Three (−)-cytisine derivatives and 1-hydroxyquinopimaric acid as acetylcholinesterase inhibitors

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In vitro acetylcholinesterase (AChE) inhibition was studied using novel derivatives of (−)-cytisine derivatives N-allylcytisine-12-carbamide (A-63), cytisine-12-carbamide (A-36), N-1-adamantylcytisine-12-thiocarbamide (U-12), and 1-hydroxyquinopimaric acid (U-201). Inhibition of acetylcholinesterase with compound A-63 was described as mixed inhibition. Substances (A-36) and (U-201) acted as competitive inhibitors with Ki equal to 6.71 mM and 3.89 mM, respectively, while (U-12) behaved as an uncompetitive inhibitor with Ki at 0.07 mM. The IC50 values were estimated at 1.47, 13.73, 3.39, and 7.81 mM, respectively. According to toxicity assessment, compound A-63 was non-toxic; it did not affect A. salina viability at a concentration less than 1000 ppm, while at 1000 ppm, only 3% mortality was observed. Mortality of A. salina was less than 50% in the same concentration range for the other three compounds that allow classifying them as moderately toxic. Although tested compounds have the characteristics of weak inhibitors, they could be useful as protectors against potent organophosphates. The present research may be fundamental to the design of new substances for acetylcholinesterase inhibition.

1. Introduction

Acetylcholinesterase (AChE, EC. 3.1.1.7) is the enzyme involved in the termination of impulse transmission. The enzyme hydrolyzes the acetylcholine, which is neurotransmitter involved in various cholinergic pathways of the peripheral and central nervous systems. Enzymatic inactivation, induced by several inhibitors, leads to the accumulation of acetylcholine causing interruption of neurotransmission, as well as the hyperstimulation of nicotinic and muscarinic receptors. Hence, reversible acetylcholinesterase inhibitors with some functional groups (carbamate, quaternary or tertiary ammonium group), interacting with the enzyme as their primary target, are applied as relevant drugs, for example in the pharmacotherapy of Alzheimer’s disease (AD), myasthenia gravis, bladder distention, glaucoma, postoperative ileus, as well as antidote to anticholinergic overdose [1–3].

Irreversible AChE inhibitors such as organophosphorus compounds (esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid) widely used as acaricides and insecticides in agriculture, as softening agents and additives to lubricants in industry. Moreover, some of them are acknowledged as dangerous chemical warfare agents (for example, sarin and VX). They join covalently with serine from the active enzyme center. The effects of poisoning with organophosphorus compounds are recognized. Oximes are unique, clinically accessible enzyme reactivators. However, Petroianu et al. [4] demonstrated in vitro and in vivo that weak AChE inhibitors, for example, metoclopramide, confer some degree of protection against poisoning by organophosphates.

So, the strong AChE inhibitors have different applications traditionally as pharmaceutical and agro-industrial preparations, while weak inhibitors could have an application as an antidote to poisoning effect of strong inhibitors. Therefore, the search for AChE inhibitors has an important impact on the development of new pharmaceutical and agro-industrial treatments.

(−)-Cytisine is a natural alkaloid extractable from Thermopsis lanceolata which possesses analeptic and anti-tobacco activity [5]. Consequently, to obtain compounds of therapeutic interest, the citisine
underwent structural modification achieving selective ligands for neuronal acetylcholine receptors. Recently, we synthesized cytisine derivatives with pronounced pharmacological effects [6–8], between them are: N-allylcytisine-12-carbamide, cytisine-12-carbamide, N-1-adamantylcytisine-12-thiocarbamide.

Moreover, 1-hydroxyquinopimaric acid is one of the framework compounds of the “bird cage” type [9]. Herz et al. [10] reported 1-hydroxyquinopimaric acid synthesis through selective reduction of quinopimaric acid, the diene adducts of levopimaric acid (Pinus sylvestris plant metabolite) and p-benzoquinone. This compound is used both as intermediates in organic synthesis and as synthons for building plant metabolite (and p-benzoquinone). This compound is used

Thus, in the present study, primary screening of these four compounds as AChE inhibitors was performed. We aimed to test the synthesized compounds as possible acetylcholinesterase inhibitors and define possible kinetic mechanisms, as well as to describe their effect on Artemia salina as the model of toxicity evaluation.

2. Materials and methods

Studied substances: N-allylcytisine-12-carbamide (A-63), cytisine-12-carbamide (A-36), N-1-adamantylcytisine-12-thiocarbamide (U-12), and 1-hydroxyquinopimaric acid (U-201) were synthesized in Ufa Institute of Chemistry of Russian Academy of Science. Acetylthiocholine iodide, acetylcholinesterase (from Electrophorus electricus), and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma- Aldrich Chemical Company.

2.1. Acetylcholinesterase assay

Acetylcholinesterase activity was assayed according to the method of Ellman et al. [12] using acetylthiocholine as substrate. The product of hydrolysis (thiocholine) was detected in the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) on a Cary model 50 spectrophotometer. The absorbance at 412 nm was recorded kinetically (ε 412 = 13,600 M⁻¹ cm⁻¹). The absorbance change was linear for 15 min for all substrate concentrations, and the slope was used to calculate the initial rate.

Enzyme activity assay was performed at 30 °C in a 1.0 mL reaction mixture containing 0.01 M 5,5′-dithiobis (2-nitrobenzoic acid) with sodium bicarbonate (1.5 mg mL⁻¹), acetylthiocholine, and inhibitors at different concentrations in 0.10 M Tris-HCl buffer, pH 8.0. All determinations were performed a minimum of three times.

2.2. Toxicology assay on Artemia salina model

To determine toxicity of the studied substances, the assay with brine shrimp Artemia salina was carried out: 25 mg of class C eggs, from the Fishery Research Center, were incubated in a hatching chamber with artificial salt water, at temperatures from 20 to 30 °C [13]. Artificial salt water contained of 23 g NaCl, 4 g Na₂SO₄, 11 g MgCl₂·6H₂O, 1.3 g CaCl₂·2H₂O, 0.7 g KCl in 1 L distilled water. The pH was adjusted to 9.0 using Na₂CO₃ to avoid the risk of Artemia larvae death by the pH decrease [14]. After 24 h, 15 mL of 0.06% yeast solution was added to every liter of saltwater to feed the larvae; 48 h after the eggs were incubated, the larvae were extracted using a Pasteur pipette and counted. For every studied substance, five concentrations (in triplicate) were tested to determine the dose-response relationship. Tested concentrations were 62.5, 125, 250, 500, and 1000 ppm, and were prepared in 10% watery DMSO solution. The 10% DMSO solution was applied in control assay performed without inhibitor. Every test tube with the sample contained 10 larvae of brine shrimp, including the control group, and was filled to 5 mL total volume with artificial saltwater. After 24 h, live larvae were counted.

3. Results

3.1. Half-maximal inhibitory concentration (IC₅₀) - a measure of the inhibition effectiveness

Table 1 shows the called number of tested compounds, their chemical structures, and molecular weights. All tested substances were not soluble in water. However, they are soluble in DMSO. So, all tested
compounds were dissolved in DMSO and then in water. After adding water to an organic solution, these substances are not precipitated. Final DMSO concentration in the reaction mixture was equal to 1.25%. The previously performed assay demonstrated that DMSO did not affect enzyme activity at this solvent concentration.

Fig. 1 shows that all studied compounds are inhibitors of AChE. The effect of these derivatives on AChE activity was not reported in previous studies. From Fig. 1 may be estimated that IC50 value of this compound is close to 1.47 mM (400 ppm $\approx 0.4$ g L$^{-1}$), 13.73 mM ($> 3000$ ppm approximated as $\approx 3.2$ g L$^{-1}$), 3.39 mM (1400 ppm $\approx 1.4$ g L$^{-1}$), and 7.81 mM (2990 ppm $\approx 2.9$ g L$^{-1}$), respectively.

3.2. Kinetic mechanism of inhibition

Michaelis-Menten assay was performed and Lineweaver- Burk coordinate was applied to define the kinetic mechanism of inhibition. The results of Lineweaver- Burk analysis are presented in the Figs. 2–5.

The applied approach led to conclude the existence of a competitive mechanism of enzyme inhibition for compounds called A-36 and U-201 (Table 1). The following classic scheme describes a competitive inhibition mechanism:

$$E + S \leftrightarrow ES \rightarrow E + P$$
$$E + I \leftrightarrow EI$$

It has been common practice to determine kinetic parameters and the inhibition constant of competitive inhibitors by the Lineweaver-Burk method. In both graphs (Figs. 2 and 3, Top), obtained lines cross the ordinate axis in the similar points, allowing to estimate $V_{\text{max}}$ value as 1.77 ± 0.02 μM min$^{-1}$ for A-36 and 3.13 ± 0.08 μM min$^{-1}$ for U-201. The $V_{\text{max}}$ is an extensive parameter, i.e., higher value corresponds to greater enzyme concentration. The analysis of abscissa in the intersection points of lines with the corresponding axis indicates that $K_m$ values increase with inhibitor concentration increasing. This behavior is typical for competitive inhibition. From the Figs. 2 and 3 (Bottom) $K_m$ values of AChE in the absence of inhibitors were estimated as similar 1.88 and 1.84 mM, respectively in an assay performed with A-36 and U-201. The values of this AChE kinetic parameter were the same in both assays.

According to the linear functions of Figs. 2 and 3 (Bottom) the inhibition constant $K_i$ values (i.e., the dissociation constant of the EI complex) were calculated as 6.71 mM and 3.91 mM, respectively.

Compounds A-63 and U-12 acted as a mixed and uncompetitive inhibitor of AChE (Figs. 4 and 5).

The following classic scheme describes an uncompetitive inhibition mechanism:

$$E + S \leftrightarrow ES \rightarrow E + P$$
$$E + I \leftrightarrow EI$$

$$EI + S \leftrightarrow EIS \rightarrow E + P$$

Mixed inhibition is described by the following scheme:

$$E + S \leftrightarrow ES \rightarrow E + P$$
$$E + I \leftrightarrow EI$$

Table 1 presents the values of inhibition parameters. The inhibition constant of the uncompetitive process is 0.07 mM, i.e., compound U-12 has a higher affinity to the enzyme in comparison with other studied substances. The parameters $\alpha = 0.38$ and $\alpha' = 0.05$ represent the effect of compound A63 on $K_m$ and $V_{\text{max}}$, respectively.

3.3. Artemia salina test performed with studied compounds

The results of the effect of studied substances on Artemia salina viability are presented in Fig. 6. The compound A-63 was not affected. A. salina viability at a concentration less than 2000 ppm, i.e., at 2000 ppm only 3% mortality was observed. So, this compound in this concentration range is non-toxic. Mortality of A. salina was less than 50% in the same concentration range (62.5–2000 ppm) for the other three compounds that confirm the inhibitory effect and allows...
classifying them as moderately toxic.

4. Discussion

Reversible AChE inhibitors have an important role in pharmacological manipulation of the enzyme activity. Assayed compounds have functional groups similar to reversible AChE inhibitors, such as carbamate, quaternary, or tertiary ammonium groups. Therefore, they may be considered as probable enzyme inhibitors, that was confirmed experimentally. Obtained results indicate that the synthesized (−)-cytisine derivatives (N-allylcytisine-12-carbamide (A-63), cytisine-12-carbamide (A-36), N-1-adamantylcytisine-12-thiocarbamide (U-12)) and 1-hydroxyquinopimaric acid (U-201) are weak acetylcholinesterase inhibitors. Compound A-63 demonstrates higher inhibition at less concentration (Fig. 1) that may be considered a sign of better inhibitory activity.

The IC_{50} values detected for A-63, A-36, U-12, and U-201 (Fig. 1) are less than reported for ethanolic and methanolic extracts from the leaves of *Rauvolfia reflexa* showed potential acetylcholinesterase (AChE) and butyryl cholinesterase (BChE) inhibitory activities, with IC_{50} values in the 8.49 to 52.23 g mL\(^{-1}\) range [15]. Feitosa et al. [16] reported active extracts from plants *Ipomoea asarifolia* (IC_{50} = 0.12 g L\(^{-1}\)), *Jatropha curcas* (IC_{50} = 0.25 g L\(^{-1}\)), *Jatropha gossypifolia* (IC_{50} = 0.05 g L\(^{-1}\)), *Kalanchoe brasiliensis* (IC_{50} = 0.16 g L\(^{-1}\)), and *Senna alata* (IC_{50} = 0.08 g L\(^{-1}\)). So, the compounds studied in the present work are characterized with IC_{50} less than for some previously reported extracts, but significantly higher than galanthamine (IC_{50} = 0.0037 g L\(^{-1}\)), which has some success in slowing down neurodegeneration in AD patients.

Kinetic mechanisms of inhibition are related to chemical structures (Figs. 2–5). Compounds A-36 and U-201 are a competitive inhibitor, while A-63 and U-12 acted as a mixed and uncompetitive inhibitor of AChE, respectively. The K_{i} values are relatively high (Table 1) that may be considered as low affinity to the enzyme. Geromichalos et al. [17]
reported for galanthamine – a competitive inhibitor of AChE – $K_i$ value at 0.0034 mM. Thus, due to higher $K_i$ estimated for studied competitive inhibitors, their affinity to AchE is less in comparison with pharmacologically applied galanthamine. López and Pascual-Villalobos [18] reported that S-carvone, estragole, and camphor provoked a mixed AChE inhibition, similarly to A-63, where the inhibitor binding to the free enzyme and the enzyme-substrate complex.

AChE is a serine hydrolase mainly found at neuromuscular junctions and cholinergic brain synapses. AChE has a remarkably high specific catalytic activity: each molecule of AChE degrades about 25,000 molecules of acetylcholine (ACh) per second, approaching the rate of a diffusion-controlled reaction [19–21]. Knowledge of AChE structure is essential for understanding its high catalytic efficacy, and the molecular basis for elucidation of the mechanism of action studied compounds [4]. The AChE molecule is characterized by an ellipsoidal form that has dimensions ~45 Å by 60 Å by 65 Å. The enzymatic monomer is an $\alpha/\beta$ protein: a mixed $\beta$ central sheet with 12 chains surrounded by 14 $\alpha$ helices. The active AChE site is located 4 Å from the bottom of the molecule and comprises two subsites ("lipophilic anionic" and "steratic") for choline binding and catalytic function, respectively [2].

It is likely that the interaction of compounds A-63, A-36, and U-201 with AChE takes place in the lipophilic anionic subsite, which interacts with the positive quaternary amine of acetylcholine. For example, quaternary ligands such as edrophonium and N-methylacridinium, as well as quaternary oximes that effectively reactivate AChE inhibited by organophosphates, act as competitive inhibitors according to a similar mechanism [2].

However, cationic substrates or inhibitors do not bind with a negatively charged amino acid at the anionic site, but by the interaction of 14 aromatic residues that line the deepening related with the active site. The 14 amino acids in aromatic deepening are highly conservative. Among the aromatic amino acids, tryptophan 84 is very relevant, thus, replacing it with alanine causes a 3000-fold reduction in enzyme activity. The steratic subsite is responsible for the hydrolysis of ACh to acetate and choline. It contains the catalytic triad of serine 200, histidine 440 and glutamate 327, similar to the catalytic subsites of other serine hydrolases [2]. In addition to two previously mentioned active center subsites, AChE contains one or more "peripheral" anionic sites other than the choline binding subsite. These serve for interaction with quaternary ligands that act as non-competitive inhibitors and are different from the site occupied by competitive monoquaternary inhibitors. It is probable that these sites are involved in the AChE...
inhibition by U-12. More accurate information about the molecular mechanism of AChE inhibition by tested substances could be obtained using molecular modeling tools (determination of free energy) or X-ray crystallography, which could be the aims of future research.

The inhibitory effect did not correlate with toxicity response observed in *Artemia salina* model: compound A-63, which shows the lowest ID50 value (Fig. 1), was not toxic, while other substances, with higher ID50, were moderately toxic (Fig. 6).

The brine shrimp lethality bioassay is a simple toxicity test for bioactive compounds with high performance. It is based on the ability of the tested compounds to affect the viability of a simple organism-brine shrimp (*Artemia salina*). The test results are interpreted according to *A. salina* nauplii percent mortality [25]: 0–10% non-toxic compound, 11–50% - moderately toxic, 51–90% highly toxic, and 100% extremely toxic compound.

Raineri and Falugi [26] studied AChE activity in embryos and larvae of *Artemia salina*. According to them, AChE activity is present in dehydrated cystic gastrulas and remains low in rehydrated developing embryos. The tendency to increase was observed at hatching and during the nauplius stages and continued in the early stages of metanauplius. The highest AChE activity is located in nerve and muscle cells and a weaker one in the differentiating metanauplius segments. Eserine and diisopropylfluorophosphonate (DFP) - irreversible AChE inhibitors, strongly inhibit the enzyme activity and cause larval paralysis [27,28].

However, in the present study, the correlation between the inhibitory effect of tested compounds and *A. salina* viability was not observed. This result correlates with reported by Alberton Magina et al. [29] for extract from *Eugenia brasiliensis* leaves and their fractions. They reported inhibitory activity on AChE between 6.7 and 63.0% at 100 mg L$^{-1}$ concentration. At the same time, in the *A. salina* test, performed with the same samples, the effect on the viability of the nauplii was observed at concentrations close to 1000 mg mL$^{-1}$, significantly greater than 100 mg L$^{-1}$. For example, the ethyl acetate fraction was the most toxic sample, with an LC50 value of 788.9 mg mL$^{-1}$. The observed trend could be related to the limited diffusion of bioactive compounds in *A. salina* nauplii, so interaction between inhibitor and enzyme is avoided and correlation with the lethal effect on *A. salina* is not observed. Different compounds may have different degrees of diffusion that are not related to their effect on AChE. Therefore, the correlation between AChE inhibition and larvae is one of the points, which requires further study.

We speculate that weak AChE inhibitors analyzed in the present study could be useful as protectors against potent organophosphates [30,31]. The possible protective action mechanism could be similar to observed for metoclopramide and consists in competing for the enzyme with the most potent organophosphates so that the enzyme is occupied by the weak inhibitor instead of the potent and, therefore, less inhibited. Future research should be conducted to offer protection against strong inhibitors and achieve superior benefits and fewer side effects.

5. Conclusion

The synthetized (---)-cytisine derivatives (N-allylcytisine-12-carbamide, cytisine-12-carbamide, N-1-adamantylecytisine-12-thiocarbamide), and 1-hydroxyquinopimaric acid are weak acetylcholinesterase inhibitors. Compound A-63 is a mixed inhibitor which demonstrates higher inhibition at less concentration in comparison with others. Kinetic mechanisms of inhibition were related to chemical structures: competitive for compounds A-36 and U-201 and uncompetitive for compound U-12. Compound A-63 was non-toxic, while other substances were moderately toxic according to *A. salina* test. The present research may be fundamental for the design of new derivatives of natural compounds for treatments involved acetylcholinesterase inhibition.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that do not have conflicts of interest.

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