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# Preparation, in vitro screening and molecular modelling of symmetrical 4-*tert*-butylpyridinium cholinesterase inhibitors—Analogues of SAD-128

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ABSTRACT

Carbamate inhibitors (e.g., pyridostimine bromide) are used as a pre-exposure treatment for the prevention of organophosphorus poisoning. They work by blocking acetylcholinesterase's (AChE) native function and thus protect AChE against irreversible inhibition by organophosphorus compounds. However, carbamate inhibitors are known for many undesirable side-effects related to the carbamylation of AChE. In this Letter, 19 analogues of SAD-128 were prepared and evaluated as cholinesterase inhibitors. The screening results showed promising inhibitory ability of four compounds better to used standards (pralidoxime, obidoxime, BW284c51, ethopropazine, SAD-128). Four most promising compounds were selected for further molecular docking studies. The SAR was stated from obtained data. The former receptor studies were reported and discussed. The further in vivo studies were recommended in the view of OP pre-exposure treatment.



Acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) are essential enzymes in human body splitting various esters.[1,2] Whereas AChE is crucial enzyme for termination of neurotransmission via splitting neuromediator acetylcholine, BChE was called non-specific esterase for its broad esterase activity (e.g., butyrylcholine, cocaine, anaesthetic esters).[3] Both AChE and BChE have many natural and artificial inhibitors blocking their native function. Among them, the organophosphorus compounds (OPs) belong to the most dangerous compounds ever developed by man.[4] They covalently bind to esterase active serine (Ser203 for human AChE and Ser198 for human BChE) and fully block their activity. They cause the permanent block of peripheral and central nervous system with cholinergic crisis development and obstruction of breathing leading to death by suffocation.[5]

Generally, the pre-exposure and post-exposure strategies were developed to counteract the effects of OPs on human.[6] The pre-exposure strategy consists in administration of reversible AChE inhibitor, stoichiometric or catalytic scavengers.[7] The post-exposure strategy is based on combination of anticholinergic drug, AChE reactivator and anticonvulsant.[8]

Concerning the pre-exposure strategies, the only currently approved method for human use is administration of reversible AChE inhibitors (e.g., pyridostigmine chloride; Fig. 1) usually in combination with centrally active anticholinergic drugs (e.g., benactyzine, trihexyphenidyl; Fig. 2).[9] The carbamate drug (pyridostigmine) is used to reversibly block AChE active site serine. Thus, it protects AChE from OPs binding and it is spontaneously decarbamylated with normal restoration of AChE activity. Additionally, it enhances the antidotal efficiency in post-exposure treatment, especially in case of nerve agent poisoning. However, the mostly used pyridostigmine bromide has charged molecule and consequently it is poorly penetrating the blood–brain barrier (BBB).[10] For this reason, the symptomatic anticholinergic drugs (Fig. 1) are used to diminish the OP effects to brain AChE.

Though the carbamate AChE inhibitors are widely used, they are well known for their increased and serious side effect.[11] These side-effects consist in carbamylation of AChE active site by higher

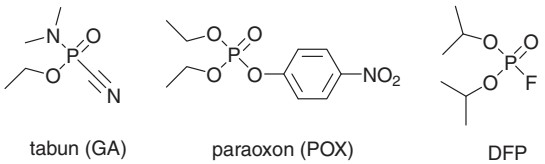


**Figure 1.** Organophosphorus compounds.

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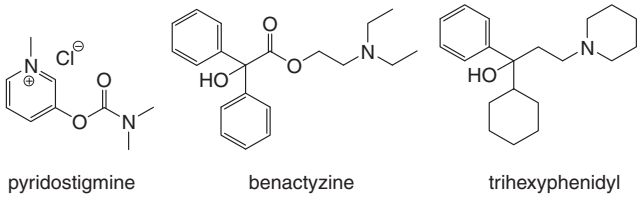



**Figure 2.** Drugs used to protect human against OP poisoning.

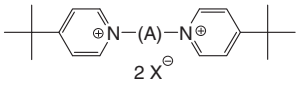
dosage of carbamate and are manifested as increased salivation, gastrointestinal motility, bronchial secretion, cardiac arrhythmia and development of cholinergic crisis. On account of these serious side-effects, other reversible AChE inhibitors might be used for combined pre-exposure strategy.[12] The main advantage of such reversible inhibitor should be the selectivity between AChE and BChE, where BChE can also act as physiological scavenger of OPs in human body. The inhibitor's reversible binding aside the active site serine (Ser203) and consequently decreased side-effects should be also requested. The inhibitor's BBB penetration remains the questionable issue. While the penetrating inhibitor may also protect brain AChE against OP attack, it may also increase the overall toxicity.[13] On the other hand, the problem with inhibitor not penetrating the BBB can be bridged by standard anticholinergics proved by clinical practise.[9]

A few decades ago, AChE inhibitor called SAD-128 (**17**; Table 1) was prepared during the development of AChE reactivators (e.g., pralidoxime, obidoxime; **1** and **2**; Fig. 3).[14] In contrast with AChE reactivators, this interesting molecule was lacking the oxime moiety and have symmetrical molecule with 4-*tert*-butylpyridinium rings. SAD-128 (**17**) as obidoxime analogue was found to be reversible AChE inhibitor able to protect against several $LD_{50}$ of various OPs.[15] Consequently, it was found also inhibitor of muscarinic and nicotinic receptors that explained the protection of experimental animals by SAD-128 against OPs.[16–18]

Concerning the promising data on SAD-128, its 19 analogues (**5**–**24**; Table 1) were prepared. Some of them were formerly reported (**6**–**14**), but they were not evaluated as cholinesterase inhibitors.[19] The design of novel symmetrical compounds originated from parent molecule with two 4-*tert*-butylpyridinium rings and variable linkage. The connecting linkers were selected from formerly published data.[20–22] Namely, the bis-pyridinium or bis-isoquinolinium compounds bearing alkylenyl and naphtylenyl linkers (**5**–**16**) showed very promising inhibitory results towards hAChE.[20,21] Differently, the linkers with heteroatom (**17** and **18**), double bonds (**19** and **20**) or xylenyl moieties (**21**–**23**) were found promising during preparation of AChE reactivators.[23–26] For these reasons, presented linkers were used in effort to exceed the properties of parent molecule (SAD-128).

The novel compounds were prepared via standard synthetic strategy.[20] The solution of 4-*tert*-butylpyridine (6.8 mmol) and corresponding dihalogenated compound (3.1 mmol) in DMF (10 ml)

**Table 1**
Newly prepared analogues of SAD-128.




| | |
|---|---|
| **5**–**16** (Br) | $(CH_2)_{1\text{-}12}$ |
| **17** (Cl) | $CH_2OCH_2$ |
| **18** (Br) | $(CH_2)_2O(CH_2)_2$ |
| **19** (Br) | (*E*)-$CH_2CH{=}CHCH_2$ |
| **20** (Cl) | (*Z*)-$CH_2CH{=}CHCH_2$ |
| **21** (Br) | 1,2-Phenylenyl |
| **22** (Br) | 1,3-Phenylenyl |
| **23** (Br) | 1,4-Phenylenyl |
| **24** (Br) | 3,6-Naphtylenyl |

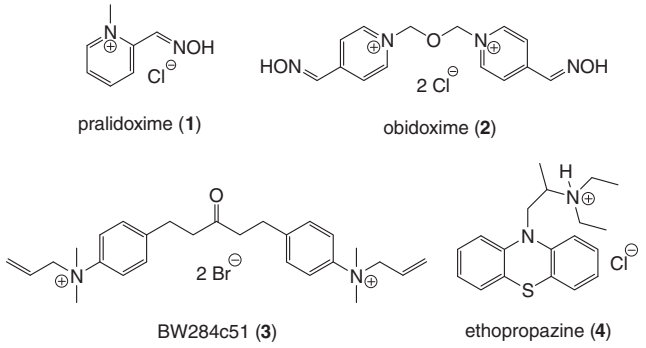
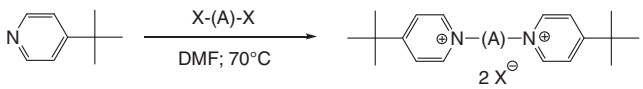



**Figure 3.** Cholinesterase reactivators and inhibitors used as standards.

was stirred at 70 °C for 14–98 h (Scheme 1). Subsequently, the reaction mixture was cooled to the room temperature, portioned with acetone (80 ml) and cooled in refrigerator overnight (5 °C). The crystalline crude product was collected by filtration. The amorphous crude product was decanted and residual solvent was poured off. The crude products were purified by boiling in ethyl acetate (50 ml). NMR, ESI-MS and elemental analysis were used to determine the entity and purity of all prepared compounds.

The former and the novel compounds were assayed for their inhibitory ability in vitro using a standard inhibition test utilising human recombinant AChE and human plasmatic BChE.[27] The hAChE was chosen as the main target for the OP pre-treatment strategy.[9] Additionally, hBChE was chosen as a member of the cholinesterase family, which is usually affected by all the compounds that are interacting with hAChE.[3] The commercial oximes (**1** and **2**) were selected as standards among AChE reactivators that may be used not only for post-exposure treatment, but also for pre-exposure treatment (e.g., prophylaxis).[9] The BW284c51 (**3**; Fig. 3) and ethopropazine hydrochloride (**4**; Fig. 3) were chosen as selective AChE or BChE inhibitors to cover the selectivity issues.[28,29] The $IC_{50}$ values of all compounds are listed in Table 2.

The commercial oximes (**1** and **2**) showed weak inhibition of hAChE on mM scale and did not seem to be relevant compounds for OP pre-exposure treatment.[22] The selective standards (**3** and **4**) presented expected results for hAChE. Whereas compound **3** was promising hAChE inhibitor on nM scale, compound **4** resulted as its weak inhibitor.[28] The newly prepared compounds presented mixed results. Compounds **5**–**9** and **17**–**23** resulted as AChE inhibitors on μM scale. More interestingly, compounds **10**–**16** and **24** were found to be nM AChE inhibitors. Some of these compounds (**11** and **12**, **14**, **15** and **16**, **24**) exceeded the commercial standard **3** (30 nM) in hAChE inhibition. Among them, compounds **12** (5 nM), **14** (12 nM) and **16** (7 nM) were the most promising AChE inhibitors bearing aliphatic linkage and compound **24** (24 nM) was the most promising inhibitor with different linkage type. For hBChE, the commercial oximes again resulted as poor inhibitors, where pralidoxime (**1**) did not showed inhibition of hBChE at all. For chosen selective compounds (**3** and **4**), compound **4** showed expected increased inhibition on μM scale, while compound **3** was found to be poor inhibitor of hBChE.[29] Similarly to hAChE results, some newly prepared compounds (**5**–**9**, **17**–**23**) showed BChE inhibition on μM scale. Furthermore, compounds **10**–**11**, **13**, **15** and **24** resulted as sub-μM hBChE inhibitors. Most interestingly,




**Scheme 1.** Preparation of SAD-128 analogues.

**Table 2**
Inhibitory potency of tested compounds towards cholinesterases and calculated lipophilicity

| Compound | AChE $IC_{50}$ ± SD[a] (μM) | BChE $IC_{50}$ ± SD[a](μM) | SI[c] BChE/AChE | $K_{i1}/K_{i2}$ (μM) | log *P* |
|---|---|---|---|---|---|
| Pralidoxime (**1**) | 878 ± 171 | –[b] | – | – | −2.46 |
| Obidoxime (**2**) | 577 ± 113 | 1910 ± 311 | 3.3 | – | −2.42 |
| BW284c51 (**3**) | 0.030 ± 0.006 | 354 ± 58 | 11,800 | 0.01/0.05 | −6.19 |
| Ethopropazine (**4**) | 1020 ± 199 | 1.6 ± 0.3 | 0.002 | 24.7/12100 | 5.11 |
| **5** | 20 ± 4 | 34 ± 6 | 1.7 | – | 2.29 |
| **6** | 17 ± 3 | 20 ± 3 | 1.2 | – | 1.35 |
| **7** | 14 ± 3 | 53 ± 9 | 3.8 | – | 0.95 |
| **8** | 3.7 ± 0.7 | 5.5 ± 0.9 | 1.5 | – | 0.68 |
| **9** | 24 ± 5 | 5.1 ± 0.8 | 0.2 | – | 0.56 |
| **10** | 0.032 ± 0.006 | 0.6 ± 0.1 | 19 | – | 0.67 |
| **11** | 0.016 ± 0.003 | 0.7 ± 0.1 | 44 | – | 0.87 |
| **12** | 0.005 ± 0.0009 | 0.016 ± 0.003 | 3.2 | 0.09/0.20 | 1.08 |
| **13** | 0.037 ± 0.007 | 0.15 ± 0.02 | 4.1 | – | 1.37 |
| **14** | 0.012 ± 0.002 | 0.06 ± 0.01 | 5.0 | – | 1.66 |
| **15** | 0.026 ± 0.005 | 0.11 ± 0.02 | 4.2 | – | 2.01 |
| **16** | 0.007 ± 0.001 | 0.019 ± 0.003 | 2.7 | – | 2.33 |
| SAD-128 (**17**) | 12 ± 2 | 75 ± 12 | 6.2 | – | 1.73 |
| **18** | 19 ± 4 | 241 ± 40 | 13 | – | 0.11 |
| **19** | 3.4 ± 0.7 | 19 ± 3 | 5.6 | – | 0.92 |
| **20** | 2.0 ± 0.4 | 4.4 ± 0.7 | 2.2 | – | 0.92 |
| **21** | 10 ± 2 | 2.4 ± 0.5 | 0.2 | – | 1.42 |
| **22** | 0.7 ± 0.1 | 1.9 ± 0.3 | 2.7 | – | 1.42 |
| **23** | 0.30 ± 0.06 | 1.3 ± 0.2 | 4.3 | – | 1.42 |
| **24** | 0.024 ± 0.005 | 0.12 ± 0.02 | 5 | 0.10/0.10 | 2.54 |

[a] Mean value of three independent determinations.
[b] No inhibition in selected concentration scale.
[c] Selectivity index.

compounds **12**, **14** and **16** were found to be nM hBChE inhibitors. Among sub-μM and nM hBChE inhibitors, compounds **10**–**16** and **24** exceeded the commercial standard **4**. The selectivity index (SI) was calculated to reveal the selectivity for one of cholinesterases. The oxime reactivator **1** displayed some selectivity towards hAChE, while it was not inhibiting hBChE. Obidoxime (**2**) didnot present higher selectivity for one cholinesterase. The standard cholinesterase inhibitors (**3** and **4**) showed anticipated results, where compound **3** was highly selective for hAChE and compound **4** for hBChE.[29] The newly prepared compounds didnot show higher selectivity with best result of 44-fold better inhibition of hAChE by compound **11**.

The novel molecules were designed as reversible AChE inhibitors. Thus, the kinetic experiments with recombinant hAChE were developed to confirm this hypothesis.[30] The AChE standard **3** was found to be its non-competitive inhibitor, whereas the BChE standard **4** showed strong competition with the substrate during enzymatic reaction and it was indicated as competitive AChE inhibitor. Two novel compounds (**12** and **24**) were highlighted for kinetic experiments. Compound **12** was chosen among the potent AChE inhibitors bearing aliphatic linkage and compound **24** as the best AChE inhibitor with different type of the linker. Both selected compounds **12** and **24** resulted as non-competitive hAChE inhibitors with almost no influence to substrate (acetylthiocholine) hydrolysis. This finding confirmed their binding aside the AChE active site serine (Ser203).

The docking studies were performed on four promising compounds after the in vitro screening (**12**, **14**, **16**, **24**) in order to rationalise their possible interactions within AChE and BChE active site. Three crystal structures were used for the docking calculations (hAChE—1b41, mAChE—2jez, 2jf0; hBChE—1p0i) and the best results were obtained for the mAChE model (2jez).[31–34] For mAChE, compounds **12**, **14** and **16** presented very similar type of interaction (Fig. 4). Thus, only the most promising compound **12** is discussed. Its top-scored docking pose (−8.58 kcal/mol) showed important cation–π interactions. First pyridinium ring was attached to Tyr124 (3.3 Å), Phe297 (3.9 Å), Tyr337 (3.4 Å) and Phe338 (3.2 Å), whereas its 4-*tert*-butyl moiety was stabilized by

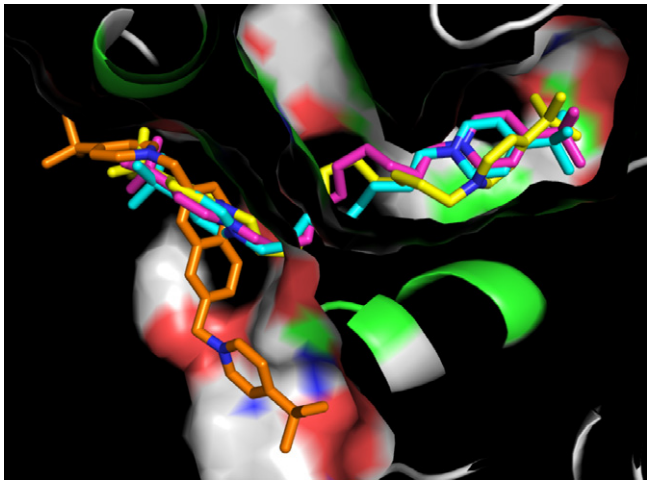


**Figure 4.** Molecular docking results for mAChE with compound **12** (blue), **14** (magenta), **16** (yellow) and **24** (orange).

CH–π interactions with Trp86 (3.4 Å), Tyr337 (3.6 Å) and His447 (3.3 Å).[35] The second pyridinium ring was attached by cation–π interaction to Tyr286 (3.3 Å) and its 4-*tert*-butyl moiety was stabilized by CH–CH interactions with Leu289 (3.2 Å) and Glu292 (3.7 Å). The whole molecule of compound **12** (as well as molecules **14** and **16**) was penetrating inside the active site gorge closely to Ser203. Differently, the top-scored docking pose of compound **24** (−8.25 kcal/mol) displayed binding on the AChE surface and was not going inside the active site gorge (Fig. 4). First of its pyridinium rings was attached by cation–π interaction to His287 (3.9 Å) and its 4-*tert*-butyl moiety presented CH–CH interactions with Leu289 (3.0 Å), Pro290 (3.8 Å) and Gln291 (4.0 Å). The second pyridinium moiety was not stabilized by aromatic interaction, but by CH–CH interactions of its 4-*tert*-butyl moiety with Val340 (3.7 Å), Val343 (3.5 Å) and Pro344 (3.8 Å).[35] Importantly, the naphtylene moiety of the connecting linker displayed strong π–π interaction with Tyr286 (3.2 Å).

For hBChE, compounds **12**, **14** and **16** again presented very similar type of interaction (Fig. 5). Concerning the most promising compound against hBChE **12**, its top-scored docking pose (−8.14 kcal/mol) showed cation–π interactions of one pyridinium moiety with Tyr332 (3.1 Å) and Phe329 (3.3 Å), while its 4-*tert*-butyl moiety was attached via CH–π interactions to Trp82 (3.5 Å) and His438 (3.6 Å). The second pyridinium moiety displayed cation–π interaction with Phe329 (3.2 Å) and its 4-*tert*-butyl moiety CH–π interactions with Trp231 (3.5 Å) and Phe398 (3.5 Å). The compounds **12**, **14** and **16** were all twisted in the BChE active site. In contrast, top-scored docking pose of compound **24** (−9.23 kcal/mol) displayed flat binding of its rigid molecule. First of its pyridinium rings was sandwiched by cation–π interactions between Trp82 (3.6 Å) and His438 (4.0 Å), whereas the corresponding 4-*tert*-butyl moiety was attached via CH–π interactions with the same residues (both 3.4 Å). The second pyridinium moiety was not attached to aromatic residues and its 4-*tert*-butyl moiety presented CH–CH interactions with Pro285 (3.4 Å) and Gly283 (3.5 Å). Importantly, the naphtylene linker was sandwiched by π–π interactions between Tyr332 (3.4 Å) and Phe329 (3.1 Å).

The structure–activity relationship (SAR) of the novel compounds generated from in vitro and docking data can be demonstrated.[36] Firstly, the pyridinium moiety was found very effective in forming strong π–cationic interaction instead of weaker π–π interactions of, for example, phenyl ring.[37] Additionally, bis-pyridinium or bis-isoquinolinium compounds were formerly found to be effective AChE or BChE inhibitors.[20,21] Their further substitution by various functional groups may be the key factor to influence the physical–chemical properties (e.g., lipophilicity). The *tert*-butyl moiety was supposed to increase the lipophilicity of the prepared molecules. From this point of view, structurally similar compounds **3** (log *P* −6.19), **18** (log *P* 0.11) and its published bispyridinium analogue without *tert*-butyl moieties (1,1′-pentane-1,5-bispyridinium dibromide; log *P* −3.52) may confirm this hypothesis.[20,38] Furthermore, additional interactions between cholinesterase and 4-*tert*-butyl moiety were expected. Subsequently, the CH-π or CH–CH interactions were found during the molecular docking of compounds **12**, **14**, **16** and **24** with AChE or BChE.[35] Thus, *tert*-butylpyridinium moiety seemed to be promising molecular tool for increasing lipophilicity and weak molecular interactions with cholinesterases.

Whereas the *tert*-butylpyridinium moiety was uniform for all novel compounds, the connecting linkage was different. The linkage was apparently influencing both cholinesterase interactions and lipophilicity. Based on in vitro data, compounds with shorter aliphatic $C_1$–$C_5$ (**5**–**9**), aliphatic with heteroatom (**17** and **18**), double bonded linkers (**19** and **20**) or linkers bearing xylene moiety (**21**–**23**) were found to be effective hAChE inhibitors only on μM scale. In contrast, compounds bearing longer aliphatic $C_6$–$C_{12}$ (**10**–**16**) and naphtylene linkers (**24**) resulted as very potent AChE inhibitors on nM scale. The assumed explanation of the increased inhibitory ability by compounds **10**–**16** and **24** consists in the accommodation of such molecules in the AChE or BChE active site gorge, where they were able to bind the essential aromatic residues (Trp, Tyr, Phe, His). Interestingly, the even linkers $C_8$–$C_{10}$–$C_{12}$ (**12**, **14**, **16**) were found to be more potent inhibitors to odd linkers (**11**, **13**, **15**). Thus, the even linkers (**12**, **14**, **16**) were supposed to better accommodate in the hAChE active site gorge. The naphtylenyl linked compound (**24**) was found slightly worse inhibitor to aliphatic linked compounds (**11** and **12**, **14**, **16**). This finding is plausibly related to its spatially rigid structure, where only limited free rotation in its molecule is available. For this reason, it showed different top-scored docking pose compared to aliphatically linked compounds. Concerning lipophilicity, the calculated log *P* values of the newly prepared compounds showed interesting results. For compounds **5**–**16**, the shortest $C_1$ linkage (**5**; log *P* 2.29) was calculated to have log *P* value close to $C_{12}$ (**16**; log *P* 2.33) or naphtylenyl (**24**; log *P* 2.54) bridge, although it was supposed to have the lowest log *P* value among all aliphatic linkers. Similarly, $C_2$ linker (**6**; log *P* 1.35) resulted close to $C_9$ linkage (**13**; log *P* 1.37). This phenomenon may be explained by the spatial shielding of quaternary nitrogen by the pyridinium rings that are very close together for the shorter aliphatic linkers (**5**–**8**). For longer aliphatic linkers (**9**–**16**), the log *P* values were found consistently increasing with the length of the linker as it was expected. Additionally, SAD-128 (**17**; 1.73) log *P* value was found superior to its aliphatic analogue (**7**; log *P* 0.95) and highly superior to its slightly longer analogue (**18**; log *P* 0.11). In this case, the same phenomenon as it was described for aliphatic linkage was supposed. And, additionally, the other participating factor should be the influence of free oxygen electron pairs that may be conjugated with aromatic rings and thus increased the lipophilicity of compound **17**. Concerning the in vitro, molecular docking and lipophilicity data, the longer even aliphatic linkers (**12**, **14**, **16**) and the naphtylenyl linkage (**24**) were highlighted for further investigation.

Compound SAD-128 (**17**) was formerly found to be receptor acting inhibitor. Namely, it was determined to be reversible inhibitor of the muscarinic (mAChR) and nicotinic (nAChR) acetylcholine receptors together with its analogues (**6**–**14**).[19] Unfortunately, the receptor's data were not connected to the cholinesterase inhibition. Currently, these data may be supplemented and summarised. The best inhibitor of hAChE from tested series (**12**) was reported as potent inhibitor of mAChR ($IC_{50}$ 37 μM) and nAChR ($IC_{50}$ 30 μM).[19] Similar data are available for compounds **10**–**14** that also resulted as potent receptor's antagonists.[19] For these reasons, presented multiple acting compounds (e.g., **12** and **24**) should be evaluated in vivo to confirm or disprove their in vitro effectiveness for OP pre-exposure treatment.

In summary, 19 analogues of SAD-128 were prepared and evaluated as cholinesterase inhibitors. The screening results showed promising inhibitory ability of four compounds better to used standards (pralidoxime, obidoxime, BW284c51, ethopropazine, SAD-128). Four most promising compounds were selected for further molecular docking studies. The SAR was stated from obtained data. The former receptor studies were reported and discussed. The further in vivo studies were recommended in the view of OP pre-exposure treatment.

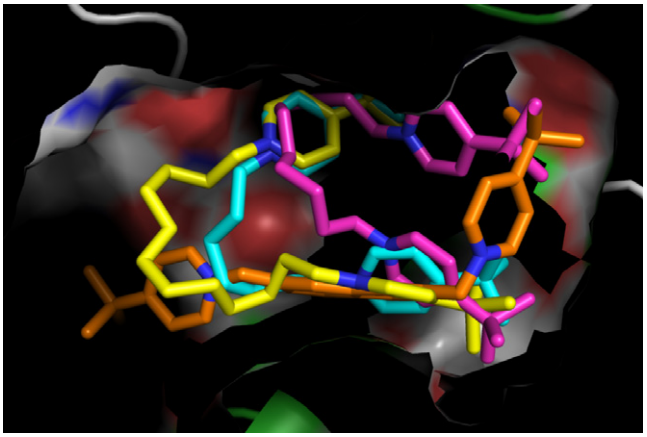


**Figure 5.** Molecular docking results for hBChE with compound **12** (blue), **14** (magenta), **16** (yellow) and **24** (orange).

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## Supplementary data

All experimental details are listed in the Supporting information. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.051.

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