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INHIBITORY EFFECT OF CARBAMATES IN TWO TYPES OF CHOLINESTERASES

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Inhibitory effect of substituted 3-(diethylaminophenyl) phenyl carbamates in two types of cholineserases (acetylcholinesterase and butyrylcholinesterase) was determined. Inhibiting effectiveness was described by the value of IC_{50} (50 % inhibitory concentration). Modified spectrophotometric Ellman's method was used. Two types of choline esters were used as substrates for cholinesterases. It was proved that the carbamates chosen are quite effective inhibitors of cholinesterases. The most effective inhibitor of acetylcholinesterase is 3-(diethylaminophenyl) phenyl carbamate with the value of IC_{50} = 1.52 μM and the most effective inhibitor of butyrylcholinesterase is 3-(diethylaminophenyl) (3-nitrophenyl) carbamate with the value of IC_{50} = 1.70 μM.

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Introduction

The cholinesterases are key enzymes in a range of important areas such as neurobiology, toxicology and pharmacology. Of these, two major groups, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), play important roles in human and animal function and health [1]. Structurally AChE and BChE consist of an active centre, which is localized in the centre of enzyme molecule, contains characteristic gorge intruding into the surface of the enzyme molecule containing catalytic and choline binding sites where the cleavage of acetylcholine (ACh) occurs. Structure of gorge of AChE and BChE is different. At the base of the gorge 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 [2]. AChE is present mainly in the central nervous system. It is bound to the cellular membranes of excitable tissues and is involved in nerve transmission processes. The main biological function of AChE is to catalyze transformation of the active neurotransmitter acetylcholine (ACh) into the inactive compounds choline and acetic acid [3].

Cholinesterase inhibitors (ChEIs) are widely used in the symptomatic treatment of Alzheimer's disease (AD) in clinical practice. They act by inhibiting one or both of the enzymes responsible for the hydrolysis of acetylcholine in the synaptic cleft, thereby increasing available acetylcholine levels and improving neurotransmission [4].

There are many criteria by which ChEIs are classified. One possible classification is using division based on the reaction mechanism. According to this criterion, ChEIs can be divided into competitive inhibitors (inhibitor and substrate compete for binding into the active site of the enzyme molecule), non competitive inhibitors (inhibitor binds at a site that is distinct from the catalytic site), acompetitive inhibitors (inhibitor reversibly binds to enzyme-substrate complex) and mixed inhibitors [2].

Alzheimer's disease, a neurodegenerative process of still uncertain etiology, is the most common type of dementia among the elderly. A promising therapeutic strategy is based on the cholinergic hypothesis, which assumes that the loss of cholinergic neurotransmission, associated with the development of the disease is responsible for memory, behaviour, and learning disorders. Therefore, selective cholinesterases inhibitors may help to halt or slow down the Alzheimer's disease progression [5].

Experimental

Principles

The effectiveness of inhibitor is usually evaluated by the value of IC_{50} (50 % inhibitory concentration, i.e., the concentration of inhibitor which is necessary for reduction of enzyme activity or reaction rate to 50 %). For determination of IC_{50} spectrophotometric Ellman's method can be used. Ellman's method is a simple, rapid and direct method to determine the SH and -S-S- groups contained in proteins [6]. This method is widely used for measuring of cholinesterase activity and effectiveness of cholinesterase inhibitors. Cholinesterase activity is measured indirectly by quantifying the concentration of 2-nitro-5-thiobenzoic acid (TNB) ion formed in the reaction between the thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid and thiocholine, a product of substrate (i.e., acetylthiocholine) hydrolysis by cholinesterase [7].

Chemicals

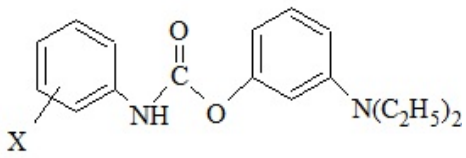
Acetylcholinesterase (type IV-S, lyophilized powder from electric eel), butyrylcholinesterase (lyophilized powder from horse plasma), acetylthiocholine iodide (ATCh), DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) were purchased from Sigma-Aldrich Prague. Dioxane p.a., KCl and KOH were purchased from Lach-Ner. KH_2PO_4 and $Na_2HPO_4.12H_2O$ were purchased from Penta.

Inhibitors: Substituted 3-(diethylamino)phenyl phenyl carbamates were synthesized at the Institute of Organic Chemistry and Technology, Faculty of Chemical Technology, University of Pardubice and their structure and used substituents are presented in Table I. Carbamates were dissolved in dioxane at the concentration of 0.01 M and then diluted with deionized water as necessary.

Determination of Cholinesterase Activity

Cholinesterase activity was determined using modified spectrophotometric Ellman's method at 25 °C in the presence of 0.1 M phosphate buffered saline (PBS, pH 7.4) in glass cuvette with 1 cm optical path. Into the glass cuvette, the following components were added: 1.184 ml PBS (0.1 M, pH 7.4), 0.8 ml DTNB (0.5 mM), 0.008 ml ATCh (0.1 M). The reaction was started by adding of 0.008 ml of enzyme (i.e., AChE or BChE). The product of given enzymatic reaction, TNB, was detected by spectrophotometer with diode array Hewlett-Packard 8453 at the wavelength of 412 nm against comparative solution, which contained the following components:

Table I The overview of tested 3-(diethylamino)phenyl phenyl carbamates



Compound	X	Nomenclature
1	-H	3-(diethylamino)phenyl phenyl carbamate
2	$-CH_3$	3-(diethylamino)phenyl (4-methylphenyl) carbamate
3	$-CH_3$	3-(diethylamino)phenyl (3-methylphenyl) carbamate
4	-Cl	3-(diethylamino)phenyl (4-chlorophenyl) carbamate
5	-Cl	3-(diethylamino)phenyl (3-chlorophenyl) carbamate
6	$-OCH_3$	3-(diethylamino)phenyl (4-methoxyphenyl) carbamate
7	$-NO_2$	3-(diethylamino)phenyl (3-nitrophenyl) carbamate

1.192 ml PBS (0.1 M, pH 7.4), 0.8 ml DTNB (0.5 mM), 0.008 ml ATCh (0.1 M). The dependence of absorbance A (412 nm) *vs.* time was observed for 70 s and actual activity of enzyme was calculated. Measurement of activity was carried out in triplicate at least. The average value of daily determined catalytic activity of given enzyme was used to calculate its suitable volume which had to be added into initial reaction mixture for determination of inhibitory effect to achieve the chosen initial activity in reaction mixture (i.e., 0.14 U).

Determination of Cholinesterase Inhibition in Presence of Substituted 3-(Diethylamino)phenyl Phenyl Carbamates

Inhibitory effect of chosen carbamates was determined using modified spectrophotometric Ellman's method at 25 °C in the presence of 0.1 M phosphate buffered saline (PBS, pH 7.4) in glass cuvette with 1 cm optical path. The activity of enzyme in reaction mixtures was 0.14 U. The total volume of reaction mixture was always 2 ml. The absorbance of product (TNB) of uninhibited or inhibited hydrolysis was detected by spectrophotometer diode array Hewlett-Packard 8453 at the wavelength of 412 nm against comparative solution.

At first, reaction rate of uninhibited hydrolysis of given substrate was determined. Into the glass cuvette the following components were placed: 0.8 ml

PBS (0.1 M, pH 7.4), 0.4 ml DTNB (0.5 mM), 0.08 ml ATCh (1 mM) and X ml PBS (0.1 M, pH 7.4). Total volume of reaction mixture after adding of all remaining components was 2 ml. The reaction was started by adding of Y ml of enzyme (i.e., AChE or BChE). The volume of enzyme was calculated according to determined actual enzyme activity to achieve the required initial activity in reaction mixture (i.e., 0.14 U). Comparative solution contained the following components: 1.52 ml PBS (0.1 M, pH 7.4), 0.4 ml DTNB (0.5 mM), 0.08 ml ATCh (1 mM). The dependence of absorbance A (412 nm) *vs.* time was observed for 150 s and reaction rate of uninhibited reaction was calculated. Measurement was done in triplicate at least, and average value of reaction rate (v_0) was calculated.

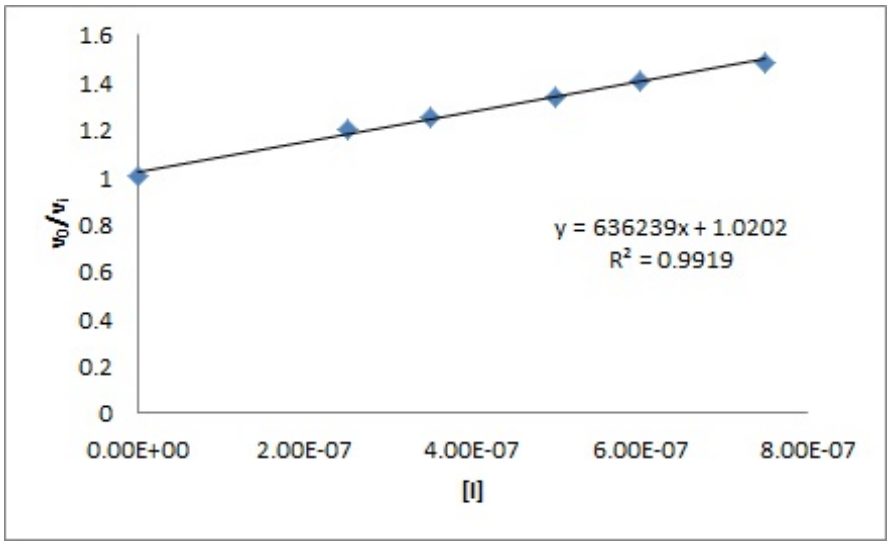


Fig. 1 The example of the dependence v_0/v_i *vs.* concentration of inhibitor. Determination was carried out under following conditions: concentration of ATCh 1 mM, activity of AChE 0.14 U, concentration of inhibitor from 0.25 to 0.75 μM, 0.1 M PBS (pH 7.4)

Then, reaction rate of inhibited hydrolysis was determined. For inhibited hydrolyses of ATCh by AChE or BChE, the initial molar concentration of inhibitor (I) was chosen so that the uninhibited and inhibited temporal dependences of determined product (TNB) were markedly different. Into the glass cuvette, the following components were placed: 0.8 ml PBS (0.1 M, pH 7.4), 0.4 ml DTNB (0.5 mM), 0.08 ml ATCh (1 mM), X ml PBS (0.1 M, pH 7.4). Total volume of reaction mixture after adding of all remaining components was 2 ml and Z ml of inhibitor to achieve required concentration of inhibitor in final reaction mixture. The reaction was started by adding of Y ml of enzyme (i.e., AChE or BChE). The volume of enzyme was calculated according to determined actual enzyme activity to achieve required initial activity in reaction mixture (i.e., 0.14 U). Comparative solution contained the following components: 1.52 ml PBS (0.1 M, pH 7.4), 0.4 ml DTNB (0.5 mM), 0.08 ml ATCh (1 mM). The dependence of absorbance A (412 nm) *vs.* time was observed for 150 s and the reaction rate of inhibited reaction

for given concentration of inhibitor was calculated. Four different concentrations of inhibitor were used. Measurements were carried out in duplicate at least, and the average value of reaction rate (v_i) was calculated.

Then the dependence v_0/v_i *vs.* concentration of inhibitor was constructed and IC_{50} was calculated from obtained equation of regression curve (based on the definition of IC_{50}). The example of determination of IC_{50} is shown in Fig. 1.

Results and Discussion

Inhibitory effect of substituted 3-(diethylamino)phenyl phenyl carbamates towards acetylcholinesterase and butyrylcholinesterase was tested. The inhibiting activities of tested carbamates were compared with previously published [8] inhibiting activities of one of four presently used drugs for treatment of Alzheimer's disease — Exelon® (rivastigmine), that is a classical acylating pseudo-reversible carbamate inhibitor of acetylcholinesterase and butyrylcholinesterase. The obtained results are summarized in Table II and are expressed as 50 % inhibitory concentration (IC_{50} [mM] with standard deviations).

Table II The determined values of IC_{50} of tested carbamates

Compound	IC_{50}, μM	
	ATCh+AChE	ATCh+BChE
1	1.52 ± 0.04	8.85 ± 0.61
2	11.81 ± 0.20	11.20 ± 2.50
3	13.82 ± 0.60	11.10 ± 0.20
4	9.10 ± 0.14	5.26 ± 0.01
5	9.05 ± 0.23	6.58 ± 0.35
6	6.24 ± 0.02	4.99 ± 0.21
7	1.70 ± 0.07	1.70 ± 0.02
Exelon®*	501 ± 3.08	19.95 ± 0.20

* The value of IC_{50} of Exelon® was published previously [8] and here is presented only for comparison

Based on obtained results, the following conclusions can be formulated: All tested substituted 3-(diethylamino)phenyl phenyl carbamates are quite potent inhibitors of acetylcholinesterase and butyrylcholinesterase, and the power of

inhibition of both enzymes is comparable.

The highest anticholinesterase activity for both cholinesterases was found in 3-(diethylamino)phenyl (3-nitrophenyl)carbamate, whereas the lowest anticholinesterase activity in both enzymes was in methyl derivatives of 3-(diethylamino)phenyl phenyl carbamate.

After comparison of obtained results of IC_{50} with previously published IC_{50} of rivastigmine, it is evident that all tested substituted 3-(diethylamino)phenyl phenyl carbamates are at least thirty-times better inhibitors of acetylcholinesterase than rivastigmine and are more potent inhibitors of butyrylcholinesterase than rivastigmine.

Conclusion

In this study seven substituted 3-(diethylamino)phenyl (3-nitrophenyl)carbamates were tested as inhibitors of two cholinergic enzymes — acetylcholinesterase and butyrylcholinesterase. The anticholinesterase activities were compared with those of Exelon®. It was proved, that chosen substituted 3-(diethylamino)phenyl (3-nitrophenyl)carbamates are quite potent inhibitors of cholinesterases and are the same or even more potent cholinesterase inhibitors in comparison with presently used drug for treatment of Alzheimer's disease Exelon®.

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