sites, a theoretical investigation was undertaken of the docking of these inhibitors to the enzyme. Molecular dynamics simulations were performed from which it resulted that the diamine diamide skeleton has enough conformational flexibility to allow the outer amine functions to contact both sites simultaneously.

Chemistry. Tetraamines **1**−**6** and diamine diamide **7**, when not available in our laboratory, were synthesized as previously described.[7,10,11] Diamine diamides **8** and **9** were synthesized by standard procedure (Scheme 1) and were characterized by ^{1}H NMR and elemental analysis.

The 2-methoxybenzyl group on the terminal nitrogens was easily introduced by condensation of diamine diamide **10**[12] with 2-methoxybenzaldehyde and reduction of

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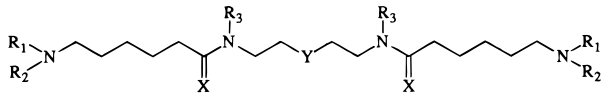
Table 1. Inhibition of AChE and BChE Activities by Polyamines



						pIC_{50}[a]		pA_2[b]		
no.	R_1	R_2	R_3	X	Y	AChE	BChE	M_1	M_2	M_3
1	2-$MeOC_6H_5CH_2$	H	H	H_2	S−S	5.14 ± 0.02	5.21 ± 0.03	nd	nd[c]	nd
2	H	H	H	H_2	S−S	3.30 ± 0.03	3.19 ± 0.02	nd	nd	nd
3	2-$MeOC_6H_5CH_2$	H	H	H_2	CH_2	5.14 ± 0.03	5.06 ± 0.02	nd	6.67 ± 0.07	5.89 ± 0.09
4	2-$MeOC_6H_5CH_2$	H	H	H_2	$(CH_2)_2$	5.19 ± 0.01	5.86 ± 0.01	nd	6.98 ± 0.12	5.76 ± 0.11
5	2-$MeOC_6H_5CH_2$	H	H	H_2	$(CH_2)_3$	5.35 ± 0.05	5.43 ± 0.02	nd	7.64 ± 0.09	5.92 ± 0.14
6	2-$MeOC_6H_5CH_2$	H	H	H_2	$(CH_2)_4$	5.27 ± 0.03	6.01 ± 0.02	6.85 ± 0.12	7.92 ± 0.08	6.06 ± 0.07
7	2-$MeOC_6H_5CH_2$	H	H	O	$(CH_2)_4$	5.73 ± 0.03	4.94 ± 0.03	nd	6.30 ± 0.10	5.35 ± 0.14
8	2-$MeOC_6H_5CH_2$	H	Me	O	$(CH_2)_4$	6.51 ± 0.02	5.22 ± 0.02	nd	6.67 ± 0.06	5.21 ± 0.12
9	2-$MeOC_6H_5CH_2$	Me	Me	O	$(CH_2)_4$	6.77 ± 0.01	4.93 ± 0.04	5.66 ± 0.02	6.39 ± 0.23[d]	5.55 ± 0.12
tacrine						6.66 ± 0.02	6.44 ± 0.01	nd	nd	nd
physostigmine						7.85 ± 0.03	7.64 ± 0.02	nd	nd	nd

[a] AChE and BChE were from human erythrocytes. pIC_{50} values [−log IC_{50} (μM)] represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements. [b] pA_2 values (±SE, $n = 5$) were calculated according to Van Rossum[21] at 1 μM (M_2 receptors) or 10 μM (M_1 and M_3 receptors) concentration. [c] nd, not determined. [d] The experiments were performed in the presence of physostigmine (0.5 μM) to inhibit AChE.

Table 2. Inhibition Constants of AChE Obtained with Caproctamine (**9**) in Comparison with Tacrine and Edrophonium[a]

compd	AChE K_i (μM)
caproctamine (**9**)	0.104 ± 0.016
edrophonium	1.63 ± 0.23
tacrine	0.151 ± 0.016

[a] Inhibition constants, expressed as K_i values, were calculated from kinetic data in Figure 1.

the intermediate Schiff base to yield diamine diamide **8**. Finally, Eschweiler−Clarke methylation of **8** gave diamine diamide **9**.[13]

Results and Discussion. Four different pharmacological actions of tetraamines **1**−**6** and diamine diamides **7**−**9** were determined, that is, AChE and BChE inhibition[14] and antagonism at muscarinic M_2 and M_3 receptor subtypes.[15] Furthermore, the most interesting compound (**9**) was evaluated also for its muscarinic M_1 antagonistic potency[15] in comparison with methoctramine (**6**). The results are summarized in Tables 1 and 2.

It is evident that all polyamines were effective inhibitors of AChE and BChE with the exception of the unsubstituted tetraamine disulfide **2**. The latter resulted as a very weak inhibitor of both enzymes, being about 2 orders of magnitude less potent than the prototype benextramine (**1**). Clearly, a 2-methoxybenzyl group on the terminal nitrogens of **1** contributes significantly to the binding with the enzyme. The replacement of the disulfide bridge of **1** with two methylenes, affording **4**, did not modify the affinity for AChE, while causing a 5-fold increase in affinity for BChE. This finding rules out the possibility that benextramine may inhibit AChE by forming a covalent bond by way of an interchange reaction between the disulfide moiety of **1** and a suitable thiol group on the enzyme. Furthermore, two methylenes served better than a disulfide functionality in the enzyme−inhibitor complex formation, as **4** was more potent than **1** at both enzymes.

Modifying the chain length between the two inner nitrogens of **4**, affording tetraamines **3**−**6**, did not affect significantly the affinity for AChE and muscarinic M_3 receptors while causing a different effect on the affinity toward BChE and muscarinic M_2 receptors. The affinity for muscarinic M_2 receptors increased, as previously observed,[10] with increasing chain length between the nitrogens, whereas the affinity for BChE did not follow a similar linear trend as **6** but not **5** was more potent than **4**. Since it was an aim of the present investigation to provide compounds useful for the treatment of neurological disorders by improving cholinergic transmission in the brain following inhibition of AChE and muscarinic M_2 receptors, we investigated diamine diamides **7**−**9**. Interestingly enough, transforming the inner amine functions of methoctramine (**6**) into amide groups, affording **7**, resulted in a significant decrease in the affinity for muscarinic M_2 and M_3 receptors and for BChE as well but produced an increase in the affinity for AChE. N-Methylation of **7**, affording **8** or **9** (caproctamine), resulted in a further increase in affinity for AChE while not affecting the affinity for BChE and muscarinic receptors.

It is evident that **9** emerges as a powerful tool for investigating the neurological disorders due to a loss in the cholinergic system. In comparison with benextramine (**1**), caproctamine (**9**) resulted as 42-fold more potent at AChE while being 2-fold less potent at BChE with a BChE/AChE selectivity ratio of 68. Furthermore, it was a weak antagonist at both muscarinic M_1 and M_3 receptors while displaying an affinity toward muscarinic M_2 receptors similar to the affinity for AChE as revealed by a comparison of pA_2 (M_2) and pIC_{50} (AChE) values which were 6.39 and 6.77, respectively. Owing to these biological properties, caproctamine (**9**) was studied further to characterize the nature of the inhibition of AChE activity. Inhibition of AChE activity by **9** was very fast and not time-dependent, as 50% of enzyme inactivation produced by a 0.17 μM concentration following a 1-min incubation was not significantly different ($p > 0.01$) from the inhibition observed up to a 40-min incubation. The graphical analysis of steady-state inhibition data for **9** in comparison with edrophonium and tacrine is shown in Figure 1, whereas the estimates of competitive inhibition constants K_i are reported in Table 2.[16] The inhibition was found to be clearly of the competitive type only for edrophonium, whereas reciprocal plots involving tacrine and **9** (Figure 1) show both

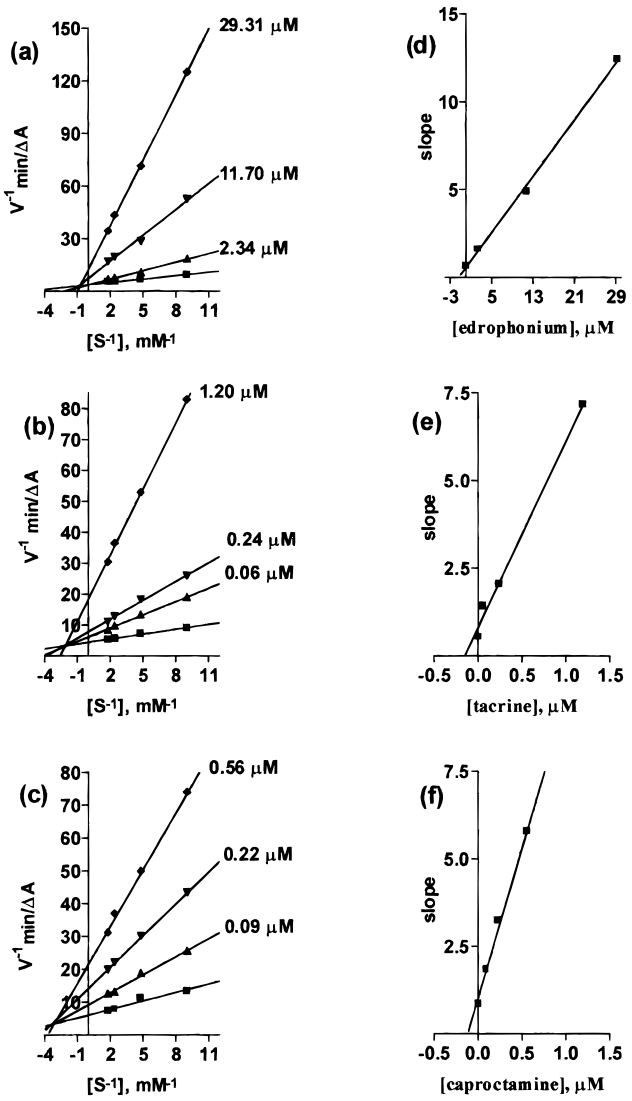


Figure 1. Steady-state inhibition by three inhibitors of AChE hydrolysis of acetylthiocholine. Reciprocal plots of initial velocity and substrate concentrations (a−c) and replots of the reciprocal plots versus inhibitor concentration (d−f) are reported. Reciprocal plots of initial velocities in the absence of inhibitors gave an estimate of k_{app} for acetylthiocholine of 170 ± 15 μM (four experiments). Lines were derived from a weighted least-squares analysis of the data points.

increasing slopes and increasing intercepts with higher inhibitor concentration. Therefore, we concluded that caproctamine (**9**) causes a mixed type of inhibition, that is, inhibition of both the active site and a second distal site of the enzyme.[17]

The possibility for caproctamine (**9**) to bind at both the active and the peripheral sites of AChE was theoretically investigated by studying the docking of its diprotonated form to the AChE gorge. Molecular dynamics simulation runs were performed,[18] and the results indicated that indeed **9** is able to simultaneously contact both sites and to establish favorable interactions with a number of residues in the gorge. The flexibility of the molecule allows it to assume many conformations, one of which is shown in Figure 2. At one end of the molecule, the *o*-methoxybenzylamine moiety can interact with a set of residues near Trp84, while, at the opposite end, the second *o*-methoxybenzylamine group can reach the peripheral binding site that was postulated to correspond to Trp279.[19] Remarkably, despite the conspicuous overall length of the molecular backbone of **9**, it can fold in such a way as to place the *o*-methoxybenzylamine functions at a distance ranging from 10 to 16 Å. This is compatible with the reaching of Trp84 and Trp279 which lie approximately 16 Å apart. The intermediate mostly lipophilic chain seems to play an important role in determining the binding of the diamine diamide **9** to the enzyme by allowing hydrophobic interactions with several aromatic residues, among which are Tyr70, Tyr121, Phe288, Phe290, Tyr330, Phe331, and Tyr334.

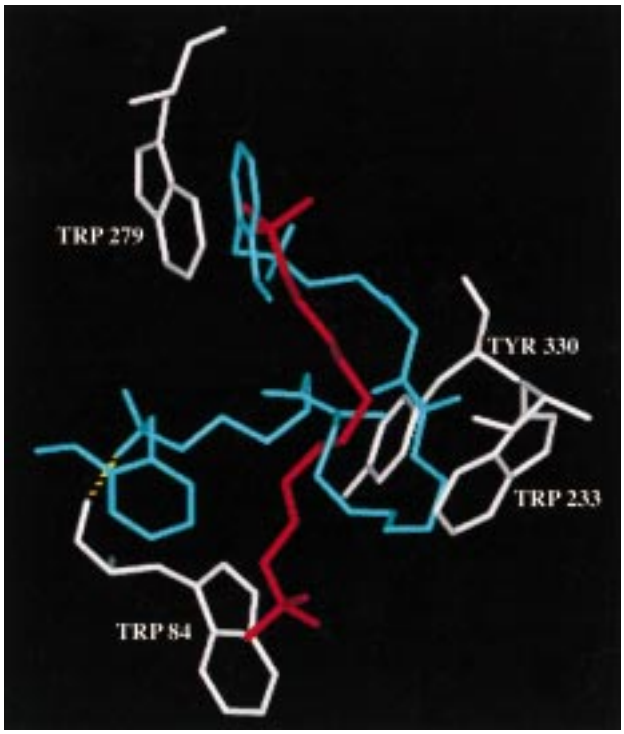


Figure 2. Representative interactions of **9** (cyan) with some selected amino acid residues (white) of the human AChE gorge: the indole of Trp279 and one *o*-methoxyphenyl of **9** form a displaced π-stacking interaction; the protonated amino group of the second *o*-methoxybenzylamine function forms an H-bond (yellow dotted line) with the backbone carbonyl of Trp84; Trp233 and Tyr330 are two of the residues contributing to the hydrophobic stabilization of the intermediate chain. The position of decamethonium (red) with respect to the two critical Trp residues as determined from the X-ray crystallography is shown for reference.[22]

In comparison with the experimentally determined (X-ray analysis)[20] AChE binding mode of decamethonium also shown in Figure 2, the way in which **9** contacts Trp84 and Trp279, although different, allows one to hypothesize the same kind of interaction. We expect that this similar binding mode leads to the same action against βA fibrillogenesis as that observed for decamethonium.[6]

In conclusion, present investigation has shown that caproctamine (**9**) is endowed with a well-balanced affinity profile as an AChE inhibitor and a competitive muscarinic M_2 receptor antagonist. Therefore, it may well be capable of stimulating cholinergic activity in the brain by decreasing ACh hydrolysis rates and, at the same time, by increasing ACh release in the synapse. It derives that caproctamine (**9**) could have potential in the investigation of AD because, besides its effects on

the cholinergic system, it might prevent also AChE-mediated βA aggregation by interacting with the peripheral anionic binding site of AChE. However, there may be significant barriers to that end use. For example, although **9** is likely more lipophilic than methoctramine (**6**) or benextramine (**1**), in vivo studies may be required to determine whether diamine diamides are able to cross the blood−brain barrier. Our future work in this area will include studies directed at gaining a better understanding of the intriguing trends noted above.

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(13) A mixture of **10** (0.4 g, 1 mmol), 2-methoxybenzaldehyde (0.3 g, 2.2 mmol), and sodium borohydride (85 mg, 2.2 mmol) in anhydrous ethanol with molecular sieves (3 Å) was stirred at room temperature for 20 h. After cooling, the mixture was made acidic with 6 N HCl, filtered to remove the sieves, and then evaporated. The residue was dissolved in water, and the resulting solution was washed with ether, made basic with 2 N NaOH, and extracted with methylene chloride. Removal of dried solvents gave **8** as the free base that was transformed into the oxalate salt: 90% yield; mp 156−157 °C (from ethanol/ether). Anal. ($C_{42}H_{66}N_4O_{12}$) C, H, N. Formic acid (96%; 0.67 mL, 17.4 mmol) was added dropwise to **8** (0.15 g, 0.23 mmol), and then formaldehyde (40%; 0.60 mL, 8.7 mmol) was added to the resulting mixture which was heated at 90 °C for 10 h, cooled (0 °C), made basic with 40% aqueous NaOH, and extracted with methylene chloride. Removal of dried solvents gave **9** as the free base that was transformed into the citrate salt: oil (95% yield); ^{1}H NMR (free base, $CDCl_3$) δ 1.16−1.78 (m, 24), 2.20−2.46 (m, 8), 2.22 (s, 6), 2.89 (s, 3), 2.94 (s, 3), 3.18−3.37 (m, 4), 3.51 (s, 4), 3.81 (s, 6), 6.82−6.94 (m, 4), 7.18−7.33 (m, 4). Anal. ($C_{52}H_{82}N_4O_{18}\cdot H_2O$) C, H, N.

(14) AChE and BChE derived from human erythrocytes were employed in this study. Anticholinesterase activity was based on measuring the hydrolysis of acetylthiocholine and the subsequent reaction of thiocholine with 4,4-dithiopyridine to form 4-thiopyridine.[23] The results are expressed as IC_{50} values which represent the concentration required to inhibit enzyme activity by 50%.

(15) Functional activity at muscarinic receptor subtypes was determined by the use of the muscarinic M_1 receptor-mediated inhibition of neurogenic twitch contractions (single pulses at 0.05 Hz), muscarinic M_2 receptor-mediated negative inotropism in driven guinea pig left atria (1 Hz), and muscarinic M_3 receptor-mediated contraction of guinea pig ileum longitudinal muscle as previously described.[24] The agonist was McN-A-343 (M_1) or arecoline propargyl ester (M_2 and M_3).

(16) Inhibition constants, expressed as K_i values, were calculated from kinetic data.[25] Three concentrations of each inhibitor and four substrate concentrations were used; acetylthiocholine concentration never exceeded 0.55 mM, to avoid substrate inhibition. Duplicate assays were conducted at each of the concentration combinations, and the mean of the enzyme velocity values was used for graphical analysis (individual velocity values were all within ±5% of the mean value).

(17) The inhibitory behavior of **9**, as deduced from plots b and c in Figure 2, is strictly similar to that displayed by some recently reported[26] bis-tetrahydroaminacridine inhibitors of AChE. These compounds bind simultaneously at both the catalytic and the peripheral sites of AChE and are characterized by "a linear mixed type of enzyme inhibition".

(18) Molecular dynamics simulations were performed using the united-atom AMBER* force field implemented in the MacroModel ver. 5.5 program.[27] The coordinates of the protein were obtained from the X-ray structure of AChE isolated from *Torpedo californica*[28] and retrieved from the Brookhaven Protein Data Bank; Phe330 was replaced by Tyr, which is present in the human AChE, with the same conformation.

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(22) The backbone atoms of Trp84 and Trp279 from the model and from the structure of AChE cocrystallized with decamethonium (Brookhaven Protein Data Bank entry 1acl) were aligned, and the resulting position of decamethonium is shown in Figure 2.

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