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BENZOTHIAZOLES AS EFFECTIVE INHIBITORS OF CHOLINESTERASES: KINETICS AND MECHANISM OF INHIBITION

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The aim of our study was to determine the inhibition efficiency of the benzothiazoles tested, representing potential inhibitors of cholinesterases. (Reaction) kinetics of hydrolyses of acetylcholine and acetylthiocholine, uninhibited and inhibited by two types of enzyme, acetylcholinesterase, and butyrylcholinesterase, has been studied. Two methods for the determination of enzymatic hydrolysis were used, Ellman's and pH-stat method and, totally, twelve inhibitors (benzothiazoles) selected to study the inhibited hydrolysis. The kinetic parameters of uninhibited and inhibited hydrolyses have been determined and, based on our current results of inhibition rate constant, 2,2,3,3-tetrafluoropropyl (S)-1-[(R)-1-(6-fluorobenzo[d]thiazole)ethyl-carbamoyl-2-

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methylpropylcarbamate found to be the best inhibitor of cholinesterases from the palette of the inhibiting substances tested.

Introduction

Cholinesterases (ChEs) constitute a group of esterases that hydrolyse choline esters at a higher rate than other esters, thus providing the hydrolysis rates that can be compared at optimum and controlled conditions. ChEs are primarily responsible for the rapid elimination of acetylcholine (ACh), proceeding within one millisecond after its release at cholinergic synapses [1]. Two types of ChEs are present in the human body, (i) acetylcholinesterase (AChE) and (ii) butyrylcholinesterase (BChE). These two forms differ from the substrate specificity, genetically, and structurally [2]. Structurally, AChE and BChE consist of active centres localized in the interior of enzyme molecule, containing characteristic gorge that intrudes into the surface of the enzyme catalytic and choline binding sites where the cleavage of ACh occurs. Structure of the gorge of AChE and BChE is different. On its base in AChE, the binding of the substrate is represented by two phenylalanine molecules whose aromatic residues protrude into the gorge. In BChE, these molecules are replaced by two smaller aminoacid molecules, such as valine and leucine [1].

Alzheimer's disease (AD) is one of the most common causes of dementia in the elderly people. AD is an age-dependent neurodegenerative disorder that destroys brain cells, causing problems with memory, thinking, and behaviour [3]. There are many hypotheses on the origination of this disease. Two main theories are invoked for the AD treatment. The cholinergic hypothesis relates the neurodegeneration with the loss of cholinergic neurotransmission and the increased activity of AChE; the amyloid hypothesis being based on the abnormal degradation of amyloid precursor protein β (APP) [4]. Currently, several approaches are applied in the treatment of AD. The main of them is administration of inhibitors of cholinesterases, which leads to the reduced activity of ChEs [5].

Inhibitors of cholinesterases (ChEIs) involve the group of chemical compounds that are able to inhibit the hydrolytic activity of cholinesterases. Due to this effect, there is the increased amount of ACh which then becomes available for the nervous and neuromuscular transmission; the mechanism of action being based on the reversible or irreversible inhibition of activity of ChEs. ChEIs differ in selectivity for AChE and BChE, in the mechanism of inhibitory effect as well as in the reversibility of response [6]. There are many criteria by which ChEIs can be classified. One possible way is a division based on the reaction mechanism, when, according to this criterion, ChEIs can be divided into (i) competitive inhibitors (inhibitor and substrate compete for binding into the active site of the enzyme molecule), (ii) non-competitive inhibitors (inhibitor binds at a site that is

distinct from the catalytic site), (iii) acompetitive inhibitors (inhibitor reversibly binds to enzyme-substrate complex), and (iv) mixed inhibitors [1].

Benzothiazoles have been reported as a class of compounds with a wide range of biological activities [7], including antibacterial and antimicrobial character [8], or anticancer [9] and antitumor [10] properties. The benzothiazole ring is also present in various marine or terrestrial natural compounds, exhibiting interesting biological activities [11].

The quality of substances intended as cholinesterase inhibitors should be judged by means of three important parameters: (i) rate of inhibition, (ii) power of inhibition, and (iii) ability to pass through the blood-brain barrier. Power of inhibition is usually represented by the value of IC_{50}; i.e., molarity of inhibitor capable of decreasing the enzyme activity down to 50 %. The ability of inhibitor to pass through the blood-brain barrier for direct inhibition of brain cholinesterases is being defined by the partition coefficient, K_{ow}, in the octan-1-ol/water system [12].

The aim of this work was to determine the rate of inhibition; i.e., the rate of reaction between enzyme and inhibitor represented by the inhibition rate constant.

Experimental

Chemicals and Reagents

Acetylcholinesterase (E.C.3.1.1.7) lyophilizate from electric eel (*Electrophorus electricus* sp.) acetylthiocholine (ATCh) iodide, acetylcholine chloride, butyrylcholinesterase (E.C.3.1.1.8) lyophilizate from horse plasma, and TNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) were all purchased from Sigma-Aldrich. The remaining chemicals were then: dioxane p.a., KCl, KOH, (all from Lach-Ner, the Czech Republic), KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$ (all from Penta, the Czech Republic).

Inhibitors, 2-substituted 6-fluorobenzo[*d*]thiazoles, were synthesised at the Institute of Organic Chemistry and Technology, Faculty of Chemical Technology, University of Pardubice. Synthesis of all the benzothiazoles tested (see Fig. 1 and Table I) and the cytotoxicity of selected ones is described in detail elsewhere [13]. Stock solutions of 0.05 M or 0.02 M benzothiazole derivatives were prepared by dissolving the appropriate amount(s) in dioxane and diluted in deionized water whenever needed.

Solution of AC and BC: were prepared by adding about 10 mg of the respective enzyme into 20 ml deionized water. These solutions were placed in plastic tubes and kept at –6 °C in the freezer.

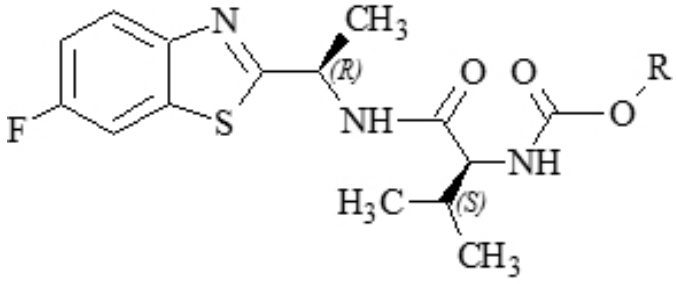


Fig. 1 General formula of the benzothiazoles tested

Table I Survey of inhibitors used 2-substituted 6-fluorobenzo[*d*]thiazoles

Inhibitor	R	Name of alkyl
1	$-CH_3$	methyl
2	$-CH_2CH_3$	ethyl
3	$-(CH_2)_2CH_3$	propyl
4	$-CH(CH_3)_2$	1-isopropyl
5	$-(CH_2)_3CH_3$	butyl
6	$-CH_2CH(CH_3)_2$	2-isobutyl
7	$-CH_2CH_2Cl$	2-chlorethyl
8	$-CH_2CH_2Br$	2-bromethyl
9	$-CH_2CCl_3$	2,2,2-trichlorethyl
10	$-CH_2CF_3$	2,2,2-trifluorethyl
11	$-CH_2CF_2CHF_2$	2,2,3,3-tetrafluorpropyl
12	$-CH_2CF_2CF_2CF_3$	2,2,3,3,4,4,4-heptafluorbutyl

Measuring Pocedures

Two different methods were used for determination of the kinetic parameters of uninhibited and inhibited hydrolyses. The first one, Ellman's method ELM), which is a simple, rapid, and direct method to determine the SH and –S–S– groups contained in proteins [14,15]. The resultant product of enzymatic reaction, 5-thio-2-nitrobenzoic acid (TNB) ion, was measured by spectrophotometer with a diode array detector (model Hewlett-Packard 8453) at the wavelength of 412 nm. The second approach pH-stat method (PHS), was based on the determination of the actual concentration of acetic acid (HA), representing the second product of

hydrolytic enzymatic reaction (for further details, see Ref. [16]). For these measurements, a 36 GP Titrino apparatus was used (Metrohm, Switzerland).

The daily determined (using Ellman's method) catalytic activity of given enzyme was needed to calculate its suitable volume which had to be added into the initial reaction mixture to achieve the initial activity chosen (i.e., 0.14 U). All experiments were done at 25 °C, pH 8, ionic strength 0.11 mol l^{-1}, and enzyme activity 0.14 U in the initial reaction mixture.

ELM Method: 0.8 ml phosphate buffer (pH 8) + 0.4 ml DTNB (0.5 mM) + ml ATCh (1 mM) + *X* ml enzyme (according to the respective activity of enzyme) + *Y* ml deionized water (at total volume of the reaction mixture of 2 ml) were pipetted into the measuring cell. The addition of enzyme solution started the reaction.

PHS Method: 5.5 ml KCl (0.5 M) + 1 ml ACh (0.1 M) + *X* ml deionized water (total volume of the reaction mixture after adding all the remaining components being 5 ml). Then, the pH value of reaction mixture was adjusted to 8 by 0.1 M KOH (pre-titration phase) and the titration sequence was started by adding such an amount of enzyme preparation which is necessary to achieve the activity 0.14 U in the reaction mixture.

For inhibited hydrolyses of ATCh (ELM method) or ACh (PHS method), the initial molar concentration of inhibitor I had been chosen so that the uninhibited and inhibited temporal dependences of reactant(s) to be determined (P, HA) were markedly different. In overall, four or five different concentrations were tested; always, in duplicate.

Theory and Calculations

The reaction scheme of enzymatic hydrolysis of substrate S (ACh or ATCh) by enzyme E (AChE or BChE) to give rise to P (Ch or TCh) and HA can be expressed by the following steps

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \tag{1}$$

$$\mathrm{ES} \xrightarrow{k_2} \mathrm{EA} + \mathrm{P} + \mathrm{HA} \tag{2}$$

where ES is the complex enzyme-substrate and EA the acylated enzyme. A steady state for the reactants E and ES exists within the whole reaction course under condition that the initial molar concentration $[\mathrm{E}]_0 << [\mathrm{S}]_0$ and the initial concentrations of all the other reactants are zero. This kind of S hydrolysis obeys the Michaelis–Menten equation

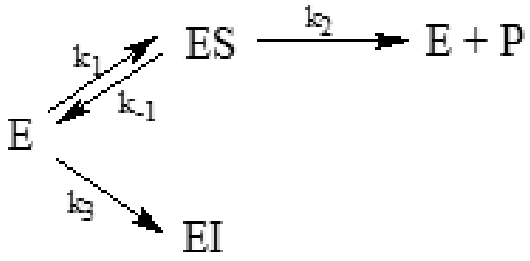
$$v = -\frac{\mathrm{d}[\mathrm{S}]}{\mathrm{d}t} = \frac{\mathrm{d}[\mathrm{P}]}{\mathrm{d}t} = \frac{\mathrm{d}[\mathrm{HA}]}{\mathrm{d}t} = \frac{V_m[\mathrm{S}]}{K_M + [\mathrm{S}]} \tag{3}$$

where v is the actual rate of the enzyme reaction under given conditions (T, pH, ionic strength etc.), symbols [S], [P], [HA] represent molar concentrations of S, P, HA, and V_m (= k_2 $[\mathrm{E}]_0$) the maximum reaction rate, when K_M (=(k_{-1} + k_2)/k_1) is Michaelis constant.

For uninhibited hydrolyses, totally four combinations E+S (ACh+AChE, ACh+BChE, ATCh+AChE and ATCh+BChE) were studied. For each type of hydrolysis of substrate by enzyme to product, the kinetic constants k_1, $k_{-1,}$ k_2 from Eqs (1), (2) and V_m and K_M from Eq. (3), an estimate of the initial molar concentration of the enzyme $[\mathrm{E}]_0$ and the standard deviation (*s.d.*)were determined using software GEPASI [17,18] being able to calculate the kinetic parameters (i.e., rate constants, K_M, and parameter V_m from one dependence concentration against time.

For inhibited hydrolyses, combinations S + E were the same as those for the uninhibited processes; moreover, reaction mixtures contained the chosen inhibitor. For each type of hydrolysis of substrate by enzyme to a product inhibited by inhibitor, the inhibition rate constant, the estimate of initial molar concentration of the enzyme $[\mathrm{E}]_0$ and the standard deviation were determined using GEPASI software. In this case, the *s.d.* is the standard deviation of experimental and simulated dependence concentration against time.

From the original measurements, the experimental dependences of the actual concentrations of products against time were calculated. These dependences were tested for validity of Eqs (1) and (2) by two independent mathematical procedures. The GEPASI software, capable of solving the kinetics of all the biochemical problems, was used for fitting the obtained data with the differential kinetic equation (3) or with the system of differential kinetic equations describing the reactions in Eqs (1) and (2). If experimental data fulfil the Michaelis–Menten equation (3), the first calculation gives the optimal values of K_M and V_m and the standard deviation, representing the concordance between the experiment and theory. From the positive solving of Eqs (1) and (2), the optimum values of rate constants k_1, k_{-1}, k_2, estimate of the initial enzyme molar concentration $[\mathrm{E}]_0$ and the standard deviation were obtained. The average values of the rate constants k_1, k_{-1}, k_2 were used for simulations of the data from experimentally inhibited hydrolyses. The inhibited hydrolyses were tested for validity of a model of the irreversible competitive inhibition using GEPASI software. In positive case, the rate constant k_3 (describing the reaction between the enzyme and inhibitor), estimate of the initial molar concentration of enzyme in the reaction mixture and the standard deviation were obtained. The reaction scheme of irreversible competitive inhibition is



Results and Discussion

The kinetics of the uninhibited and inhibited enzymatic hydrolyses was studied for the above-mentioned combination of the "substrate + enzyme + inhibitor" type observed by Ellman's and pH-stat methods. Every experiment was tested for validity of Eqs (1), (2), and (3). The standard deviation and graphical comparison of experimental and theoretical curves served as the validation criteria selected. At first, uninhibited hydrolysis of ACh (or ATCh) was performed to determine the values of the rate constants k_1, k_{-1}, and k_2 valid for the enzyme(s) used (AChE or BChE). From several independent hydrolyses, the average values of rate constants were obtained and the example of uninhibited hydrolysis is shown in Fig. 2, making comparison of experimental and theoretical curves. The average values of K_M, V_m, k_1, k_{-1}, and k_2, the estimate of the initial molar concentration of enzyme $[E]_0$ and the standard deviation for uninhibited hydrolyses are presented in Table II.

The average values of the rate constant, k_2, and the maximum reaction rate, V_M , presented in Table II indicate that hydrolysis of ACh catalysed by BChE is faster than catalysis by AChE. Similarly, the hydrolysis of ATCh catalysed by AChE is faster. Otherwise, it is difficult to compare the results obtained by the analytical methods used because of different conditions, including the substrate used. It is not possible to use ACh for the ELM method and the use of ATCh for

Table II Average values of rate constants k_1, k_{-1}, and k_2, estimated for initial molar concentration of enzyme $[E]_0$, maximum reaction rate V_m and Michaelis constant K_M of uninhibited hydrolyses of ACh and ATCh catalysed by AChE and BChE and observed by Ellman's and pH-stat methods in combination with GEPASI software

E + S	k_1 $mM^{-1}\,s^{-1}$	k_{-1} s^{-1}	k_2 s^{-1}	E_0 μM	V_m μM s^{-1}	K_M mM	*s.d.** μM
ACh-AChE	0.146	17.391	32.308	0.098	3.187	3.517	0.264
ACh-BChE	0.159	26.327	47.396	0.118	5.632	4.986	0.144
ATCh-AChE	0.039	2.974	13.514	0.105	16.013	418.82	30.17
ATCh-BChE	0.024	2.096	9.514	0.121	11.535	317.14	26.91

PHS has not been good, too, because of slightly higher acidity of ATCh compared to ACh, which also explains somewhat larger consumption of KOH for titration. Also, the initial concentration of substrate could affect the qualitative and/or quantitative parameters of the reaction mechanism. The selected ELM method needed $[S]_0$ = 0.04 mM because of a high absorption coefficient of intensively yellow product (TNB), while PHS method allowed to use ten-fold higher concentration, $[S]_0$ = 4 mM.

The average values obtained for the rate constants k_1, k_{-1}, and k_2 were used as the data of choice in simulations from experimental inhibited hydrolyses.

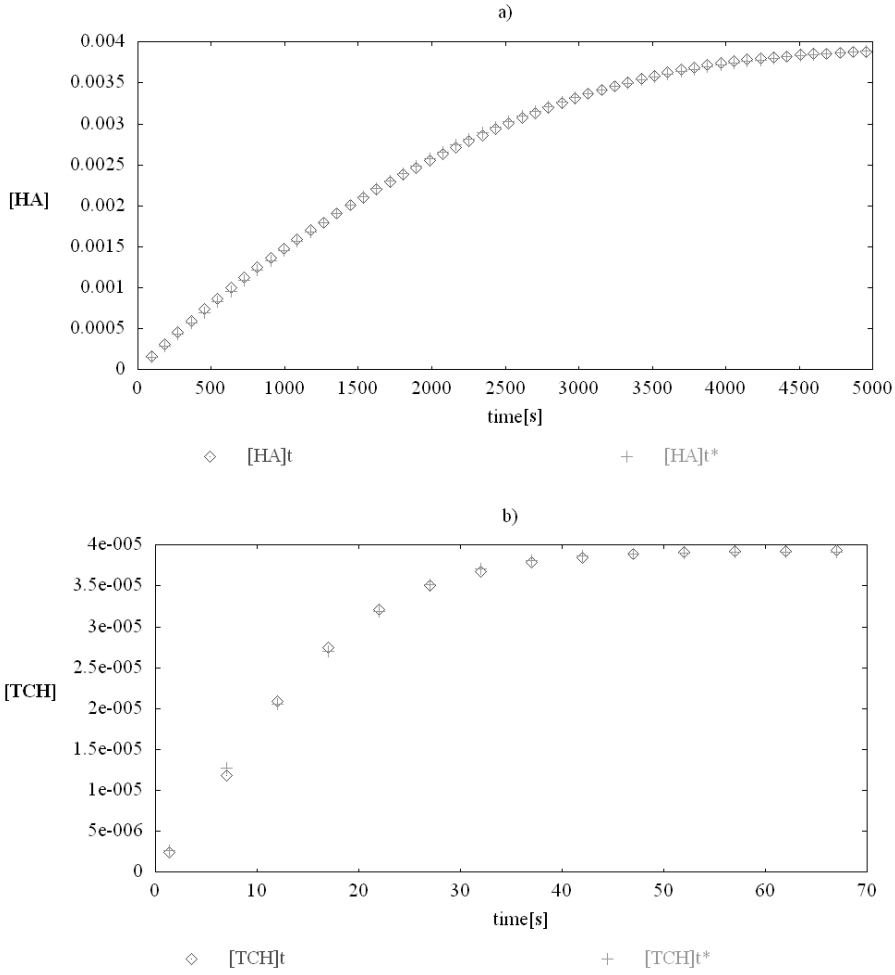


Fig. 2 Examples of simulation of experimental and theoretical dependences [X]t and [X]t* (X = HA or TCh, t = reaction time) for uninhibited hydrolyses calculated by GEPASI software. a) The course of uninhibited hydrolysis of ACh catalysed by AChE and observed using PHS method. [HA]t is experimental dependence and [HA]t* is theoretical dependence. $[S]_0$ = 4 mM, k_1 = 16 170 M^{-1} s^{-1}, k_{-1} = 12.1 s^{-1}, k_2 = 27.21 s^{-1}, $[E]_0$ = 0.094 mM, *s.d.* = 21.72 mM. b) The course of uninhibited hydrolysis of ATCh catalysed by AChE and observed using ELM method. [TCH]t is experimental dependence and [TCH]t* is theoretical dependence. $[S]_0$ = 0.04 mM, k_1 = 272 600 M^{-1} s^{-1}, k_{-1} = 1.42 s^{-1}, k_2 = 19.23 s^{-1}, $[E]_0$= 0.123 mM, *s.d.* = 0.312 mM. In this case, *s.d.* is the standard deviation of experimental and simulated dependence concentration *vs*. time

In inhibited hydrolyses, the initial concentration of all tested inhibitors was chosen so that the uninhibited and inhibited temporal dependences of the reaction components to be determined (TCh and HA) were markedly different. Every performed inhibited hydrolysis was reproduced minimally one time. For all the inhibitors tested, simulations of the experimental data by means of a model for the competitive inhibition with irreversible inhibition step were evaluated as positive. The example of inhibited hydrolysis is shown in Fig. 3, comparing again the experimental and theoretical curves. In Table III, the average values of the inhibition rate constants k_3 are presented. For the given initial molar concentra-

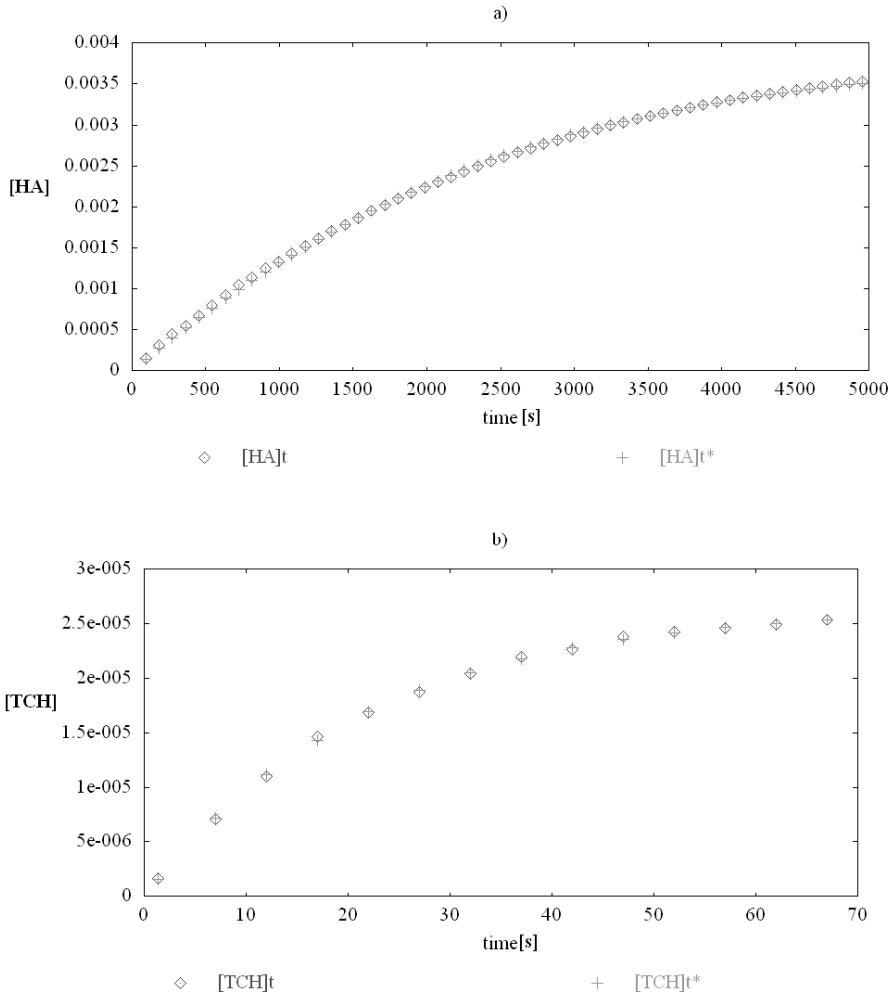


Fig. 3 Examples of simulation of experimental and theoretical dependences [X]t and [X]t* (X = HA or TCh, t = reaction time) for inhibited hydrolyses calculated by GEPASI software. a) The course of hydrolysis of ACh by AChE inhibited by inhibitor 11, observed using PHS method. [HA]t is experimental dependence and [HA]t* is theoretical dependence. $[S]_0 = 4$ mM, $[I]_0 = 16$ mM, $k_3 = 2.294$ M^{-1} s^{-1}, $[E]_0 = 0.095$ mM, $s.d.$ = 24.57 mM. b) The course of hydrolysis of ATCh by AChE inhibited by inhibitor 11, observed using ELM method. [P]t is experimental dependence and [P]t* is theoretical dependence. $[S]_0 = 0.04$ mM, $[I]_0 = 10$ mM, $k_3 = 299.36$ M^{-1} s^{-1}, $[E]_0 = 0.145$ mM, $s.d.$ = 0.237 mM. In this case, $s.d.$ is the standard deviation of experimental and simulated dependence concentration *vs.* time

Table III Average values of inhibition rate constants k_3 of hydrolyses of ACh and ATCh catalysed by AChE and BChE, inhibited by twelve 2-substituted 6-fluorobenzo[*d*]thiazoles and observed by Ellman's and pH-stat methods in combination with GEPASI software

Inhibitor	Measuring procedure			
	PHS		ELM	
	ACh-AChE	ACh-BChE	ATCh-AChE	ATCh-BChE
	k_3 M^{-1} s^{-1}	k_3 M^{-1} s^{-1}	k_3 M^{-1} s^{-1}	k_3 M^{-1} s^{-1}
1	1.147	1.871	244.4	254
2	2.963	6.816	152.4	286.8
3	3.447	3.999	113.3	235.4
4	0.951	6.016	236.3	265.3
5	4.852	7.983	204	265.7
6	3.258	5.375	289.3	269.5
7	4.301	6.991	278.4	299.2
8	1.997	6.372	269.4	254.3
9	2.814	7.264	299.9	235.3
10	9.154	5.241	273.9	223.7
11	5.409	12.04	351.3	289.6
12	5.167	4.111	246.9	236.4

tions of inhibitor, the estimate of the initial molar concentration of enzyme varied from 0.1 to 0.15 mmol l^{-1}. The standard deviation (and the corresponding concentration) of experimental and simulated dependences concentration vs. time was lower than 30 mmol l^{-1} (for PHS method) and lower than 0.3 mmol l^{-1} (for ELM method).

The rate constant k_3 represents the rate, for which the inhibitor is binding to the active site of enzyme. Based on the data listed above, the following conclusions can be expressed:

(1) The uninhibited hydrolyses of ATCh catalysed by AChE or BChE and observed by Ellman's method and of ACh catalysed by AChE or BChE and observed by pH-stat method fulfil the Michaelis–Menten model. The average values of rate constants k_1, k_2 of ATCh hydrolysis (presented in Table II) show that the hydrolysis of ATCh catalysed by AChE is faster than that catalysed

by BChE. For hydrolysis of ACh, there is different situation: according the average value of k_1 (see Table II), the rate of the first step of uninhibited hydrolysis leading to formation of the complex enzyme-substrate is comparable for both hydrolyses (catalysed by AChE and BChE). Nevertheless, the second step leading to the product(s) is faster for hydrolysis catalysed by BChE.

It is obvious that kinetic parameters surveyed in Table II and obtained from the experiments by two different analytical methods (ELM, PHS) differ substantially. We suppose that this fact is caused by variations in the compositions of reaction mixtures used for the analytical methods mentioned: ELM method requires the surplus of DTNB and a constant pH value is adjusted by phosphate buffer in contrast to the PHS method, where no DTNB is present and the constant pH value is set up with solution of KOH, when different substrates are used, of course.

(2) The inhibited hydrolyses of ATCh catalysed by AChE or BChE, inhibited by chosen benzothiazoles and observed by Ellman's method and of ACh catalysed by AChE or BChE, inhibited by the benzothiazoles chosen and monitored with the pH-stat method obey again the Michaelis–Menten model, as well as the model of irreversible competitive inhibition. The values of inhibition rate constant k_3 obtained by Ellman's method and pH-stat method for all the tested substrate + enzyme + inhibitor combinations are gathered in Table III. It is evident that inhibition rate constants obtained by two different analytical methods (ELM, PHS) differ substantially — in some cases, more than hundred times. The most probable explanation — at least, according to our knowledge — that concerns different composition of the reaction mixture is mentioned above.

PHS method: For the ACh+AChE combination, the highest average value of inhibition rate constant k_3 was reached for inhibitor 10 (9.145 M^{-1} s^{-1}) and inhibitor 11 (5.409 M^{-1} s^{-1}). Regarding the ACh + BChE combination, the highest average value of inhibition rate constant k_3 belongs to the inhibitor 5 (7.983 M^{-1} s^{-1}) and the inhibitor 11 (12.04 M^{-1} s^{-1}).

ELM method: For the ATCh + AChE combination, the highest average value of inhibition rate constant k_3 is attained by the inhibitor 11 (351.3 M^{-1} s^{-1}) and the inhibitor 9 (299.9 M^{-1} s^{-1}), whereas the ATCh + BChE combination, the highest average value of inhibition rate constant k_3 can be attributed to the inhibitor 7 (299.2 M^{-1} s^{-1}) and to the inhibitor 11 (289.6 M^{-1} s^{-1}), respectively.

It is necessary to keep in mind that the rate of inhibition is the only contributing condition to use the substance as cholinesterase inhibitor. Other important parameters are, e.g., the effectiveness of inhibition defined by IC_{50} (50% inhibitory concentration), ability of the inhibitor to cross the blood-brain barrier (characterized by K_{ow}; i.e., the partition coefficient between octan-1-ol and water) and its toxicity, of course. A useful new inhibitor must have the IC_{50} value as low

as possible. The lowest value of IC_{50} for inhibition of AChE was reached by the inhibitor 11 (28.97 ± 0.25 :M) and, for inhibition of BChE, by the inhibitor 9 (26.56 ± 0.2 :M). The average values of IC_{50} and K_{ow} of tested benzothiazoles are published in our previous study [13]. Also, the determination of the above-mentioned parameters is principal to determine the inhibition efficiency of the inhibitors tested, when the respective results have already been published [13].

A second step was to determine kinetic parameters k_1, k_{-1}, k_2, and k_3 of the individual enzymatic steps of so-catalysed reaction which are important for the determination of the type of inhibition. Nowadays, the use of molecular docking is very attractive and all the inhibitors tested have been used for molecular modelling by means of the GOLD 5.0.1 program (CCDC Co., UK) run in a 64-bit Linux version. It can be hypothesised that the compounds studied could act as the 'bulky'- blockers at the entrance of the normal ionic substrate (ACh) into the active gorge [13].

Conclusion

The group of 2-substituted 6-fluorobenzo[*d*]thiazoles was tested *in vitro* as potential inhibitors of cholinesterases. For all chosen benzothiazoles, it can be stated that hydrolyses of acetylcholine or acetylthiocholine catalysed by cholinesterases under given reaction conditions fulfil the Michaelis–Menten model, as well as the model of irreversible competitive inhibition.

Based on the current results of inhibition rate constant and the previously published data of IC_{50} and K_{ow} (Imramovský et al., 2013), the best inhibitor of cholinesterases among the compounds tested is the inhibitor 11 — i.e., 2,2,3,3-tetra-fluoropropyl(*S*)-1-[(*R*)-1-(6-fluorobenzo[*d*]thiazole)ethylcarbamoyl-2-methyl-propylcarbamate.

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