Half-inhibition Concentrations of New Cholinesterase Inhibitors

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Z. Naturforsch. **59 c,** 293–296 (2004); received July 7/September 26, 2003

The power of chosen carbamates and hydrazinium derivatives (carbazates) to inhibit the hydrolysis of acetylthiocholine by butyrylcholinesterase or acetylcholinesterase was tested. The determined pI_{50} values (= negative logarithm of the molar concentration inhibiting the enzyme activity by 50%) of the tested substances were compared with pI_{50} values of the commercially used drugs for the Alzheimer's disease treatment – rivastigmine and galanthamine.

Key words: Cholinesterases, Inhibitors, Index pI_{50}

Introduction

The aim of our research was to find a suitable inhibitor decreasing the concentration and/or activity of acetylcholinesterase (ACHE) and/or butyrylcholinesterase (BCHE) and to compare its inhibiting power with the drugs for Alzheimer's disease (AD) treatment already in use.

As one of the most common reasons for AD lack of acetylcholine is considered, which acts as neuromediator of the cholinergic nerve system in the human brain (Scheibel *et al.*, 1986). This theory is supported by relatively effective therapeutics increasing the concentration of brain acetylcholine.

Acetylcholine is hydrolyzed by several forms of cholinesterase, which can be differentially inhibited by various cholinesterase inhibitors. There are two major classes of cholinesterases: ACHE and BCHE, both present in the central and peripheral brain compartments. In comparison with the narrow specifity of ACHE, BCHE hydrolyzes a broader range of substrates (Krall *et al.*, 1999).

Two classes of agents, cholinergic agonists and cholinesterase inhibitors have shown efficacy in treating the symptoms of AD. Many cholinesterase inhibitors were examined as potential drugs for the treatment of AD (Kulhavý *et al.*, 2002).

In our work we tested the inhibiting properties of *N*-alkyl carbamates of 3-(diethylamino)phenol, chlorine derivatives of 2-phenoxycarbonyl-2-methylhydrazinium-chloride and 2-phenoxycarbonyl-1,2-dimethylhydrazinium-chloride. Their inhibiting properties were compared with the same properties of the commercially used drugs – rivastigmine and galanthamine.

Materials and Methods

Chemicals

Butyrylcholinesterase (BCHE): lyophilizate from horse plasma, pressed in pellets of ca. 6 g; it was obtained from the Department of Toxicology, Purkyně Military Medical Academy, Hradec Králové, CZ. Acetylcholinesterase ($ACHE_1$): lyophilizate from electric eel. Acetylcholinesterase ($ACHE_2$): lyophilizate from bovine erythrocytes. Acetylthiocholine (ATCH) iodide: substrate. All from Sigma-Aldrich, kept at 5 °C. 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent): Sigma-Aldrich, kept at laboratory temperature.

Inhibitors: The used carbamates (Table I, part A) and hydrazinium derivatives (carbazates, Table I, part B) were synthesized in the Department of Organic Chemistry, Faculty of Chemical Technology, University of Pardubice. The melting points of all inhibitors agree with those given in the literature.

Sevin (1-naphthyl-*N*-methylcarbamate, Fig. 1, C) was obtained from Merck-Schuchard, Munich,

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Germany. Rivastigmine (Exelon®, Novartis, Switzerland, Fig. 1, D) and galanthamine (Reminyl®, Janssen Pharmaceutica, Belgium, Fig. 1, E) were obtained from the Department of Toxicology, Purkyně Military Medical Academy, Hradec Králové, CZ. All inhibitors were kept at 5 °C.

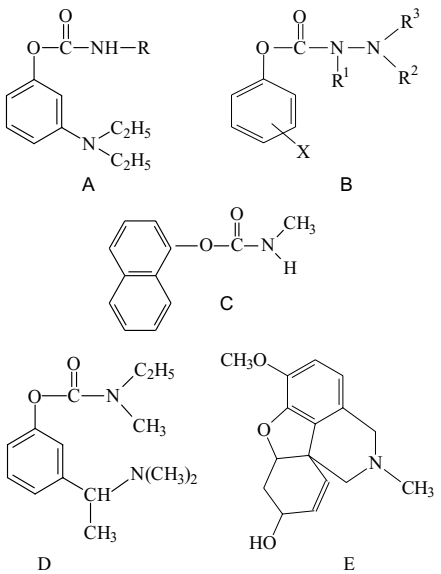

Fig. 1. Structures of the used carbamates (A) and hydrazinium derivatives (carbazates, B) of sevin (1-naphthyl-*N*-methylcarbamate, C), rivastigmine (Exelon®, (+)(*S*)-*N*-ethyl-3-[(1-dimethylamino)ethyl]-*N*-methylphenylcarbamate hydrogentartrate, D) and galanthamine (Reminyl®, natural product, E).

Analytical solutions

BCHE preparation

One pellet was dissolved in ca. 250 ml of phosphate buffer, pH 7.6. The enzymatic activity of the resulting solution was 0.25 U/ml (U = international catalytic unit, 1 ml of enzyme preparation transforms 0.25 μmol of substrate within 1 min). The solution was kept at 5 °C. For the daily experiment this activity was adjusted by means of the inhibitor sevin with a known pI_{50} value of 4.3 (Kulhavý *et al.*, 2002).

$ACHE_1$ and $ACHE_2$ preparations

The original lyophilisate was dissolved in the phosphate buffer so that the activity of this solution was 0.25 U/ml. The solution was kept at 5 °C. For the daily experiment this activity was adjusted by means of sevin (pI_{50} = 5.05) (Metcalf, 1971 ; Martinez *et al.*, 2000).

Indicating solution for Ellman's method

6.7 mg ATCH iodide and 8.3 mg DTNB (Ellman's reagent) were solved in 100 ml of the phosphate buffer. A fresh solution was prepared for the measurement every day.

0.05 M analytical solutions of all carbamates or hydrazinium derivatives were prepared by dissolving them in dioxane and/or deionized water. Solution of 0.05 M sevin: 0.05 g of the solid sevin was dissolved in 5 ml of dioxane. Solution of 0.005 M rivastigmine: One pellet was dissolved in 3 ml of deionized water. Solution of 0.005 M galanthamine: One pellet was dissolved in 6.7 ml of deionized water. Solutions with lower concentrations were prepared by dilution with deionized water. All presented solutions were kept at 5 °C.

Methods and apparatus

Determination of the index pI_{50}

The mixture of the chosen concentrations of an inhibitor (Table I, I–XII, sevin, rivastigmine or galanthamine) and enzyme ($ACHE_1$, $ACHE_2$ or BCHE) in aequous phosphate buffer, pH 7.6, was quickly homogenized and then thermostated 5 min at 25 °C. During this time interval a part of the used enzyme was blocked by the given inhibitor according to its inhibition power. Then a chosen amount of indicating solution (substrate ATCH + Ellman's reagent DTNB) was added, the mixture quickly homogenized again and thermostated 10 min at 25 °C. Then the absorbance *A* at 412 nm was measured. This value shows, according to Ellman's spectrometric method, the actual degree of the given enzymatic hydrolysis.

Ellman's spectrometric method

The surplus of DTNB from the indicating solution reacts practically immediately with the product of thiocholine (TCH) hydrolysis, forming a yellow substance with the maximum absorbance *A* at 412 nm. This absorbance is proportional to the actual concentration of TCH in the reaction mixture (Ellman *et al.*, 1961).

Realisation

Into the glass test tubes were placed 0.1, 0.2, 0.3, 0.4 and 0.5 ml of a 5×10^{-5}, 5×10^{-6} and 5×10^{-7} M

solution of the tested carbamate or 5×10^{-4}, 5×10^{-5} and 5×10^{-7} M of the hydrazinium derivative. All solutions were filled up to 1 ml with phosphate buffer and 0.5 ml of the enzyme preparation ($ACHE_1$, $ACHE_2$ or BCHE) was added, homogenized and thermostated 5 min at 25 °C. Then 0.5 ml of the indicating solution was added to all test tubes, the mixtures were homogenized again and thermostated 10 min at 25 °C. Then *A* (412 nm) of all mixtures was measured and compared with the standard solution (1 ml phosphate buffer, 0.5 ml indicating solution, 0.5 ml water). From the dependence inhibition in percent (%I) *vs.* pI, the pI_{50} value was determined graphically.

Results and Discussion

In Table II the pI_{50} values of the tested inhibitors and comparative drugs are presented, determined at given conditions (25 °C; pH 7.6 – phosphate buffer; t = 10 min; enzyme activity of $ACHE_1$, $ACHE_2$ and BCHE = 0.25/8 U/ml = 0.03125 U/ml; 1 ml of the reaction mixture converts 0.03 μmol of substrate in 1 min). The comparison of the pI_{50} values shows, that some tested carbamates and hydrazinium derivatives proved to be the same or even better cholinesterase inhibitors as the presently used drugs Exelon® and Reminyl®.

The increasing number of C-atoms in the *N′*-alkyl group of 3-(diethanolamino)phenyl carbamates (Table I, inhibitors I–V) does not have an uniform effect on the cholinesterase inhibition. According to BCHE, their inhibition power increases up to derivative V except III. In the case of $ACHE_1$, the anticholinesterase activity shows the unambiguous decrease with increasing number of C-atoms in the *N′*-alkyl group. While using $ACHE_2$, the carbamate analogue II appears as the strongest inhibitor, then the inhibitory activity decreases with the number of C-atoms in the *N′*-alkyl group.

Table II. The determined pI_{50} values (= negative logarithm of the molar concentration inhibiting the enzyme activity to one half) of the tested inhibitors and compared drugs.

Inhibitor	pI_{50} (BCHE)	pI_{50} ($ACHE_1$)	pI_{50} ($ACHE_2$)
I	6.0	6.2	4.8
II	6.2	6.0	6.3
III	5.5	5.7	6.0
IV	6.5	5.3	5.6
V	6.9	4.8	4.9
VI	4.4	3.4	3.3
VII	5.5	3.7	5.0
VIII	5.3	3.8	4.3
IX	3.45	3.1	3.3
X	4.1	2.7	2.9
XI	4.9	3.7	4.2
XII	4.2	3.1	3.1
XIII	2.9	2.5	2.3
Rivastigmine	4.7	3.3	4.1
Galanthamine	5.1	6.4	6.4

Table I. An overview of used inhibitors. Part A – carbamates, part B – hydrazinium derivatives (carbazates). See Fig. 1 (A, B).

A	R	Nomenclature
I	C_2H_5	3-(Diethylamino)phenyl-*N′*-1-ethylcarbamate
II	C_3H_7	3-(Diethylamino)phenyl-*N′*-1-propylcarbamate
III	C_4H_9	3-(Diethylamino)phenyl-*N′*-1-butylcarbamate
IV	C_6H_{13}	3-(Diethylamino)phenyl-*N′*-1-hexylcarbamate
V	C_8H_{17}	3-(Diethylamino)phenyl-*N′*-1-octylcarbamate

B	R^1	R^2	R^3	X	Nomenclature
VI	CH_3	H	H	–	2-Phenoxycarbonyl-2-methylhydrazinium-chloride
VII	CH_3	H	H	Cl	2-(2-Chlorophenoxy)carbonyl-2-methylhydrazinium-chloride
VIII	CH_3	H	H	Cl	2-(3-Chlorophenoxy)carbonyl-2-methylhydrazinium-chloride
IX	CH_3	H	H	Cl	2-(4-Chlorophenoxy)carbonyl-2-methylhydrazinium-chloride
X	CH_3	H	CH_3	–	2-Phenoxycarbonyl-1,2-dimethylhydrazinium-chloride
XI	CH_3	H	CH_3	Cl	2-(2-Chlorophenoxy)carbonyl-1,2-dimethylhydrazinium-chloride
XII	CH_3	H	CH_3	Cl	2-(3-Chlorophenoxy)carbonyl-1,2-dimethylhydrazinium-chloride
XIII	CH_3	H	CH_3	Cl	2-(4-Chlorophenoxy)carbonyl-1,2-dimethylhydraziniumchloride

In the tested group of hydrazinium derivatives (Table I, substances VI–XIII) the 2- and 3-chloro derivatives of 2-phenoxycarbonyl-2-methylhydrazinium-chloride (substance VII and VIII) and 2-chloro derivatives of 2-phenoxycarbonyl-1,2-dimethylhydrazinium-chloride (substance XI) are the relatively best inhibitors. But the pI_{50} values of this group are of 1–3 units smaller than of the best carbamates.

According to the pI_{50} values, determined at the conditions described above *in vitro*, the tested carbamates are equal or more effective inhibitors of the used esterases in comparison to the currently applied drugs against the Alzheimer's disease. But their pI_{50} values are not dramatically (*i.e.* more than 2–3 units) greater than that of the compared drugs. The tested hydrazinium derivatives (carbazates) appear, according to the same criteria, as less effective inhibitors of the used esterases than these drugs.

Acknowledgement

This work was financially supported by the Ministerium of Education, Youth and Sports, research project 253 100 002.

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