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Design, synthesis and biological evaluation of ambenonium derivatives as AChE inhibitors

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Abstract

Ambenonium (**1**), an old AChE inhibitor, is endowed with an outstanding affinity and a peculiar mechanism of action that, taken together, make it a very promising pharmacological tool for the treatment of Alzheimer's disease (AD). Unfortunately, the bisquaternary structure of **1** prevents its passage through the blood brain barrier. In a search of centrally active ambenonium derivatives, we planned to synthesize tertiary amines of **1**, such as **2** and **3**. In addition, to add new insights into the binding mechanism of the inhibitor, we designed constrained analogues of ambenonium by incorporating the diamine functions into cyclic moieties (**4**–**12**). The biological evaluation of the new compounds has been assessed in vitro against human AChE and BChE. All tertiary amine derivatives resulted more than 1000-fold less potent than **1** and, unlike prototype, did not show any selectivity between the two enzymes. This result, because of recent findings concerning the role of BChE in AD, makes our compounds, endowed with a well-balanced profile of AChE/BChE inhibition, valuable candidates for further development. To better clarify the interactions that account for the high affinity of **1**, docking simulations and molecular dynamics studies on the AChE-**1** complex were also carried out.
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Keywords: Alzheimer's disease; Acetylcholinesterase inhibitor; Acetylcholinesterase; Butyrylcholinesterase; Ambenonium analogues

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder and, with the continuing increase of the elderly population, it has become an urgent public health problem in western countries. Paradoxically, this devastating disease is still of unknown etiology, with the exception of the rare familial case of autosomal dominant inheritance of gene defects [1]. A key biochemical hallmark of AD is a selective loss of cholinergic neurons in the forebrain with a marked decrease in cholineacetyltransferase (ChAT) in the hippocampus and cerebral cortex, which correlates with brain pathology and cognitive dysfunction. Associated with this neurochemical changes are the extracellular deposition of β-amyloid peptide and the accumulation of intracellular neurofibrillary tangles that remain the definitive diagnostic markers for the disease. In the absence of a definitive cure for AD, the treatment has focused on therapy to restore the cholinergic tone in the brain, providing symptomatic benefits and slowing progression of the disease [2].

Among the possible therapeutic approaches toward improving cholinergic transmission in AD, acetylcholinesterase inhibitors (AChEIs) are the only FDA-approved pharmacological treatment and the drugs tacrine, donepezil, rivastigmine, and galantamine have been shown to be really effective in the treatment of the cognitive, behavioral, and functional deficits of AD.

Ambenonium (**1**) is an old AChEI, which is structurally dissimilar from those above mentioned and endowed with one of the highest known affinity (sub nanomolar range) [3,4]. This outstanding potency is unique for a compound that does not form any covalent bond at the active site of the enzyme, although its bisquaternary oxamide structure prevents the passage

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through the blood brain barrier, after conventional oral or intravenous routes of administration. In addition, as recently suggested for other AChEIs, it has been reported that **1** promotes the non-amyloidogenic route of amyloid precursor protein (APP) processing, due to a stimulatory effect on protein kinase C [5,6]. Taken together, these two possible mechanisms of action should make ambenonium a very promising pharmacological tool in the treatment of AD, and a suitable lead in the development of a new family of AChEIs.

To investigate the binding mode of **1** with AChE, we have first carried out docking simulations, providing the molecular basis of its inhibitory activity. Furthermore, in a search of ambenonium derivatives able to penetrate the blood brain barrier, we planned to synthesize correspondent tertiary amines of **1**, by deleting one ethyl group from each quaternary ammonium center (**2** and **3**). In particular in **3**, the chloro substituent on the phenyl ring of ambenonium has been substituted with an *O*-methoxy, to verify whether the substituent at position 2 might have any influence on the basicity and, as a consequence, on the extent of protonation of the benzylic nitrogen atom, as already demonstrated for caproctamine derivatives [7]. In addition, to add new insights into the binding mechanism of ambenonium and its tertiary derivatives with AChE, we designed constrained analogues by incorporating the diamine functions into cyclic moieties. In compounds **4**–**6** the diamine functions of the lead have been included in a piperazine cycle and the distance between the nitrogens has been varied by introducing methylene units between the carbonyls. Furthermore, we focused on rigid analogs **7**–**12**, in which the oxamide function has been reversed and the amide nitrogen included in or linked to a cycle. In particular, to investigate if additional interaction with the enzyme might take place, a derivative of amino acid leucine with a lipophilic isobutyl tail was synthesized (**12**). Finally, with the aim of improving the access of the molecule to the CNS modulating the lipophilicity, the amide moieties of **3** were transformed into trifluoroacetamides, as in derivative **13**. The design strategy of our compounds is shown in Fig. 1.

2. Chemistry

Compounds **2**–**6** were synthesized through acylation reaction of the oxalyl, malonyl or succinyl chlorides and the appropriate diamine.

The diamine bearing a 2-chlorobenzyl substituent (**18**) was prepared as outlined in Scheme 1, via the key intermediate **16** [8] that represented a useful synthon also in the synthesis of **7**–**11**. Thus, protection of the amino function of 2-aminoethyl bromide (**14**) with CBZ group yielded the derivative **15** that alkylated amine **16** to provide intermediate **17**. Cleavage of the protecting moiety of **17** with HBr afforded the desired diamine **18**, which upon reaction with oxalyl chloride gave the corresponding tertiary amine (**2**) of **1**.

For the synthesis of diamine **21**, carrying a 2-methoxybenzyl group on one nitrogen, a more straightforward synthetic approach was followed (Scheme 2). Reductive amination of *N*-CBZ-1,2-diamino ethane with 2-methoxybenzaldehyde furnished compound **19**, which, in turn, was alkylated with diethylsulfate and deprotected in acidic medium to give **21**. Again, acylation of **21** with oxalyl chloride provided final compound **3**.

Alkylation of 1-BOC-piperazine with 2-bromobenzyl bromide and subsequent deprotection of the carbamate moiety of **22** with CF_3COOH gave **23** in two steps, which, in turn, was acylated with the appropriate acidic chloride to give **4**–**6** (Scheme 3).

Amides **7**–**9** were synthesized by reaction of the proper 1,2-diaminocyclohexane with chloroacetyl chloride followed by nucleophilic substitution with amine **16** (Scheme 4).

Similarly, starting from piperazine and amines **16** and **28** [8], derivatives **10** and **11** were obtained (Scheme 5).

The commercial availability of an appropriate (*S*)-leucine derivative dictated a slightly different approach for the synthesis of analogue **12**, as shown in Scheme 6. *N*-BOC-leucine-*N*-hydroxysuccinimide was amidated with piperazine to give **29**, which, upon treatment with CF_3COOH to remove the protecting groups and reductive amination with 2-bromobenzaldehyde, provided **12**.

The synthesis of the trifluoroacetyl derivative **13** was accomplished as illustrated in Scheme 7. Derivative **32**, obtained through reductive amination and subsequent ethylation of glycine sodium salt, was amidated with 1,2-diamino ethane to **33**, which, upon reduction of the carbonyl functions with borane and acetylation with ethyl trifluoroacetate, afforded **13**.

3. Results and discussion

Before starting the design of this series of ambenomium (**1**) analogues, we thought it of interest to carry out docking simulations and molecular dynamics (MD) studies on the AChE-**1** complex, with the aim to rationalize at molecular level the interactions between **1** and AChE that account for its high potency. **1** was firstly docked into the enzyme gorge and then the complex was geometrically relaxed by means of MD simulations. In Fig. 2 is reported a minimized average conformation taken from the last 60 ps of MD simulations. This picture indeed shows that **1** is able to contact both the catalytic and the peripheral AChE sites. Actually, in our docking model, **1** interacted with Trp84 of the catalytic site by means of a π–π stacking,

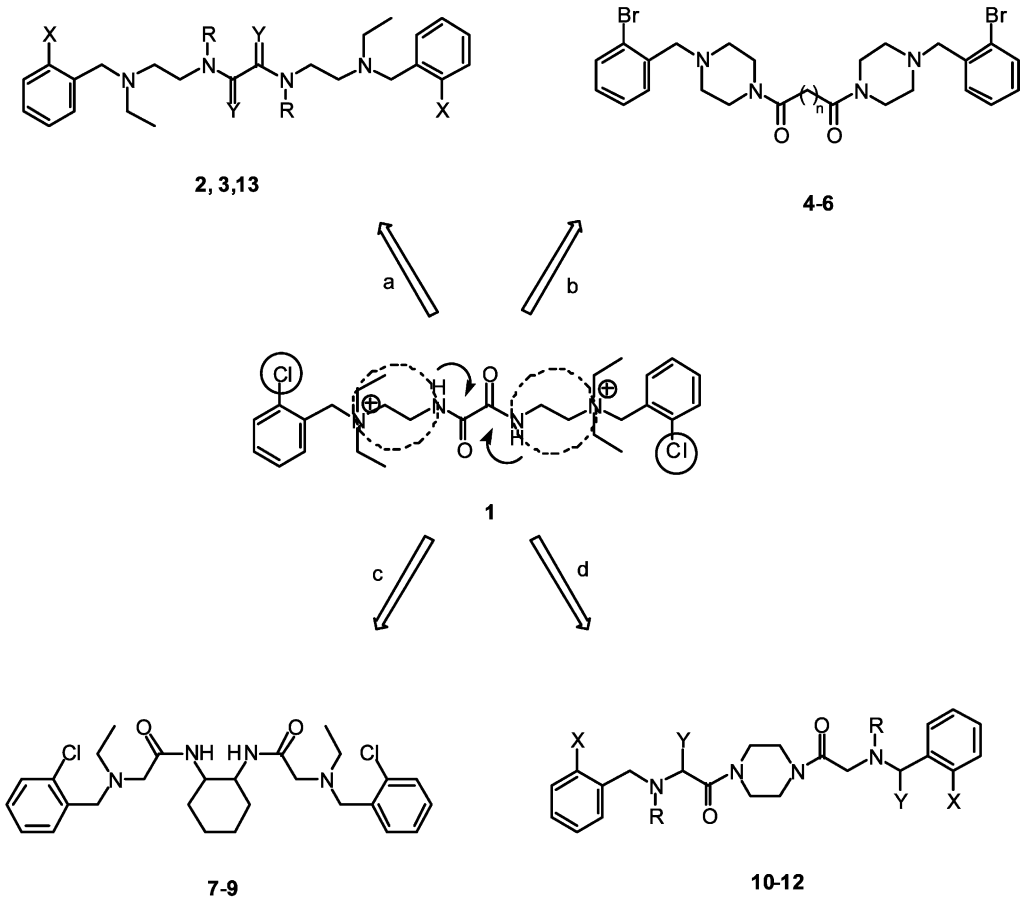
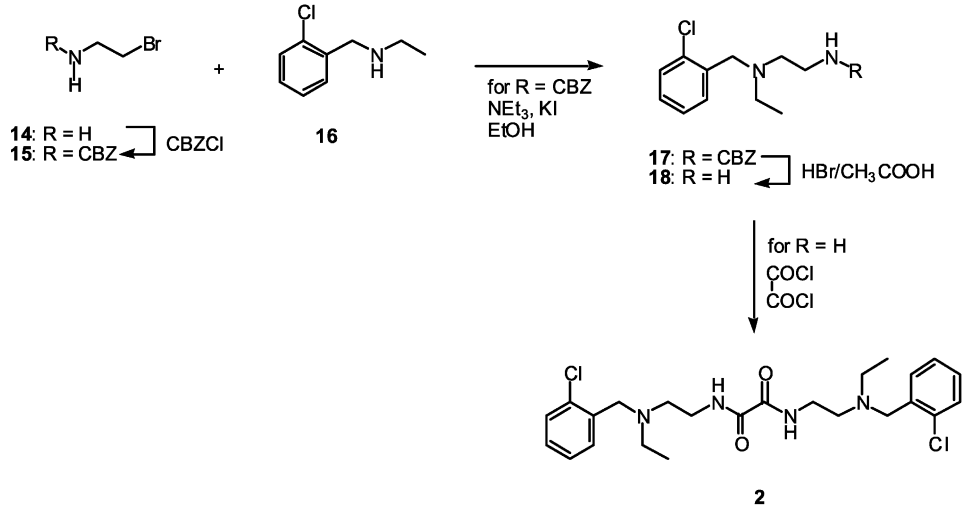
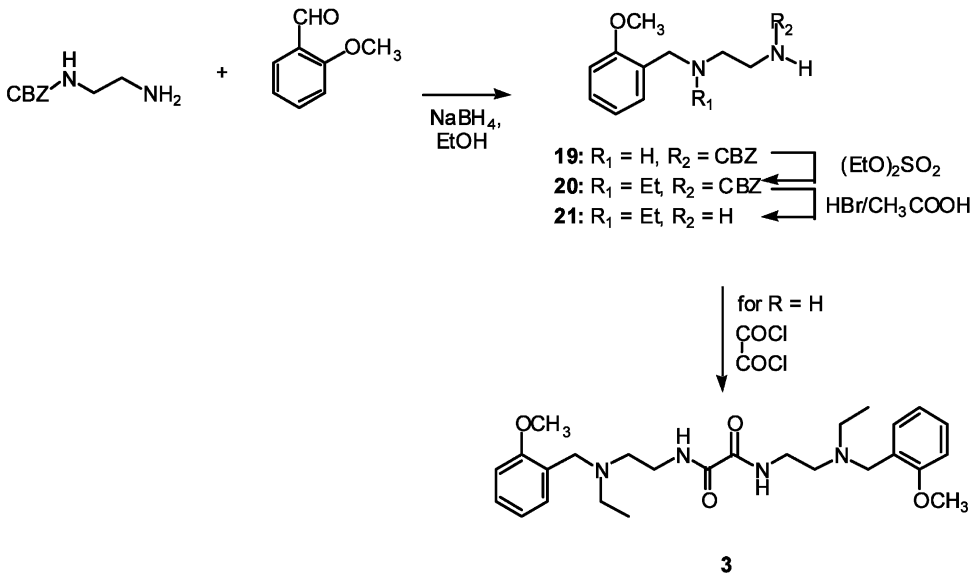


Fig. 1. Design strategy for the synthesis of ambenonium (**1**) derivatives: one ethyl group has been deleted from each ammonium quaternary center (a); the diamine functions have been incorporated into a piperazine cycle and the distance between the nitrogens has been varied (b); the oxamide function has been reversed and the amide nitrogen included in or linked to a cycle (c and d).

and with Trp279 of the peripheral site by means of a π-cation interaction. Furthermore, other relevant interactions between **1** and AChE were detected, providing the molecular basis of **1** inhibitory activity. In particular, we pointed out further π-cation interactions with Tyr330, and Tyr70, whereas H-bonds were identified with Tyr121, Tyr70, and Asp72. In the latter, the interaction was also reinforced by the formal charge of −1 of



Scheme 1.



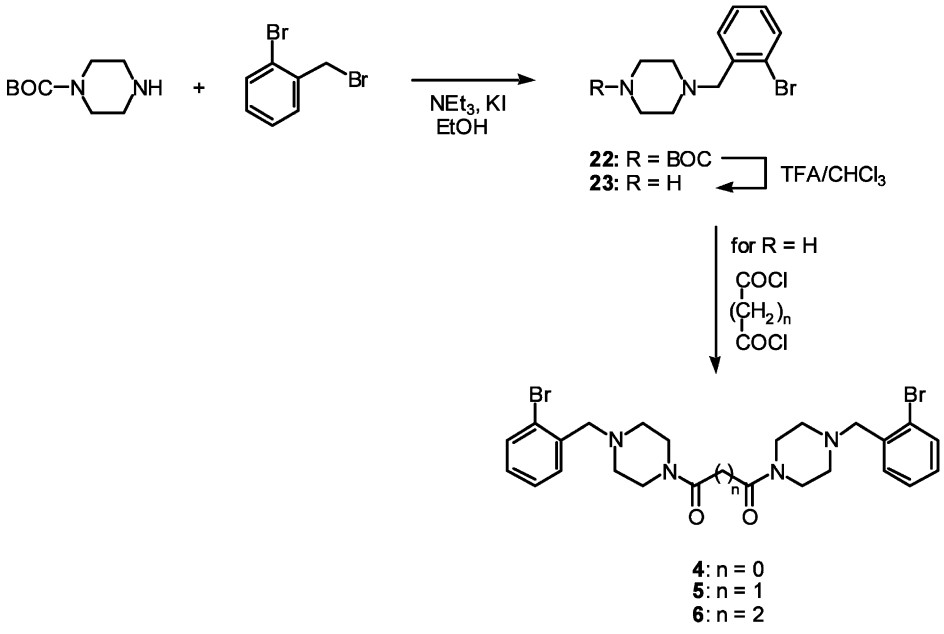
Scheme 2.

Asp72, providing a tighter H-bond. Eventually, an electrostatic interaction was detected between a quaternary ammonium group of **1** and Asp276.

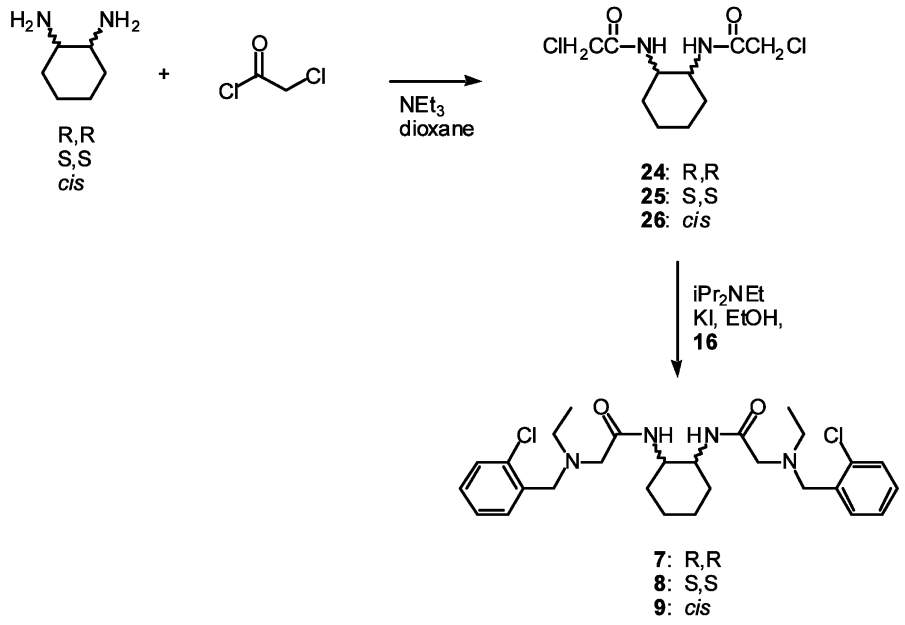
Fig. 2 clearly points out that **1** is able establishing highly favorable contacts with several AChE amino acids of both the central and the peripheral enzyme sites, thus providing a possible explanation for the striking potency of such inhibitor.

These theoretical studies strengthened our basic idea to design a new series of lipophilic ambenonium derivatives, having access to the central nervous system. The biological properties of the new compounds **2**–**13** were investigated by determining the inhibitory potencies on AChE and BChE from human erythrocytes and serum and are reported in Table 1 in comparison with that of prototype **1**.

An analysis of the results reveals that unfortunately the replacement of the quaternary ammonium functions of **1** with tertiary amines, affording **2** (tertiary ambenonium) and **3**, caused a dramatic loss of affinity, being the new derivative more than four orders of magnitude less potent than the lead. This finding clearly indicates that the presence of two ammonium centers is an essential requirement for the binding with the enzyme and that



Scheme 3.



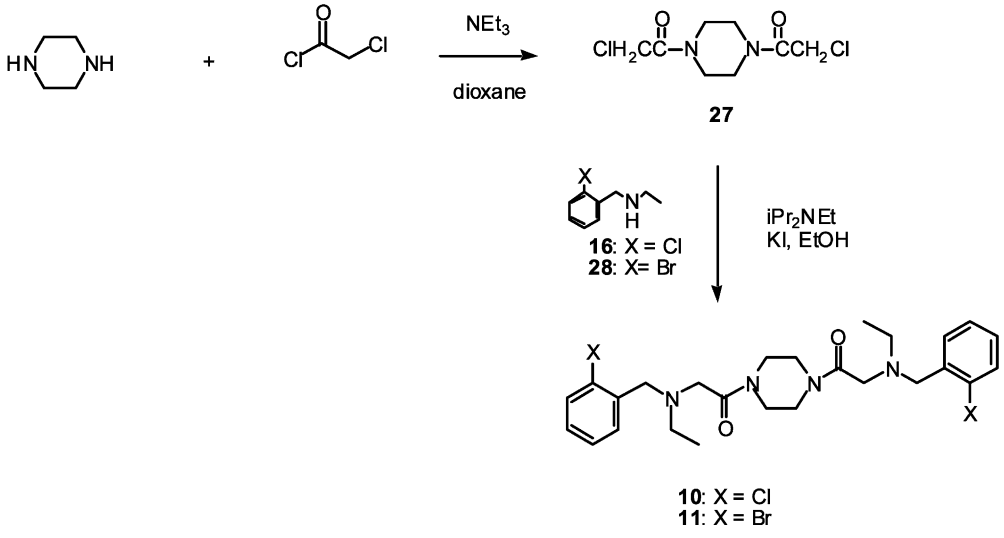
Scheme 4.

tertiary amine functions, although protonated at physiological pH [7], might establish a different binding mode with the target.

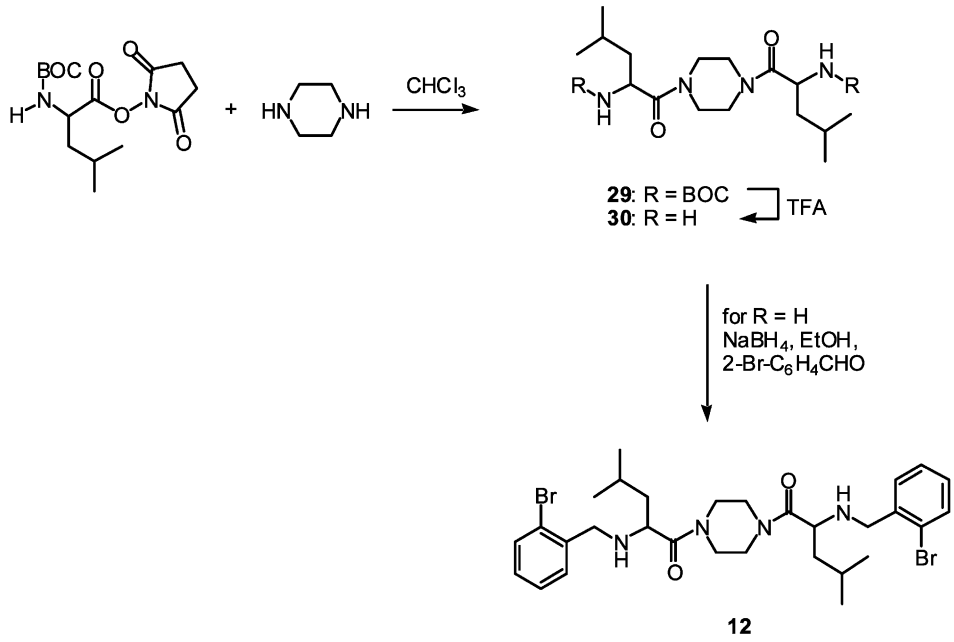
The insertion of the ethylenediamine functions into a piperazine cycle was detrimental for the activity: compounds **4**–**6**, with different distances between the nitrogens, were not able to inhibit the enzyme up to 1 mM concentration, suggesting that a certain degree of flexibility in the diamine moiety is an important determinant for the recognition of AChE.

Next, the transformation of the oxamide in cyclic amides gave raise to interesting results: in the series of the cyclohexane diamides **7**–**9**, only the *cis* stereoisomer **9** retained good inhibitory potency, comparable to that exhibited by tertiary ambenonium (**2**), whereas the other stereoisomers were devoid of affinity. To explain these experimental data, a superimposition between the conformation of **1**, as obtained from MDs simulations, and the *cis* (**9**) and the *trans* (**8**) derivatives was carried out. Low energy conformations of each molecule were selected and fitted onto **1**, as shown in Fig. 3. It is evident that the overlap is not perfect for the *trans* stereoisomer, whereas a good overall superimposition is shown for **1** and **9**, confirming that the spatial arrangement of the diamide moiety of **9** better resembles that of **1**.

Cyclization of the oxamide moiety in a piperazine ring afforded compounds **10** and **11**, differing in the sub-



Scheme 5.

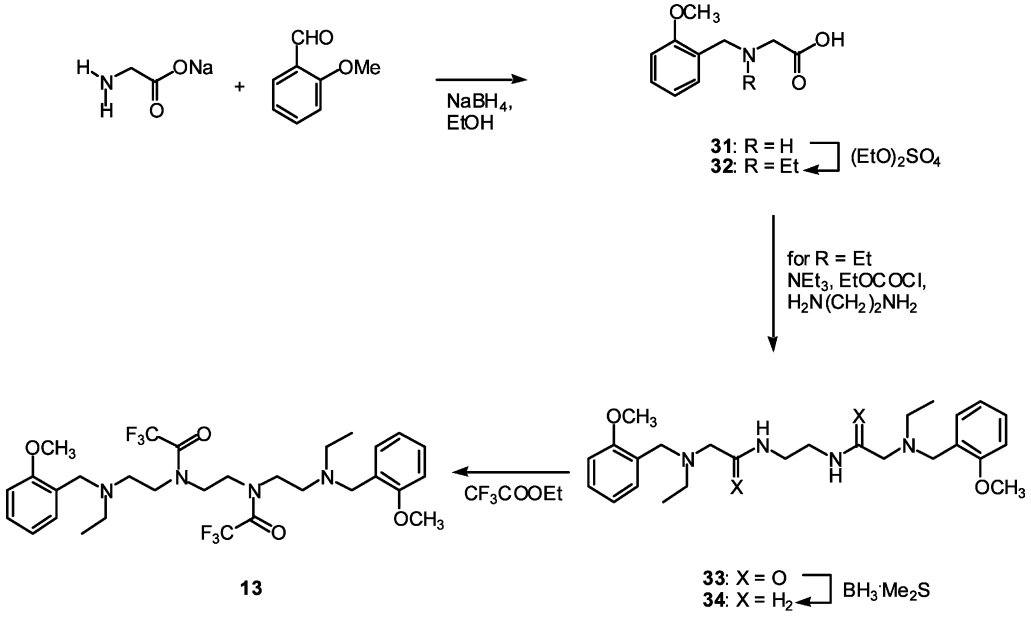


Scheme 6.

stituent at ortho position of the phenyl. Again, these compounds displayed similar antagonist potencies for AChE and comparable to that of **2**.

Derivative **12**, obtained through amidation of the amino acid Leu with piperazine, showed an intriguing affinity profile: in fact, notwithstanding the presence of a secondary amine, which is known not conferring optimum interaction with the enzyme, it is endowed of inhibitory activity in the micromolar range. This finding suggests that the isobutyl tail may interact with apolar residues of the enzyme.

Interestingly enough, transformation of the oxamide function of **3** in trifuoroacetamides, affording **13**, did not affect potency negatively: **13** was slightly more active than **3** at both AChE and BChE. Regarding the inhibitory potency toward BChE, all of the tested compounds, unlike the prototype, were not able to discriminate between the two enzymes. The most potent compound of the series was the *cis*-derivative **9**, with an IC_{50} value of 3.73 ± 0.24 μM. This result may be interesting because of the recent findings [9,10] on the role of BChE in AD, which is viewed as a 'pathological



Scheme 7.

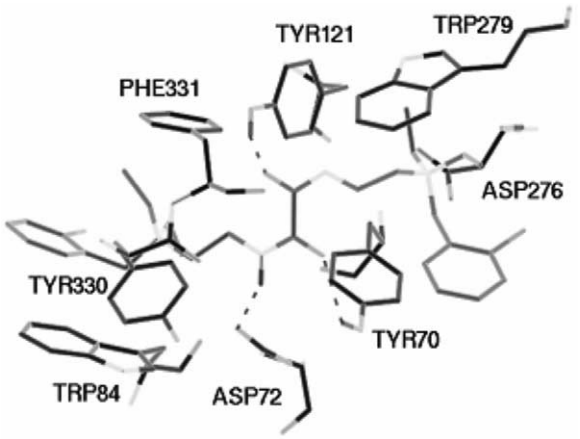


Fig. 2. The binding mode of ambenonium (**1**) within the AChE gorge. A minimized average conformation over the last 60 ps of MD simulations is shown. Hydrogen atoms explicitly involved in H-bonds (dotted lines) are shown.

cholinesterase', acting under conditions of decreased AChE activity. To this end, our compounds, that are endowed with a well-balanced profile of AChE/BChE inhibition, may represent valuable candidates for further development.

4. Experimental

4.1. Chemistry

Melting points were taken in glass capillary tubes on a Buechi SMP-20 apparatus and are uncorrected. IR, electron impact (EI) mass and NMR spectra were recorded on Perkin–Elmer 297, VG 7070E, and Varian VXR 300 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to TMS, and spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Although IR spectra data are not included (because of the lack of unusual features), they were obtained for all compounds reported and were consistent with the assigned structures. The elemental compositions of the compounds agreed to within $\pm 0.4\%$ of the calculated value. When the elemental analysis is not included, crude compounds were used in the next step without further purification. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F254) that were visualized in an iodine chamber. The term 'dried' refers to the use of anhydrous Na_2SO_4.

4.1.1. (2-Bromo-ethyl)-carbamic acid benzyl ester (**15**)

A solution of benzyl chloroformate (1.56 ml, 10 mmol) was added dropwise to a solution of 2-bromo-ethylamine hydrobromide (**14**) (2 g, 10 mmol) in 1 M NaOH (20 ml) and the reaction mixture was vigorously stirred for 30 min. The formed solid was collected and dissolved in $CHCl_3$ (40 ml). The obtained solution was then washed with 1 N HCl (2×30 ml) and water (2×30 ml). The organic phase was dried and evaporated to give **15** as a solid, which was triturated with ether: 66% yield; m.p. 42 °C; ^{1}H NMR ($CDCl_3$): δ 3.48 (t, 2H), 3.62 (q, 2H), 5.13 (s, 2H), 5.17 (br s, 1H exchangeable with D_2O), 7.39–7.50 (m, 5H).

4.1.2. {2-[(2-Chloro-benzyl)-ethyl-amino]-ethyl}-carbamic acid benzyl ester (**17**)

A mixture of **15** (550 mg, 2.13 mmol), **16** (399 mg, 1.94 mmol), NEt_3 (0.28 ml, 2.13 mmol) and KI (161 mg, 0.97 mmol) in absolute EtOH was refluxed for 16 h. Removal of the solvent gave a residue that was purified by flash chromatography. Eluting with EtOAc/cyclohexane (3:7) gave **17** as transparent oil: 26% yield; ^{1}H NMR ($CDCl_3$): δ 0.94 (t, 3H), 2.40–2.57 (m, 4H), 3.16 (q, 2H), 3.58 (s, 2H), 5.00 (s, 2H), 5.21 (br s, 1H exchangeable with D_2O), 7.02–7.37 (m, 9H).

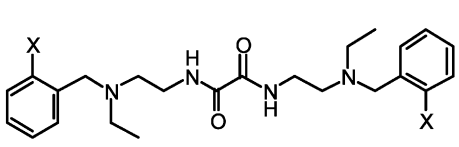
4.1.3. N1-(2-Chloro-benzyl)-N1-ethyl-ethane-1,2-diamine (**18**)

A solution of **17** (175 mg, 0.51 mmol) and 30% HBr in CH_3COOH (2 ml) in CH_3COOH (6 ml) was stirred for 3 h. Ether (10 ml) was added yielding a solid that was dissolved in water. The aqueous solution was washed with CH_2Cl_2 (1×10 ml), made basic with K_2CO_3 and extracted with CH_2Cl_2 (3×15 ml). The combined organic extracts were dried and evaporated to give **18** as yellow oil: 95% yield; ^{1}H NMR ($CDCl_3$): δ 1.03 (t, 3H), 2.45–2.60 (m, 4H), 2.69 (t, 2H), 3.63 (s, 2H), 7.08–7.32 (m, 3H), 7.46 (d, 1H).

4.1.4. N,N′-Bis-{2-[(2-chloro-benzyl)-ethyl-amino]-ethyl}-oxalamide dihydrochloride (**2**)

A solution of oxalyl chloride (0.022 ml, 0.25 mmol) in dioxane (2 ml) was added dropwise at 10 °C to a solution of **18** (95 mg, 0.45 mmol) in dioxane (3 ml). The mixture was stirred for 1 h and the formed solid was collected by filtration and purified by flash chromatography. Eluting with $CHCl_3$/MeOH/aq. 28% NH_3 (9:1:0.1) gave **2** as the free base that was converted into the dihydrochloride salt: 59% yield; m.p. 197 °C (EtOH/Et_2O); ^{1}H NMR ($CDCl_3$; free base): δ 1.04 (t, 6H), 2.53 (q, 8H), 2.62–2.70 (m, 4H), 3.66 (s, 4H), 7.12–7.34 (m, 6H), 7.51 (d, 2H). EI MS *m/e* (relative intensity) 478 (M^+, 1), 182 (100).

Table 1
Inhibition of AChE and BChE activities by ambenonium-related compounds



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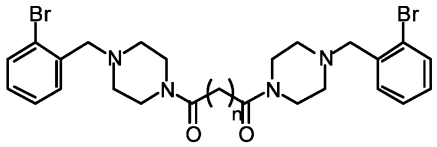

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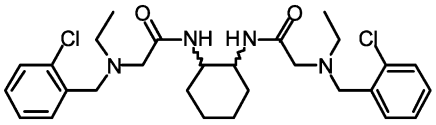

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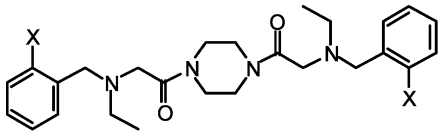

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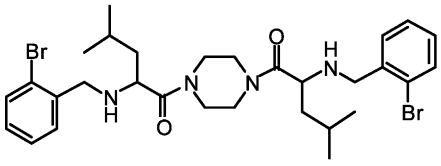

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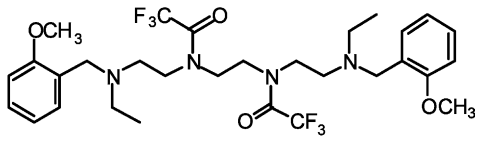

13

no.	Stereoisomer	X	*n*	IC_{50} (μM) [a]	
				AChE	BchE
1				0.000698 ± 0.00004	8.20 ± 0.44
2		Cl		28.0 ± 3.1	nd [b]
3		OMe		11.0 ± 1.2	21.5 ± 0.80
4		Br	0	> 1000	Nd
5		Br	1	> 1000	Nd
6		Br	2	> 1000	Nd
7	*R*,*R*			> 1000	Nd
8	*S*,*S*			> 1000	Nd
9	*cis*			64.8 ± 7.0	3.73 ± 0.24
10		Cl		4.81 ± 0.35	4.96 ± 0.31
11		Br		4.72 ± 0.55	4.09 ± 0.25
12				8.02 ± 0.37	Nd
13				4.4 ± 0.40	6.10 ± 0.25

[a] AChE and BChE were from human erythrocytes. pIC_{50} values represent the negative logarithm of the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements, each performed in triplicate.
[b] nd, not determined.

*4.1.5. [2-(2-Methoxy-benzylamino)-ethyl]-carbamic acid benzyl ester (**19**)*

A mixture of *N*-CBZ-1,2-diaminoethane (720 mg, 3.7 mmol), molecular sieves (3 Å) and 2-methoxybenzaldehyde (760 mg, 5.6 mmol) was stirred for 30 min at room temperature (r.t.), and then $NaBH_4$ (0.21 g, 5.6 mmol) was added and the stirring was continued for further 14 h. Following removal of molecular sieves, the solution was made acidic with 3 N HCl. Removal of the solvent gave a residue which was dissolved in water (50 ml). The aqueous solution was washed with ether (3 × 30 ml), made basic with 35% NaOH and extracted with CH_2Cl_2 (3 × 30 ml). The combined organic extracts were dried and evaporated to give **19** as yellow oil: 80% yield; ^1H NMR ($CDCl_3$): δ 1.59 (br s, 1H exchangeable with D_2O), 2.71 (t, 2H), 3.29 (q, 2H), 3.75 (s, 2H), 3.80 (s, 3H), 5.09 (s, 2H), 5.38 (br s, 1H exchangeable with D_2O), 6.84–6.92 (m, 2H), 7.13–7.36 (m, 7H).

*4.1.6. {2-[Ethyl-(2-methoxy-benzyl)-amino]-ethyl}-carbamic acid benzyl ester (**20**)*

Diethyl sulfate (0.75 ml, 5.7 mmol) was added dropwise to a stirred solution of **19** (890 mg, 6.5 mmol) in toluene (50 ml) and the resulting mixture was refluxed for 24 h. After cooling at r.t., the mixture was shaken with concd. NaOH (50 ml). The organic layer was separated, dried and evaporated to give a residue, which was purified by flash chromatography. Eluting with CH_2Cl_2/petroleum ether/MeOH/aq. 28% NH_3 (4.6:5.0:0.35:0.035) afforded **20** as an oil: 60%

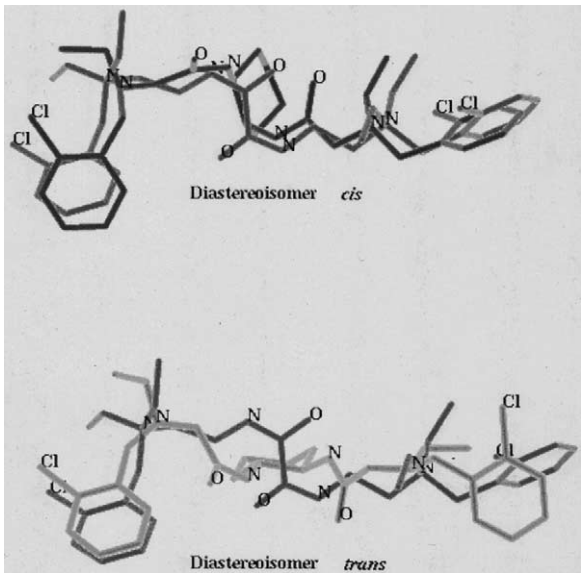

Fig. 3. Superimposition of **9** and **8** (trans) onto **1** (black).

yield; ^{1}H NMR ($CDCl_3$, free base): δ 1.04 (t, 3H), 2.50–2.66 (m, 4H), 3.34 (q, 2H), 3.61 (s, 2H), 3.81 (s, 3H), 5.14 (s, 2H), 5.70 (br s, 1H exchangeable with D_2O), 6.86–6.98 (m, 2H), 7.23–7.43 (m, 7H).

*4.1.7. N1-Ethyl-N1-(2-methoxy-benzyl)-ethane-1,2-diamine (**21**)*

It was obtained as an oil by deprotection of **20** following the procedure described for **18**: 75% yield; ^{1}H NMR ($CDCl_3$): δ 1.05 (t, 3H), 2.09 (br s, 2H exchangeable with D_2O), 2.50–2.61 (m, 4H), 2.72–2.82 (m, 2H), 3.60 (s, 2H), 3.82 (s, 3H), 6.83–6.98 (m, 2H), 7.18–7.43 (m, 2H).

*4.1.8. N,N′-Bis-{2-[ethyl-(2-methoxy-benzyl)-amino]-ethyl}-oxalamide dioxalate (**3**)*

It was obtained by reaction of **21** and oxalyl chloride following the procedure described for **2**. Eluting with CH_2Cl_2/EtOH/aq. 28% NH_3 (9.5:0.5:0.05) gave **3** as the free base that was converted into the dioxalate salt: 30% yield; m.p. 197 °C (EtOH/Et_2O); ^{1}H NMR ($CDCl_3$; free base): δ 1.04 (t, 6H), 2.45–2.65 (m, 8H), 3.41 (q, 4H), 3.63 (s, 4H), 3.84 (s, 6H), 6.84–6.99 (m, 4H), 7.20–7.42 (m, 4H), 7.98 (br s, 2H exchangeable with D_2O).

*4.1.9. 4-(2-Bromo-benzyl)-piperazine-1-carboxylic acid tert-butyl ester (**22**)*

It was obtained as an oil by reaction of 1-BOC-piperazine and 2-bromo benzyl bromide following the procedure described for **17**: 85% yield; ^{1}H NMR ($CDCl_3$): δ 1.39 (s, 9H), 2.82 (s, 4H), 3.63 (s, 4H), 4.10 (s, 2H), 7.13 (t, 1H), 7.31 (q, 1H), 7.51 (d, 1H), 7.82 (d, 1H).

*4.1.10. 1-(2-Bromo-benzyl)-piperazine (**23**)*

A solution of **22** (780 mg, 2.2 mmol) and TFA (5.5 ml) in $CHCl_3$ was stirred at r.t. for 3 h. The solvent was evaporated yielding a solid that was dissolved in water (30 ml). The resulting solution was then washed with ether (3 × 20 ml), made basic with K_2CO_3, and extracted with $CHCl_3$ (3 × 20 ml). The organic extracts were dried and evaporated to give **23** as an oil: 77% yield; ^{1}H NMR ($CDCl_3$): δ 1.50 (s, 1H exchangeable with D_2O), 2.38 (t, 4H), 2.57 (t, 4H), 3.46 (s, 2H), 7.00 (t, 1H), 7.18 (t, 1H), 7.37 (t, 2H).

*4.1.11. 1,2-Bis-[4-(2-bromo-benzyl)-piperazin-1-yl]-ethane-1,2-dione dihydrochloride (**4**)*

It was obtained by reaction of **23** and oxalyl chloride following the procedure described for **2**. The formed solid was purified by crystallization from *i*-PrOH/MeOH: 50% yield; m.p. 185 °C; ^{1}H NMR ($CDCl_3$; free base): δ 2.53 (t, 8H), 3.43 (t, 4H), 3.65 (t, 8H), 7.07–7.19 (t, 2H), 7.24–7.35 (t, 2H), 7.39–7.47 (d, 2H), 7.52–7.90 (d, 2H). EI MS *m/e* (relative intensity) 564 (M^+, 4), 169 (100).

*4.1.12. 1,3-Bis-[4-(2-bromo-benzyl)-piperazin-1-yl]-propane-1,3-dione dihydrochloride (**5**)*

It was obtained by reaction of **23** and malonyl chloride following the procedure described for **2**. Eluting with EtOAc/petroleum ether/MeOH/aq. 28% NH_3 (5:4:1:0.05) gave **5** as the free base that was converted into the dihydrochloride salt: 53% yield; m.p. 205 °C (EtOH/Et_2O); ^{1}H NMR ($CDCl_3$; free base): δ 2.50 (q, 8H), 3.50 (s, 2H), 3.53–3.68 (m, 12H), 7.10 (t, 2H), 7.21 (t, 2H), 7.42 (d, 2H), 7.53 (d, 2H). EI MS *m/e* (relative intensity) 578 (M^+, 1), 169 (100).

*4.1.13. 1,4-Bis-[4-(2-bromo-benzyl)-piperazin-1-yl]-butane-1,4-dione dihydrochloride (**6**)*

It was obtained by reaction of **23** and succinyl chloride following the procedure described for **2**. The formed solid was purified by crystallization from *i*-PrOH/MeOH; 69% yield; m.p. 270 °C; ^{1}H NMR (D_2O): δ 2.71 (s, 4H), 3.34–3.57 (m, 8H), 3.61–3.98 (m, 8H), 4.55 (s, 4H), 7.33–7.52 (d, 4H), 7.73–7.81 (d, 4H). EI MS *m/e* (relative intensity) 592 (M^+, 10), 169 (100).

*4.1.14. 2-Chloro-N-[2-(2-chloro-acetylamino)-cyclohexyl]-acetamide (R,R) (**24**)*

A solution of chloroacetyl chloride (0.66 ml, 8.3 mmol) in dioxane (5 ml) was added at 5 °C to a solution of (1*R*,2*R*)-1,2-diaminocyclohexane (500 mg, 4.4 mmol) and NEt_3 (1.16 ml, 8.3 mmol) in dioxane (15 ml) and the reaction mixture was stirred at r.t. for 2 h. The formed NEt_3 hydrochloride was filtered off and the solution was evaporated in vacuo yielding a residue that was dissolved in CH_2Cl_2 (30 ml). The obtained solution was then washed with water (2 × 20 ml), with 20% aq.

Na_2CO_3 (2 × 20 ml) and 0.5 N HCl (2 × 20 ml). The organic phase was dried and evaporated to give **24** as a solid: 47% yield; m.p. 230 °C; ^{1}H NMR ($CDCl_3$): δ 1.33 (d, 4H), 1.73–1.84 (m, 2H), 1.98–2.11 (m, 2H), 3.66–3.71 (m, 2H), 4.01 (s, 4H), 6.80 (br s, 2H).

*4.1.15. 2-Chloro-N-[2-(2-chloro-acetylamino)-cyclohexyl]-acetamide (S,S) (**25**)*

It was obtained by reaction of (1*S*,2*S*)-1,2-diaminocyclohexane and chloroacetyl chloride following the procedure described for **24**: 70% yield.

*4.1.16. 2-Chloro-N-[2-(2-chloro-acetylamino)-cyclohexyl]-acetamide (cis) (**26**)*

It was obtained by reaction of *cis*-1,2-diaminocyclohexane and chloroacetyl chloride following the procedure described for **24**: 25% yield; m.p. 137 °C; ^{1}H NMR ($CDCl_3$): δ 1.49–1.72 (m, 6H), 1.84 (br s, 2H), 4.00–4.26 (m, 6H), 7.00 (br s, 2H).

*4.1.17. 2-[(2-Chloro-benzyl)-ethyl-amino]-N-(2-{2-[(2-chloro-benzyl)-ethyl-amino]-acetylamino}-cyclohexyl)-acetamide (R,R) dihydrochloride (**7**)*

A mixture of **16** (300 mg, 1.46 mmol), **24** (194 mg, 0.73 mmol), *i*-Pr_2NEt (0.51 ml, 2.92 mmol) and KI (61 mg, 0.37 mmol) in absolute EtOH (15 ml) was refluxed for 72 h. Removal of the solvent gave a residue that was dissolved in CH_2Cl_2 (30 ml). The obtained solution was then washed with water (2 × 20 ml) and extracted with 1 N HCl (2 × 20 ml). The aqueous phase was made basic with K_2CO_3 and extracted with CH_2Cl_2 (3 × 30 ml). Evaporation of the dried solvent gave **7** as the free base that was converted into the dihydrochloride salt (hygroscopic): 40% yield; ^{1}H NMR (DMSO-d_6/D_2O): δ 1.16 (t, 10H), 1.54–1.66 (m, 4H), 3.12 (d, 4H), 3.41 (br s, 2H), 3.64 (q, 4H), 4.53 (s, 4H), 7.32–7.48 (m, 6H), 7.57 (d, 2H). EI MS *m/e* (relative intensity) 532 (M^+, 3), 182 (100).

*4.1.18. 2-[(2-Chloro-benzyl)-ethyl-amino]-N-(2-{2-[(2-chloro-benzyl)-ethyl-amino]-acetylamino}-cyclohexyl)-acetamide dihydrochloride (S,S) (**8**)*

It was obtained by reaction of **16** and **25** following the procedure described for **7**: 70% yield.

*4.1.19. 2-[(2-Chloro-benzyl)-ethyl-amino]-N-(2-{2-[(2-chloro-benzyl)-ethyl-amino]-acetylamino}-cyclohexyl)-acetamide dihydrochloride (cis) (**9**)*

It was obtained by reaction of **16** and **26** following the procedure described for **7**: 57% yield; ^{1}H NMR (DMSO-d_6/D_2O): δ 1.13–1.42 (m, 10H), 1.56 (br s, 6H), 3.20 (br s, 2H), 4.00 (br s, 6H), 4.59 (br s, 4H), 7.39–7.61 (m, 6H), 7.81 (br s, 2H). EI MS *m/e* (relative intensity) 532 (M^+, 2), 182 (100).

*4.1.20. 2-Chloro-1-(4-chloroacetyl-piperazin-1-yl)-ethanone (**27**)*

It was obtained by reaction of piperazine and chloroacetyl chloride following the procedure described for **24**: 30% yield; m.p. 118 °C; ^{1}H NMR ($CDCl_3$): δ 3.43–3.73 (m, 8H), 4.09 (s, 4H).

*4.1.21. 2-[(2-Chloro-benzyl)-ethyl-amino]-1-(4-{[(2-chloro-benzyl)-ethyl-amino]-acetyl}-piperazin-1-yl)-ethanone dihydrochloride (**10**)*

It was obtained by reaction of **16** and **27** following the procedure described for **7**: 41% yield; m.p. 200 °C; ^{1}H NMR (DMSO-d_6): δ 1.29–1.35 (m, 6H), 3.20–3.36 (m, 12H), 4.40–4.55 (m, 8H), 7.49–7.58 (m, 6H), 7.78–7.89 (m, 2H), 9.95 (br s, 2H exchangeable with D_2O).

*4.1.22. 2-[(2-Bromo-benzyl)-ethyl-amino]-1-(4-{[(2-chloro-benzyl)-ethyl-amino]-acetyl}-piperazin-1-yl)-ethanone dihydrochloride (**11**)*

It was obtained by reaction of **28** and **27** following the procedure described for **7**: 45% yield; m.p. 290 °C; ^{1}H NMR (DMSO-d_6): δ 1.32 (t, 6H), 3.22–3.57 (m, 12H), 4.45 (s, 4H), 4.57 (s, 4H), 7.42–7.94 (m, 8H), 9.96 (br s, 2H exchangeable with D_2O). EI MS *m/e* (relative intensity) 594 (M^+, 2), 226 (100).

*4.1.23. {1-[4-(2-tert-Butoxycarbonylamino-4-methyl-pentanoyl)-piperazine-1-carbonyl]-3-methyl-butyl}-carbamic acid tert-butyl ester (**29**)*

A solution of *N*-terbutyloxycarbonyleucine-*N*-hydroxy-succinimmide (500 mg, 1.5 mmol) in $CHCl_3$ (10 ml) was added dropwise to a solution of piperazine (72 mg, 0.84 mmol) in 15 ml of $CHCl_3$. The resulting mixture was stirred at r.t. for 18 h, then washed with 1 M $KHSO_4$ (2 × 10 ml) and water (2 × 10 ml), dried and evaporated to give **29** as an oil: 72% yield; ^{1}H NMR ($CDCl_3$): δ 0.87 (q, 12H), 1.52–1.75 (m, 20H), 2.75 (s, 2H), 3.12–3.90 (m, 8H), 4.48–4.67 (m, 2H), 5.31 (d, 2H exchangeable with D_2O).

*4.1.24. 2-Amino-1-[4-(2-amino-4-methyl-pentanoyl)-piperazin-1-yl]-4-methyl-pentan-1-one (**30**)*

It was obtained as an oil by deprotection of **29** following the procedure described for **23**: 95% yield; ^{1}H NMR ($CDCl_3$): δ 0.87 (d, 12H), 1.12–1.36 (m, 6H), 1.70 (s, 4H exchangeable with D_2O), 3.32–3.70 (m, 10H).

*4.1.25. 2-(2-Bromo-benzylamino)-1-{4-[2-(2-bromo-benzylamino)-4-methyl-pentanoyl]-piperazin-1-yl}-4-methyl-pentan-1-one dihydrochloride (**12**)*

It was obtained by reaction of **30** and 2-bromobenzaldehyde following the procedure described for **19**: 45% yield; m.p. > 290 °C (MeOH/*i*-PrOH); ^{1}H NMR (D_2O): δ 0.89 (q, 12H), 1.67 (m, 6H), 3.20–3.53 (m, 8H), 4.20–

4.50 (m, 6H), 7.29–7.52 (m, 6H), 7.63–7.89 (t, 2H). EI MS *m/e* (relative intensity) 397 (M^+, 4), 254 (100).

4.1.26. (2-Methoxy-benzylamino)-acetic acid (**31**)

It was obtained by reaction of glycine sodium salt and 2-methoxybenzaldehyde following the procedure described for **19**: 35% yield; m.p. 141–143 °C; ^{1}H NMR (D_2O): δ 3.38 (s, 2H), 3.71 (s, 3H), 4.06 (s, 2H), 6.80–6.97 (m, 2H), 7.11–7.18 (m, 1H), 7.21–7.33 (1H).

4.1.27. [Ethyl-(2-methoxy-benzyl)-amino]-acetic acid (**32**)

It was obtained by reaction of **31** and diethyl sulfate following the procedure described for **20**: 20% yield; ^{1}H NMR ($CDCl_3$): δ 1.09 (t, 3H), 2.78 (q, 2H), 3.22 (s, 2H), 3.80 (s, 3H), 3.92 (s, 2H), 6.92–7.04 (m, 4H), 7.27–7.42 (m, 1H).

4.1.28. 2-(2-Methoxy-benzylamino)-N-{2-[2-(2-methoxy-benzylamino)-acetylamino]-ethyl}-acetamide (**33**)

Ethyl chlorocarbonate (0.30 ml, 2.9 mmol) in dry dioxane (10 ml) was added dropwise to a stirred and cooled (5 °C) solution of **32** (0.63 g, 2.9 mmol) and NEt_3 (0.81 ml, 5.8 mmol) in dioxane (50 ml), followed after standing for 15 min by the addition of ethylenediamine (0.097 ml, 1.5 mmol) in dioxane (20 ml). After stirring at r.t. for 24 h, the mixture was evaporated, affording a residue that was suspended in water (100 ml). The aqueous mixture was extracted with $CHCl_3$ (4 × 20 ml). The organic phase was washed with 2 N NaOH, 2 N HCl and brine. Removal of the dried solvent gave a yellow oil that was purified by flash chromatography. Eluting with CH_2Cl_2/EtOH/aq. 28% NH_3 (9.5:0.5:0.04) afforded **33** as a low melting solid: 65% yield; ^{1}H NMR ($CDCl_3$): δ 1.03 (t, 6H), 2.56 (q, 4H), 3.08 (s, 4H), 3.13–3.19 (m, 4H), 3.61 (s, 4H), 3.89 (s, 6H), 6.87–7.00 (m, 4H), 7.19–7.38 (m, 4H), 7.92 (br s, 2H exchangeable with D_2O).

4.1.29. N-(2-Methoxy-benzyl)-N′-{2-[2-(2-methoxy-benzylamino)-ethylamino]-ethyl}-ethane-1,2-diamine (**34**)

A solution of 10 M $BH_3 \cdot CH_3SCH_3$ (0.55 ml, 5.5 mmol) in dry diglyme (5 ml) was added dropwise at 5 °C to a solution of **33** (460 mg, 0.98 mmol) in dry diglyme (35 ml) with stirring under a stream of dry nitrogen. When the addition was completed, the reaction mixture was heated at 120 °C for 10 h. After cooling at 0 °C, excess borane was destroyed by cautious addition of MeOH (5 ml). The resulting mixture was left to stand overnight at r.t., cooled at 0 °C, treated with HCl gas for 10 min, and then heated at 120 °C for 3 h. After cooling, the formed solid was filtered, dissolved in water. The aqueous solution was made basic with NaOH pellets, extracted with CH_2Cl_2 (4 × 20 ml), dried and evaporated to give a residue that was purified by flash chromatography. Eluting with CH_2Cl_2/EtOH/aq. 28% NH_3 (9:1:0.1) gave **34** as an oil: 30% yield; ^{1}H NMR ($CDCl_3$): δ 1.02 (t, 6H), 2.18 (br s, 2H exchangeable with D_2O), 2.48–2.68 (m, 16H), 3.58 (s, 4H), 3.80 (s, 6H), 6.82–6.93 (m, 4H), 7.19–7.36 (m, 4H).

4.1.30. 2,2,2-Trifluoro-N-[2-(2-methoxy-benzylamino)-ethyl]-N-(2-{[2-(2-methoxy-benzylamino)-ethyl]-trifluoroacetyl-amino}-ethyl)-acetamide dioxalate (**13**)

Ethyl trifluoroacetate (0.24 ml, 2.04 mmol) was added to a solution of **34** (90 mg, 0.2 mmol) in dry MeOH (10 ml) and the reaction mixture was stirred at r.t. for 3 days. Evaporation of the solvent gave a residue that was purified by flash chromatography. Eluting with CH_2Cl_2/EtOH/aq. 28% NH_3 (9.5:0.5:0.05) afforded **13** as a free base which was transformed into the dioxalate salt: 30% yield; ^{1}H NMR ($CDCl_3$, free base): δ 1.03 (t, 6H), 2.56–2.68 (m, 12H), 3.40–3.60 (m, 8H), 3.80 (s, 6H), 6.87–7.00 (m, 4H), 7.19–7.38 (m, 4H).

4.2. Biology

4.2.1. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)

The method of Ellmann et al. was followed [11]. Five different concentrations of each compound were used in order to obtain inhibition of AChE or BChE activity comprised between 20 and 80%. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,5′-dithio-bis(2-nitrobenzoic acid), 0.035 U/ml AChE or BChE derived from human erythrocytes or human serum (0.5 and 3.4 U.I./mg, respectively; Sigma Chemical), and 550 μM acetylthiocoline iodide. Test compounds were added to the assay solution and preincubated with the enzyme for 20 min followed by the addition of substrate. Assays were done with a blank containing all components except AChE or BChE in order to account for non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analysed in triplicate, and IC_{50} values were determined graphically from log concentration–inhibition curves.

4.3. Molecular modeling

The modeling procedure of the AChE–ambenonium complex was performed by means of the MacroModel Ver 5.5 software package [12]. The energy calculations were carried out with MacroModel implementation of AMBER force field, i.e. AMBER*, that uses by default the united atom model [13], providing a special scheme to incorporate specific structural and energetic data directly into the force field. This feature allows to treat explicitly (all-atom model [14]) hydrogen atoms bound

to heteroatoms and aromatic carbons. In this way, we have been able taking into account also the multiple-ion contributions of the π-cation interaction. Actually, these are fundamental when modeling AChE–inhibitor complexes [15], and their major contribution is indeed accounted for by the interaction between quadrupole of aromatic rings and charge of positive groups [16].

The coordinates of the AChE–decamethonium complex were retrieved from the Protein Data Bank (PDB code 1ACL [17]). Ambenonium was properly built and docked into the enzyme active site, taking advantage of AChE–decamethonium interaction model. The AChE–ambenonium complex was then submitted to a molecular modeling protocol aimed at refining the position of the inhibitor in the active site pocket. Minimization and MD simulations were carried out on a core of unconstrained atoms around the active site (8 Å) and on a shell of constrained atoms (energy penalty force constant of 100 kJ/Å^2/mol^1) surrounding the core (6 Å). An initial minimization (2000 steps, steepest descent) and a subsequent temperature constant MD simulation (100 ps, 298 K, 1.0 fs time step) were carried out. An equilibration time of 40 ps was allowed before starting the data collection. The MD average structure of the last 60 ps was energy minimized first by steepest descent and then by conjugate gradient with a derivative convergence criterion of 0.05 kJ/Å^2/mol^1. This molecular modeling protocol has already provided reliable results, when studying AChE–inhibitor complexes [18,19].

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