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Short communication

Preparation and *in vitro* screening of symmetrical bis-isoquinolinium cholinesterase inhibitors bearing various connecting linkage – Implications for early Myasthenia gravis treatment

Kamil Musilek [a,d,*], Marketa Komloova [b], Ondrej Holas [b], Martina Hrabinova [c], Miroslav Pohanka [b], Vlastimil Dohnal [d], Florian Nachon [e], Martin Dolezal [b], Kamil Kuca [a,c]

[a] University of Defence, Faculty of Military Health Sciences, Department of Toxicology, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
[b] Charles University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry and Drug Control, Heyrovskeho 1203, 500 05 Hradec Kralove, Czech Republic
[c] University of Defence, Faculty of Military Health Sciences, Centre of Advanced Studies, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
[d] University of Jan Evangelista Purkynje, Faculty of Science, Department of Chemistry, Ceske mladeze 8, 400 96 Usti nad Labem, Czech Republic
[e] Toxicology Department, Enzymology Unit, Centre de Recherches du Service de Sante des Armees, BP 87, 38702 La Tronche Cedex, France

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ABSTRACT

Inhibitors of acetylcholinesterase are compounds widely used in the treatment of various diseases, such as Alzheimer's disease, glaucoma and Myasthenia gravis (MG). Compounds used in the therapy of MG posses a positive charge in the molecule to ensure peripheral effect of action and minimal blood–brain barrier penetration. The most prescribed carbamate inhibitors are however known for many severe side effects related to the carbamylation of AChE. This paper describes preparation and *in vitro* evaluation of 20 newly prepared bis-isoquinolinium inhibitors of potential concern for MG.

The newly prepared compounds were evaluated *in vitro* on human recombinant AChE and human plasmatic butyrylcholinesterase (BChE). Their inhibitory ability was expressed as IC_{50} and compared to chosen standards ambenonium dichloride, edrophonium chloride, BW284c51 and ethopropazine hydrochloride. Three novel compounds presented promising inhibition (in nM range) of both enzymes *in vitro* better or similar to edrophonium and BW284c51, but worse to ambenonium. The novel inhibitors did not present higher selectivity toward AChE or BChE. The kinetic assay confirmed non-competitive inhibition of hAChE by two selected promising novel compounds. Two newly prepared compounds were also chosen for docking studies that confirmed apparent π–π or π–cationic interactions aside the cholinesterases catalytic sites. The SAR findings were discussed.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder which is caused by antibody-mediated destruction of postsynaptic membrane in neuromuscular junction. In most cases antibodies target the nicotinic acetylcholine receptors (nAChRs), although other components of postsynaptic membrane can be affected as well. Impaired neuromuscular transmission results in a painless weakness and fatigue of striated muscles [1].

Peripheral inhibitors of acetylcholinesterase (EC 3.1.1.7; AChE) are a first-line treatment in early stages and in the mild forms of MG, where there are still sufficient AChRs presented [2]. They enhance cholinergic transmission by inhibiting the hydrolysis of acetylcholine (ACh) and thus increasing its concentration in the synaptic cleft. Current early MG treatment involves AChE carbamate inhibitors such as pyridostigmine bromide or neostigmine bromide (Fig. 1). Pyridostigmine bromide is better tolerated and more widely used compared to neostigmine [3]. However, the usage of both carbamate compounds often leads to development of serious gastrointestinal side effects (nausea, diarrhea, abdominal cramping), increased bronchial secretion and cardiac arrhythmia [4]. Additionally, high concentration of these drugs may evolve into a cholinergic crisis, characterized by even more severe weakness [5]. Mentioned side effects of carbamates are related to the carbamylation of AChE. This type of inhibition is common for carbamate inhibitors that bind to serine oxygen in the AChE active site [6]. The resulting carbamylated enzyme intermediate inhibits AChE activity until a water molecule attacks the carbonyl to reactivate enzyme and produces a carbamic

* Corresponding author. University of Defence, Faculty of Military Health Sciences, Department of Toxicology, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic. Tel.: +420 973 251 523; fax: +420 495 518 094.
E-mail address: kamil.musilek@gmail.com (K. Musilek).

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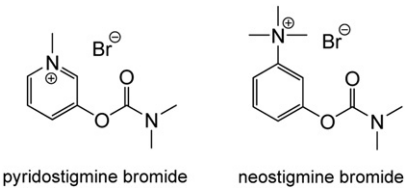


Fig. 1. Carbamate inhibitors used for MG treatment.

acid derivative. The spontaneous regeneration of carbamylated enzyme proceeds in the range of minutes.

Importantly for MG treatment, the AChE inhibitors are not required to penetrate through the blood–brain barrier (BBB) to decrease their central side effects [7]. Thus, both carbamate drugs contain quaternary nitrogen. This structural feature is important for increased peripheral effect of mentioned drugs, where the charged compounds are penetrating in minor ratio [8]. The BBB crossing of pyridostigmine or neostigmine is limited, but may proceed through disruptive mechanisms [9]. Hence, these compounds may still exhibit central side effects related to carbamylation of brain AChE that could result in cholinergic crisis [6].

Additionally, structurally dissimilar compounds from above mentioned carbamates are used for the MG treatment. Bisquaternary inhibitor ambenonium dichloride (**1**; Fig. 2) is favored with one of the highest known inhibitory ability toward AChE (in sub-nM range) [10]. Its outstanding potency is unique for the compound that does not form any covalent bond with the active site of the enzyme. Docking studies on AChE–ambenonium complex showed that **1** is able to establish highly favorable contacts with amino acids of the catalytic and the peripheral AChE sites [11]. Ambenonium produces fewer muscarinic side effects compared to carbamates and it is advantageous because of prolonged action that results in a greater therapeutic effect during the night and awakening in contrast to short-acting anti-AChE compounds. Moreover, its bisquaternary structure better prevents the passage through the BBB after conventional oral or intravenous route of administration [11]. Other drug, edrophonium chloride (**2**; Fig. 2), is used as diagnostic tool for MG. It has rapid onset and short pharmacologic action, thus it cannot be used for treatment purposes [12].

There is a huge variety of compounds reversibly inhibiting AChE that might be used in the MG treatment [13]. In contrast to carbamates, their effect should originate in competitive reversible inhibition of AChE aside the active serine (S203 for human AChE) [14]. The selective AChE inhibition instead of dual AChE/BChE (butyrylcholinesterase; EC 3.1.1.8) inhibition might keep the non-specific esterase (BChE) active for other toxic substrates [15]. Our approach originates from design, synthesis, *in vitro* screening and docking studies of selected bisquaternary bis-isoquinolinium compounds connected by various linkages.

2. Design and synthesis bis-isoquinolinium compounds

Formerly, the bispyridinium compounds bearing various linkers were prepared and evaluated as AChE and BChE inhibitors [16]. This paper describes the preparation of 20 symmetrical bis-

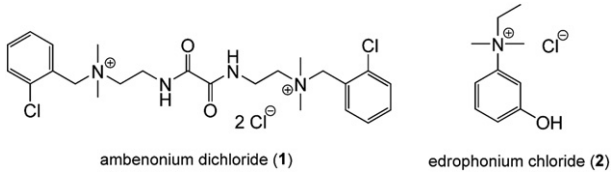
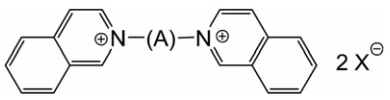
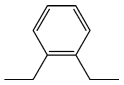
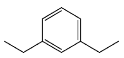
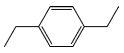
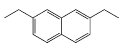


Fig. 2. Non-covalent AChE inhibitors used for MG treatment or diagnosis.

Table 1
Newly prepared AChE/BChE inhibitors.



(A)	Compound (anion)
$(CH_2)_{1-12}$	**3**–**14** (Br)
CH_2OCH_2	**15** (Cl)
$(CH_2)_2O(CH_2)_2$	**16** (Br)
(*E*)-$CH_2CH{=}CHCH_2$	**17** (Br)
(*Z*)-$CH_2CH{=}CHCH_2$	**18** (Cl)
	**19** (Br)
	**20** (Br)
	**21** (Br)
	**22** (Br)

isoquinolinium AChE inhibitors (**3**–**22**; Table 1). Some of these compounds (**3**–**14**) were previously evaluated as inhibitors of rat brain homogenate with moderate results [17]. The further evaluation of these compounds on the source of human AChE and their selectivity toward AChE/BChE was considered to be important for future design of novel compounds. Some experimental data were added to better characterize the synthesized compounds.

Though the synthesis of such compounds is trivial, their structural differences were found important for comprehension of key factors influencing inhibition of AChE and/or BChE [18]. Namely, presented compounds differ in the structure of the connecting linker. The key length and/or spatial orientation of connecting linker are highly important factors for molecular interactions among the enzyme active sites. Subsequently, the optimal structure of the linker depends not only on the length, but is usually related to presented π-electrons (double bond or aromatic residues) or heteroatom (hydrogen-bonding interactions), too [19]. The bis-isoquinolinium part of the molecule was chosen as an extension of previously prepared bispyridinium compounds [16]. While the lipophilicity of the best AChE inhibitor from bispyridinium series with decylenyl linker was very low (log *P* −1.97), similar compound from bis-isoquinolinium series was calculated to have still low lipophilicity (log *P* 0.40) to cross BBB and thus may act as peripheral AChE inhibitor. Presumably, bis-isoquinolinium compounds may extend the π–π or π–cationic interactions with aromatic residues (His, Trp, Tyr, Phe) of the enzyme active sites found for bispyridinium ones. These amino-acid residues are well known for their principal function in the enzyme active sites via non-covalent interactions (AChE or BChE) [20]. Moreover, the isoquinolinium, quinolinium or acridinium compounds were formerly found to be very effective inhibitors of AChE [21]. Hence, higher AChE and/or BChE affinity was hypothesized for bis-isoquinolinium moiety. Moreover, the related bispyridinium compounds were found to be non-competitive inhibitors of both enzymes aside the active serine (S203) in contrast to carbamate compounds [16]. Additionally, the peripheral effect of AChE inhibitors is preferred for MG treatment in

order to decrease central side effects caused by interactions with the brain AChE. For this reason, the charged molecules were chosen. Moreover, the monoquaternary compounds were penetrating BBB at least in 10%, whereas the bisquaternary compounds were previously found to penetrate the blood–brain barrier (BBB) in less than 2% [8,22,23]. For these reasons, the bisquaternary molecules were designed to maintain the peripheral inhibitory activity against AChE.

The new inhibitors (**3**–**22**; Fig. 3) were prepared via standard synthetic strategy [24,25]. The solution of isoquinoline (1.6 g, 12.4 mmol) and corresponding alkylating agent (5.6 mmol) in DMF (10 ml) was stirred at 70 °C. The reaction mixture remained at the room temperature. It was portioned with acetone (50 ml) and cooled in refrigerator (5 °C) overnight. The crystalline or amorphous crude product was collected by filtration, washed with acetone (3 × 20 ml) and recrystallized from MeCN. NMR, ESI-MS and elemental analysis determined the entity and purity of all prepared compounds.

3. AChE and BChE inhibition results

The bisquaternary bis-isoquinolinium compounds (**3**–**22**) were assayed for their inhibitory ability in standard inhibition test *in vitro* using human recombinant AChE (hAChE) and human plasmatic BChE (hBChE) [26]. The IC50 values and selectivity index for IC_{50} BChE/IC50 AChE ratio (SI) of all newly prepared compounds along with the standards **1**–**2** are listed in Table 2. The selective AChE inhibitor BW284c51 (**23**; 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide; Fig. 4) and selective BChE inhibitor ethopropazine hydrochloride (**24**) were also determined to better depict the selectivity issues [27,28].

The standard compounds used for MG treatment or diagnosis **1**–**2** were found to be potent hAChE inhibitors, where compound **1** exhibited inhibitory ability on sub-nM range (0.7 nM). Both compounds also presented very high selectivity toward AChE required for therapeutic use. Additionally, the kinetic experiment confirmed their inhibitory ability and showed that the presence of substrate did not affect non-competitive inhibition by compound **1** [29]. Thus compound **1** was supposed to bind aside the AChE active serine, whereas inhibition by compound **2** was affected by presence of the substrate. Compound **2** was hypothesized to bind competitively closely to active serine and consequently influenced by the substrate [30,31]. The selective standard compounds **23**–**24** were found to be potent and also selective AChE or BChE inhibitors. Compound **23** showed high and selective inhibition of hAChE (30 nM), whereas kinetic experiments confirmed its binding to AChE peripheral active site without influence of substrate to inhibition kinetics. Compound **24** resulted as potent and selective inhibitor of hBChE (1.6 μM) [32]. The kinetic experiments for hAChE displayed strong competition of inhibitor **24** and substrate during enzymatic reaction.

Concerning the newly prepared compounds, there may be seen at least three trends of inhibitory ability within the whole series. Firstly, some compounds (**3**, **5**, **14**, **16**, **19**) showed only minor inhibition of AChE in mM scale. Surprisingly, some of these compounds (**14**, **16**, **19**) presented better inhibition of BChE than AChE, while compound **14** remained the most selective BChE inhibitor among all tested compounds with 4 orders of magnitude difference between AChE and BChE. Though compound **4** was not soluble in screening medium (not even by addition of DMSO), inhibition of both enzymes similar to compounds **3** or **5** was supposed. The other group of compounds (**6**, **7**, **15**, **17**, **18**, **20**, **21**) displayed inhibition of AChE in μM scale. Again, compounds **7** and **20** inhibited BChE more than AChE, though the SI was lower when compared to compound **14**. The third group of prepared compounds (**8**–**13**, **22**) was able to inhibit AChE on nM scale, although none of these compounds exceeded the inhibitory ability of MG standard **1**. Among them, compound **10** with an aliphatic linker presented the best inhibition results toward AChE from the newly prepared compounds; however, the SI for hAChE remained poor. Interestingly, compound **22** also resulted as potent inhibitor of AChE, while the structure of its connecting linker was completely different. In this case, the different weak (π–π or π–cationic) interactions were supposed to be crucial for the inhibitory ability of such compound [16]. Unfortunately, the most potent compounds **10**–**12** displayed only poor selectivity between AChE and BChE. This lack of selectivity may limit their further *in vivo* evaluation and possible use due to interference with BChE that is responsible for unspecific esterase activity in the organism [15]. Though BChE has not direct effect on physiological functions, its pharmacological and toxicological importance have been formerly recognized. Namely, BChE is involved in degradation of numerous drugs and poisons [33]. While many drugs are mixed inhibitors of

Table 2
Inhibitory potency of tested compounds toward cholinesterases.

Compound	AChE IC50 ± SD (μM)[a]	BChE IC50 ± SD (μM)[a]	SI BChE/AChE	K_{i1}/K_{i2} (μM)
Ambenonium (**1**)	0.0007 ± 0.0001	6.82 ± 1.11	9743	0.005/0.006
Edrophonium (**2**)	5.17 ± 1.0	1370 ± 223	265	0.79/4.80
3	654 ± 128	1400 ± 228	2.1	–
4	–[b]	–[b]	–	–
5	446 ± 87	2600 ± 424	5.8	–
6	36 ± 7	40 ± 6.5	1.1	–
7	13 ± 2.5	0.7 ± 0.1	0.05	–
8	0.3 ± 0.06	2 ± 0.3	6.7	–
9	0.5 ± 0.1	3 ± 0.6	6.0	–
10	0.005 ± 0.001	0.4 ± 0.06	80	0.62/0.98
11	0.04 ± 0.008	0.6 ± 0.09	15	–
12	0.05 ± 0.01	1.6 ± 0.3	32	–
13	0.1 ± 0.02	9 ± 1.5	90	–
14	137 ± 33	0.1 ± 0.02	0.0007	–
15	2 ± 0.5	154 ± 25	77	–
16	1310 ± 255	51 ± 8	0.04	–
17	7 ± 1.5	64 ± 10	9.1	–
18	17 ± 3	25 ± 4	1.5	–
19	250 ± 49	0.6 ± 0.1	0.002	–
20	8 ± 2	1 ± 0.2	0.1	–
21	12 ± 2	840 ± 137	70	–
22	0.3 ± 0.06	4 ± 0.7	13	1.25/0.75
BW284c51 (**23**)	0.03 ± 0.006	354 ± 58	11800	0.01/0.05
Ethopropazine (**24**)	1020 ± 199	1.6 ± 0.3	0.002	24.7/12100

[a] Mean value of three independent determinations.
[b] Not soluble in screening medium.

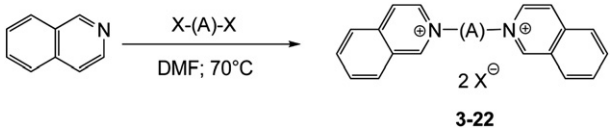


Fig. 3. Preparation of bis-isoquinolinium salts.

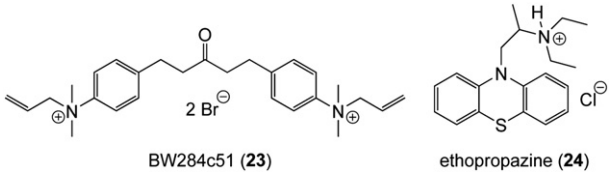


Fig. 4. Chosen selective AChE/BChE inhibitors.

AChE and BChE, truly selective AChE inhibitors are compounds of interest to not affect non-specific function of BChE [34].

4. Molecular docking results and SAR discussion

Regarding the AChE, top-scored docking pose of compound **10** (−11.09 kcal/mol) showed apparent interactions with aromatic residues of peripheral anionic site (PAS) (Fig. 5) [35–38]. Namely, strong π−π or cation−π interactions between both isoquinolinium moieties and Trp286 (3.7 and 3.8 Å) occurred. First isoquinolinium moiety was sandwiched among Tyr72 (3.9 Å), Tyr124 (3.5 Å), Trp286 (3.8 Å) and Phe297 (3.5 Å). Besides Trp286, second isoquinolinium moiety displayed some interaction with His287 (4.2 Å). Compound **10** did not penetrate closely to active Ser203 and blocked the AChE gorge entrance. Similarly to inhibitor **10**, compound **22** (−12.04 kcal/mol) presented sandwiching of one isoquinolinium moiety among Tyr72 (4.3 Å), Tyr124 (3.5 Å), Trp286 (3.4 Å) and Phe297 (3.3 Å). Second isoquinolinium moiety was again attached to Trp286 (4.0 Å) and His287 (4.2 Å). Differently from inhibitor **10**, the naphtylenyl moiety displayed strong π−π interaction with Trp286 (3.0 Å).

Concerning BChE, top-scored docking pose of compound **10** (−9.49 kcal/mol) showed interactions with aromatic Phe, Trp and Tyr residues (Fig. 6). The π−π or cation−π interaction with Trp82 (3.9 Å) and the T-stacking with Trp231 (3.6 Å) occurred. Moreover, one isoquinolinium ring presented additional T-stacking with His438 (3.6 Å), Phe329 (3.5 Å) and Phe398 (3.7 Å). Similarly to inhibitor **10**, compound **22** (−10.18 kcal/mol) presented the π−π or cation−π interaction with Trp82 (3.5 Å). The same isoquinolinium moiety was also T-stacked toward His438 (3.7 Å). The naphtylenyl moiety showed strong π−π interaction with Phe329 (3.1 Å). Differently to compound **10**, the second isoquinolinium moiety was not attached to aromatic residue and remained close to Pro285 (3.7 Å).

Some SAR, which originated from the docking studies and *in vitro* data, can be demonstrated [39]. Firstly, the isoquinolinium moiety showed its importance from the point of view of π−π and

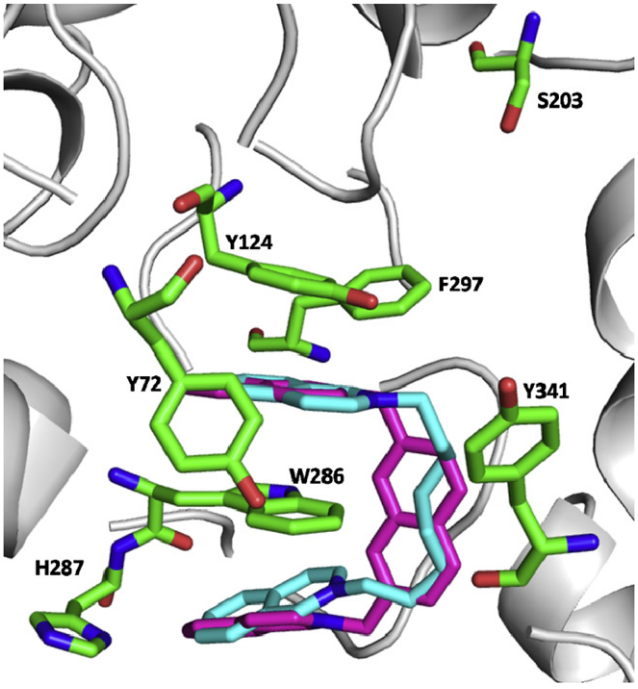


Fig. 5. Molecular docking for mAChE with compound 10 (blue) and 22 (magenta). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

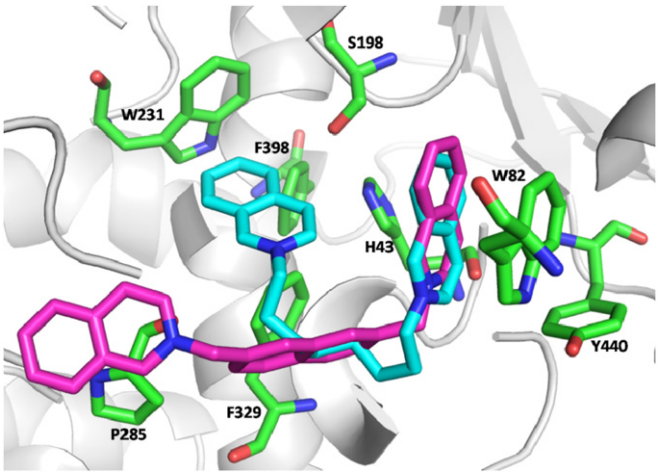


Fig. 6. Molecular docking for hBChE with compound 10 (blue) and 22 (magenta). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

π−cationic interactions with aromatic residues of ChE active sites and the results obtained for analogous bispyridinium compounds were confirmed. Additionally, the isoquinolinium compounds were found to be more potent inhibitors of hAChE compared to former pyridinium ones. Since the pyridinium moiety was previously successfully used, the quinolinium compounds will be the aim of further interest [16,17].

Secondly, the used linkage highly influenced the inhibitory ability of prepared compounds. The length of the connecting linkage remained the most important factor within whole series of compounds. The compounds with 6–11 methylene units (**8**–**13**) revealed the best inhibition of hAChE. These results apparently correlate with the distance of important aromatic residues of hAChE and thus with interactions of isoquinolinium moiety. The situation changed for hBChE, where less aromatic residues are presented. Compounds **10**–**14** were plausibly stacked between two Trp residues and consequently resulted as potent BChE inhibitors. Compounds bearing shorter linkers (**3**–**6**) were found inefficient for both enzymes. The similar results were previously obtained for bispyridinium series [16].

Differently, heteroatom (**15**–**16**), double bond (**17**–**18**), xylene (**19**–**21**) and naphtylene linkage (**22**) were introduced to find possible additional interactions [27]. Not surprisingly, most of these compounds (**15**–**21**) displayed only minor inhibitory activity of both enzymes (mM range). The linkage length of such compounds varied from 4 to 6 carbon−carbon (C−C) bonds and thus they were found insufficient to interact similarly as the potent inhibitors **8**–**13**. Due to the poor *in vitro* results of **15**–**21**, the plausible interactions with both enzymes were not further studied. More interestingly, compound **22** with naphtylenyl linkage exhibited the promising inhibitory ability for both used enzymes. Its linker was similar length to compound **9** (7 C−C bonds) and thus displayed the similar binding to AChE or BChE [16]. Though the compound **22** was not the best inhibitor in the tested series, it presented important interactions of its linker with aromatic residues. Such interactions may help in further design of more selective AChE inhibitors suitable for MG treatment.

The docking studies further improved the *in vitro* findings and confirmed the SAR conclusions. Both chosen compounds **10** and **22** presented similar binding toward AChE aside active site (S203) and the docking results were confirmed by kinetic experiments, where the presence of substrate did not affect inhibition by these compounds. The higher IC_{50} of compound **22** might be explained by conformational and spatial rigidity of the naphtylenyl linkage as its

accommodation in the narrow AChE gorge was difficult. Though the binding of compounds **10** and **22** was different for BChE, their IC_{50} resulted only with one order of magnitude difference. This finding might be explained by closing of BChE gorge, where compound **10** better protect the active site (S198) from accommodation of the substrate.

5. Conclusion

In summary, 20 novel bis-isoquinolinium cholinesterase inhibitors were prepared in effort to compare their *in vitro* ability to standard MG drugs. At least three compounds (**10**–**12**) showed promising inhibitory (IC_{50}) ability toward hAChE on nM scale better or similar to standards edrophonium and BW284c51, but did not present high selectivity between hAChE and hBChE. None of prepared compounds was able to exceed ambenonium in hAChE inhibition. The kinetic experiments confirmed non-competitive inhibition of hAChE by two chosen novel compounds. Consequently, docking studies confirmed their binding of two selected inhibitors aside the hAChE active site via π–π or π–cationic interactions. The SAR findings were determined. Though the bis-isoquinolinium cholinesterase inhibitors exceeded the formerly prepared bispyridinium compounds, the bis-quinolinium compounds will be the aim of further interest.

6. Experimental section

6.1. Chemical preparation

Solvents (acetone, DMF, MeCN) and reagents were purchased from Fluka and Sigma–Aldrich (Czech Republic) and used without further purification. Reactions were monitored by TLC using DC-Alufolien Cellulose F (Merck, Germany) and mobile phase BuOH–CH_3-COOH–H_2O 5:1:2, detection by solution of Dragendorff reagent (solution containing 10 ml CH_3COOH, 50 ml H_2O and 5 ml of basic solution prepared by mixing of two fractions – fraction A: 850 mg Bi $(NO_3)_3$, 40 ml H_2O, 10 ml CH_3COOH; fraction B: 8 g KI, 20 ml H_2O). Melting points were measured on micro heating stage PHMK 05 (VEB Kombinat Nagema, Radebeul, Germany) and were uncorrected.

NMR spectra were generally recorded at Varian Gemini 300 (^1H 300 MHz, ^{13}C 75 MHz, Palo Alto CA, USA). In all cases, the chemical shift values for ^1H spectra are reported in ppm (δ) relative to residual $CHD_2SO_2CD_3$ (δ 2.50) or D_2O (δ 4.79), shift values for ^{13}C spectra are reported in ppm (δ) relative to solvent peak dimethylsulfoxide-d_6 δ 39.43. Signals are quoted as s (singlet), d (doublet), t (triplet) and m (multiplet).

The mass spectra (MS respectively MSn) were measured on an LCQ FLEET ion trap and evaluated using Xcalibur v 2.5.0 software (both Thermo Fisher Scientific, San Jose, CA, USA). The sample was dissolved in deionized water (Goro, s.r.o., Prague, Czech Republic), and injected continuously (8 μl/min) by Hamilton syringe into electrospray ion source. The parameters of electrospray were set up as follows: sheath gas flow rate 20 arbitrary units, aux gas flow rate 5 arbitrary units, sweep gas flow rate 0 arbitrary units, spray voltage 5 kV, capillary temperature 275 °C, capillary voltage 13 V, tube lens 100 V.

6.2. Prepared bisquaternary salts

1,1-Bis(isoquinolinium)-meth-1,1-diyl dibromide (**3**) [17]. M.p. 257–258 °C. ^1H NMR (300 MHz, D_2O) δ ppm 10.34 (s, 2H, H-1,1′), 8.90 (d, 2H, J = 6.7 Hz, H-3,3′), 8.59 (d, 4H, J = 7.0 Hz, H-4,4′,8,8′), 8.36–8.31 (m, 4H, H-5,5′,7,7′), 8.17–8.09 (m, 2H, H-6,6′), 7.66 (s, 2H, N–CH_2–). ^{13}C NMR (75 MHz, D_2O) δ ppm 150.68, 139.18, 138.57, 136.55, 132.64, 132.05, 130.99, 127.31, 127.24, 77.56. ESI-MS: m/z 136.1 [M^{2+}/2] (calculated for [$C_{19}H_{16}N_2^{2+}$/2] 136.07). EA: calculated 52.81% C, 3.73% H, 6.48% N; found 52.42% C, 4.15% H, 6.38% N.

1,2-Bis(isoquinolinium)-eth-1,2-diyl dibromide (**4**) [17]. M.p. 269–271 °C. ^1H NMR (300 MHz, D_2O) δ ppm 9.70 (s, 2H, H-1,1′), 8.49–8.43 (m, 4H, H-3,3′,8,8′), 8.33–8.24 (m, 6H, H-4,4′,5,5′,7,7′), 8.08–8.01 (m, 2H, H-6,6′), 5.56 (s, 4H, N–CH_2–). ^{13}C NMR (75 MHz, D_2O) δ ppm 159.35, 147.76, 147.45, 143.11, 141.54, 139.73, 137.19, 136.94, 136.90, 69.61. ESI-MS: m/z 143.1 [M^{2+}/2] (calculated for [$C_{20}H_{18}N_2^{2+}$/2] 143.08). EA: calculated 53.84% C, 4.07% H, 6.28% N; found 54.06% C, 4.42% H, 5.88% N.

1,3-Bis(isoquinolinium)-prop-1,3-diyl dibromide (**5**) [17]. M.p. 244–246 °C. ^1H NMR (300 MHz, D_2O) δ ppm 9.74 (s, 2H, H-1,1′), 8.55 (d, 2H, J = 6.7 Hz, H-3,3′), 8.36–8.27 (m, 4H, H-4,4′,8,8′), 8.19–8.08 (m, 4H, H-5,5′,7,7′), 7.98 (t, 2H, J = 7.3 Hz, H-6,6′), 5.04 (t, 4H, J = 7.0 Hz, N–CH_2–), 3.13–2.98 (m, 2H, N–CH_2–CH_2–). ^{13}C NMR (75 MHz, D_2O) 148.66, 137.07, 136.86, 133.0, 131.19, 129.39, 126.92, 126.66, 126.29, 57.96, 29.70. ESI-MS: m/z 150.1 [M^{2+}/2] (calculated for [$C_{21}H_{20}N_2^{2+}$/2] 150.08). EA: calculated 54.81% C, 4.38% H, 6.09% N; found 54.79% C, 4.32% H, 5.81% N.

1,4-Bis(isoquinolinium)-but-1,4-diyl dibromide (**6**) [17]. M.p. 263–265 °C. ^1H NMR (300 MHz, D_2O) δ ppm 9.72 (s, 2H, H-1,1′), 8.54–8.45 (d, 2H, J = 6.4 Hz, H-3,3′), 8.39–8.33 (m, 4H, H-4,4′,8,8′), 8.22–8.16 (m, 4H, H-5,5′,7,7′), 8.04–7.97 (m, 2H, H-6,6′), 4.87–4.82 (m, 4H, –N–CH_2–), 2.31–2.24 (m, 4H, N–CH_2–CH_2–). ^{13}C NMR (75 MHz, D_2O) 148.55, 136.97, 136.72, 133.96, 129.49, 127.08, 126.69, 126.07, 60.04, 26.69. ESI-MS: m/z 157.1 [M^{2+}/2] (calculated for [$C_{22}H_{22}N_2^{2+}$/2] 157.09). EA: calculated 55.72% C, 4.68% H, 5.91% N; found 55.85% C, 5.11% H, 5.56% N.

1,5-Bis(isoquinolinium)-pent-1,5-diyl dibromide (**7**) [17]. M.p. 207–209 °C. ^1H NMR (300 MHz, D_2O) δ ppm 9.61 (s, 2H, H-1,1′), 8.42 (d, 2H, J = 7.0 Hz, H-3,3′), 8.30–8.24 (m, 4H, H-4,4′,8,8′), 8.16–8.11 (m, 4H, H-5,5′,7,7′), 7.99–7.92 (m, 2H, H-6,6′), 7.72–4.68 (m, 4H, N–CH_2–), 2.17–2.10 (m, 4H, –N–CH_2–CH_2–), 1.40–1.27 (m, 2H, –N–$(CH_2)_2$–CH_2–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.46, 136.83, 136.75, 133.35, 131.04, 129.43, 126.92, 126.73, 125.97, 60.57, 29.08, 21.29. ESI-MS: m/z 164.2 [M^{2+}/2] (calculated for [$C_{23}H_{24}N_2^{2+}$/2] 164.10). EA: calculated 56.58% C, 4.95% H, 5.74% N; found 56.12% C, 5.55% H, 5.43% N.

1,6-Bis(isoquinolinium)-hex-1,6-diyl dibromide (**8**) [17,24]. M.p. 233–235 °C. ^1H NMR (300 MHz, D_2O) δ ppm 9.66 (s, 2H, H-1,1′), 8.47 (d, 2H, J = 6.4 Hz, H-3,3′), 8.39–8.30 (m, 4H, H-4,4′,8,8′), 8.24–8.12 (m, 4H, H-5,5′,7,7′), 8.05–7.93 (m, 2H, H-6,6′), 4.73 (t, 4H, J = 7.3 Hz, N–CH_2–), 2.17–2.03 (m, 4H, –N–CH_2–CH_2–), 1.49–1.39 (m, 4H, –N–$(CH_2)_2$–CH_2–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.47, 136.95, 136.65, 133.45, 130.96, 129.53, 127.10, 126.75, 125.98, 61.09, 29.70, 24.58. ESI-MS: m/z 171.2 [M^{2+}/2] (calculated for [$C_{24}H_{26}N_2^{2+}$/2] 171.11). EA: calculated 57.39% C, 5.22% H, 5.58% N; found 57.55% C, 5.67% H, 5.12% N.

1,7-Bis(isoquinolinium)-hept-1,7-diyl dibromide (**9**) [17]. M.p. 205–207 °C. ^1H NMR (300 MHz, D_2O) δ ppm 9.66 (s, 2H, H-1,1′), 8.47 (d, 2H, J = 6.4 Hz, H-3,3′), 8.33 (t, 4H, J = 7.3 Hz, H-4,4′,8,8′), 8.18–8.08 (m, 4H, H-5,5′,7,7′), 7.98–7.91 (m, 2H, H-6,6′), 4.72 (t, 4H, J = 7.3 Hz, N–CH_2–), 2.13–2.02 (m, 4H, N–CH_2–CH_2–), 1.50–1.30 (m, 6H, N–$(CH_2)_2$–$(CH_2)_2$–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.32, 136.81, 136.41, 133.37, 130.76, 129.38, 126.98, 126.58, 125.86, 60.97, 26.92, 24.45. ESI-MS: m/z 178.2 [M^{2+}/2] (calculated for [$C_{25}H_{28}N_2^{2+}$/2] 178.11). EA: calculated 58.16% C, 5.47% H, 5.43% N; found 58.41% C, 5.86% H, 4.97% N.

1,8-Bis(isoquinolinium)-oct-1,8-diyl dibromide (**10**) [17]. M.p. 221–223 °C. ^1H NMR (300 MHz, D_2O) δ ppm 9.67 (s, 2H, H-1,1′), 8.48 (d, 2H, J = 6.7 Hz, H-3,3′), 8.40–8.33 (m, 4H, H-4, 4′,8,8′), 8.23–8.12 (m, 4H, H-5,5′,7,7′), 8.03–7.96 (m, 2H, H-6,6′), 4.72 (t, 4H, J = 7.3 Hz, N–CH_2–), 2.13–2.00 (m, 4H, N–CH_2–CH_2–), 1.38–1.25 (m, 8H, –N–$(CH_2)_2$–$(CH_2)_2$–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.35, 136.85, 136.44, 133.37, 130.78, 129.39, 126.61, 125.84, 61.06, 29.63, 27.23, 24.55.

ESI-MS: *m/z* 185.2 [M^{2+}/2] (calculated for [$C_{26}H_{30}N_2^{2+}$/2] 185.12). EA: calculated 58.88% C, 5.70% H, 5.28% N; found 58.59% C, 6.18% H, 4.76% N.

1,9-Bis(isoquinolinium)-non-1,9-diyl dibromide (**11**) [17]. M.p. 157–159 °C. ^{1}H NMR (300 MHz, D_2O) δ ppm 9.67 (s, 2H, H-1,1′), 8.48 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.39–8.33 (m, 4H, H-4, 4′,8,8′), 8.22–8.11 (m, 4H, H-5,5′,7,7′), 8.01–7.95 (m, 2H, H-6,6′), 4.70 (t, 4H, *J* = 7.3 Hz, N–*CH_2*–), 2.12–1.98 (m, 4H, N–CH_2–*CH_2*–), 1.38–1.21 (m, 10H, N–(CH_2)$_2$–*$(CH_2)_5$*–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.21, 136.73, 136.33, 133.31, 130.68, 129.30, 126.91, 126.51, 125.75, 60.98, 29.61, 27.40, 27.17, 24.47. ESI-MS: *m/z* 192.2 [M^{2+}/2] (calculated for [$C_{27}H_{32}N_2^{2+}$/2] 192.13). EA: calculated 59.57% C, 5.93% H, 5.15% N; found 59.07% C, 6.12% H, 4.97% N.

1,10-Bis(isoquinolinium)-dec-1,10-diyl dibromide (**12**) [17,24]. M.p. 222–224 °C. ^{1}H NMR (300 MHz, D_2O) δ ppm 9.68 (s, 2H, H-1,1′), 8.49 (d, 2H, *J* = 6.2 Hz, H-3,3′), 8.44–8.34 (m, 4H, H-4, 4′,8,8′), 8.26–8.13 (m, 4H, H-5,5′,7,7′), 8.08–7.95 (m, 2H, H-6,6′), 4.70 (t, 4H, *J* = 7.3 Hz, N–*CH_2*–), 2.11–1.96 (m, 4H, N–CH_2–*CH_2*–), 1.37–1.10 (m, 12H, N–(CH_2)$_2$–*$(CH_2)_6$*–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.37, 136.90, 136.46, 133.42, 130.81, 129.43, 127.06, 126.64, 125.87, 61.10, 29.70, 27.60, 27.34, 24.58. ESI-MS: *m/z* 199.2 [M^{2+}/2] (calculated for [$C_{28}H_{34}N_2^{2+}$/2] 199.14). EA: calculated 60.23% C, 6.14% H, 5.02% N; found 60.40% C, 6.48% H, 4.68% N.

1,11-Bis(isoquinolinium)-undec-1,11-diyl dibromide (**13**) [17]. M.p. 180–182 °C. ^{1}H NMR (300 MHz, D_2O) δ ppm 9.69 (s, 2H, H-1,1′), 8.49 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.40–8.34 (m, 4H, H-4,4′,8,8′), 8.22–8.11 (m, 4H, H-5,5′,7,7′), 8.03–7.94 (m, 2H, H-6,6′), 4.70 (t, 4H, *J* = 7.1 Hz, N–*CH_2*–), 2.08–1.96 (m, 4H, N–CH_2–*CH_2*–), 1.33–0.99 (m, 14H, N–(CH_2)$_2$–*$(CH_2)_7$*–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.23, 136.74, 136.41, 133.35, 130.77, 129.34, 126.91, 126.56, 125.79, 61.01, 29.64, 27.62, 27.31, 24.48. ESI-MS: *m/z* 206.2 [M^{2+}/2] (calculated for [$C_{29}H_{36}N_2^{2+}$/2] 206.15). EA: calculated 60.85% C, 6.34% H, 4.89% N; found 60.43% C, 6.68% H, 4.67% N.

1,12-Bis(isoquinolinium)-dodec-1,12-diyl dibromide (**14**) [17,24]. M.p. 225–227 °C. ^{1}H NMR (300 MHz, D_2O) δ ppm 9.71 (s, 2H, H-1,1′), 8.50 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.43–8.34 (m, 4H, H-4,4′,8,8′), 8.24–8.12 (m, 4H, H-5,5′,7,7′), 8.03–7.94 (m, 2H, H-6,6′), 4.71 (t, 4H, *J* = 7.0 Hz, N–*CH_2*–), 2.07–1.94 (m, 4H, N–CH_2–*CH_2*–), 1.28–0.90 (m, 16H, N–(CH_2)$_2$–*$(CH_2)_8$*–). ^{13}C NMR (75 MHz, D_2O) δ ppm 148.26, 136.78, 136.48, 133.36, 130.83, 129.37, 126.93, 126.60, 125.83, 61.03, 29.65, 27.71, 27.69, 27.33, 24.51. ESI-MS: *m/z* 213.2 [M^{2+}/2] (calculated for [$C_{30}H_{38}N_2^{2+}$/2] 213.15). EA: calculated 61.44% C, 6.53% H, 4.78% N; found 61.25% C, 6.62% H, 4.85% N.

1,3-Bis(isoquinolinium)-2-oxaprop-1,3-diyl dichloride (**15**). M.p. 158–160 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.85 (s, 2H, H-1,1′), 8.63 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.32–8.27 (m, 4H, H-4,4′,8,8′), 8.20–8.14 (m, 2H, H-7,7′), 8.07 (d, 2H, *J* = 7.9 Hz, H-5,5′), 8.01–7.94 (m, 2H, H-6,6′), 6.49 (s, 4H, –*CH_2*–O). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.29, 138.32, 137.79, 131.89, 131.73, 130.00, 126.90, 126.48, 126.34, 87.85. ESI-MS: *m/z* 302.0 [M^{2+}] (calculated for [$C_{20}H_{18}N_2O^{2+}$] 302.14). EA: calculated 64.35% C, 4.86% H, 7.50% N; found 63.91% C, 5.20% H, 7.08% N.

1,5-Bis(isoquinolinium)-3-oxapent-1,5-diyl dibromide (**16**). M.p. 203–205 °C. ^{1}H NMR (300 MHz, DMSO, d_6): δ (ppm) 9.39 (s, 2H, H-1, 1′), 8.26 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.14–8.06 (m, 2H, H-4,4′), 8.02 (d, 2H, *J* = 7.2 Hz, H-8,8′), 7.97–7.89 (m, 4H, H-5,5′,7,7′), 7.88–7.81 (m, 2H, H-6,6′), 4.88–4.82 (m, 4H, –*CH_2*–O), 4.19–4.12 (m, 4H, N–*CH_2*–). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.69, 137.30, 136.62, 133.28, 131.41, 129.35, 126.96, 126.37, 125.46, 67.58, 60.15. ESI-MS: *m/z* 329.1 [M^{2+}] (calculated for [$C_{22}H_{22}N_2O^{2+}$] 330.17). EA: calculated 53.90% C, 4.52% H, 5.71% N; found 53.98% C, 4.94% H, 5.47% N.

1,4-Bis(isoquinolinium)-but-(2*E*)-ene-1,4-diyl dibromide (**17**). M.p. 275–277 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 10.17 (s, 2H, H-1,1′), 8.78 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.63 (d, 2H, *J* = 6.7 Hz, H-4,4′), 8.24 (d, 2H, *J* = 7.9 Hz, H-8,8′), 8.37 (d, 2H, *J* = 7.9 Hz, H-5,5′), 8.28 (t, 2H, *J* = 7.2 Hz, H-7,7′), 8.09 (t, 2H, *J* = 7.2 Hz, H-6,6′), 6.36–6.32 (m, 2H, =*CH*–), 5.51–5.48 (m, 4H, –*CH_2*–). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 150.26, 137.03, 134.86, 131.20, 130.43, 130.10, 127.23, 127.12, 125.82, 60.83. ESI-MS: *m/z* 156.1 [M^{2+}/2] (calculated for [$C_{22}H_{20}N_2^{2+}$/2] 156.08). EA: calculated 55.96% C, 4.27% H, 5.93% N; found 55.83% C, 4.35% H, 5.94% N.

1,4-Bis(isoquinolinium)-but-(2*Z*)-ene-1,4-diyl dichloride (**18**). M.p. 229–230 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 10.51 (s, 2H, H-1,1′), 9.05 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.66 (d, 2H, *J* = 6.7 Hz, H-4,4′), 8.61 (d, 2H, *J* = 7.8 Hz, H-8,8′), 8.39 (d, 2H, *J* = 7.8 Hz, H-5,5′), 8.28 (t, 2H, *J* = 7.1 Hz, H-7,7′), 8.10 (t, 2H, *J* = 7.1 Hz, H-6,6′), 6.33–6.21 (m, 2H, =*CH*–), 5.94–5.85 (m, 4H, –*CH_2*–). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 150.53, 137.02, 136.94, 135.16, 131.11, 130.51, 128.76, 127.24, 125.80, 57.24. ESI-MS: *m/z* 311.1 [M^{2+}] (calculated for [$C_{22}H_{20}N_2^{2+}$] 312.16). EA: calculated 68.93% C, 5.26% H, 7.31% N; found 68.86% C, 7.04% H, 7.64% N.

1,1′-Bis(isoquinolinium)-1,2-phenyldimethylenyl dibromide (**19**). M.p. 288–289 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.14 (s, 2H, H-1,1′), 8.34 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.02 (d, 2H, *J* = 6.6 Hz, H-4,4′), 7.97–7.89 (m, 4H, H-5,5′,8,8′), 7.88–7.85 (m, 4H, Ph), 7.81–7.70 (m, 4H, H-6,6′,7,7′), 6.17 (s, 4H, –*CH_2*–). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 146.73, 137.32, 136.08, 134.27, 132.45, 131.61, 131.30, 130.43, 128.95, 126.45, 125.95, 125.88, 61.06. ESI-MS: *m/z* 363.2 [M^{2+}] (calculated for [$C_{26}H_{22}N_2^{2+}$] 362.18). EA: calculated 59.79% C, 4.25% H, 5.36% N; found 59.59% C, 4.17% H, 5.66% N.

1,1′-Bis(isoquinolinium)-1,3-phenyldimethylenyl dibromide (**20**). M.p. 286–288 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.73 (s, 2H, H-1,1′), 8.45 (d, 2H, *J* = 6.5 Hz, H-3,3′), 8.33–8.26 (m, 4H, H-4,4′,8,8′), 8.23–8.13 (m, 4H, H-5,5′,7,7′), 8.04–7.95 (m, 2H, H-6,6′), 7.62–7.58 (m, 3H, Ph), 7.27 (s, 1H, Ph), 5.96 (s, 4H, –*CH_2*–). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 149.11, 137.31, 137.24, 134.43, 133.72, 131.38, 130.31, 126.83, 139.66, 127.74, 127.23, 126.99, 126.31, 63.44. ESI-MS: *m/z* 361.8 [M^{2+}] (calculated for [$C_{26}H_{22}N_2^{2+}$] 362.18). EA: calculated 59.79% C, 4.25% H, 5.36% N; found 59.32% C, 4.31% H, 5.46% N.

1,1′-Bis(isoquinolinium)-1,4-phenyldimethylenyl dibromide (**21**). M.p. decomp. 316 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 10.37 (s, 2H, H-1,1′), 8.85 (d, 2H, *J* = 6.7 Hz, H-3,3′), 8.60 (d, 2H, *J* = 6.8 Hz, H-4,4′), 8.52 (d, 2H, *J* = 8.2 Hz, H-8,8′), 8.34 (d, 2H, *J* = 8.1 Hz, H-5,5′), 8.27 (t, 2H, *J* = 7.1 Hz, H-7,7′), 8.08 (t, 2H, *J* = 7.2 Hz, H-6,6′), 7.70 (s, 4H, Ph), 6.01 (s, 4H, –*CH_2*–). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 150.25, 137.12, 136.95, 135.25, 134.68, 131.29, 130.51, 129.59, 127.26, 127.20, 126.23, 62.60. ESI-MS: *m/z* 361.9 [M^{2+}] (calculated for [$C_{26}H_{22}N_2^{2+}$] 362.18). EA: calculated 59.79% C, 4.25% H, 5.36% N; found 59.58% C, 4.34% H, 5.50% N.

1,1′-Bis(isoquinolinium)-naphtyl-3,6-dimethylenyl dibromide (**22**). M.p. 241–243 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 10.21 (s, 2H, H-1,1′), 8.77 (d, 2H, *J* = 6.4 Hz, H-3,3′), 8.57–8.47 (m, 4H, H-4,4′,8,8′), 8.33–8.21 (m, 6H, H-5,5′,7,7′, Nph), 8.11–7.97 (m, 4H, H-6,6′, Nph) 7.71 (d, *J* = 7.5 Hz, Nph), 6.20 (s, 4H, –*CH_2*–). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 151.35, 139.24, 138.75, 135.76, 135.15, 134.65, 133.48, 132.83, 131.89, 130.69, 130.56, 129.34, 128.65, 128.10, 127.91, 65.48. ESI-MS: *m/z* 412.0 [M^{2+}] (calculated for [$C_{30}H_{24}N_2^{2+}$] 412.19). EA: calculated 62.96% C, 4.23% H, 4.89% N; found 62.59% C, 4.30% H, 4.63% N.

6.3. In vitro reactivation assay

Multichannel spectrophotometer Sunrise (Tecan, Salzburg, Austria) was used for all measurements of cholinesterases activity. Standard conditions for temperature and pressure (SATP; 25 °C, 100 kPa) were used. Previously optimized Ellman's procedure was slightly adopted in order to estimate anticholinergic properties [26]. 96-wells photometric microplates made from polystyrene (Nunc, Rockilde, Denmark) were used for measuring purposes. Human

erythrocyte AChE or human plasmatic BChE (Aldrich; commercially purified by affinity chromatography) was suspended into phosphate buffer (pH 7.4) up to final activity 0.002 U/μl. Protein solution of cholinesterase (5 μl) was dissolved in buffer (20 μl) and freshly mixed solution of 0.4 mg/ml 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB; 20 μl) was added. Appropriate concentration of inhibitor (10 mM–1 nM; 5 μl) was injected per well and mixture was incubated for 5 min 1 mM acetylthiocholine chloride (ATChCl) in phosphate buffer (20 μl) was added. After additional 5 min incubation, absorbance was measured at 412 nm using automatic shaking of the microplate.

Percentage of inhibition (I) was calculated from the measured data as follows:

$$I = \left(1 - \frac{\Delta A_i}{\Delta A_0}\right) \times 100$$

ΔA_i indicates absorbance change provided by cholinesterase exposed to anticholinergic compound. ΔA_0 indicates absorbance change caused by intact cholinesterase, where phosphate buffer was applied in the same way as the anticholinergic compound.

IC_{50} was determined using Origin 6.1 (Northampton, MA, USA). Percentage of inhibition was calculated by Hill plot ($n = 1$). The other plot variants were not optimal and the correlation coefficient was lower compared to chosen method. Subsequently, IC_{50} was computed.

6.4. In vitro inhibition assay

Human erythrocyte AChE (Aldrich) was used throughout the experiments. The adopted photometrical Ellman's method was used for AChE activity evaluation [29]. A polystyrene cuvette was filled with 0.4 mg/ml DTNB (0.4 ml), AChE solution with overall activity 0.5 μkat in phosphate buffered saline (PBS; 25 μl), tested inhibitor (25 μl), and PBS (450 μl). The mixture was gently shaken and the reaction was started by addition of varying concentration (0.1 mM–1 M) of ATChCl in PBS (100 μl). The arising yellow color of 5-thio-2-nitrobenzoic acid was measured at 412 nm against blank (mixture of DTNB and ATChCl in given concentrations). The spontaneous interaction between tested inhibitor and DTNB was excluded after incubation of whole reaction mixture without AChE that was replaced by PBS (25 μl). The inhibition was evaluated by Lineweaver–Burk plot for inhibitor concentration (10^{-8}–10^{-2} mol/l) and ATChCl concentration (10^{-5}–10^{-1} mol/l). The measurements were carried out in triplicate and the average value was used for the plot construction.

The obtained data were processed by Origin 8.0 (Northampton, MA, USA). The constants were calculated from enzyme kinetics using Lineweaver–Burk plot (double reciprocal plot). The AChE dissociation constant for enzyme–inhibitor complex (K_{i1}) and dissociation constant for enzyme–inhibitor–substrate complex (K_{i2}) were calculated using following equations:

$$K_{i1} = \frac{[E][I]}{[EI]} \quad K_{i2} = \frac{[ES][I]}{[ESI]}$$

6.5. Molecular docking

Docking calculations were carried out using Autodock 4.0.1 [35]. A Lamarckian genetic algorithm (Amber force field) was used. A population of 150 individuals and 2,500,000 function evaluations were applied. The structure optimization was performed for 27,000 generations. Electrostatic energies were calculated for all non-bonds between moving atoms. Minimum electrostatic potential (−41.82 kcal/mol) and maximum electrostatic potential (40.09 kcal/mol) were set up. Docking calculations were set to 50 runs. At the end of calculation, Autodock performed cluster analysis.

The structure of mus musculus AChE and human BChE was prepared using Pymol 1.1 from crystal structure (pdb code 2gyv and 1p0i) using Autodock Tools 1.5.2 [36–38]. The 3D affinity grid box was designed to include the full active and peripheral site of AChE. The number of grid points in the x-, y- and z-axes was 110, 110 and 110 with grid points separated by 0.253 Å. The molecular models of ligands were built using ChemDraw 11.1 and minimized with UCSF Chimera 1.3 (Amber Force field) in charged form [36]. The maximum root mean square tolerance for conformational cluster analysis was 2.0 Å. The visualization of enzyme–ligand interactions (Figs. 5 and 6) was prepared using Pymol 1.1 [37].

Conflict of interest

Authors declare that there are no conflicts of interest.

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