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Annulated bicyclic isothioureas: identification of active and selective butyrylcholinesterase inhibitors

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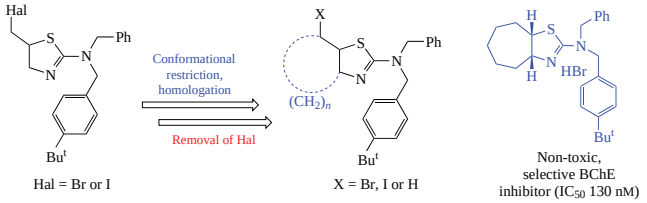
Structural optimization of butyrylcholinesterase inhibitors, 5-bromomethyl- and 5-iodomethyl-*N*,*N*-disubstituted 2-aminothiazolines, led to a series of their annulated bicyclic analogues, obtained by intramolecular cyclization of cycloalkenylthioureas. The most active compound in this series, cyclohepta[*d*]thiazol-2-amine, is a mixed-type butyrylcholinesterase inhibitor with IC_{50} = 130 nM, highly selective compared to acetylcholinesterase and non-toxic at 100 μM concentrations.



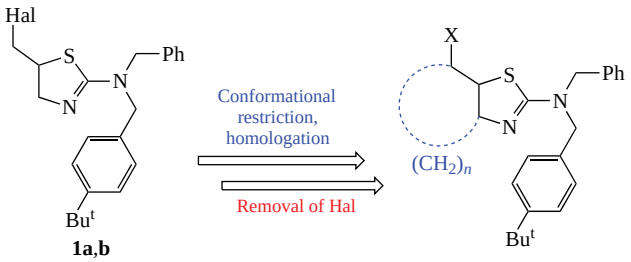
Keywords: bicyclic isothioureas, cycloalka[*d*]thiazole-2-amine, thioureas, 2-aminothiazolines, heterocyclization, butyrylcholinesterase inhibitors, Alzheimer's disease, cytotoxicity.

Butyrylcholinesterase (BChE, EC 3.1.1.8) is a serine hydrolase that, along with acetylcholinesterase (AChE, EC 3.1.1.7) participates in cholinergic neurotransmission catalyzing hydrolysis of acetylcholine (ACh). In recent decades, development of specific BChE inhibitors has attracted attention, primarily due to their therapeutic potential for the treatment of Alzheimer's disease (AD).[1–7] Cognitive deficiency of AD is assumed to be a consequence of the cholinergic deficit within the brain. Anticholinesterase drugs help to restore brain acetylcholine levels by inhibiting cholinesterases. In healthy brain AChE plays a dominant role in regulating ACh levels, but in the ACh deficient brain of the patients with AD activity of BChE progressively increases. This makes BChE a reasonable therapeutic target for the replenishment of ACh deficit of AD.

Both AChE and BChE are also known to be involved in cell proliferation and differentiation,[4] and the role of the enzymes in carcinogenesis has been studied. Recently there has been convincing evidence of a decrease in the level of BChE in prostate, pancreatic and other types of cancer,[8,9] but the reason for the decrease is still unclear. This also makes it relevant to design active and selective BChE inhibitors for their use as molecular tools in biological research aimed to clarify the role of the enzyme in the etiology of tumor diseases.[10]

In our previous work, during the screening of a series of *N*,*N*-disubstituted 2-amino-5-halomethyl-2-thiazolines we found several BChE inhibitors with pronounced selectivity *versus* AChE.[11,12] The most active compounds **1a**,**b** (Figure 1) inhibited BChE at low micromolar and submicromolar concentrations (IC_{50} 2.34 and 0.77 μM, respectively).[11] In the present work, we performed the optimization of the leads **1a**,**b** aimed at increasing their BChE inhibitory potency. For this, classical drug design methods were used, namely, conformational restriction and homologation, that allow one in many cases to increase inhibitory activity of the lead.[13] We proposed to obtain bicyclic analogues of the lead molecules **1a**,**b** in which rotation around the bond C(5)–CHal is restricted (see Figure 1, structure **A**). The degree of rigidity of the bond incorporated into alicycle varied depending on the size of the latter. Though the presence of halogen atoms in structures **1a**,**b** and **A** might be helpful for the radiolabeling of BChE inhibitors, analogues without halogen atoms of general formula **B** were also obtained in order to improve toxicological profile and to compensate for the increase of lipophilicity with the introduction of an additional cycle.

Target sets of compounds **5**–**7** with *cis*-orientation of the annulated rings were synthesized from bromocyloalkenes **2** (*n* = 1–3) through the corresponding cycloalkenyl isothiocyanates **3** (*n* = 1–3, Scheme 1) based on previously elaborated procedures.[14–17] The reaction of cycloalkenylthioureas **4a**–**c** with Br_2 and I_2 gave bicyclic bromine- and iodine-containing isothioureas **5a**–**c** and **6a**–**c** in good yields and proceeded with the predominate formation of isomers with *trans*-orientation of halogen atom to heterocyclic fragment. Only in the case of

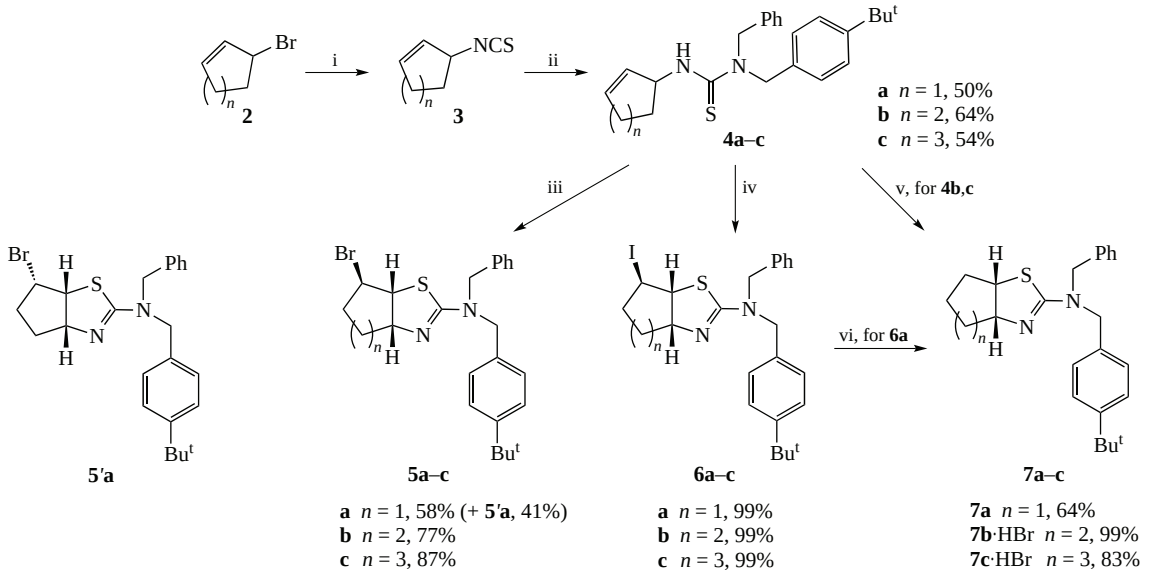


1a,**b**

a Hal = Br
b Hal = I

A X = Hal (Br, I)
B X = H

Figure 1 Lead compounds and the ways of their optimization carried out in the present work.



Scheme 1 *Reagents and conditions*: i, KSCN, MeOH, room temperature, 2 h; ii, Bn(4-Bu^{t}C$_6$H$_4$CH$_2$)NH, DIPEA, CH_2Cl_2, room temperature, 12 h; iii, Br_2, CH_2Cl_2, room temperature, 12 h, then $NaHCO_3$; iv, I_2, CH_2Cl_2, room temperature, 12 h, then $NaHCO_3$; v, AcBr, MeOH, CH_2Cl_2, room temperature, 12 h; vi, *n*-Bu_3SnH, toluene, reflux. The marked configuration of compounds is relative (they represent racemic mixtures).

synthesis of compound **5a** its *cis*-isomer **5′a** was also isolated with 41% yield. Compounds **7b**,**c** were prepared by treatment of thioureas **4b**,**c** with AcBr/MeOH, while thiourea **4a** was unreactive under these conditions. The target isothiourea **7a** was obtained through reductive deiodination of the corresponding iodo derivative **6a** with *n*-Bu_3SnH (see Online Supplementary Materials).

Initial assessment of inhibitory activity and selectivity of all new bicyclic isothioureas **5a–c**, **5′a**, **6a–c** and **7a–c** and their synthetic precursors, thioureas **4a–c**, was performed using human erythrocyte AChE and equine serum BChE as described.[18] The known AChE and BChE inhibitor Tacrine served as a positive control (Table 1). As can be seen from Table 1, all cyclic isothioureas effectively inhibited BChE with IC_{50} values in mid-nanomolar to low micromolar range, while thioureas **4a–c** demonstrated moderate BChE inhibitory properties. Importantly, all new compounds were highly selective towards BChE, none of them showed significant inhibition of AChE.

Table 1 hAChE and eqBChE inhibitory potential of novel compounds.[a]

Compound	Inhibitionof hAChE, % inhibition at 20 μM ± SEM	Inhibition of eqBChE, IC_{50} (μM) or % inhibition at 20 μM ± SEM
4a	2.4 ± 0.3%	16.3 ± 0.2%
4b	n.a.	42.0 ± 1.5%
4c	n.a.	36.1 ± 4.0%
5a	3.4 ± 1.1%	0.738 ± 0.073
5′a	1.8 ± 0.2%	0.717 ± 0.022
5b	12.6 ± 1.9%	1.33 ± 0.02
5c	n.a.	0.702 ± 0.029
6a	n.a.	2.61 ± 0.23
6b	n.a.	5.83 ± 0.23
6c	1.5 ± 0.5%	0.244 ± 0.026
7a	11.3 ± 1.2%	1.15 ± 0.02
7b	13.6 ± 0.3%	0.597 ± 0.042
7c	5.7 ± 0.7%	0.130 ± 0.012
1a	<10%	2.3 ± 0.2
1b	<10%	0.77 ± 0.07
Tacrine	IC_{50}: 0.74 ± 0.05 μM	0.0311 ± 0.0003

[a] n.a. – not active. Data are means ± SEM from three independent experiments.

Analysis of the data obtained shows that the introduction of a seven-membered ring (**5c**, **6c**) into lead-molecules **1a** and **1b** increases their activity. Inhibitory properties of compounds with an additional five- or six-membered ring depend on the nature of the halogen atom: both iodine derivatives **6a** and **6b** are less active than the parent molecule **1b**, but the activity of the bromine derivatives **5a** and **5b** increases by one and a half to three times compared to the parent molecule **1b**. In this case, the configuration of the bromine atom does not play a significant role, because IC_{50} values are very close for both diastereomers **5a** and **5′a**.

Bicyclic isothioureas without a halogen atom **7a–c** exhibited the highest inhibitory activity against BChE in each homologous series and an increase in the cycle size **7a**–**7b**–**7c** enhances the degree of inhibition. Compound **7c** is the most potent BChE inhibitor of the series with an IC_{50} value of 130 nM and it is about six time more active than lead molecule **1b**. Since this compound demonstrates very low anti-AChE activity (see Table 1), it is also a highly selective BChE inhibitor.

Next, we carried out the kinetics studies for compound **7c** to evaluate the mechanism of BChE inhibition. Analysis with Lineweaver–Burk double reciprocal plots (see Online Supplementary Materials) showed that binding of compound **7c** to BChE changed both V_{max} and K_m values, consistent with mixed-type inhibition. The obtained constants for eqBChE inhibition are: $K_i = 77.6 \pm 2.1$ nM (competitive inhibition constant), $\alpha K_i = 129 \pm 5$ nM (non-competitive inhibition constant). Previously we showed that the replacement of bromine in the lead molecule **1a** with iodine (**1b**) leads to a change in the mechanism (from competitive to mixed-type) of BChE inhibition. Thus, novel bicyclic isothiourea **7c** without a halogen atom exhibits mechanism of inhibition similar to that of iodine-substituted lead **1b**, and not to the bromine-substituted lead **1a**.

Proposed binding mode of two possible protonated forms of (3a*R*,8a*S*)-isomer of isothiourea **7c** in the BChE gorge (PDB: 6R6W) obtained by molecular docking is presented in Figure 2 (for detailed description of the docking procedure, see Online Supplementary Materials). As can be seen, regardless of the position of the protonated nitrogen, the latter is located close to the Trp82 residue of the choline-binding pocket of BChE to give the important cation–π interaction in the active site. In turn, unsubstituted benzyl ring of compound **7c** is involved in the π–π stacking interaction with Trp82 and is located near the catalytic triad Ser198–His438–Glu197. Another benzyl ring also has π–π

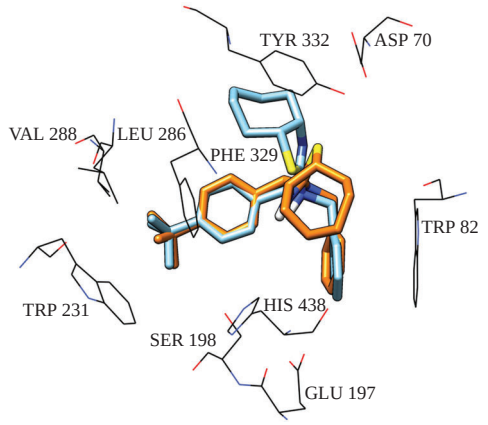


Figure 2 The position of $N^{+}_{endocyclic}$ (orange) and $N^{+}_{exocyclic}$ (blue) protonated forms of (3a*R*,8a*S*)-**7c** in the complex with hBChE (PDB: 6R6W) predicted by molecular docking in the AutoDock Vina 1.1.2 software[19] (only key amino acid residues are shown and hydrogen atoms are omitted for clarity).

interactions (T-stacking) with the aromatic cycle of Phe329 and its bulky *tert*-butyl-substituent is exposed within the acyl pocket close to the residues of Leu286 and Trp231.† This explains the selectivity of ligand **7c**, because the volume of this area is one of the structural differences between active sites of BChE and AChE.[5] Annulated bicyclic fragments of two protonated forms of isothiourea **7c** occupy a mirror image position and are exposed to the area adjacent to the active site (see Figure 2). Thus, molecular modeling results are in accordance with the kinetic data on the mixed type inhibition of compound **7c** and its selectivity to BChE compared to AChE.

Additional 50% cytotoxic concentration testing of the compound **7c** and its iodine-containing analogue **6c** on the conditionally normal breast epithelial cells MCF-10A as described[20] has shown that the values of CC_{50} are ≫100 μM and 62.2 ± 0.4 μM, respectively. Isothiourea **7c** was also non-toxic to non-cancer lung fibroblasts VA13 (CC_{50} ≫ 100 μM).

In conclusion, the primary optimization of previously discovered BChE inhibitors of the structural type of *N*,*N*-disubstituted 2-amino-5-halomethyl-2-thiazolines led to cyclohepta[*d*]thiazol-2-amine, which is non-toxic, effective and selective (compared to AChE) BChE inhibitor.

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† Similar location of *tert*-butylbenzyl residue as well as cation–π interaction of protonated nitrogen with Trp82, are predicted for (3a*S*,8a*R*)-enantiomer of **7c** (both protonated forms of which have very close positions). However, unsubstituted benzyl ring of the latter is involved in π–π stacking not with Trp82, but with Tyr 332 (see the image in Online Supplementary Materials).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.01.024.

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