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ABSTRACT

A new series of substituted tacrine/acridine and tacrine/tacrine dimers with aliphatic or alkylene-thiourea linkers was synthesized and the potential of these compounds as novel human acetylcholinesterase (*h*AChE) and human butyrylcholinesterase (*h*BChE) inhibitors with nanomolar inhibition activity was evaluated. The most potent AChE inhibitor was found to be homodimeric tacrine derivative **14a**, which demonstrated an IC_{50} value of 2 nM; this value indicates an activity rate which is 250-times higher than that of tacrine **1** and 7500-times higher than 7-MEOTA **15**, the compounds which were used as standards in the study. IC_{50} values of derivatives **1**, **9**, **10**, **14b** and **15** were compared with the dissociation constants of the enzyme-inhibitor complex, K_{i1}, and the enzyme-substrate-inhibitor complex, K_{i2}, for. A dual binding site is presumed for the synthesized compounds which possess two tacrines or tacrine and acridine as terminal moieties show evidence of dual site binding. DFT calculations of theoretical desolvation free energies, $\Delta\Delta G_{theor}$, and docking studies elucidate these suggestions in more detail.

1. Introduction

Alzheimer's disease (AD) is a common neurological disorder which is characterized by progressive memory loss and a wide range of cognitive impairments. The ever increasing spread of this disorder is already placing huge strains on the economies and health systems of modern aging societies. Intensive research over the last three decades has confirmed that the pathophysiology of AD has a multifactorial nature. Despite the huge effort and resources invested in the development of drugs which could treat AD, the most widely used palliative treatment is still based on the well established cholinergic process in which the concentration of acetylcholine (ACh) neurotransmitters in the brain and central nervous system (CNS) is increased using cholinesterases inhibitors (ChEIs) [3–5]; an overview of cholinesterase inhibitors and their mode of action have been summarized in reviews [1,2]. In addition to acetylcholinesterase (AChE) which hydrolyzes about 80% of acetylcholine in the healthy brain, attention has also focused on butyrylcholinesterase (BChE), whereas the function of this enzym is less clearly defined due to its ability to hydrolyze ACh and other esters [6–10].

Various anti-AChE agents such as tacrine **1** (Fig. 1), donepezil, rivastigmine, galantamine, and N-methyl-D-aspartate (NMDA) receptor antagonist, memantine have shown to cause a slight improvement in cognitive and memory disorders [5,11]. Crystallographic studies have demonstrated that these anti-AChE agents all bind to AChE within the catalytic active site (CAS) in a 20 Å deep gorge, while the larger donepezil compound also binds at a peripheral anionic binding site (PAS) at the rim of the AChE gorge. The peripheral anionic centre of AChE has also been proven to take

Abbreviations: AD, Alzheimer's disease; ACh, acetylcholine; CNS, central nervous system; ChE, cholinesterase; ChEI, cholinesterase inhibitor; *h*AChE, human acetylcholinesterase; *h*BChE, human butyrylcholinesterase; THA, 1,2,3,4-tetrahydroacridine; NMDA, *N*-methyl-D-aspartate; CAS, catalytic active site; PAS, peripheral anionic site; APP, amyloid precursor protein; Aβ, amyloid β-protein; E, enzyme; S, substrate; I, inhibitor; DFT, density functional theory; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ATChCl, acetylthiocholine chloride; PBS, phosphate buffered saline.

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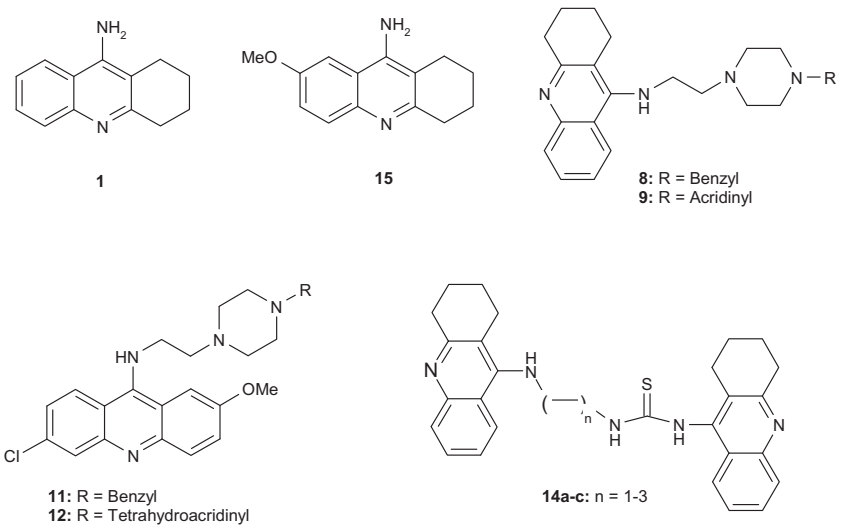

Fig. 1. Structure of tacrine **1**, homo and hetero tacrine/acridine hybrids **8, 9**, **11**, **12**, **14a–c**, and 7-MEOTA **15**.

part in amyloid-β peptide aggregation into neuric plagues and neurofibrilary tangles [12,13], effects which are important hallmarks of AD and key pathological events which trigger neurodegenerative processes in AD.

Several papers have emphasized the importance of heteroatoms in the linkers, in particular nitrogens or amide moieties, which form additional cation-π or H-bond interactions of inhibitors with aminoacids lining the enzyme gorge. This optimization of the linker's structure [14] has gradually shifted the observed anti-ChE activity to the attractive sub-nanomolar or even picomolar range [14–16]. The development of a hydrazide linker for tacrine-derived heterodimers has led to the practical application of these agents in the inhibition of cholinesterases, the bivalent binding to nicotinic and muscarinic acetylcholine receptors, as well as the histochemical imaging of acetylcholinesterase and amyloid-β [17].

As an integration of the above approaches and in a continuation of our previous study concerning the development of new AChE inhibitors [18–21], this paper is devoted to the synthesis of tacrine-piperazine/acridine hybrids as novel ChEIs with aliphatic or alkylene-thiourea linkers, and an evaluation of the AChE/BChE inhibition evaluation of these derivatives combined with binding mode studies using quantum chemistry and a molecular docking study. Piperazine was used in combination with benzyl as a substitute for the well-known piperidine-benzyl moiety of donepezil in an attempt to examine the effect of a second protonable tertiary nitrogen inside the piperazine ring. Additionally, a little explored thiourea compound [22,23] connected to an alkylenediamine spacer of variable length was included as the linker in order to examine its role in inhibition.

2. Experimental methods

2.1. Materials

All chemicals and solvents were obtained commercially and used without purification. Human erythrocytal AChE, human plasmatic BChE, 5,5′-dithiobis(2-nitrobenzoic acid) [DTNB] were purchased from Sigma-Aldrich, St. Louis, MO, USA. Thin-layer chromatography was performed on Macherey-Nagel Alugram ®Sil G/UV254 plates and spots were visualized with UV light. Column chromatography was performed using silica gel from Merck (0.063–0.040 mm).

2.2. Chemistry

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Mercury Plus NMR spectrometer in $CDCl_3$ or DMSO-D_6 with tetrametylsilane as an internal standard. Chemical shifts, δ, are expressed in parts per million (ppm), and coupling constants, J, in Hz. Melting points were recorded on a Boetius hotplate apparatus and are uncorrected. Yields refer to isolated pure products. CHN analysis was performed on a CHN analyzer Perkin-Elmer 2400. The obtained values matched the calculated ones within a range of ±0.3%. A multichannel Sunrise spectrophotometer (Tecan, Salzburg, Austria) and 96-well polystyrene photometric microplates (Nunc, Rockilde, Denmark) were used to measure the anticholinesterase activity.

2.3. Preparation of tacrine/acridine dimers

The reaction course of the synthesis of derivatives **7–9** is depicted in Scheme 1, derivatives **11** and **12** in Scheme 2, and derivatives **14a–c** in Scheme 3. Schemes 1–3 are given in Supplementary material. Four starting compounds, 9-chloro-1,2,3,4-tetrahydroacridine (**2**) [24], 9-chloroacridine (**3**) [25], 6,9- dichloro-2-methoxyacridine (**4**) [26], and 9-isothiocyanato-1,2,3,4-tetrahydroacridine (**5**) [24], were employed in the synthesis of the studied derivatives. Reaction conditions and compound characterisation are given in the Supplementary material.

2.4. Measurement of AChE and BChE activity

A multichannel Sunrise spectrophotometer was used to measure anticholinesterase activity. The potency was expressed in IC_{50} values which represent an inhibitor concentration which reduces cholinesterase activity by 50%. A previously optimized procedure [27] was slightly adapted to estimate the anticholinergic properties, and 96-well polystyrene photometric microplates were used. AChE and BChE were suspended in the phosphate buffer (pH 7.4) up to the final activity of 0.002 U/μL. Cholinesterase (5 μL), a freshly mixed solution of 5,5′-dithiobis(2- nitrobenzoic acid) in the phosphate buffer (0.4 mg/mL, 40 μL), and a given concentration of the inhibitor (10^{-3}–10^{-10} M) dissolved in iso-propanol were injected into each well. The enzymatic reaction was initiated with acetylthiocholine chloride (ATChCl) in the phosphate buffer (1 mM, 20 μL). The absorbance was measured at 412 nm after 5 min incubation upon a vibrating microplate.

2.5. Theoretical calculations

2.5.1. DFT calculations

In order to assess the factors governing the ligand-enzyme complexation, we have computed desolvation free energies [28] were calculated for all ligands and both standards using the DFT (B3LYP/6—311 + G(d,p) method [29,30]. Optimized structures of all compounds were determined for isolated *N*-mono- or *N*,*N*-diprotonated molecules in vacuum ($\varepsilon = 1.0$), water ($\varepsilon = 78.4$) and *n*-octanol ($\varepsilon = 9.9$). Atomic charges were assigned using a CHELPG option included in Gaussian 09. Solvation of the molecules was estimated using a CPCM scheme [31] within the Gaussian 09 package [29]. Desolvation free energies were calculated as the difference between the pertinent free energies of non-solvated and solvated molecules.

2.5.2. Molecular modeling

Molecular models for derivatives **9**, **14b** were built using the building options included in Marvin 5.1.4 2008 software from ChemAxon [32]. The same software was used to determine the overall protonation of compounds. Docking simulations were carried out using an AUTODOCK ver. 4.2, and MGL TOOLS 1.4.5 (revision 30) was used to prepare input files [33–35]. Molecules of water and co-crystallized fasciculin with other non-enzymatic molecules were removed and hydrogens were added. A united atom representation was used for ligands and the enzyme. Gasteiger partial atomic charges for the protein and ligands were added. A grid for energy was set with coordinates $x = 116.4$, $y = 104.3$, $z = -130.6$ within the *h*AChE (PDB ID: 1B41) active site with dimensions 80 points × 80 points × 80 points and with a spacing of 0.375 A. Docking runs were performed using a Lamarckian genetic algorithm. Docking began with a population of random ligand conformations in random orientations and at random translations. Each docking experiment was derived from 50 different runs which were set to terminate after a maximum of 5000,000 energy evaluations or 27,000 generations. The population size was set to 500. All other parameters used in the experiments were default values.

3. Results and discussion

3.1. Synthesis of substituted tacrine, acridine, tacrine/acridine dimers, and tacrine/tacrine dimers

A new series of compounds with piperazine and thiourea linkers, derivatives **8**, **9**, **11**, **12**, **14a–c** were synthesized according to the processes outlined in Schemes 1–3 see Supplementary material. Compounds **6**, **7** have been described previously [19]. *N*-(2-(Piperazin-1-yl)ethyl)-1,2,3,4-tetrahydroacridin-9-ylamine (**7**) produced *N*-[2-(4-benzylpiperazin-1-yl)ethyl]-1,2,3,4-tetrahydroacridin-9-ylamine (**8**) after treatment with benzylchloride in CH_2Cl_2. The coupling of amine **7** with 9-chloroacridine (**3**) through aromatic nucleophilic substitution at the 9-position to led to the synthesis of *N*-{2-[4-(acridin-9-yl)piperazin-1-yl]ethyl}-1,2,3,4-tetrahydroacridin-9-ylamine (**9**). Substituted acridine derivatives **11** and **12** were prepared from the starting base of 6,9- dichloro-2-methoxyacridine (**4**). 6,9-Dichloro-2-methoxyacridine (**4**) was first converted to 6-chloro-2-methoxy-*N*-[2-(piperazin-1-yl)ethyl]acridin-9-ylamine (**10**) with the addition of 1-(2-aminoethyl)piperazine; the desired compound, *N*-[2-(4-benzylpiperazin-1-yl)ethyl]-6-chloro-2-methoxyacridin-9-ylamine (**11**) was then obtained following the further addition of benzyl chloride. Finally, amine **10** reacted with 9-chloro-1,2,3,4-tetrahydroacridine (**2**) in order to produce the tacrine/acridine dimer, 6-chloro-2-methoxy-*N*-{2-[4-(1,2,3,4-tetrahydroacridin-9-yl)piperazin-1-yl]ethyl}acridin-9-ylamine

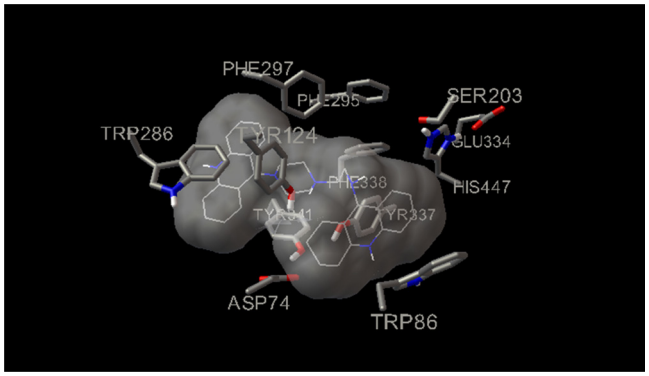

Fig. 2. Top-score docking poses of derivative **9** depict putative structural orientation in the gorge of *h*AChE. The figure was made using PyMOL (http://pymol.sourceforge.net).

(**12**). The compounds which possess extended alkylene-thiourea tethers **14a–c** were synthesized from 9-isothiocyanato-1,2,3,4-tetrahydroacridine (**5**) and tetrahydroacridine derivatives **13a–c**. The intermediates *N*-(1,2,3,4-tetrahydroacridin-9-yl)alkanediamines **13a–c** [36] were obtained through the reaction of 9-chloro-1,2,3,4-tetrahydroacridine (**2**) with ethylene-, butylene- and hexylene-1,6-diamine, respectively, in phenol. All of the synthesized compounds were identified using ^{1}H NMR, ^{13}C NMR, and elementary analysis.

3.2. Inhibition of human AChE and BChE activities

The cholinesterase activity of the studied compounds was evaluated using spectrophotometry according to the method established by Ellman [37]. The target compounds were evaluated for their in vitro inhibitory activities against human erythrocytal AChE and human plasmatic BChE in comparison to those obtained using the standard compounds tacrine **1** and 7-MEOTA **15**. The obtained values are shown in Table 1.

The IC_{50} values obtained during the experiments show that all of the compounds demonstrate an inhibitory effect on both enzymes AChE and BChE. As can be seen in Table 1, derivatives **9**, **12** and **14b** & **c** exhibited the highest levels of nanomolar *h*AChE inhibition. These compounds share a combination of terminal acridine or tacrine units joined with alkylene-piperazine or alkylene-thiourea linkers, and the results suggest that this arrangement is highly effective. Ligands **9**, **12** and **14b** and **c** can act as both mono and dual binding site inhibitors. The inhibition potency of these four inhibitors is almost one hundred times greater than that of compounds **7**, **8**, **10**, and **14a**; it is also between 60 and 250 times greater than that of the tacrine standard, and exceeds that of the standard 7-MEOTA (**15**) by an incredible 1800–7500-times. Very interesting low-nanomolar activity was also observed for dual binding tacrine homodimers **14a–c** with incorporated alkylene-thiourea linkers. As the docking models of **14b** prove unambiguously that the inhibitor was inserted into the catalytic site through its THA-thiourea terminal moiety (Fig. 3), it would be assumed that the tacrine-alkylene end of the molecule would point outward of the gorge. A comparison of the inhibition potency within the series **14a–c** stressed the importance of linker length. Whereas the two ethylene units in the **14b** molecule or the three units in **14c** ensure the low-nanomolar efficacy of both inhibitors, the single ethylene unit in **14a** provides only a medium-nanomolar level of activity (260 nM). This clearly proves that the length of molecule **14a** is too short to reach both enzyme binding sites at once. As can be seen, the high level of inhibition exhibited by derivatives **14b** and **c** remains unchanged following the substitution of the pure alkylene linker of bis(7)-tacrine with the alkylene-thiourea system. The only compound

Table 1
Inhibition of AChE and BChE (IC50)[a], selectivity index[b] and dissociation constants[c,d] of tested compounds.

Compound	IC^a_{50} (*h*AChE) ± SD (nM)	IC^a_{50} (*h*BChE) ± SD (nM)	SI[b]	K^c_{il} (Nm)	K^d_{i2} (nM)	α^e
1	500 ± 100	23 ± 4	21.7	225	101	0.49
7	150 ± 30	5 290 ± 860	0.03	–	–	
8	160 ± 30	0.4 ± 0.07	400	–	–	
9	3 ± 0.6	20 ± 3	0.15	77.4	32.2	0.42
10	140 ± 30	341,000 ± 55,000	0.0004	420	nd	
12	6 ± 1	2 580 ± 420	0.002	–	–	
14a	260 ± 50	20 ± 3	13	–	–	
14b	2 ± 0.4	8 ± 1	0.25	7.45	6.68	0.90
14c	8 ± 2	7 ± 1	1.14	–	–	
15	15,000 ± 2900	21 000 ± 3 400	0.71	2090	6340	3.03

[a] IC_{50}: The concentration required to elicit a 50% inhibition of the enzyme.
[b] Selectivity index SI = IC_{50}(*h*AChE)/IC_{50}(*h*BChE).
[c] K_{i1}: Dissociation constant for the *h*AChE-inhibitor complex.
[d] K_{i2}: Dissociation constant for the *h*AChE-substrate-inhibitor complex.
[e] $\alpha = K_{i2}/K_{i1}$ [38].

which does not contain tacrine within its structure, derivative **10**, showed only minimal activity (IC_{50} = 341,000 nM). Derivatives, **8**, **9**, and **14a–c**, in which tacrine is connected to an ethylene unit, possess an excellent low, even sub-nanomolar efficacy (for BuChE: 0.4–20 nM), better than that of tacrine itself. The key factor which enhances the binding in the case of this series of compounds is the presence of another aromate on the opposite side of the molecules (Bn, Acr, or THA). When this aromate was missing (e.g. derivative **10**, R = H), a substantial decrease in inhibitory activity was observed (5290 nM). In conclusion, the comparison of AChE/BChE selectivities within the series shows that compound **10** is the most selective for AChE (though its activity is just moderate), while compound **8** demonstrates the best selectivity for BChE.

3.3. Theoretical study

Studies of tacrine **1** and 6-chloro-tacrine have shown that enzyme-ligand inhibition potency depends on the electron distribution within ligand molecules [39]. Our DFT calculations for tacrine (**1**), 7-MEOTA (**15**) and 6-Cl-tacrine confirm the theoretical prediction [39] that a decrease in the total electron charge on the ligand leads to a deterioration in the bindings and confirms the prevailing anionic character of the catalytic centre (Supplementary material, Table 1).

Desolvation energy is another important factor governing ligand-enzyme complexation. Optimized conformations were used to compute desolvation free energies, $\Delta\Delta G_{1theor}$ and $\Delta\Delta G_{2theor}$ (Table 2, Supplementary), for particular compounds, values which describe the transfer of the substrate solvated by water and *n*-octanol, respectively, to a vacuum. We believe that calculations and simulations of this nature may simulate the real processes occurring in neuron cells, in which the ligand transfers from the hydrophilic cell cytoplasm to the hydrophobic active centre of AChE. The higher values of ligand stabilization by water ($\Delta\Delta G_{1theo}$, first row of Table 2 in supldata) than by *n*-octanol ($\Delta\Delta G_{2theor}$, second row) obtained in these simulations show that all of the ligands examined in this study are more hydrophilic than lipophilic. The obtained desolvation free energies follow trends which were evident in the IC_{50} values obtained in the previous experiments, e.g. more potent dual-site binding AChEIs possess magnitudes of $\Delta\Delta G_{theor}$ more than twice as great as those of mono-site binding AChEIs. The moderate deviations may be related to the allosteric properties of AChE towards ligands containing various tacrine moieties linked by alkylene-piperazine or alkylene-thiourea spacers.

3.4. Studies of molecular modeling

Docking studies of the binding of the two most active compounds, derivatives **9** and **14b,** into a *h*AChE crystal structure (PDB ID: 1B41) were carried out in order to provide better insight into the inhibition process. The proposed binding mode of derivative **9** is shown in Fig. 2, and suggests that stacking of the positively charged acridine ring occurs between TYR341and TRP286 residues of the peripheral anionic site, and that this binding is stabilized by cation-π interactions. The protonated tacrine sceleton of compound **9** interacts with TRP86 in the catalytic site through cation-π interactions, although there is no interaction with the catalytic triad. Additional stabilization of the complex may be attributed to a hydrogen bond between the amino group of tacrine and the oxygen atom of TYR337. Docking models of the binding of thiourea **14b** into *h*AChE show a sandwiching of N^1-tacrine between TYR337 and TRP86 (cationic π-site) stabilized by π–π interaction (Fig. 3). The stacking of the second charged tacrine ring between TYR341 and TRP286 (PAS) could also provide an additional stabilizing effect. The results are shown in more detail in the Supplementary Material.

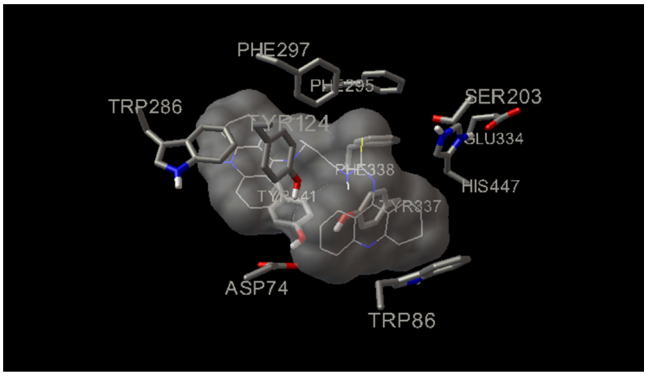

Fig. 3. Top-score docking poses of derivative **14b** depict putative structural orientation in the gorge of *h*AChE. The figure was made using PyMOL (http://pymol.sourceforge.net).

4. Conclusion

Novel tacrine/acridine monomers with alkylene-piperazine tethers, tacrine/acridine-benzyl/acridine heterodimers, and tacrine-tacrine homodimers joined together with non-symmetrical alkylene- piperazine or alkylene-thiourea linkers were prepared in order to examine their inhibitory effects on cholinesterase. The target compounds demonstrated low or even sub-nanomolar IC_{50} inhibition constants, results which are approximately twice as effective as those of tacrine. Derivatives **9**, **12** and **14b** and **c** were particularly effective against *h*AChE (IC_{50} = 3 – 8 nM), while compounds **8**, **9** and **14a–c** proved to be especially potent against *h*BChE (IC_{50} = 0.4 – 20 nM). The compounds synthesized in this

study can act both as mono or dual binding site inhibitors. The inhibitory effect of THA moiety on *h*AChE can be replaced by the effect of acridine, however acridine ligands are poor inhibitors of *h*BChE unless a tacrine-alkylene system is present in the molecule. These findings are further evidence of the structural dissimilarity of the two types of cholinesterase, a fact which is further proven by the differing effects of derivatives **7**, **8**, and **10** on both enzymes; the inhibitory activity of these compounds on *h*AChE is largely similar (150, 160, 140 nM, resp.), but there are dramatic differences in their effects on *h*BChE (5290, 4, 341000 nM, resp.). Hydrophobic and stacking interactions in derivative **8** may be responsible for the extraordinary sub-nanomolar activity which was observed during the study. The importance of tether length was also demonstrated by extending the (ethylene)*n*-thiourea chains ($n = 1, 2, 3$) in homodimeric inhibitors **14a–c**; the **14a** molecule was too short to allow dual site binding ($n = 1$, 260 nM), whereas derivatives **14b** ($n = 2$, 2 nM) and **14c** ($n = 3$, 8 nM) were of sufficient length to permit this. Extreme rates of selectivity were found for compounds **8** (400) and **10** (0.0004). An examination of the dissociation constants K_i proved that derivatives **9** and **14b** demonstrate a higher affinity to the ES complex than to the enzyme itself. Docking models of the binding of the two most active compounds, derivatives **9** and **14b**, into the pertinent crystal structure of the *h*AChE (PDB ID: 1B41) revealed that the nonpolar hydrophobic interactions between the dimers and the lipophilic part of the enzyme are the principal driving forces of *h*AChE inhibition. Within the *h*AChE molecule, ligand **9** associates with the catalytic centre at the tacrine rest and ligand **14b** at the tacrine-thiourea moiety.

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