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Original article

Synthesis, design and biological evaluation of novel highly potent tacrine congeners for the treatment of Alzheimer's disease

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ABSTRACT

New tacrine derivatives **5a**–**d**, **6a**–**d** with piperazino-ethyl spacer linked with corresponding secondary amines and tacrine homodimer **8** were synthesized and tested as cholinesterase inhibitors on human acetylcholinesterase (*h*AChE) and human plasmatic butyrylcholinesterase (*h*BChE). In most cases the majority of synthesized derivatives exhibit a high AChE and BChE inhibitory activity with IC_{50} values in the low-nanomolar range, being clearly more potent than the reference standard tacrine (9-amino-1,2,3,4-tetrahydroacridine, **1**) and 7-MEOTA (7-methoxy-9-amino-1,2,3,4-tetrahydroacridine). Among them, inhibitors **8** and **5c**, showed a strong inhibitory activity against *h*AChE, with an IC_{50} value of 4.49 nM and 4.97, nM resp., and a high selectivity to *h*AChE. The compound **5d** acted as the most potent inhibitor against *h*BChE with an IC_{50} value of 33.7 nM and exhibited also a good selectivity towards *h*BChE. The dissociation constants K_i of the selected inhibitors were compared with their IC_{50} values. Molecular modeling studies were performed to predict the binding modes between individual derivatives and *h*AChE/*h*BChE.

1. Introduction

Alzheimer's disease (AD)[2] is a progressive and neurodegenerative disease characterized by progressive memory impairment, related to a disordered cognitive function [1]. Histological changes underlying this disorder give rise to a presence of numerous amyloid plaques of β-amyloid peptide (Aβ), neurofibrillary tangles (NFT) and a dramatic loss of synapses, a decrease in a number of neurons [2,3]. Scientists have proposed several theories explaining the mechanism of AD development [4–8]. The oldest of the AD hypothesis is the cholinergic hypothesis [4,5,9] which has become the leading strategy for the development of cholinesterase inhibitors (ChEIs) aimed to increasing of levels of acetylcholine (ACh) through inhibition of cholinesterases (ChEs) [10,11]. The most prevailing hypothesis, the amyloid hypothesis posits that an increased production of β-amyloid peptide and its aggregation and accumulation in a brain lead to a neuronal cell death [6,12]. Two classes of drugs, namely tacrine (**1**, Cognex®), donepezil (Aricept®), rivastigmine (Exelon®), galantamine (Reminyl®) as AChE inhibitors (AChEIs) and memantine as ann N-methyl-D-aspartate (NMDA) receptor antagonist, were approved to treat AD [11–13]. Tacrine (THA, 9-amino-1,2,3,4-tetrahydroacridine) with a major selectivity towards BChE instead of AChE, was the first ChEI approved for the treatment of AD [14,15]. The use of tacrine in AD has been limited by serious side effects such as hepatotoxicity, gastrointestinal disturbance and hypotension [16–18]. The multifactorial nature of AD supports new therapeutic strategies based on a multipotent approach (one molecule, multiple targets) paradigm [9,19,20]. Such a strategy has been described in a review that summarized the research development of tacrine derivatives with a cholinesterase and amyloid aggregation inhibiting activity from a bioorganic aspect [21]. As potent acetylcholinesterase inhibitors with

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; NFT, neurofibrillary tangles; AChEIs, acetylcholinesterase inhibitors; AChE, acetylcholinesterase; ACh, acetylcholine; THA, 9-amino-1,2,3,4-tetrahydroacridine; BChE, butyrylcholinesterase; ChEI, cholinesterase inhibitors; NMDA, N-methyl-D-aspartate receptor; BACE, β-secretase; APP, amyloid precursor protein; PAS, peripheral anionic site; CAS, catalytic active site; MTDLs, multi-target-directed ligands; M_2, muscarinic receptors; CPC, chloropropionylchloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ATC, acetylthiocholine; BTC, butylthiocholine; PB, phosphate buffer.

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antioxidant properties, tacrine-melatonin hybrids [16], tacrine-ferulic acid hybrids [22], tacrine-carvedilol heterodimers [23] have been synthesized. Some other tacrine heterodimers,such as tacrine-cystamine dimer [24], tacrine-oxoisoaporphine congeners [25], heterobivalent tacrine derivatives containing aromatic groups [26] and tacrine-lipoic acid dimers [27] exhibiting acetylcholinesterase and acetylcholinesterase-induced Aβ aggregation inhibition activity. Tacrine-gallamine hybrids which act as AChEI and M_2 muscarinic receptor modulators [28], tacrine–NO–donors, designed as hepatoprotective anti-Alzheimer drug candidates [29], tacrine-propidium heterodimers with an inhibition effect on cholinesterase/Aβ aggregation and BACE-1 [30] present a further class of multi-target-directed ligands (Fig. 1).

In the view of the above mentioned reasons, we focused on the development of more active and selective ligands which are capable of interacting with a catalytic anionic side (CAS) and also with a peripheral anionic side (PAS) of AChE. In our previous paper we reported a synthesis of a novel AChEIs, 2-[(1,2,3,4-tetrahydroacridin-9-yl)imino]-3-substituted 1,3-thiazolidin-4-ones [31] and tacrine ligands with piperazine and thiourea linkers, most of them with a remarkable *h*AChE/*h*BChE inhibition activity [32]. Given the fact that amines are widely known as biologically active compounds or the important building blocks for the synthesis of biologically active compounds [33,34] we decided to incorporate appropriate secondary aminesinto thetacrine moiety.Here, we report the synthesis, biological evaluation and molecular modeling of ChEIs, represented by tacrine derivatives (Fig. 2) designed by a combination of tacrine, piperazino-ethyl side chain and appropriate secondary amines **5a**–**d**, **6a**–**d** (Fig. 2) and tacrine homodimer **8**. An interaction with both CAS and PAS of AChE are confirmed by docking simulation.

2. Results and discussion

2.1. Chemistry

Tacrine derivatives **4**, **5a**–**d**, **6a**–**d** and tacrine homodimer **8** were obtained by the method summarized in Scheme 1. As a starting compound for the synthesis of a new tacrine ligands, 9-chloro-1,2,3,4-tetrahydroacridine (**2**) was used. The synthetic route of *N*-(piperazinoethyl)-*N*-(1,2,3,4-tetrahydroacridin-9-yl)amine (**3**) was reported in our previous paper [32]. Piperazinoethyl-tetrahydroacridinylamine **3** was further acylated by the corresponding 3-chloropropionylchloride (CPC) in excess of triethylamine [35,36], to give halogenated intermediate chloropropionamide **4** (Scheme 1). Subsequent aminolysis of **4** with appropriate secondary amines (piperidine, methylcyclohexylamine, ethylcyclohexylamine and *N*-methylaniline) in acetone/ethanol afforded the final products **5a**–**d** in 50–65% yield. Compounds **6a**–**d** were prepared from derivatives **5a**–**d** by a reduction of their amide group with $LiAlH_4$ (Scheme 1). The reaction of 9-chloro-1,2,3,4-tetrahydroacridine (**2**) with 1,2-diaminoethane in phenol gave known compound **7** [37,38]. For the synthesis of tacrine homodimer **8**, *N*-(1,2,3,4-tetrahydroacridin-9-yl)-1,2-ethanediamine (**7**) was reacted with chloropropionamide **4** in the presence of N,

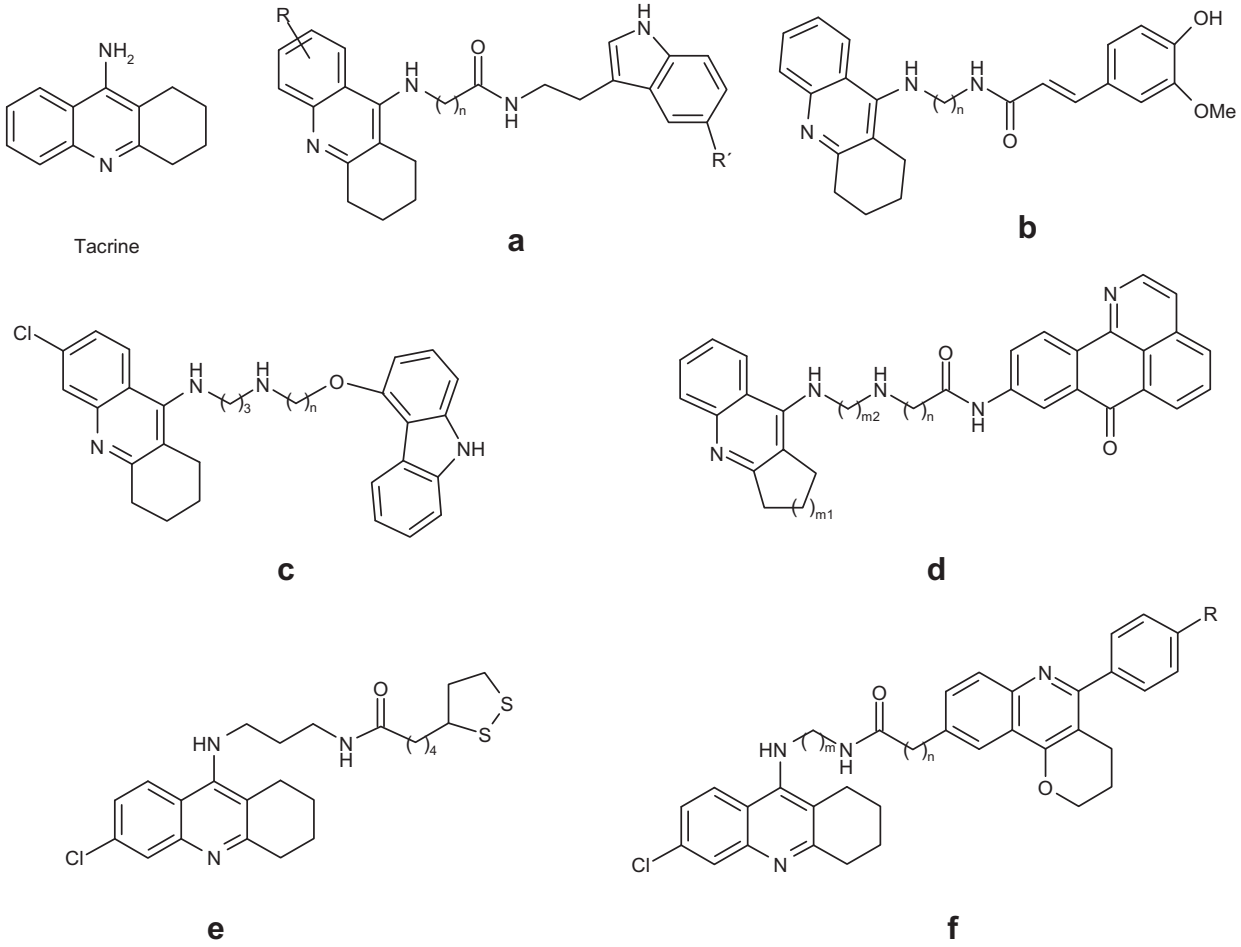


Fig. 1. Selected multi-target-directed ligands bearing tacrine moiety: a) tacrine-melatonin, b) tacrine-ferulic acid, c) tacrine-carvedilol, d) tacrine-oxoisoaporphine, e) tacrine-lipoic acid, f) tacrine-propidium.

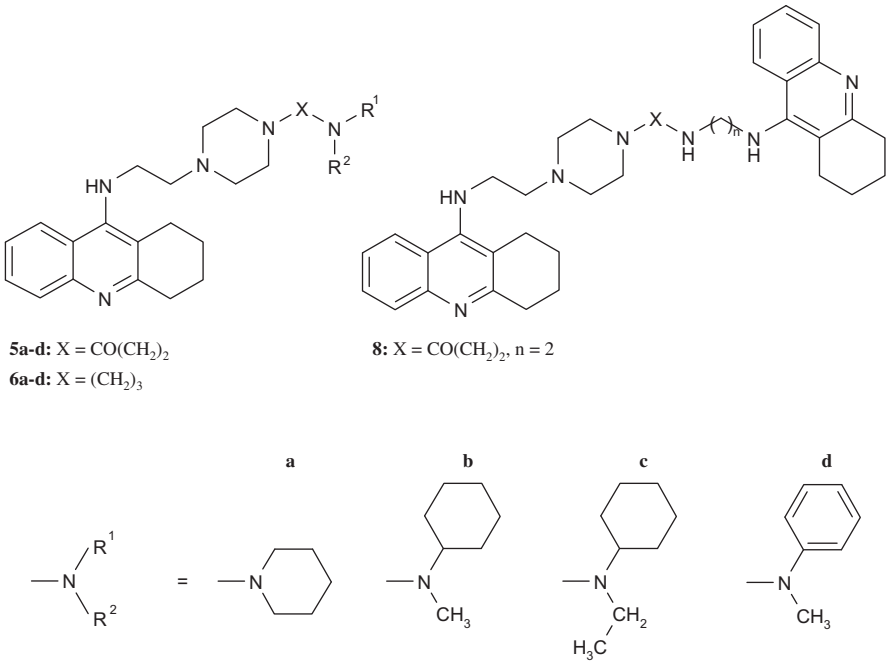
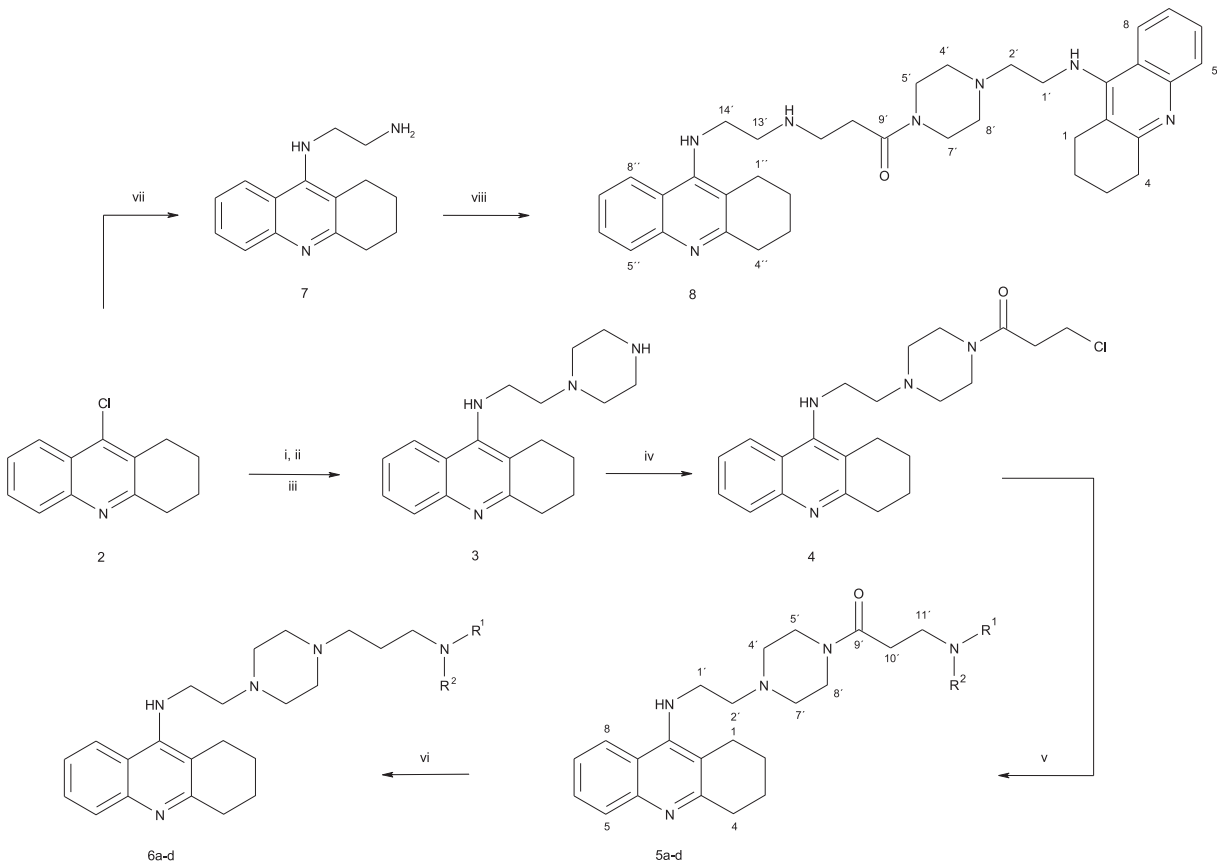


Fig. 2. Chemical structure of synthesized tacrine derivatives **5a**–**d**, **6a**–**d** and tacrine homodimer **8**.



Scheme 1. Synthesis of designed inhibitors **5a**–**d**, **6a**–**d**, **8**. Reagents and conditions: (i) 4-(2-aminoethyl)piperazine-1-carboxylic acid *tert*-buthyl ester, phenol, reflux, 4 h; (ii) AcOH/HCl, 1.5 h; (iii) NH_4OH, 15 min; (iv) CPC, Et_3N, CH_2Cl_2, O °C, 1 h; (v) corresponding secondary amine, K_2CO_3, acetone/ethanol, reflux, 9–13 h; (vi) $LiAlH_4$, THF, reflux, 2 h; (vii) 1, 2-diaminoethane, phenol, reflux, 2h; (viii) **4**, N, N-diisopropylethylamine, CH_2Cl_2, reflux, 72 h.

N-diisopropylethylamine (Hunig's base) in CH_2Cl_2 (Scheme 1). Target product **8** was obtained in 63% yield.

2.2. Inhibition studies on hAChE and hBuChE

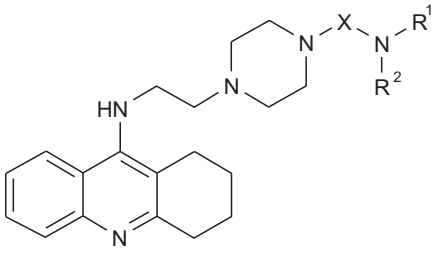
To determine a therapeutic potential of the new series of tacrine derivatives for the treatment of AD, the inhibition constants, IC_{50} of compounds **5a**–**d**, **6a**–**d** and **8** was determined against human erythrocytal AChE and human plasmatic BChE using the method of Ellman et al. [39]. For comparison, tacrine **1** and 7-MEOTA were used as a reference compounds. 7-MEOTA is a weaker inhibitor compared with tacrine but was found to be less toxic and pharmacologically equally active to tacrine [40]. IC_{50} values and selectivity index [SI = IC_{50}(*h*BChE)/IC_{50}(*h*AChE)] of tacrine derivatives **5a**–**d**, **6a**–**d**, **8** and dissociation constants K_{i1} and K_{i2} of selected derivatives **5a**–**d**, **6b**, **6d** are summarized in Table 1.

The synthesized tacrine compounds showed an inhibitory activity against *h*AChE in the low–nanomolar range of IC_{50} values. They are two orders of magnitude more potent than tacrine **1**, and four orders of magnitude more effective in inhibiting AChE than 7-MEOTA. The derivatives **8** and **5c** exhibited the most promising inhibition towards *h*AChE (**8**, IC_{50} = 4.49 nM and **5c**, IC_{50} = 4.97 nM). The changes of substituents on the terminal side chain e.g. (**5b**→**5c**) evidently influence the increase of their AChE inhibitory effect (see Table 1). The derivatives with the amide group (**5b**–**d**) exhibit a higher activity compared to the compounds in which the amide group was reduced to a methylene group (**6b**–**d**). It is notable that some of the tested compounds exhibited a 1.5- to 338.5-fold higher inhibitory activity towards AChE than towards BChE. The best selectivity for *h*AChE was shown by tacrine homodimer **8** (SI = 338.5) and for *h*BChE by **5d** (SI = 0.48). The IC_{50} values were compared with two dissociation constants K_{i1} and K_{i2} which were measured for selected ligands **5a**–**d** and **6b**, **6d** (Table 1). The Lineweaver–Burk plot revealed that tacrine congeners **5a**–**d** and **6b**, **6d** inhibited *h*AChE non-competitively. The K_{i1} values of 0.38 nM for the ligand **5b** indicate a strongest affinity towards *h*AChE that is 600-times higher compared to tacrine and 5500-times higher related to 7-MEOTA. The K_{i1} values of 1.35 nM for the ligand **5c** with affinity towards *h*AChE was 4-times higher compared to methyl derivative **5b**.

2.3. Molecular modeling studies

The compound **5c** and its structurally related compounds **5b**, **6b** were treated in silico towards *h*AChE in order to find an explanation for their inhibition potential. A similar study was done for *h*BChE with **5b**–**d** and **6b**. Docking simulations were performed to reveal possible intermolecular interactions that might drive an inhibition activity of novel tacrine derivatives (for more details see Experimental section). To overcome a software limitation, we simulated flipping of spacer's terminal nitrogen by building two structures for each derivate with the opposite configuration of substituents (see Supplementary data, Fig. S1). Subsequently, we solved the orientation of a carbonyl group attached to a piperazine ring. We did not choose to set up a rotable amide bond within a software option, but rather we built two structures with an opposite carbonyl orientation to restrain a planar conformation of an amide group (see Supplementary data, Fig. S1). Thus, we obtained four structures for compounds **5b**–**d** and two for derivative **6b**. In order to find a degree of protonization of our compounds, we applied software options within Marvin software packed [http://www.chemaxon.com]. As a consequence of the prediction with bis/tris protonization falling into the ratio 1:1, we decided to study bis and tris protonated compounds separately (see Experimental section). Thus, each structure was once treated as bis protonated in a docking run and then in a tris protonated charged molecule with protonated positions in accordance to a software prediction. So, we gained the library of eight items for compound **5b**–**d** and four items

Table 1
Inhibition of *h*AChE and *h*BChE by target compounds their selectivity index and dissociation constants K_{i1} and K_{i2}.



Compound	—N(R1)(R2)	X	IC_{50}(*h*AChE)[a] ± SD (nM)	IC_{50}(*h*BChE)[a] ± SD (nM)	SI[b]	K_{i1}[c] (nM)	K_{i2}[d] (nM)
5a	Piperidine-1-yl	$CO(CH_2)_2$	51.7 ± 10.3	77.8 ± 13.5	1.51	16.8	76.1
5b	Methylcyclohexylamino	$CO(CH_2)_2$	14.9 ± 2.9	63.7 ± 11.0	4.28	0.38	0.318
5c	Ethylcyclohexylamino	$CO(CH_2)_2$	4.97 ± 1	41.2 ± 7.16	8.29	1.35	3.73
5d	Methylphenylamino	$CO(CH_2)_2$	70.3 ± 14.06	33.7 ± 5.86	0,48	13.7	85.5
6a	Piperidine-1-yl	$(CH_2)_3$	nd	nd	nd	nd	nd
6b	Methylcyclohexylamino	$(CH_2)_3$	40.7 ± 8.14	137 ± 23.8	3.37	10.3	47.6
6c	Ethylcyclohexylamino	$(CH_2)_3$	194 ± 0.11	126 ± 0.07	0.65	–	–
6d	Methylphenylamino	$(CH_2)_3$	84.8 ± 16.9	211 ± 36.7	2.49	80.0	24.7
8	–	$CO(CH_2)_2$	4.49 ± 0.34	1520 ± 0.14	338.5	–	–
1	–	–	500 ± 100	23 ± 4	0,05	225	101
7-MEOTA	–	–	15 000 ± 2900	21 000 ± 3400	1,4	2090	6340

nd: not determined.
[a] IC_{50}, inhibitor concentration (means ± SD of three experiments) for 50% inhibition of enzyme.
[b] Selectivity index for AChE = IC_{50}(*h*BChE)/IC_{50}(*h*AChE).
[c] K_{i1}: Dissociation constant for AChE - inhibitor complex.
[d] K_{i2}: Dissociation constant for AChE - inhibitor–substrate complex.

set for **6b** (see Supplementary data, Fig. S1). Conformation of a piperazine ring remained the same as in an x-ray structure for a donepezil inhibition complex with *Tc*AChE (PDB: 1EVE). The poses with the lowest binding energy within *h*AChE for derivative **5b**, **5c**, **6b** are depicted in Fig. 3.

There are five hydrogen bonds between **5b** and TRP86, TYR124, TYR337 (Fig. 4). A similar binding orientation could be found for compound **6b** (Fig. 4). There are predicted formations of three hydrogen bonds with TRP86, TYR124, TYR337 amino acids. Derivate **5c** adopts a pose directly when interacting with amino acids of catalytic triad, SER203 and HIS447, which might be the reason for an overall higher biological activity over other derivatives. An additional stabilization might be gained by a formation of hydrogen bonds with TYR124 and TYR337 (Fig. 4).

The most active derivative **5d** with related **5b**, **5c**, **6b** were studied by a docking simulation toward *h*BChE. An overall view of docking poses with the lowest binding energy proposed by a simulation is depicted in Fig. 5. There we can see a very close orientation of derivatives **5b**–**d** in the enzymatic cavity with a different orientation of piperazine of **6b**. All derivatives, except **6b**, form hydrogen bonds with ASP70, TYR337 and HIS438 as it is shown in Fig. 4. A higher inhibition of **5d** could be prescribed to a presence of an additional aromatic system, phenyl group, that is able to provide an additional stabilization by a π–π interaction with PHE329. This interaction might be missing in derivates **5b**, **5c**, **6b** where phenyl is substituted by cyclohexyl.

3. Conclusion

In summary, we have reported the synthesis and the results for an acetylcholinesterase and butyrylcholinesterase inhibition activity of eight new tacrine ligands (**5a**–**d**, **6a**–**d**) and tacrine homodimer **8**. Our results showed that synthetized compounds had a high cholinesterases inhibitory activity with IC_{50} values less than 15 nM against *h*AChE, and less than 40 nM toward *h*BChE, being clearly more potent than tacrine **1** and 7-MEOTA. The most promising inhibition activity of two orders of magnitude better than tacrine and four orders of magnitude better than 7-MEOTA was found for derivatives **8** and **5c** against *h*AChE (IC_{50} = 4.49, 4.97 nM). Derivatives **5b**–**d** with an amide group in tether showed better inhibitory activities against *h*AChE than derivatives with a methylene group **6b**–**d**. The reduction of amide groups in tether significantly reduced an anti-*h*BChE activity as indicated by a comparison of **5b**–**d** with **6b**–**d**. Among all tested compounds, derivative **8** showed the best selectivity against *h*AChE (338.5) and compound **5d** to *h*BChE (0.48). Presumed binding modes between individual derivatives and ChEs were predicted by docking calculations. Molecular modeling studies confirmed that these hybrids target both, the catalytic active site and peripheral anionic site of AChE.

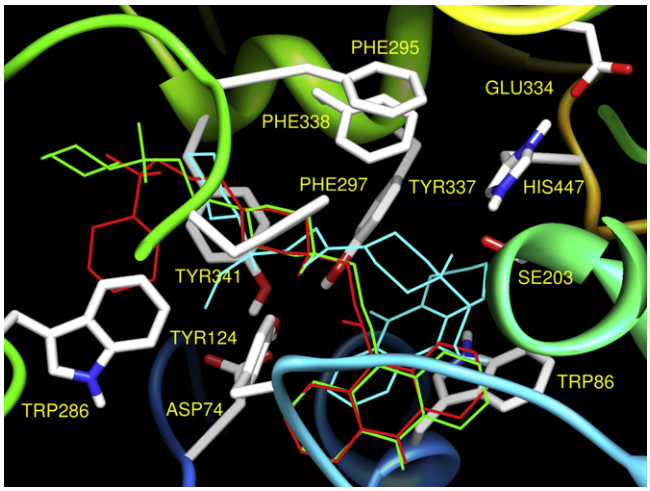


Fig. 3. Top-score docking pose of derivatives **5b** (red), **5c** (cyan), **6b** (green) depicted their putative structural orientations in the active-site gorge of the *h*AChE in a ribbon style [41]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Experimental section

4.1. Synthesis

All solvents, chemicals, and reagents were obtained commercially and used without purification. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Mercury Plus NMR spectrometer using $CDCl_3$ or DMSO-D_6 as solvents with tetramethylsilane as an internal standard. Chemical shifts, *δ*, are given in parts per million (ppm), and spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet) or m (multiplet). Coupling constants, *J*, are expressed in hertz (Hz). Thin-layer chromatography was performed on Macherey–Nagel Alugram ®Sil G/UV254 plates, and spots were visualized with UV light. Chromatographic separations were performed on silica gel 60 (0.063–0.040 mm, Merck) column chromatography. Melting points were recorded on a Boetius hot-plate apparatus and are uncorrected. Yields refer to isolated pure products and were not maximized. CHN analysis was performed on a CHN analyzer Perkin–Elmer 2400.

4.1.1. Synthesis of 3-chloro-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino) ethyl]piperazino}-1-propanone (*4*)

A solution of *N*-(piperazinoethyl)-*N*-(1,2,3,4-tetrahydroacridin-9-yl)amine (**3**, 100 mg, 0.33 mmol) and Et_3N (0.11 ml, 0.82 mmol) in CH_2Cl_2 (5 ml) was added dropwise to a mixture of chloropropionyl chloride (0.078 ml, 0.82 mmol) in CH_2Cl_2 (2 ml) at 0 °C. The mixture was stirred at 0 °C for 1 h and then at rt for 10 min. A saturated solution of NH_3 in CH_3OH (0.3 ml) was added. The mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (9 ml) and then washed with brine (6 × 3 ml). After drying over anhydrous $MgSO_4$, the organic layer was concentrated. The residue was purified by column chromatography, eluent $CHCl_3$/MeOH (9:1). Compound **4** (0.1 g, 77% yield) was obtained as a yellow powder, m.p. = 180–183 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) *δ* 1.83–2.0 (m, 4H, 2 × CH_2, H-2,3), 2.48–2.56 (m, 4H, 2 × CH_2, H-4′,8′), 2.66 (t, 2H, CH_2, H-2′, *J* = 5.4 Hz), 2.72–2.76 (m, 2H, CH_2, H-1), 2.82 (t, 2H, CH_2, H-10′, *J* = 7.2 Hz), 3.08–3.13 (m, 2H, CH_2, H-4), 3.51–3.55 and 3.66–3.74 (m, 4H, 2 × CH_2, H-5′,7′), 3.64 (t, 2H, CH_2, H-1′, *J* = 5.6 Hz), 3.84 (t, 2H, CH_2, H-11′, *J* = 7.0 Hz), 5.30 (bs, 1H, NH), 7.35 (dd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 8.4 Hz), 7.60 (dd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 8.0 Hz), 7.98–8.04 (m, 2H, 2 × CH, H-5,8). ^{13}C NMR ($CDCl_3$) *δ* 22.5, 22.9 (C-2, 3), 24.7 (C-1), 33.1 (C-4), 36.0 (C-10′), 39.8 (C-11′), 42.0, 45.5 (C-5′, 7′), 45.0 (C-1′), 52.3, 52.6 (C-4′, 8′), 57.4 (C-2′), 115.4 (C-9a), 119.6 (C-8a), 122.6 (C-8), 123.8 (C-7), 128.1 (C-5), 128.8 (C-6), 146.5 (C-10a), 151.3 (C-9), 157.6 (C-4a), 168.1 (C-9′). Anal. Calcd for $C_{22}H_{29}ClN_4O$ (400.96): C, 65.90; H, 7.29; N, 13.97. Found: C, 66.02; H, 7.37; N, 14.06.

4.1.2. General procedures for the synthesis of 3-substituted-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]piperazino}-1-propanones ***5a-5d***

Secondary amine (0.25 mmol), K_2CO_3 (70 mg, 0.5 mmol) and 1:1 mixture of acetone/ethanol (8 ml) were added to a solution of 3-chloro-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]

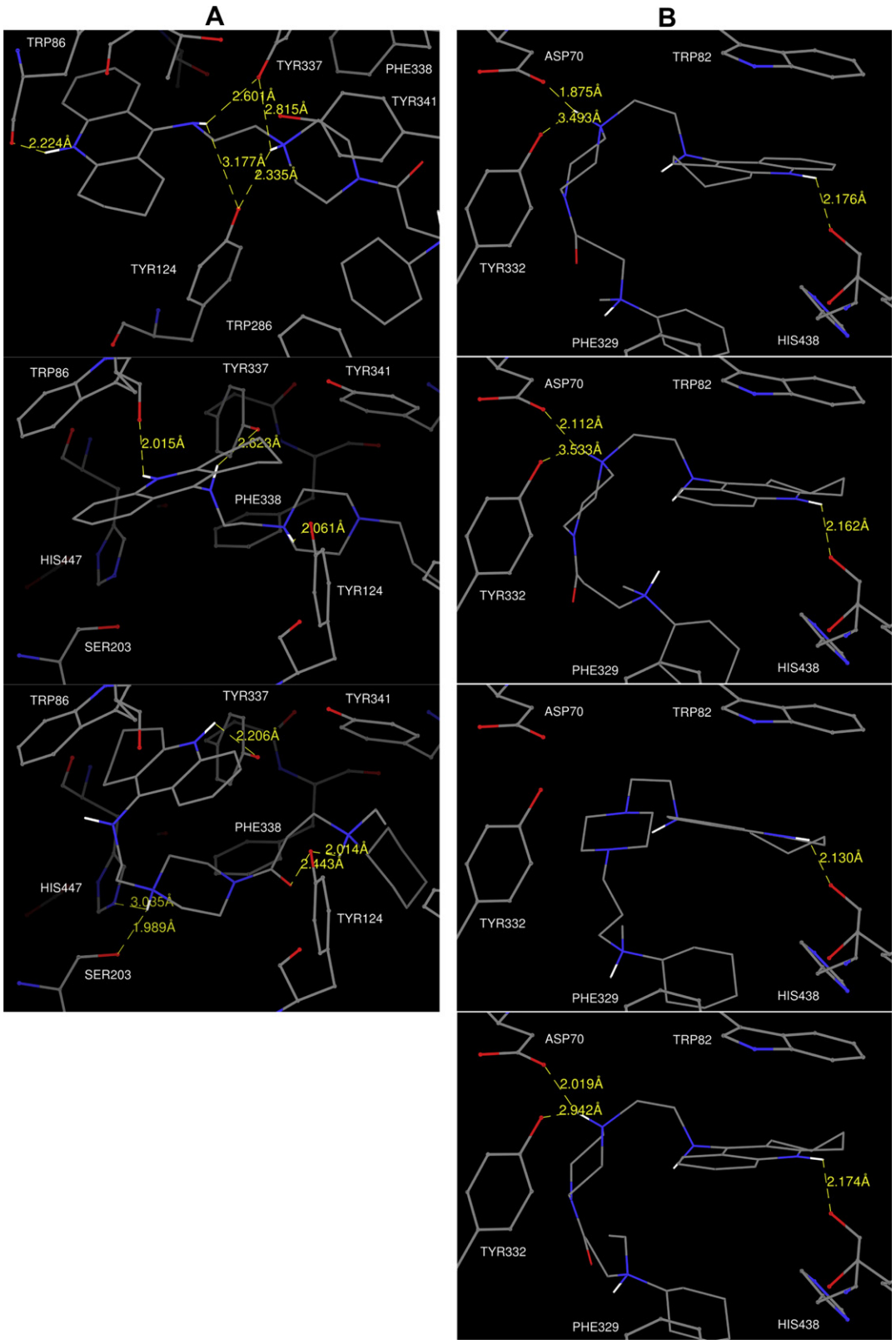


Fig. 4. Column A: Top-score docking pose of derivative **5b**, **6b**, **5c** depicted its putative hydrogen bonds formed with amino acid residues in the active-site gorge of the *h*AChE. Column B: Top-score docking pose of derivative **5d**, **5b**, **6b**, **5c** depicted its putative hydrogen bonds formed with amino acid residues in the active-site gorge of the *h*BChE. Atoms' color palette: C–gray; H–white; N–blue, O–red [41]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

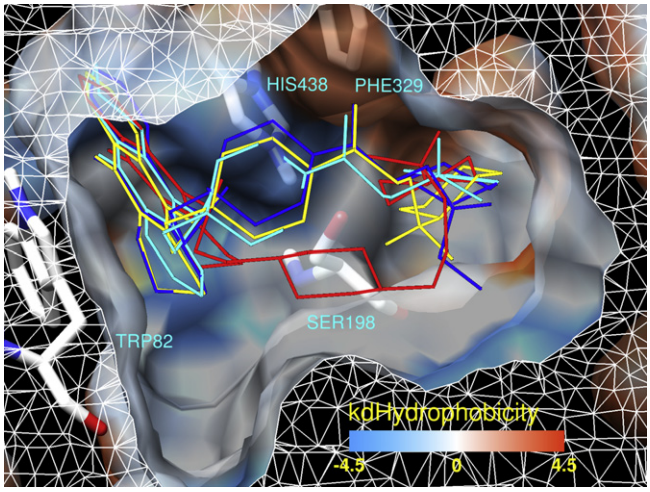


Fig. 5. Top-score docking pose for derivatives **5b** (yellow), **5c** (blue), **5d** (cyan), **6b** (red) depicted putative structural orientations in the active-site gorge of the *h*BChE. For clarity only enzyme active site is shown as a hydrophobic surface pocket [41,42]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

piperazino-1-yl}-1-propanone (**4**, 100 mg, 0.25 mmol),. The mixture was refluxed and stirred for 9–13 h, then cooled to rt, filtered, and evaporated.

*4.1.2.1. Synthesis of 3-(piperidino)-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]-piperazino}-1-propanone (**5a**).* Intermediate **4** was reacted with piperidine following the general procedure to give 60 mg (60%) of **5a** as oil after column chromatograpghy ($CHCl_3$/MeOH/Et_3N, 4/1/0.05). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.51–1.70 (m, 6H, 3 × CH_2, H-14′,15′,16′), 1.88–2.0 (m, 4H, 2 × CH_2, H-2,3), 2.40–2.55 (m, 8H, 4 × CH_2, H-4′,8′,13′,17′), 2.56–2.62 (m, 2H, CH_2, H-10′), 2.64 (t, 2H, CH_2, H-2′, J = 5.8 Hz), 2.68–2.82 (m, 4H, 2 × CH_2, H-1,11′), 3.05–3.15 (m, 2H, CH_2, H-4), 3.50–3.60 and 3.66–3.70 (m, 4H, 2 × CH_2, H-5′,7′), 3.63 (t, 2H, CH_2, H-1′, J = 5.6 Hz), 7.35 (dd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 7.2 Hz), 7.57 (dd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 7.2 Hz), 7.97 (d, 2H, CH_2, H-5, J = 8.4 Hz), 8.0 (d, 2H, CH_2, H-8, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ 22.7, 23.0 (C-2, 3), 24.4 (C-1,15′), 25.7 (C-14′,16′), 31.0 (C-10′), 33.4 (C-4), 41.7, 45.6 (C-5′,7′), 45.0 (C-1′), 52.4, 52.8 (C-4′,8′,11′), 54.6 (C-13′,17′), 57.5 (C-2′), 115.7 (C-9a), 119.9 (C-8a), 122.7 (C-8), 123.8 (C-7), 128.0 (C-6), 128.7 (C-5), 146.6 (C-10a), 151.3 (C-9), 158.0 (C-4a), 167.8 (C 9′). Anal. Calcd for $C_{27}H_{39}N_5O$ (449.64): C, 72.12; H, 8.74; N, 15.58. Found: C, 72.18; H, 8.89; N, 15.64.

*4.1.2.2. Synthesis of 3-[cyclohexyl(methyl)amino]-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]piperazino}-1-propanone (**5b**).* Intermediate **4** was reacted with methylcyclohexylamine following the general procedure to give 77 mg (65%) of **5b** as oil after column chromatograpghy ($CHCl_3$/MeOH/Et_3N, 4/1/0.05). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.20–1.30 (m, 10H, 5 × CH_2, H-14′,15′,16′,17′,18′), 1.87–1.97 (m, 4H, 2 × CH_2, H-2,3), 2.3 (s, 3H, CH_3, H-1″), 2.35–2.44 (m, 1H, CH, H-13′), 2.46–2.56 (m, 6H, 3 × CH_2, H-4′,8′,10′), 2.63 (t, 2H, CH_2, H-2′, J = 6.0 Hz), 2.75–2.80 (m, 2H, CH_2, H-1), 2.82 (t, 2H, CH_2, H-11′, J = 6.8 Hz), 3.07 (t, 2H, CH_2, H-4, J = 5.8 Hz), 3.53–3.55 and 3.67–3.72 (m, 4H, 2 × CH_2, H-5′,7′), 3.55–3.63 (m, 2H, CH_2, H-1′), 7.34 (dd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 8.2 Hz), 7.56 (dd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 8.2 Hz), 7.92 (d, 1H, CH, H-5, J = 8.2 Hz), 8.0 (d, 1H, CH, H-8, J = 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ 22.8, 22.9 (C-2,3), 25.0 (C-1), 26.0 (C-15′,17′), 26.2 (C-16′), 29.0 (C-14′, 18′), 32.0 (C-10′), 33.9 (C-4), 37.8 (C-1″), 41.6, 45.7 (C-5′,7′), 45.0 (C-1′), 49.6 (C-11′), 52.5, 52.8 (C-4′,8′), 57.7 (C-2′), 63.3 (C-13′), 116.1 (C-9a), 120.3 (C-8a), 122.6 (C-8), 123.7 (C-7), 128.4 (C-6), 128.8 (C-5), 147.3 (C-10a), 151.0 (C-9), 158.5 (C-4a), 167.8 (C-9′). Anal. Calcd for $C_{29}H_{43}N_5O$ (477.70): C, 72.92; H, 9.07; N, 14.66. Found: C, 73.1; H, 9.13; N, 14.86.

*4.1.2.3. Synthesis of 3-[cyclohexyl(ethyl)amino]-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]piperazino}-1-propanone (**5c**).* Intermediate **4** was reacted with ethylcyclohexylamine following the general procedure to give 61 mg (50%) of **5c** as oil after column chromatograpghy ($CHCl_3$/MeOH/Et_3N, 4/1/0.05). ^1H NMR ($CDCl_3$, 400 MHz) δ 0.9 (t, 3H, CH_3, H-2″, J = 7.2 Hz), 1.18–1.28 (m, 10H, 5 × CH_2, H-14′,15′,16′,17′,18′), 1.89–1.96 (m, 4H, 2 × CH_2, H-2,3), 2.30–2.40 (m, 1H, CH, H-13′), 2.45–2.55 (m, 6H, 3 × CH_2, H-4′,8′,10′), 2.56–2.62 (m, 2H, CH_2, H-1″), 2.63–2.66 (m, 2H, CH_2, H-2′), 2.74–2.80 (m, 2H, CH_2, H-1), 2.83 (t, 2H, CH_2, H-11′, J = 6.8 Hz), 3.07 (t, 2H, CH_2, H-4, J = 5.8 Hz), 3.52–3.56 and 3.66–3.70 (m, 4H, 2 × CH_2, H-5′,7′), 3.56–3.62 (m, 2H, CH_2, H-1′), 7.34 (ddd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 6.8 Hz, J_3 = 8.4 Hz), 7.55 (ddd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 7.0 Hz, J_3 = 8.4 Hz), 7.91 (d, 1H, CH, H-5, J = 8.4 Hz), 8.0 (d, 1H, CH, H-8, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ 14.1 (C-2″), 22.8, 23.1 (C-2,3), 24.9 (C-1), 26.1 (C-15′,17′), 26.3 (C-16′), 29.1 (C-14′,18′), 32.0 (C-10′), 33.9 (C-4), 41.6, 45.7 (C-5′,7′), 45.1 (C-1′), 45.6 (C-1″), 50.6 (C-11′), 52.4, 52.8 (C-4′,8′), 57.6 (C-2′), 60.1 (C-13′), 116.1 (C-9a), 120.3 (C-8a), 122.6 (C-8), 123.7 (C-7), 128.7 (C-6), 128.8 (C-5), 147.3 (C-10a), 150.9 (C-9), 158.5 (C-4a), 167.8 (C-9′). Anal. Calcd for $C_{30}H_{45}N_5O$ (491.73): C, 73.28; H, 9.22; N, 14.24. Found: C, 73.33; H, 9.29; N, 14.34.

*4.1.2.4. Synthesis of 3-(methylanilino)-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]-piperazino}-1-propanone (**5d**).* Intermediate **4** was reacted with *N*-methyl-*N*-phenylamine following the general procedure to give 72 mg (61%) of **5d** as oil after column chromatograpghy ($CHCl_3$/MeOH/Et_3N, 4/1/0.05). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.88–1.98 (m, 4H, 2 × CH_2, H-2,3), 2.47–2.57 (m, 6H, 3 × CH_2, H-4′,8′,11′), 2.62–2.66 (m, 4H, 2 × CH_2, H-2′,10′), 2.77 (t, 2H, CH_2, H-1, J = 6.0 Hz), 2.83 (s, 3H, CH_3, H-1″), 3.07 (t, 2H, CH_2, H-4, J = 5.8 Hz), 3.53–3.66 and 3.73–3.77 (m, 6H, 3 × CH_2, H-1′,5′,7′), 5.06 (bs, 1H, NH), 6.60 (dd, 2H, 2 × CH, H-14′,18′, J_1 = 2.0 Hz, J_2 = 8.0 Hz), 6.70 (ddd, 1H, CH, H-16′, J_1 = 2.0 Hz, J_2 = 7.2 Hz, J_3 = 8.0 Hz), 7.20 (ddd, 2H, 2 × CH, H-15′,17′, J_1 = 2.0 Hz, J_2 = 7.2 Hz, J_3 = 8.0 Hz), 7.34 (dd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 7.2 Hz), 7.55 (dd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 7.2 Hz), 7.91 (d, 1H, CH, H-5, J = 8.4 Hz), 8.0 (d, 1H, CH, H-8, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ 22.8, 23.1 (C-2,3), 24.9 (C-1), 33.9 (C-4,10′), 38.0 (C-1″), 42.0, 45.7 (C-5′,7′), 45.0 (C-1′), 52.3, 52.8 (C-4′,8′), 53.7 (C-11′), 57.6 (C-2′), 112.3 (C-14′,18′), 116.2 (C-9a), 117.1 (C-16′), 120.3 (C-8a), 122.5 (C-8), 123.6 (C-7), 128.3 (C-6), 128.8 (C-5), 129.1 (C-15′,17′), 147.4 (C-10a), 149.3 (C-13′), 150.7 (C-9), 158.6 (C-4a), 167.8 (C-9′). Anal. Calcd for $C_{29}H_{37}N_5O$ (471.65): C, 73.85; H, 7.91; N, 14.85. Found: C, 73.92; H, 7.98; N, 15.05.

*4.1.3. General procedure for the synthesis of N-(2-{4-[3-(substituted) propyl]piperazino}ethyl)-(1,2,3,4-tetrahydroacridin-9-yl) amines **6a–6d***

Propanone **5** (0.39 mmol) in THF (10 ml) was added to $LiAlH_4$ (230 mg, 1.56 mmol) suspended in THF (26 ml). The reaction mixture was stirred under reflux for 2 h. The excess of $LiAlH_4$ was decomposed by a careful subsequent addition of water (5 drops) and NaOH (1N, 5 drops) and stirred for further 0.5 h. Then Na_2SO_4 was added and we continued to stir for final 2 h. After cooling to rt the product was filtered.

*4.1.3.1. Synthesis of N-(2-{4-[3-(piperidinopropyl)]piperazino}ethyl)-(1,2,3,4-tetrahydroacridin-9-yl)amine (**6a**).* Starting with **5a** and $LiAlH_4$, followed by the general procedure and column chromatograpghy (eluent $CHCl_3$/MeOH/Et_3N, 4/1/0.05), led to 42 mg (25%) of **6a** as oil. ^1H NMR ($CDCl_3$, 400 MHz) δ 1.51–1.63 (m, 6H, 3 × CH_2,

H-14′,15′,16′), 1.67–1.74 (m, 2H, CH_2, H-10′), 1.88–1.98 (m, 4H, 2 × CH_2, H-2,3), 2.28–2.25-2.46 (m, 8H, 4 × CH_2, H-9′,11′,13′,17′), 2.46–2.58 (m, 8H, 4 × CH_2, H-4′,8′,5′,7′), 2.60 (t, 2H, CH_2, H-2′, J = 6.0 Hz), 2.74–2.82 (m, 2H, CH_2, H-1), 3.04–3.12 (m, 2H, CH_2, H-4), 3.53–3.60 (m, 2H, CH_2, H-1′), 7.33 (ddd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 6.8 Hz, J_3 = 8.0 Hz), 7.54 (ddd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 6.8 Hz, J_3 = 8.0 Hz), 7.91 (d, 1H, CH, H-5, J = 8.0 Hz), 8.02 (d, 1H, CH, H-8, J = 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 22.8, 23.1 (C-2,3), 24.4 (C-15′), 24.9 (C-1), 26.0 (C-14′,16′), 30.0 (C-10′), 33.8 (C-4), 45.2 (C-1′), 52.6, 53.4 (C-4′,5′,7′,8′,9′,11′), 54.6 (C-13′,17′), 57.5 (C-2′), 115.9 (C-9a), 120.2 (C-8a), 122.8 (C-8), 123.5 (C-7), 128.3 (C-6), 128.5 (C-5), 147.2 (C-10a), 151.2 (C-9), 158.3 (C-4a). Anal. Calcd for $C_{27}H_{41}N_5$ (435.66): C, 74.44; H, 9.49; N, 16.08. Found: C, 74.56; H, 9.64; N, 16.09.

*4.1.3.2. Synthesis of N-(2-{4-[3-(cyclohexyl(methyl)amino)propyl] piperazino}ethyl)-(1,2,3,4-tetrahydroacridin-9-yl)amine (**6b**).* Starting with **5b** and $LiAlH_4$, followed by the general procedure and column chromatograpghy (eluent $CHCl_3$/MeOH/Et_3N, 4/1/0.05), led to 45 mg (25%) of **6b** as oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.18–1.28 (m, 10H, 5 × CH_2, H-14′,15′,16′,17′,18′), 1.68–1.71 (m, 2H, CH_2, H-10′), 1.89–1.95 (m, 4H, 2 × CH_2, H-2,3), 2.30 (s, 3H, CH_3, H-1″), 2.35–2.42 (m, 1H, CH, H-13′), 2.45–2.58 (m, 12H, 6 × CH_2, H-4′,5′,7′,8′,9′,11′) 2.61 (t, 2H, CH_2, H-2′, J = 6.0 Hz), 2.76 (t, 2H, CH_2, H-1, J = 5.6 Hz), 3.07 (t, 2H, CH_2, H-4, J = 6.0 Hz), 3.56 (t, 2H, CH_2, H-1′, J = 6.0 Hz), 7.33 (ddd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 7.2 Hz, J_3 = 8.4 Hz), 7.54 (ddd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 7.2 Hz, J_3 = 8.4 Hz), 7.91 (d, 1H, CH, H-5, J = 8.2 Hz), 8.02 (d, 1H, CH, H-8, J = 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ 22.8, 23.1 (C-2,3), 25.2 (C-1), 26.0 (C-15′,17′), 26.3 (C-16′), 28.5 (C-14′,18′), 30.0 (C-10′), 33.8 (C-4), 37.8 (C-1″), 45.1 (C-1′), 51.6 (C-11′), 52.6, 53.5 (C-4′,5′,7′,8′,9′), 57.5 (C-2′), 63.0 (C-13′), 115.9 (C-9a), 120.2 (C-8a), 122.8 (C-8), 123.5 (C-7), 128.3 (C-6), 128.5 (C-5), 147.2 (C-10a), 151.2 (C-9), 158.4 (C-4a). Anal. Calcd for $C_{29}H_{45}N_5$ (463.72): C, 75.12; H, 9.78; N, 15.10. Found: C, 75.29; H, 9.85; N, 15.30.

*4.1.3.3. Synthesis of N-(2-{4-[3-(cyclohexyl(ethyl)amino)propyl]piperazino}ethyl)-(1,2,3,4-tetrahydroacridin-9-yl)amine (**6c**).* Starting with **5c** and $LiAlH_4$, followed by the general procedure and column chromatograpghy (eluent $CHCl_3$/MeOH/Et_3N, 4/1/0.05), led to 39 mg (21%) of **6c** as oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.90 (m, 3H, CH_3, H-2″), 1.18–1.28 (m, 10H, 5 × CH_2, H-14′,15′,16′,17′,18′), 1.64–1.73 (m, 2H, CH_2, H-10′), 1.81–1.96 (m, 10H, 4 × CH_2, H-2,3), 2.23–2.28 (m, 2H, CH_2, H-1″), 2.35–2.42 (m, 1H, CH, H-13′), 2.47–2.62 (m, 12H, 6 × CH_2, H-4′,5′,7′,8′,9′,11′), 2.63–2.73 (m, 4H, 2 × CH_2, H-1,2′), 3.16–3.23 (m, 2H, CH_2, H-4), 3.62–3.72 (m, 2H, CH_2, H-1′), 7.38 (dd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 7.2 Hz),7.62 (dd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 7.2 Hz), 8.08 (d, 1H, CH, H-5, J = 8.2 Hz), 8.21 (d, 1H, CH, H-8, J = 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ 14.1 (C-2″), 22.5, 22.6 (C-2,3), 24.2 (C-1), 26.1 (C-15′,17′), 26.4 (C-16′), 29.7 (C-14′,18′), 30.0 (C-10′), 32.9 (C-4), 44.4 (C-1′), 45.3 (C-1″), 52.4, 53.3 (C-4′,5′,7′,8′,9′,11′), 57.6 (C-2′), 60.7 (C-13′), 116.2 (C-9a), 120.3 (C-8a), 123.3 (C-8) 124.2 (C-7), 125.4, (C-6), 132.3 (C-5), 147.3 (C-10a), 151.3 (C-9), 158.2 (C-4a). Anal. Calcd for $C_{30}H_{47}N_5$ (477.74): C, 75.42; H, 9.92; N, 14.66. Found: C, 75.46; H, 10.07; N, 14.71.

*4.1.3.4. Synthesis of N-(2-{4-[3-(methylanilino)propyl]piperazino} ethyl)-(1,2,3,4-tetrahydroacridin-9-yl)amine (**6d**).* Starting with **5d** and $LiAlH_4$, followed by the general procedure and column chromatograpghy (eluent $CHCl_3$/MeOH/Et_3N, 4/1/0.05), led to 35 mg (20%) of **6d** as oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.65–1.70 (m, 2H, CH_2, H-10′), 1.88–1.94 (m, 4H, 2 × CH_2, H-2,3), 2.45–2.58 (m, 10H, 5 × CH_2, H-4′,5′,7′, 8′,9′,11′), 2.59–2.67 (m, 2H, CH_2, H-2′), 2.72–2.77 (m, 2H, CH_2, H-1), 2.83 (s, 3H, CH_3, H-1″), 3.04–3.09 (m, 2H, CH_2, H-4), 3.55–3.61 (m, 2H, CH_2, H-1′), 6.61 (dd, 2H, 2 × CH, H-14′,18′, J_1 = 2.0 Hz, J_2 = 8.0 Hz), 6.70 (ddd, 1H, CH, H-16′, J_1 = 2.0. Hz, J_2 = 7.2 Hz, J_3 = 8.0 Hz), 7.20 (ddd, 2H, 2 × CH, H-15′,17′, J_1 = 2.0 Hz, J_2 = 7.2 Hz, J_3 = 8.0 Hz), 7.34 (ddd, 1H, CH, H-7, J_1 = 1.2 Hz, J_2 = 6.8 Hz, J_3 = 8.2 Hz), 7.55 (ddd, 1H, CH, H-6, J_1 = 1.2 Hz, J_2 = 6.8 Hz, J_3 = 8.2 Hz), 7.94 (d, 1H, CH, H-5, J = 8.2 Hz), 8.05 (d, 2H, CH_2, H-8, J = 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ 22.7, 23.0 (C-2,3), 24.7 (C-1), 29.8 (C-10′), 33.4 (C-4), 38.2 (C-1″), 45.0 (C-1′), 52.5, 53.4 (C-4′,5′,7′,8′), 52.6 (C-9′,11′), 57.4 (C-2′), 112.4 (C-14′,18′), 115.6 (C-9a), 117.2 (C-16′), 120.0 (C-8a), 122.9 (C-8), 123.6 (C-7), 128.4 (C-6), 128.6 (C-5), 129.1 (C-15′,17′), 147.4 (C-10a), 149.3 (C-13′), 151.4 (C-9), 158.5 (C-4a). Anal. Calcd for $C_{29}H_{39}N_5$ (457.67): C, 76.11; H, 8.59; N, 15.30. Found: C, 76.15; H, 8.84; N, 15.37.

*4.1.4. Synthesis of 3-{[2-(1,2,3,4-tetrahydroacridin-9-ylamino) ethyl]amino}-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl] piperazino}-1-propanone (**8**)*

A solution of *N*-(2-aminoethyl)-1,2,3,4-tetrahydroacridin-9-ylamine (**7**, 90 mg, 0.37 mmol) in CH_2Cl_2 (2 ml), 3-chloro-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]-piperazino}-1-propanone (**4**, 160 mg, 0.40 mmol) in CH_2Cl_2 (2 ml), and DIPEA (70 mg, 0.55 mmol) was stirred at rt for 72 h. The reaction mixture was cooled and evaporation of the solvent afforded a residue which was dissolved in CH_2Cl_2 (5 ml) and washed with water (10 ml) and NaCl (10 ml). The collected organic fractions were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography over silica gel in eluent EtOAc/MeOH/NH_4OH, 6/2/0.2. Compound **7** (150 mg, 63% yield) was obtained as yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.89–1.96 (m, 8H, 4 × CH_2, H-2,3,2″,3″), 2.47–2.55 (m, 8H, 4 × CH_2, H-4′,8′,11′,13′), 2.63 (t, 2H, CH_2, H-2′, J = 6.0 Hz), 2.75–2.83 (m, 4H, 2 × CH_2, H-1,1″), 3.01–3.09 (m, 4H, 2 × CH_2, H-4,4″), 3.54–3.63 (m, 6H, 3 × CH_2, H-1′,10′,14′), 3.67–3.79 (m, 4H, 2 × CH_2, H-5′,7′), 4.76 (bs, 1H, NH), 5.04 (bs, 2H, 2 × NH), 7.31–7.38 (m, 2H, 2 × CH, H-7,7″), 7.52–7.59 (m, 2H, 2 × CH, H-6,6″), 7.88–7.95 (m, 2H, 2 × CH, H-5,5″), 7.97–8.20 (m, 2H, 2 × CH, H-8,8″). ^{13}C NMR ($CDCl_3$) δ 22.8, 23.1 (C-2,3,2″,3″), 25.0 (C-1,1″), 34.0 (C-4,4″), 41.7, 42.0 (C-5′,7′), 45.0 (C-1′), 45.5 (C-10′), 45.8 (C-14′), 52.3 (C-11′,13′), 52.7, 52.8 (C-4′,8′), 57.6 (C-2′), 116.1 (C-9a,9a″), 120.3 (C-8a,8a″), 122.5 (C-8,8″), 123.6 (C-7,7″), 128.3 (C-6,6″), 128.7 (C-5,5″), 147.4 (C-10a,10a″), 150.7 (C-9,9″), 158.5 (C-4a,4a″), 168.1 (C-9′). Anal. Calcd for $C_{37}H_{47}N_7O$ (605.83): C, 73.36; H, 7.82; N, 16.18. Found: C, 73.56; H, 7.88; N, 16.38.

4.2. Pharmacological studies

4.2.1. Determination of inhibitory potency on hAChE and hBChE

An AChE and BChE inhibitory activity of the tested drugs was determined using Ellman's method [39] and was expressed as IC_{50}, i.e. concentration that reduces the cholinesterase activity by 50%. Human recombinant AChE (AChE; EC 3.1.1.7), human plasmatic BChE (BChE; EC 3.1.1.8), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB), phosphate buffer (PB, pH 7.4), acetylthiocholine (ATC), and butylthiocholine (BTC), and tacrine hydrochloride were purchased from Sigma–Aldrich, Praque, Czech Republic. For measuring purposes – polystyrene cuvette (-Brand GmbH + Co. KG, Denmark) was utilized. All the assays were carried out in a 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 7.4. Enzyme solutions were prepared at 2.0 units/ml in 2 ml aliquots. The assay medium (1 ml) consisted of 650 μL of 0.1 M phosphate buffer (pH 7.4), 200 μL of 0.01 M DTNB, 25 μL of enzyme, and 100 μL of 0.01 M substrate (ATC chloride solution). Assay solutions with inhibitor (10^{-3}–10^{-10} M) were preincubated for 5 min. The reaction was started by an immediate addition of 100 μL of substrate. The activity was determined by measuring the increase in absorbance at 412 nm at 1 min intervals using a spectrophotometer Helios-Zeta (Thermospectronic, Cambridge UK). Each concentration was

assayed in triplicate. In vitro BChE assay was similar with the method described above. In addition we calculated the corresponding selectivity index (Ratio = [IC_{50} (BChE)]/[IC_{50} (AChE)]). This method has been optimized to determine the constants K_{i1} and K_{i2}. Software Origin 6.1 (Northamption USA) was used for the statistical data evaluation.

4.2.2. Molecular modeling studies

Molecular models of the derivatives **5b**–**d** and **6b** were computer-built by the means of building options in the Marvin 5.1.4 2008, ChemAxon [http://www.chemaxon.com]. The same software was used to determine the overall protonization of compounds. In order to prepare the input files, docking simulations were carried out using AUTODOCK ver. 4.2. MGL TOOLS 1.4.5 (revision 30) [43,44]. Molecules of water with other nonenzymatic molecules were removed and hydrogens were added. For ligands and enzymes, a united atom representation was used. Gasteiger partial atomic charges for proteins and ligands were added. For the initial docking, the grid for energy was set in the coordinates $x = 116.4$, $y = 104.3$, $z = -130.6$ within the *h*AChE (PDB ID: 1B41) and $x = 138.7$, $y = 116.3$, $z = 41.0$ within the *h*BChE (PDB ID: 1P01) active site with dimensions 80 points *x* 80 points *x* 80 points and with spacing of 0.375 Å. Thus, a ligand pose with the lowest energy was chosen as the space for the construction of the redocking energy grid with coordinates $x = 117.0$, $y = 109.5$, $z = -132.2$ and with dimensions 42 points × 42 points × 42 points within *h*AChE (PDB ID: 1B41). Likewise for *h*BChE (PDB ID: 1P01), coordinates $x = 136.5$, $y = 111.1$, $z = 40.8$ with dimensions 49 points × 55 points × 43 points were set. Spacing of 0.375 Å was set for both enzymes, which was used to define rotatable bonds in ligands. A flexible ligand docking was performed for the compounds. Docking runs were performed using the Lamarckian genetic algorithm. Docking began with a population of random ligand conformations in random orientation and at random translation. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 5,000,000 energy evaluations or 27,000 generations. The population size was set to 500. Other parameters were used as default. Pictures were prepared using Chimera software [41].

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.06.051.

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