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Preparation, in vitro screening and molecular modelling of symmetrical bis-quinolinium cholinesterase inhibitors—implications for early Myasthenia gravis treatment

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ABSTRACT

This paper describes the preparation and in vitro evaluation of 18 newly prepared bis-quinolinium inhibitors on human recombinant acetylcholinesterase (AChE) and human plasmatic butyrylcholinesterase (BChE). Their inhibitory (IC_{50}) and was compared to the chosen standards ambenonium dichloride, edrophonium chloride, BW284c51 and ethopropazine hydrochloride. One novel compound was found to be a promising inhibitor of hAChE (in nM range) and was better than edrophonium chloride or BW284c51, but was worse than ambenonium chloride. This compound also showed selectivity towards hAChE and it was confirmed as a non-competitive inhibitor of hAChE by kinetic analysis. A molecular modelling study further confirmed its binding to the peripheral active site of hAChE via apparent π–π or π–cationic interactions.

Acetylcholinesterase inhibitors are used in the treatment of disorders with impaired cholinergic transmission. The inhibition of acetylcholinesterase (EC 3.1.1.7; AChE) is a key treatment strategy for Alzheimer's disease, the most common form of dementia in the elderly population.[1] In contrast, peripherally acting AChE inhibitors are used in various conditions, such as glaucoma, constipation, spasmolysis and also to antagonise muscle relaxation in anaesthesiology.[2,3] In addition, a significant use of peripheral AChE inhibitors is to mitigate the symptoms of early Myasthenia gravis (MG), an autoimmune disorder resulting from the destruction of the post-synaptic membrane in the neuromuscular junction.[4]

In most MG cases, human antibodies are produced to the nicotinic acetylcholine receptor (nAChR), but other neuromuscular junction proteins (e.g., muscle-specific tyrosine kinase, MuSK) can be also targetted.[5,6] these antibodies initiate the autoimmune attack to the endplate region of neuromuscular junction resulting in reduced density of nAChR, destruction of the synaptic folds and the general simplification of the post-synaptic membrane. The decreased probability of the acetylcholine (ACh)–nAChR interaction causing a reduced transmission in the neuromuscular junction results in a characteristic symptom of MG, that is, weakness of the striated muscles.[7] The AChE inhibitors work by increasing the concentration of ACh in the synaptic junction and thus enhance the cholinergic transmission in spite of the nAChR depletion.[8] Though the application of AChE inhibitors is only a symptomatic approach and it does not resolve the original cause of the disease, it plays a significant role in early/mild MG treatment. The peripheral AChE inhibitors are usually the initial drugs for MG treatment and have been successfully used in the sole treatment of mildly presenting MG. However, progressive MG forms require additional immunosuppressive treatment.[9]

Compounds that are currently commercially available for MG treatment belong to carbamate drugs, for example, neostigmine bromide (**A**; Prostigmin®, Vagostigmin®, Fig. 1) and the most frequently used drug—pyridostigmine bromide (**B**; Mestinon®, Fig. 1), which is slightly better tolerated than neostigmine.[10] Edrophonium chloride (**1**; Tensilon®, Fig. 1), is used as a diagnostic tool for MG. It has a rapid onset and short pharmacological action, thus it cannot be used for treatment purposes.[11] The use of a

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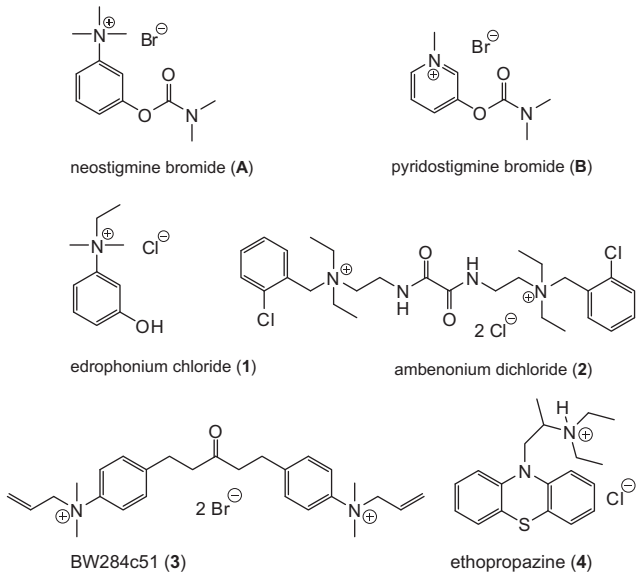


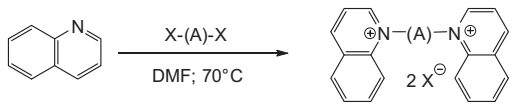
Figure 1. AChE inhibitors used for MG treatment (**A** and **B**, **1** and **2**) and selective cholinesterase inhibitors (**3** and **4**).

non-carbamate bisquaternary drug with prolonged effect, such as ambenonium dichloride (Mytelase®, Fig. 1) is another treatment option. However, the application of MG drugs can be followed by serious side-effects caused by the increased muscarinic activity (gastrointestinal discomfort, increased salivation, lacrimation and bronchial secretion).[12,13] These side-effects are dose-dependent and can be treated with anticholinergic drugs (e.g., diphenoxylate hydrochloride, atropine or loperamide hydrochloride).[14] An excessive dosing of MG commercial drugs may also lead to the cholinergic crisis, resulting in even greater muscle weakness, diarrhoea, bradycardia, excessive oropharyngeal and bronchial secretion.[15]

In order to avoid central nervous system AChE-related side-effects, it is recommended to treat MG with compounds that do not penetrate across the blood–brain barrier (BBB). All MG commercial used compounds contain a positive charge in their structure which should restrict their action to the periphery. However, some recent studies have suggested that pyridostigmine may affect centrally mediated AChE due to stress-induced BBB penetration.[16] Additionally, compounds designed for MG treatment should be selective for AChE leaving other non-specific cholinesterases (butyrylcholinesterase; BChE, EC 3.1.1.8) unaffected. This AChE selectivity will provide a notably decrease in the appearance of side-effects and potential dosing difficulties.[17]

Previously, the preparation and evaluation of the inhibitory effect of bis-pyridinium and bis-isoquinolinium compounds were investigated.[18,19] Since the AChE esteratic site (Ser203) is located on the bottom of the narrow gorge and a peripheral anionic/aromatic site (PAS) lies in its entrance, the length of the connecting linker as well as a spatial orientation of the inhibitor molecule were considered as being the most important factors for the interaction between inhibitor and enzyme.[20] The bis-quinolinium compounds (Table 1) described in this paper were prepared and investigated to support these formerly reported findings. Hence, various aliphatic or aromatic linkers between two quinolinium moieties were used to study their molecular interaction with cholinesterases. A quinolinium moiety was chosen to follow the influence of the heteroaromatic part of the molecule towards inhibition of the cholinesterases, because previously bis-isoquinolinium compounds showed a very promising inhibitory ability.[19] Assuming that the enzyme–inhibitor interactions depend largely on the formation of π–π or π–cationic interactions between the heteroaromatic part of the inhibitor and the enzyme aromatic residues for this type of compounds, the bis-quinolinium compounds seem to bridge the gap between bis-pyridinium and bis-isoquinolinium series.[21] The bisquaternary structure of prepared compounds was selected to promote a peripheral inhibitory effect. Since there is a possibility that monoquaternary compounds could penetrate the BBB, the addition of another charge is predicted to benefit minimal BBB penetration. In addition, though some compounds from bis-quinolinium series had been previously prepared, they had not been evaluated for their cholinesterase inhibition activity.[22–24]

Table 1
Prepared bis-quinolinium salts bearing different linkers



5–14 (Br)[a]	$(CH_2)_{3-12}$
15 (Cl)	CH_2OCH_2
16 (Br)	$(CH_2)_2O(CH_2)_2$
17 (Br)	(*E*)–$CH_2CH{=}CHCH_2$
18 (Cl)	(*Z*)–$CH_2CH{=}CHCH_2$
19 (Br)	1,2-Phenylenyl
20 (Br)	1,3-Phenylenyl
21 (Br)	1,4-Phenylenyl
22 (Br)	3,6-Naphtalenyl

[a] All compounds are described in detail in the Supplementary data.

The novel inhibitors were prepared by quaternisation of quinoline (4.2 mmol) with a corresponding alkylating agent (1.9 mmol) in MeCN (30 ml). The solution was stirred at 70 °C for 10–20 h and then cooled to room temperature. The crude product was collected by filtration, washed with acetone and re-crystallized from MeCN. NMR, ESI-MS and elemental analysis were used to determine the structure and purity of all compounds.

The inhibitory ability of the newly prepared compounds was determined in vitro on human recombinant AChE (hAChE) and human plasmatic BChE (hBChE) using a modified Ellman's procedure.[25] The IC_{50} values were compared to those obtained for edrophonium chloride (**1**), ambenonium dichloride (**2**) and selective AChE/BChE inhibitors BW284c51 (**3**, Fig. 1) or ethopropazine hydrochloride (**4**, Fig. 1). Edrophonium (**1**) and ambenonium (**2**) were chosen as standards that are currently used for MG diagnosis and treatment, and which are non-carbamate related drugs. BW284c51 (**3**) and ethopropazine (**4**) were determined as selective AChE/BChE inhibitors to cover selectivity issues.[26,27] The selectivity index was calculated as a ratio between the IC_{50} of BChE/AChE. The kinetic experiments on recombinant hAChE were done for chosen inhibitors of interest. The results are summarised in Table 2.

The in vitro inhibitory ability of the MG standards was confirmed. While **1** (IC_{50} = 5 μM) was found to be a weak AChE inhibitor with high selectivity for AChE, compound **2** (0.7 nM) was the best inhibitor among all the tested compounds with five orders of magnitude selectivity for AChE. Moreover, the kinetic experiments for hAChE showed that compound **1** influenced the substrate (acetylthiocholine) decomposition rate, whereas inhibitor **2** was found to be a non-competitive AChE inhibitor. The selective inhibitors (**3, 4**) also confirmed older data with a strong inhibition of cholinesterases (30 nm for hAChE by **3**, 1.6 μM for hBChE by **4**) and a high selectivity for tested enzymes. Compound **3** was found to be a non-competitive hAChE inhibitor, while the selective hBChE inhibitor **4** influenced the hAChE substrate decomposition rate.

The novel compounds showed various inhibitory capabilities of the tested enzymes. The lowest inhibitory ability towards hAChE was observed in compounds bearing an oxygen in the alkylenyl linker (**13, 14**) with an IC_{50} value in the mM or <mM range. Compounds with xylenyl linkers (**19–21**) did not demonstrate a

Table 2
Screening of standard and prepared hAChE/hBChE inhibitors

Inhibitor	AChE IC_{50} ± SD[a] (μM)	BChE IC_{50} ± SD[a] (μM)	SI[c] BChE/AChE	K_{i1}/K_{i2}[d] (μM)
Edrophonium (**1**)	5 ± 1	1370 ± 223	274	0.8/4.8
Ambenonium (**2**)	0.0007 ± 0.0001	7 ± 1	10000	0.005/0.006
BW-284c51 (**3**)	0.03 ± 0.006	354 ± 58	11800	0.01/0.05
Ethopropazine (**4**)	1020 ± 199	1.6 ± 0.3	0.002	25/12100
5	9 ± 2	13 ± 2	1.6	–
6	38 ± 8	32 ± 5	0.8	–
7	4 ± 0.8	34 ± 6	8.5	–
8	25 ± 5	5 ± 0.8	0.2	–
9	0.09 ± 0.02	0.4 ± 0.06	4.4	–
10	0.08 ± 0.01	0.7 ± 0.1	8.8	–
11	0.08 ± 0.02	0.6 ± 0.1	7.5	–
12	0.001 ± 0.0002	0.1 ± 0.02	100	0.04/0.06
13	0.01 ± 0.002	0.5 ± 0.07	50	–
14	0.02 ± 0.004	0.4 ± 0.07	20	–
15	418 ± 82	871 ± 141	2.1	–
16	35 ± 7	48 ± 8	1.4	–
17	6 ± 1	308 ± 5	51	–
18	2 ± 0.4	0.8 ± 0.1	0.4	–
19	35 ± 7	6 ± 9	0.2	–
20	40 ± 8	20 ± 3	0.5	–
21	–[b]	–[b]	–	–
22	0.8 ± 0.2	0.2 ± 0.03	0.3	2.3/8.2

[a] Mean value of three independent determinations ± standard deviation (SD).
[b] No inhibition in selected concentration scale.
[c] Selectivity index.
[d] K_{i1}-dissociation constant for enzyme-inhibitor complex; K_{i2}-dissociation constant for enzyme–inhibitor–substrate complex.

significant inhibitory ability. Though compound **19** was not soluble in the screening medium, no notable difference in hAChE inhibition compared to the structurally similar compounds **17** and **18** are predicted. Compounds with but-2-enyl linkers or with C3–C6 methylenyl units showed only moderate hAChE inhibition (up to 2 μM) which is similar to the standard compound **1** and above those achieved by the other standards (**2, 3**).

The most interesting inhibitory ability was observed for novel compounds with C7–C12 methylenyl units (**9–14**) and a naphthalenyl linker (**22**). Two compounds (**13 and 14**; 10–20 nM) showed comparable hAChE inhibition compared to standard **3**, whereas compound **12** (1 nM) resulted as a hAChE inhibitor one order of magnitude better than the standard **3**. Three compounds (**12–14**) also presented a higher selectivity for hAChE, although their selectivity index (SI) was lower than standards **2, 3**. Two structurally different novel inhibitors (alkylenyl and naphtalenyl) were further used for kinetic experiments. Both compound **12** bearing a C10 methylenyl linkage and compound **22** with naphtalenyl linkage, were found to be non-competitive hAChE inhibitors. The in vitro results for these newly prepared compounds highlighted compound **12** as a promising non-competitive and selective hAChE inhibitor, and therefore warranted further investigation.

Molecular docking studies were performed on two promising compounds after the above in vitro screening (**12, 22**) in order to rationalise possible interactions within AChE and BChE.[28] Specifically, compound **12** was chosen among the novel inhibitors bearing polymethylenyl linkers and a sub-μM inhibitory ability (**9–14**). For other compounds of similar origin (**9–14**), a similar binding to cholinesterases was hypothesised and docking studies were not performed. In contrast, compound **22** bearing a naphtalenyl linkage but still with sub-μM inhibitory ability, was chosen for docking studies to determine additional interactions within the enzymes' active sites and important SAR features. Three AChE structures (1b41, 2gyv, 2jez) and one BChE (1p0i) structure were used.[29–36]

Regarding AChE, the best results were obtained for the hAChE structure (1b41). The top-scored docking pose of **12** (−8.27 kcal/mol) displayed binding in the PAS region without penetrating the oxyanion hole (Fig. 2). First quinolinium moiety was attached by π–cationic interactions to aromatic residues of Tyr124 (3.8 Å), Trp286 (3.7 Å), Phe297 (3.2 Å), Phe338 (3.4 Å) and Tyr341 (3.4 Å), whereas the second quinolinium moiety displayed only distant binding to Trp286 (4.6 Å). Similarly, the top-scoring docking pose of **22** (−9.44 kcal/mol) showed binding to the PAS and was not affected by the catalytic triad (Fig. 2). First quinolinium moiety was stacked among the aromatic residues of Tyr72 (4.0 Å), Tyr124 (2.9 Å), Trp286 (3.2 Å), Phe297 (3.6 Å), Phe338 (3.5 Å) and Tyr341 (3.1 Å). The naphtalenyl linker displayed some weak π–π interactions with Trp286 (4.2 Å) and Tyr341 (4.0 Å), but the other quinolinium moiety was found to be at a large distance from PAS Trp286 (6.2 Å). Based on this molecular modelling and concerning the in vitro data, compound **12** (1 nM) probably resulted as two orders of magnitude more potent hAChE inhibitor to compound **22** (0.8 μM) because of its spatial and conformational flexibility which

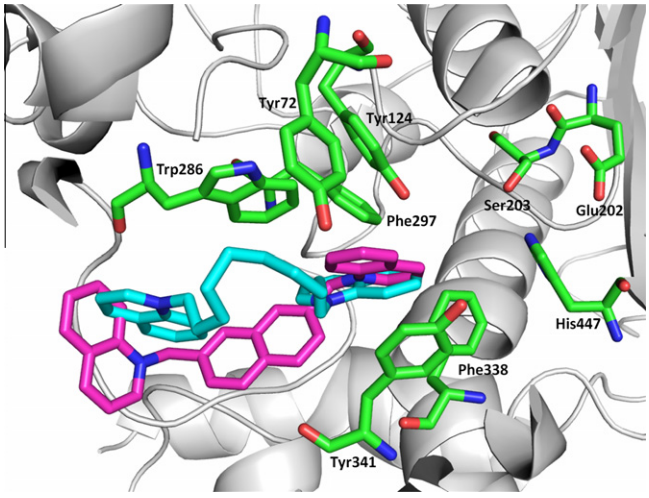


Figure 2. Molecular docking results for hAChE interactions with compound **12** (blue) and **22** (magenta).

would help in its accommodation in the hAChE peripheral active site, when compared to a structurally rigid molecule **22** with a naphtalenyl linkage.

The molecular modelling results for hBChE are depicted in Figure 3. Similar to the AChE results, the top-scored docking pose of compound **12** (−9.22 kcal/mol) displayed important interactions with aromatic residues. Namely, both quinolinium moieties were accommodated by π–cationic interactions between Trp82 (3.5 Å) and Trp231 (4.1 Å) and was further stabilized by interactions with Phe329 (3.4 Å), Phe398 (3.8 Å), His438 (3.7 Å), Trp430 (3.6 Å) or Tyr440 (3.1 Å). The top-scored docking pose of compound **22** (−9.48 kcal/mol) was stabilized differently to compound **12**. Its first quinolinium moiety was attached to Trp82 (3.3 Å) via a π–cationic interaction and stabilized by additional interactions with aromatic residues of His438 (3.7 Å), Trp430 (3.8 Å) or Tyr440 (3.0 Å). The naphtalenyl linker was sandwiched between Phe329 (3.4 Å) and Tyr332 (3.4 Å) by π–π interactions, whereas the second quinolinium moiety was found to be not interacting with hBChE aromatic residues. Concerning the in vitro results, both compounds **12** (0.1 μM) and **22** (0.2 μM) resulted in similar inhibitory abilities towards hBChE, although both compounds were found to bind in different manners to hBChE. A plausible explanation of their similar inhibitory ability may consist in the accommodation of compound **12** in the hBChE cavity which closes the entrance of the active site Ser198, while compound **22** closes the narrow entrance to this hBChE cavity between Phe329 and Tyr332 because it is a rigid molecule.

The SAR results with the bis-quinolinium series confirmed our previous findings with bis-pyridinium and bis-isoquinolinium compounds.[18,19] Whereas the compounds bearing connecting linkers with heteroatoms, double bond or xylenyl moiety were found to be inefficient cholinesterase inhibitors, compounds bearing a methylenyl linkage produced promising AChE inhibitors, especially compounds with a C7–C12 methylenyl linkage. The best in vitro inhibitory results towards hAChE were obtained with a C10 linker (1 nM) that corresponds with the best results for the bis-pyridinium series with a C10 linker (0.4 μM) and this is also favourable to the best results with the bis-isoquinolinium series with a C8 linkage (5 nM).[18,19] However, bis-quinolinium compounds were found to be slightly better than the bis-isoquinolinium series and thus they will be the subject of further studies in the design and preparation of non-symmetrical bisquaternary AChE inhibitors.

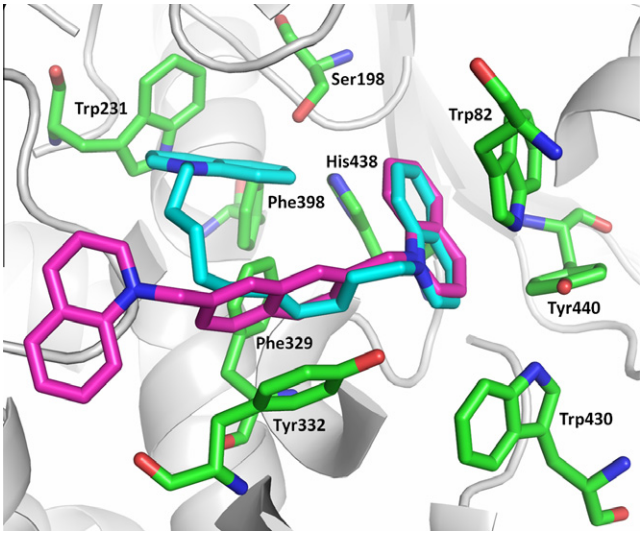


Figure 3. Molecular docking results for hBChE interactions with compound **12** (blue) and **22** (magenta).

In summary, 18 symmetrical bis-quinolinium compounds were prepared and their inhibitory abilities were tested in vitro on hAChE and hBChE. Three compounds showed inhibitory results better or comparable to the standards of edrophonium chloride and BW284c51 and in addition one compound (**12**) also presented selectivity for hAChE. None of the prepared compounds were able to exceed the ambenonium chloride in hAChE inhibition. The kinetic experiments confirmed non-competitive inhibition of hAChE by two chosen bis-quinolinium compounds. The binding of novel compounds to the active site Ser203 was further suggested by molecular modelling studies. The SAR findings were also comparable to previously prepared bis-pyridinium and bis-isoquinolinium series of compounds, but highlighted that the bis-quinolinium series will be the focus of future studies on the design of non-symmetrical bisquaternary molecules.

Experimental section: All experimental details are listed in the Supplementary data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.047.

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