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Acetylcholinesterase inhibition activity of peptide analogues of galanthamine with potential application for treatment of Alzheimer`s disease

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An acetylcholinesterase inhibitor (AChEI) or anti-cholinesterase is a compound that inhibits the cholinesterase enzyme from breaking down acetylcholine, increasing both level and duration of action of the neurotransmitter ACh. AChEIs occur naturally as venoms and poisons; they are used as weapons in the form of nerve agents, and as constituents of medicines for Myasthenia Gravis treatment. They are used to increase neuromuscular transmission to treat Glaucoma and Alzheimer disease (AD) as well as an antidote to anticholinergic poisoning.
Herein, we report the kinetic investigation of five peptide amide and esters of galanthamine Boc-Val-Asn-Leu-Ala-Gly-Ogal, Boc-Val-Asn-Leu-Ala-Val-Gly-Ogal, Boc-Asp-(norGal)-Asp-Leu-Ala-Val-NH-Bzl, Boc-Asp-(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl, Boc-Asp-(norGal)-Val-Asn-Leu-β-Ala-Val-NH-Bzl, inhibitors of AChE. In addition, IC_{50} values (50 % inhibition effect on the enzyme) according to AChE were determined. Finally, we compare the obtained IC_{50} values for synthetic peptides with those of two pesticides Parathion and Carbofuran, well know ACHEI's.

Key words: peptides, enzymes, inhibitors; pharmaceutical application

INTRODUCTION

Acetylcholinesterase (AChE) (E.C.3.1.1.7) is a serine hydrolase that catalyzes the hydrolytic degradation of acetylcholine to choline and acetic acid. According to cholinergic hypothesis AChE is one of both choline esterases (together with butyrylcholine esterase BuChE) which plays key role for progression of Alzheimer's disease [1]. One of possible approaches for treatment of patients with Alzheimer's disease is using of acetylcholinesterase inhibitors (AChEIs) [2]. AChEIs could have different origin, extracted from natural sources (galanthamine, huperzine A, uleine etc.) [3-6] or synthetic (including organophosphates, carbamates, peptides, etc.) one [7-10]. Parathion and carbofuran are compounds that belongs to two main groups of pesticides-organophosphorus (OPs) and carbamates. They are still widely used in veterinary practice and in agriculture as fungicides, insecticides and herbicides. Since carbamates, as well as OPs, are AChE inhibitors, both compounds cause similar toxic acute effects and symptoms derived from poisoning. The principal difference between OPs and carbamate induced inhibitory action is that the AChE-OP complex is much more stable than AChE-carbamate, making carbamates the potential candidates for the treatment of Alzheimer's disease [8].

Herein we report the acetylcholine esterase inhibition activity of several synthetic peptide amide and esters, derivatives of natural AChEI galanthamine. We also obtained IC_{50} values for synthesized hybrid structures and additionally, we compared these data with those for two synthetic pesticides: one from the OP group and one carbamate.

EXPERIMENTALS

AChE inhibition activity

All kinetic investigations and IC_{50} determinations were done using an optical biosensor with Acetylcholinesterase (AChE) (EC.3.1.1.7) from *Electrophorus electricus* (electric eel), Type VI-S, AChE from electric eel, immobilized onto hybrid membranes synthesized by sol-gel technology. Synthesis of used membranes containing cellulose acetate propionate with high molecule weight (~25 000) (CAP), methyl triethoxysilane (MTES) and Polyamidoamine (PAMAM) dendrimers is described in [11]. The quantity of protein immobilized onto the membranes, was determine using Lowry's methodology [12]. Initially, the activity of the immobilized AChE was measured

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without presence of inhibitor. The experimental procedure was run at 25° C in 1 ml of 0.1M sodium phosphate buffer (pH 8) containing 90 µl of ACh iodide (7.5 AM) and 45 µl of 5,5'- dithiobis-2-nitrobenzoic acid (DTNB, 10 µM) and stirring. Further, newly synthesized compounds at different concentrations from 5 µM to 100µM were diluted in 1,5 ml of 0,1M sodium phosphate buffer (pH 8). 50mg of membranes with the immobilized enzyme were added directly to the solution and were incubated together for 30 min at 25° C. The membranes with immobilized AChE, were moved out from the solution and the residual activity was measured following the procedure according to Elman's method with DTNB reagent [13].

Peptide inhibitors were synthesized according to methodology described in [14]. They all are amides or esters of natural galanthamine with following structures:

Boc-Asp-(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl (**I_1**), Boc-Asp-(norGal)-Asp-Leu-Ala-Val-NH-Bzl (**I_2**), Boc-Asp-(norGal)-Val-Asn-Leu-s-Ala-Val-NH-Bzl (**I_3**), Boc-Val-Asn-Leu-Ala-Val-Gly-OGal (**I_4**), Boc-Val-Asn-Leu-Ala-Gly-OGal (**I_5**)

The inhibitory effects of all the analyzed AChEIs (the newly synthesized peptides, as well as the pesticides) was calculated by measuring the difference in the enzyme activity before and after incubation with inhibitor. The measurement was done at 412 nm for 8 min.

The inhibition percentage was calculated according to equation.

$$\text{Inhibition (\%)} = [(E_0 - Ei)/E_0]*100,$$

Where E_0 is the initial inhibited sensor activity and E is the inhibited sensor activity. The sensitivity of the biosensor toward ACh was measured.

RESULTS AND DISCUSSION

Galantamine is one of the most selectively inhibitors of AChE which is one of the commonly used inhibitors to treat patients with mild to moderate AD. Therefore, syntheses of novel peptides compounds containing galantamine analogues are very important.

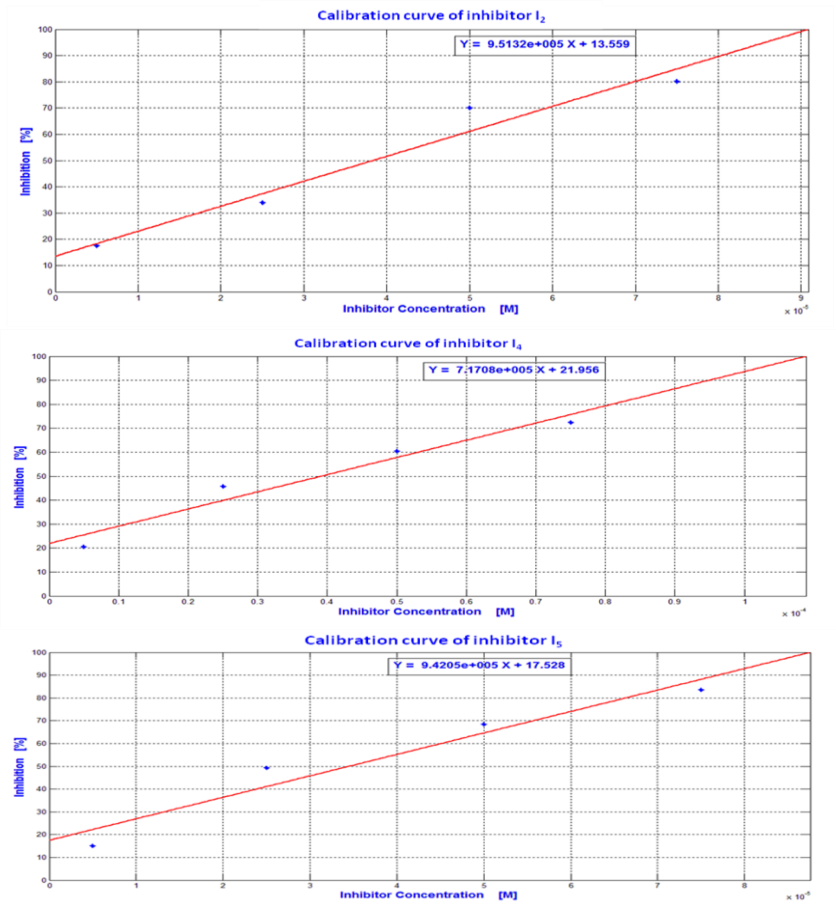

Fig. 1. Calibration curves for free AChE and the peptide inhibitors I_2, I_4 and I_5

Table 2. IC_{50} values for the synthesized and the some pesticides

Symbols	Amino acid sequence	IC_{50} [M x10^{-6}]
I_1	Boc-Asp(norGal)-Val-Asn-Leu-β-Ala-Val-NH-Bzl	-
I_2	Boc-Asp(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl	40.70±0,10
I_3	Boc-Asp(norGal)-Asp-Leu-Ala-Val-NH-Bzl	38.30±0,18
I_4	Boc-Val-Asn-Leu-Ala-Val-Gly-OGal	39.11±0,12
I_5	Boc-Val-Asn-Leu-Ala-Gly-OGal	34.46±0,07
Gal*	galanthamine	5.00
	Parathion	1.75**
	Carbofuran	0.65**

* data from literature [15], **both values are x10^{-10}

Table 3. The values of Ki for different newly inhibitors concentrations for immobilized AChE

Inhibitor concentration, (Mx10^{-6})	*Ki (Mx10^{-6}) MTES (AChE)*			
	I_1	*I_2*	*I_4*	*I_5*
5	0.45624	0.26209	41.482	0.21175
25	3.85745	1.51692	2.28114	1.43737
50	5.62258	4.79799	5.46817	3.36793
75	9.28964	6.96592	8.72065	9.52302

In this part examination of the effect of some types of those newly synthesized inhibitors that were designed in [14] was achieved. In addition, their IC_{50} values (50% inhibition effect on the enzyme) against AChE were determined. Five different peptide inhibitors, galantamine derivatives were investigated. The results showed that four of them (I_5, I_4, I_2 and I_1) have inhibitory effect towards the enzyme AChE. Surprisingly compound I_3 has no inhibitory effect towards enzyme, but it reacts as an activator. Therefore, the study was continued only with the other four inhibitors (I_5, I_4, I_2 and I_1). Initially, we made the calibration curves with inhibitors I_1, I_2, I_4 and I_5. They are illustrated on Fig. 1 in the presence of ACh at 7,5μM concentration. The plots appear linear response for concentration of the inhibitor from 5 μM to 100μM.

After drawing the calibration curves, the IC_{50} values for the newly synthesized inhibitors are determined and they are summarized in Table 2.
The IC_{50} values for the inhibitors I_1, I_2, I_4, and I_5, for immobilized AChE on MTES hybrid membranes is presented in Table 2. These values are obtained from the experimental work and compared to the obtained ones from the mathematical model.

The values of Ki for different newly inhibitors concentrations for immobilized AChE are presented at Table 3. The results showed that all newly inhibitors compound I_1, I_2, I_4 and I_5 act as competitive inhibitor for immobilized AChE.

IC_{50} values for the novel peptides compounds inhibitors for immobilized AChE on MTES hybrid membranes, obtained from experimental work and theoretical mathematical model were compared using simple regression analysis. The results are shown on a regression line in Figure 2 The obtained regression equations for different inhibitors are as follows:
I_1: Y = 3.4243 X – 251.95, correlation (r) = 0.782.
I_2: Y = 5.1909 X – 405.52, correlation (r) = 0.696;
I_4: Y = 0.47351 X + 7.0091, correlation (r) = 0.239;
I_5: Y = 3.854 X – 261.21, correlation (r) = 0.7501;

A good correlation existed between the results of experimental method and theoretical mathematical model for inhibitors I_5 and I_1. Data from these statistical calculations confirmed the precision of the proposed model.
As it can be seen from the table 1 four of five galanthamine derivatives have inhibition activity and the IC_{50} values are in micromolar range, but they are 7-8 times lower than those of natural galanthamine. The comparison of obtained data shows that carbamate and phosphororganic pesticides are one million times more potent inhibitors of AChE. Eventhough, looking to the IC_{50} values for both pesticides, carbofuran seems

stronger inhibitor than parathion, it is actually less toxic because of the inhibition mechanism. Carbamates are considered to be safer than OP insecticides that irreversibly inhibit AChE causing more severe cholinergic poisoning.

It is proved that OP as well as the organochlorine pesticides that are also irreversible inhibitors, are toxic to the nervous system, reproductive organs, and endocrine system. Moreover, they can cause cancer and increase the risk of developing Alzheimer's disease. As a result of their wide use in agriculture, traces of them can further be found into animal tissues, milk, honey, eggs, etc.

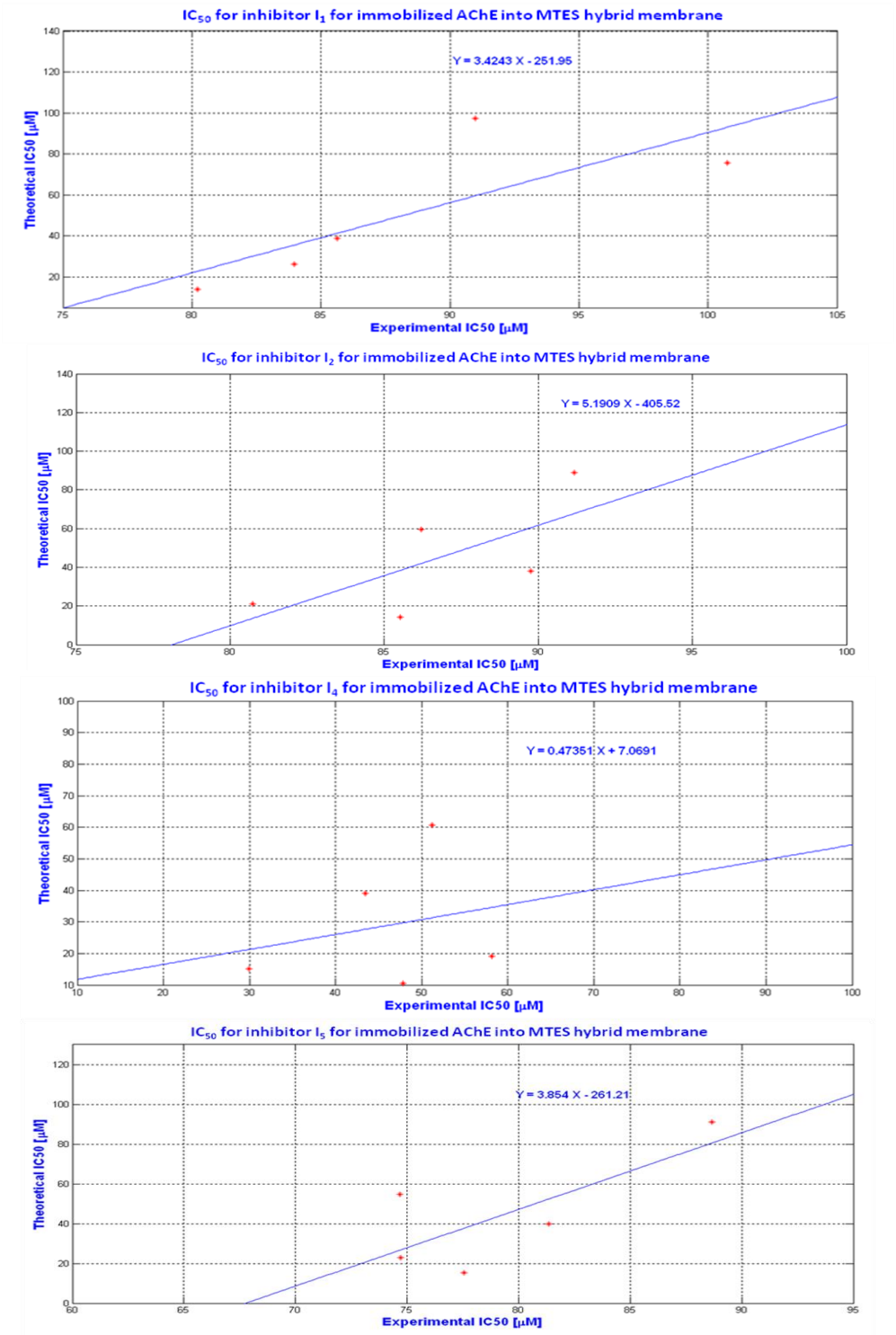

Fig. 2. IC_{50} correlation curve for the newly compounds inhibitor I_1, I_2, I_4 and I_5 and immobilized AChE onto MTES hybrid membrane

CONCLUSION

Therefore, food safety is an integral part of the EU policy for protection of consumer's health and maximum residue levels for pesticides are defined in specific Regulations. However, some carbamates, due to their reversible AChE inhibitory action, found an important application in human medicine as pharmacologically active compounds. For example, rivastigmine is a carbamate with probably the most meaningful pharmacological application, being validated in the symptomatic treatment of Alzheimer's disease.

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ИЗСЛЕДВАНЕ ИНХИБИТОРНАТА АКТИВНОСТ ВЪРХУ АЦЕТИЛХОЛИНЕСТЕРАЗА НА ПЕПТИДНИ АНАЛОЗИ НА ГАЛАНТАМИН, С ПОТЕНЦИАЛНО ПРИЛОЖЕНИЕ ПРИ ПАЦИЕНТИ С БОЛЕСТТА НА АЛЦХАЙМЕР

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(Резюме)

Ацетилхолинестеразен инхибитор или анти-холинестеразно вещество е съединение, което инхибира ензима холинестераза като блокира ацетилхолина, което води до увеличаване както на нивото, така и продължителността на действие на невротрансмитера ацетилхолин.

Ацетилхолинестеразното инхибиране може да възникне при въздейсвтие на различни отрови и токсини върху организма. Свойствата на този тип инхибитори са изследвани и прилагани като оръжие за масово поразяване, а от друга страна се включват в състава на лекарства за различни заболявания като миастения гравис. Също така се прилагат за увеличаване на нервномускулнния пренос на импулси, при лечение на глаукома, болестта на Алцхаймер, както и като противоотрова при антихолинергично отравяне.

В настоящата работа, ние докладваме резултатите от кинетичните изследвания на пет пептида, амидни и естерни аналози на галантамин: Boc-Val-Asn-Leu-Ala-Gly-Ogal, Boc-Val-Asn-Leu-Ala-Val-Gly-Ogal, Boc-Asp-(norGal)-Asp-Leu-Ala-Val-NH-Bzl, Boc-Asp-(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl, Boc-Asp-(norGal)-Val-Asn-Leu-β-Ala-Val-NH-Bzl, като потенциални инхибитори на ацетлихолинестераза. Определени са стойностите на IC_{50} (50% инхибиране активността на ензима) спрямо ацетилхолинестераза. В допълнение ние сравняваме получените стойности за IC_{50} с тези на два моделни пестицида, които са добре известни мощни инхибитори на ацетилхолинестеразата.