Homo- and hetero-bivalent edrophonium-like ammonium salts as highly potent, dual binding site AChE inhibitors

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A B S T R A C T

A number of mono- and bis-quaternary ammonium salts, containing edrophonium-like and coumarin moieties tethered by an appropriate linker, proved to be highly potent and selective dual binding site acetylcholinesterase inhibitors with good selectivity over butyrylcholinesterase. Homobivalent bis-quaternary inhibitors differing by only one methylene unit in the linker, were the most potent and selective inhibitors exhibiting a sub-nanomolar affinity (IC_{50} < 0.49 and 0.17 nM, respectively) and a high butyryl-/acetylcholinesterase affinity ratio (SI = 1465 and 4165, respectively). The corresponding hetero-bivalent coumarinic inhibitors were also endowed with excellent inhibitory potency but a lower AChE selectivity (IC_{50} = 2.1 and 1.0 nM, and SI = 505 and 708, respectively). Docking simulations enabled clear interpretation of the structure–affinity relationships and detection of key binding interactions at the primary and peripheral AChE binding sites.

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1. Introduction

In vertebrates two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), efficiently catalyze acetylcholine hydrolysis. They are distinguished on the basis of substrate specificity, tissue distribution, and sensitivity to inhibitors. AChE is predominant in the muscle and nervous system, and plays a fundamental role in impulse transmission by terminating the action of the neurotransmitter acetylcholine at the cholinergic synapses and neuromuscular junctions.

AChE inhibitors have found widespread use in the treatment of different pathologies, such as Alzheimer’s disease (AD), glaucoma, neuromuscular blockade in surgical anesthesia, and myasthenia gravis. Although the degree of similarity between AChE and BChE is high (51–54% of identity and 70–72% homology), their physiological and pathological roles only partly overlap. Therefore, the discovery of selective AChE or BChE inhibitors, and of dual inhibitors as well, is intensely pursued. Nowadays, these studies are extremely facilitated by the availability of high-resolution X-ray crystal structures of many AChE–inhibitor complexes. The three-dimensional structure of AChE has been first determined on Torpedo californica (Tc), and since then many other complexes with structurally diverse inhibitors and AChE from different species have been determined and reported in the Protein Brookhaven Database (PDB). The most interesting structural aspects of these isoenzymes are the presence of a deep narrow gorge, at the bottom of which the catalytic triad is found, and of a regulatory site, called the peripheral anionic site (PAS), at the entrance of the gorge. PAS is absent in BChE, which might explain, at least in part, some observed substrate/inhibitor specificities. Most of the reported AChE inhibitors interact with the primary or peripheral binding site, or both. Compounds such as edrophonium and tacrine (Chart 1) act exclusively at the primary binding site, whereas others such as propidium and fasciculin act at the PAS, and bis-quaternary ammonium salts (e.g., decamethonium) as well as diverse homo- and hetero-bivalent mono- and bis-protonated amines act at both.

Recently, different groups have successfully improved the enzyme affinity of monovalent AChE inhibitors, such as tacrine and (−)-huperzine A, by synthesizing homo- and hetero-bivalent derivatives with binding moieties placed at the appropriate distance to efficiently interact with both binding sites. It is worth noting that binding at the PAS can be triggered also by non-ionic interactions, that is, π-π stacking and hydrophobic interactions, as in the case of donepezil, an efficient AChE inhibitor currently used in the treatment of AD.

Some years ago, some of us published the synthesis and biological evaluation of a series of 7-substituted coumarins displaying dual AChE-monoamineoxidase (MAO) inhibitory activity. The absence of basic (protonable) or quaternary nitrogen atoms in these compounds, the prevalent hydrophobic character of the...
substituted coumarin ring, and preliminary molecular docking simulations suggested that their AChE inhibitory activity might arise from an interaction at the PAS. To recover additional and efficient binding at the primary binding site potentially capable of enhancing the low AChE inhibitory potency of our coumarin derivatives, we designed and prepared a small library of potential dual binding site AChE inhibitors, depicted in Chart 2, through a solid-phase approach.

We reasoned that an additional and efficient interaction with the primary binding site might be triggered by the introduction of an edrophonium-like moiety (a trimethyl- instead of the dimethyl-ethyl-ammonium group of edrophonium was used) at a suitable distance from the coumarin nucleus. Indeed, tethering two low-affinity ligands with a linker of appropriate chemical nature and length has resulted in a successful strategy to strongly improve protein binding affinity and lays the groundwork for a ‘fragment-based’ design strategy.15 Moreover, we designed and tested also homobivalent bis-quaternary ammonium salts containing edrophonium-like cationic moiety.

2. Chemistry

Within the strategic framework delineated above, we designed and prepared compounds 1–15 (Table 1) through solid-phase synthesis on a Wang resin, starting from a mono-TiPS-protected 3,5-dihydroxy-N-methyl (or -benzyl) aniline, as reported in Scheme 1 and already previously described by our group.16 Compound 16, a dideoxy-analogue of 14 was prepared according to a traditional solution phase synthesis as reported in Scheme 2. Since data from literature suggested that an appropriate distance between the two potentially binding moieties (i.e., coumarin and edrophonium-like

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Table 1. AChE and BChE inhibition data of compounds 1–16 and reference leads 3-HBT and DMC.

CHART 1. Chemical structures of some cited AChE inhibitors.

CHART 2. Chemical structures of targeted homo- and hetero-bivalent AChE inhibitors 1–16 and reference leads 3-HBT and DMC. (Y = H, OH; and R1 = CH3 and C6H5CH2; n = 3, 4; see Table 1).
moieties) might be reached by linking the two phenolic hydroxyls with 3 or 4 methylene groups, compounds 1–16 were prepared and tested as cholinesterase inhibitors along with two reference compounds 3-hydroxy-N,N,N-trimethylbenzamminium iodide (3-HTB) and 3,4-dimethyl-7-methoxycoumarin (DMC) (Chart 2).

3. Biological assays

Compounds 1–16 and reference leads 3-HTB and DMC, were tested as cholinesterase inhibitors on bovine acetylcholinesterase and equine serum butyrylcholinesterase according to the spectrophotometric method of Ellman. Inhibition data are reported in Table 1 as IC<sub>50</sub> mean values resulting from at least three independent measures. Less active compounds were tested at 50 or 10 μM, according to their solubility in the assay medium, and their activity, expressed as percent of inhibition at a given concentration are reported in Table 1 along with the chemical structures of all the compounds examined.

4. Results and discussion

At a glance, the data in Table 1 show that our design led to an outstanding improvement of the AChE inhibitory potencies of the separate 3-HTB and DMC moieties, which presented IC<sub>50</sub> values equal to 12,000 and 42,000 nM, respectively. Indeed, mono-quaternary (13 and 14) and bis-quaternary (11 and 12) ammonium salts were endowed with an outstanding AChE affinity (from nanomolar to sub-nanomolar IC<sub>50</sub>) and an excellent AChE selectivity (from 505 to 4165 SI, where SI is the selectivity index, that is, the IC<sub>50</sub>BChE/IC<sub>50</sub> AChE affinity ratio). Noticeably, the most active and selective AChE bis-quaternary homobivalent inhibitors 11 and 12 differ only by one methylene unit in the spacer length and their sub-nanomolar affinities are close (IC<sub>50</sub> = 0.49 and 0.17 nM, respectively). Interestingly, bis-quaternary homobivalent inhibitor 12 showed an inhibitory potency almost identical to that of Ambenonium, (IC<sub>50</sub> = 0.17 and 0.12 nM, respectively) and much better than BW284C51 (IC<sub>50</sub> = 8 nM), two of the most potent bis-quaternary ammonium salts described so far (Chart 1).

Moreover, the hetero-bivalent mono-quaternary ammonium salts 13 and 14 also showed impressive AChE affinity likely arising from strong interactions between both the coumarin and 3-HTB moieties and the PAS and catalytic binding site, respectively. In particular, the AChE inhibitory activity of 14 (IC<sub>50</sub> = 1.0 nM) was 12,000- and 42,000-fold higher than that of the single separate moieties, 3-HTB and DMC, respectively.

The strategy of designing dual binding site AChE inhibitors by joining two AChE binding molecules, used previously by diverse authors, led also to the discovery of very potent inhibitors, but their starting separate moieties (e.g., tacrine and (-)-huperzine A) already displayed an affinity in the high nanomolar range.

Scheme 1. Solid-phase synthesis of compounds 1–15. Reagents and conditions: (a) PPh<sub>3</sub>, DIAD, THF; (b) TBAF, THF; (c) PBu<sub>3</sub>, ADDP, CH<sub>3</sub>Cl<sub>2</sub>, 3-[(tert-butyldimethyl)silyloxy]propan-1-ol or 4-[(tert-butyldimethyl)silyloxy]butan-1-ol; (d) PBu<sub>3</sub>, ADDP, CH<sub>3</sub>Cl<sub>2</sub>, 3-(dimethylamino)-5-[(triisopropylsilyl)oxy]phenol or 3,4-dimethyl-7-hydroxycoumarin; (e) TFA, CH<sub>2</sub>Cl<sub>2</sub>; (f) CH<sub>3</sub>I, CH<sub>3</sub>CN, 1, 2 and 4, or 7 and 8. G: 3-hydroxy-5-(trimethylammonium)phenyl iodide (11 and 12), 3-hydroxy-5-(N-benzyl-N,N-dimethylammonium)phenyl iodide (15) and 3,4-dimethylcoumarin (13 and 14).

Scheme 2. Synthesis of compound 16. Reagents and conditions: (a) K<sub>2</sub>CO<sub>3</sub>, CH<sub>3</sub>CN, reflux; (b) H<sub>2</sub>, Pd ‘black’, EtOH/dioxane 1:1, rt; (c) K<sub>2</sub>CO<sub>3</sub>, CH<sub>3</sub>I, EtoH, reflux.
activity of 16 to the synthetic pathways shown in Scheme 2. The AChE inhibitory end, we prepared compound played by the phenolic hydroxyl(s) of the 3-HTB moieties in the AChE binding sites.

The lowest active AChE inhibitors were the bis-β-methyl- and β-methylamine derivatives may be ascribed to possible steric effects in the corresponding derivatives with three methylene units (Fig. 2). However, the top-scored docking pose (58.13 kJ/mol) of the most active hetero-bivalent congener 12, which displayed an inhibitory potency in the sub-nanomolar range (IC50 = 0.17 nM) and a very high AChE selectivity (SI = 4165). Homobivalent bis-quinuclidinyl ammonium salts 11 and 12 were more active than the corresponding mono-quinuclidinyl hetero-bivalent congeners 13 and 14, whereas opposite results were observed when comparing parent aminic derivatives (i.e., 1 and 7, and 2 and 8).

The length of the spacer linking the two moieties binding at the catalytic and peripheral binding sites influenced the observed AChE affinity. Among medium- and highly potent inhibitors, those with four-methylene units were always slightly more potent than the corresponding derivatives with three methylene units (8 > 7; 12 > 11 and 14 > 13). This result suggested that an appropriate distance for a more efficient binding at both the catalytic and peripheral binding sites was provided by a linker of that length. The lower active AChE inhibitors were the bis-N-methyl-N-benzylamine derivatives 5 and 6, with the former yielding only 3% AChE inhibition at 50 μM. Mono N-methyl-N-benzylamine derivatives were also less active than the corresponding N,N-dimethylamine derivatives, as can be observed from the following comparisons: 3 < 1, 4 < 2, 9 < 7, and 10 < 8. The low inhibitory potencies of N-benzylamine derivatives may be ascribed to possible steric effects at the AChE binding sites.

An additional investigation was undertaken to assess the role played by the phenolic hydroxyl(s) of the 3-HTB moieties in the binding at the primary and peripheral binding sites of AChE. To this end, we prepared compound 16, which lacked both the phenolic hydroxyls compared to the highly potent inhibitor 12 according to the synthetic pathways shown in Scheme 2. The AChE inhibitory activity of 16 was dramatically lowered (IC50 = 158 nM from 0.17 nM of 12), while the BChE inhibitory activity was decreased to a much lower extent (IC50 = 2344 nM from 715 nM). These findings suggested that the phenolic hydroxyl group of the 3-HTB moieties plays indeed a key role in ligand binding at the catalytic site of AChE, as already observed in the X-ray crystallographic structure of edrophonium with TcAChE.

5. Docking studies

To support the interpretation of the structure–affinity relationships and to gain more insights on the molecular determinants responsible for the observed high affinities, a careful modeling study was undertaken through molecular docking.

Docking runs basically addressed the effects on affinity of cation–π, π–π stacking, and other non-bonded interactions involving charged and aromatic molecular moieties of our inhibitors and the electron-rich W86 and W286 amino acid side chains located in the catalytic and peripheral AChE binding sites, respectively.

Moreover, we attempted to explain the dramatic loss of affinity observed by removing the two hydroxyl groups from the highly potent homobivalent inhibitor 12 yielding the low active inhibitor 16.

Docking was first executed on the most active AChE inhibitor 12 (IC50 = 0.17 nM), while scaffold constraint was adopted to perform docking simulations with the other selected inhibitors. Top-scored docking pose of 12 (50.16 kJ/mol) displayed a cation–π interaction between the trimethylammonium groups and the electron-rich side chain of W86, a highly specific hydrogen bond between the phenolic hydroxyl and an oxygen atom of the hydroxyl group of S203 (indicated by a red dashed line in Fig. 1) and a potential π–π stacking interaction between the aromatic moiety of the ligand and the aromatic ring(s) of W286 in the PAS.

Figure 1. Top-scored docking pose of 12 into the hAChE binding sites.

Similarly, docking simulations revealed that the top-scored docking pose (58.13 kJ/mol) of the most active hetero-bivalent inhibitor 14, displayed a binding pattern similar to that of 12 (Fig. 2). However, the π–π stacking interaction of the coumarin moiety was probably slightly weaker than the combined π–π and π–cation interactions involving the phenyl-trimethylammonium moiety. The key interactions underlying the binding of the strong inhibitors 12 and 14 took place at an optimal distance assured by a four methylene linker, in full agreement with the observed experimental affinities.

Docking results from the other analyzed inhibitors provided easily interpretable binding models (data not shown). However, the correlation of the GOLDScore values with the observed inhibitory potencies (expressed as pIC50) for inhibitors 2, 6, 8, 12, 14, and 16 was quite poor (r² = 0.32), confirming that docking scores are not well suited to correctly predict free binding energies.
Docking poses were therefore subjected to a rescoring process according to a protocol recently proposed by Jacobson.25 Prime 1.5 module, available within Schrödinger-Maestro 7.5,26 was used to minimize in implicit solvent (generalised Born) the protein–ligand complex ($E_{\text{lig-prot}}$) together with free ligand ($E_{\text{lig}}$) and protein ($E_{\text{prot}}$). For each ligand, the relative binding energy (RBE) was then calculated by subtracting from the energy of the ligand–protein complex the sum of the energy of the isolated protein and ligand. As expected, the linear correlation of RBE with pIC50 was considerably improved as can be easily seen in Figure 3 reporting both the worse ($r^2 = 0.32$) and the improved ($r^2 = 0.79$) linear regression derived by plotting experimental $pC_{50}$ versus normalized data (0–1) from GOLDScore (dashed line and solid triangles) and RBE (whole line and empty circles) values, respectively.

6. Conclusions

In summary, the very potent AChE inhibitors described in this work, carrying one or two quaternary ammonium groups, might have potential in the treatment of myasthenia gravis, neuromuscular blockade, and glaucoma. Taken together, our results confirm and reinforce the strategic validity of the ‘fragment-based’ design for the preparation of highly potent AChE inhibitors. By tethering low-affinity inhibitors with a spacer of an appropriate length, it was possible to obtain AChE inhibitors with low-to-sub-nanomolar affinity. In particular, a properly substituted coumarin ring proved to be an ideal molecular entity for an optimal interaction at the PAS of AChE, as already observed by us14 and Recanatini and co-workers.27 This observation should be adequately considered to design dual binding site AChE inhibitors presenting a basic amino group, in the moiety binding at the catalytic site, in place of the quaternary ammonium groups examined in this work. Dual binding site AChE inhibitors of this kind might play an important role in the symptomatic treatment of AD since the interaction at PAS may inhibit the AChE induced beta-amyloid aggregation,28 a characteristic pathological event in AD.

Lastly, modeling studies allowed an in depth analysis and interpretation of the structure–affinity relationships and increased our understanding of the main binding interactions taking place at the AChE binding sites. Besides an expected important role played by cation–π,29 π–π stacking, hydrophobic, and other non-bonded interactions,30 the key role of a phenolic hydroxyl forming a highly specific hydrogen bond with an oxygen atom of the hydroxyl group of Ser 203, as already observed with edrophonium,21 was confirmed. The analysis of all these important binding interactions, well supported by a more accurate calculation of the energy of the enzyme–inhibitor complex formation, provided valuable insights for the design of new classes of potent and selective AChE inhibitors.

7. Experimental

7.1. Chemistry

Compounds 1–15 were prepared according to the reaction pathways illustrated in Scheme 1.16 Amines 7–10 were purified before the final quaternization reaction by flash chromatography on silica gel columns eluting with binary ethyl acetate-n-hexane mixtures. The purity of all the tested compounds, checked by HPLC, 1H NMR, and ESI mass, was always >96%. Starting materials, reagents, and analytical grade solvents were from commercial sources. Melting point (mp) was determined only for target compound 16 by the capillary method on a Stuart Scientific SMP3 electrothermal apparatus and is uncorrected. HPLC analyses were carried with a Waters 1585 system, equipped with a model 2487 UV detector, on a Waters Xterra C8 column (3 mm × 250 mm), and different MeOH:H2O mixtures as the mobile phase. ESI mass spectra were performed on a Agilent 1100 series LC-MSD trap system VL apparatus. Microanalyses were made only on the target final product 16 in a Euroag 3000 microanalyzer instrument; C, H, and N were within ±0.4% of the calculated values. 1H NMR spectra were recorded at 300 MHz on a Varian Mercury 300 instrument. Chemical shifts are expressed in δ (ppm) and coupling constants J in hertz (Hz). The following abbreviations were used: s (singlet), br (broad signal), m (multiplet).

The synthesis of compound 16 was carried out according to the reaction steps depicted in Scheme 2, as follows:

7.1.1. Synthesis of 1,4-bis(3-nitrophenoxy)butane (18)

m-Nitrophenol (1.0 g, 7.2 mmol) was dissolved in 22 mL of dry CH2CN, and then anhydrous K2CO3 (498 mg, 3.6 mmol) and 1,4-dibromobutane (287 µL, 2.4 mmol) were added. The mixture was refluxed for 24 h and the solvent was removed under reduced pressure. The resulting crude solid mixture was triturated with CHCl3 (100 mL) and the inorganic solid residue was filtered off. The organic phase was extracted with NaOH 3 N (3 × 30 mL), dried over anhydrous Na2SO4, and concentrated to dryness. The resulting solid was washed with n-hexane and filtered, yielding 698 mg (87%) of a white-off solid with a sufficient purity for the subsequent reaction. MS (ESI) m/z 333 (M+H)+; 1H NMR (CDCl3) δ 2.05 (br, 4H), 4.13 (br, 4H), 7.20–7.26 (m, 3H), 7.40–7.46 (m, 2H), 7.72–7.84 (m, 3H).
7.12. Synthesis of 3,3’-(butane-1,4-diylbis(oxy))dibenzenamine (17)

Compound 18 (332 mg, 1.0 mmol) was dissolved in 60 mL of a 1:1 mixture of ethanol/dioxane, and then Pd ‘black’ (60 mg) was added. The mixture was stirred for 7 h at room temperature under H₂ pressure (4 bar). The catalyst was removed by filtration through a pad of Celite®, and the solvent was removed under vacuum yielded the desired product as a yellow oil with acceptable high purity (248 mg, 91% yield). MS (ESI) m/z 318 (M+2Na)+, 1H NMR (CDCl₃) δ 1.93 (br, 4H), 3.98 (br, 4H), 3.50 (br, 4H), 6.24–6.33 (m, 6H), 7.02–7.07 (m, 2H).

7.13. Synthesis of 3,3’-(butane-1,4-diylbis(oxy))bis(N,N,N-trimethylbenzenaminium iodide) (16)

Diamine 17 (100 mg, 0.37 mmol) was dissolved in ethanol (3.0 mL), and then anhydrous K₂CO₃ (102 mg, 0.74 mmol) and iodides were used as substrates and 5,5’-phosphate buffer 0.1 M, at pH 8.0. Acetyl- and butyryl-thiocoline (0.36 U/mg), and BChE from equine serum (13 U/mg) were run in a pad of Celite purity (248 mg, 91% yield). MS (ESI) yielded the desired product as a yellow oil with acceptably high purity (84 mg (37%) of the title ammonium salt. Mp 172–175 °C. 1H NMR (DMSO-d₆) δ 4.13 (br, 4H), 7.15–7.17 (m, 2H), 7.45–7.56 (m, 6H).

7.2. Cholinesterase inhibition assay

The inhibition assays on AChE, from bovine erythrocytes (0.36 U/mL), and BChE from equine serum (13 U/mL) were run in phosphate buffer 0.1 M, at pH 8.0. Acetyl- and butyryl-thiocoline iodides were used as substrates and 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) as the chromophoric reagent. Inhibition assays were carried out on an Agilent 8453E UV–visible spectrophotometer equipped with a cell changer. AChE inhibitory activity was determined in a reaction mixture containing 200 µL of a solution of AChE (0.415 U/mL in 0.1 M phosphate buffer, pH 8.0), 100 µL of a 3.3 mM solution of DTNB in 0.1 M phosphate buffer (pH 7.0) containing 6 mM NaHCO₃, 100 µL of a solution of the inhibitor (five to seven concentrations ranging from 1 × 10⁻¹¹ to 1 × 10⁻³ M), and 500 µL of phosphate buffer, pH 8.0. After incubation for 20 min at 25 °C, acetylthiocholine iodide (100 µL of 0.05 mM water solution) was added as the substrate, and AChE-catalyzed hydrolysis was followed by measuring the increase of absorbance at 412 nm for 30 min at 25 °C. The concentration of compound which determined 50% inhibition of the AChE activity (IC₅₀) was calculated by non-linear regression of the response–concentration (log) curve, using GraphPad Prism v. 4.0. BChE inhibitory activity was assessed similarly using butyrylthiocholine iodide (0.05 mM) as the substrate.

7.3. Computational studies

Computational analyses were conducted on a 16 nodes Linux Cluster employing an openMosix® architecture composed by AMD Athlon XP 2400+ and Intel Xeon 2600 cpu. All the molecules were built from the Sybyl fragment libraries. Geometrical optimisation and charge calculation were carried out by means of a quantum mechanical method with the PM3 Hamiltonian. Molecules and models were displayed and manipulated on a Silicon Graphics O₂ machine. The docking poses reported in Figures 1 and 2 were prepared with the graphical system PyMol.²²

7.4. Docking simulations

The target protein was prepared by adding hydrogen atoms, completing and optimizing missing residues, removing water and the cocrystallized fasciculin molecule from the AChE crystallo-
26. Maestro7.5, Schrödinger Inc., Portland, OR 97201, USA.
31. SYBYL7.2 Tripos Inc., St. Louis, MO 63144.
32. The PyMOL Molecular Graphics System; DeLano Scientific: San Carlos, CA, USA.