Esterase profiles of hexafluoropropan-2-ol-based dialkyl phosphates as a major determinant of their effects in mouse brain *in vivo**

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The relationship between the esterase profiles of two *O*-phosphorylated hexafluoropropan-2-ols and their inhibitory activities and selectivities against mouse brain esterases was studied on brain preparations and in the whole animals. The predictions made from the analysis of esterase profiles of the studied organophosphorus compounds were found to be in complete agreement with their *in vivo* inhibitory activities against acetylcholinesterase, butyrylcholinesterase, and neuropathy target esterase.

Key words: organophosphorus compounds, esterase profile, acetylcholinesterase, butyrylcholinesterase, neuropathy target esterase, carboxylesterase, mice, brain, *in vivo*.

Organophosphorus compounds (OPCs) possess a wide spectrum of bioactivities and are applied in various fields of industry, agriculture, and medicine. The necessity for the design of new compounds and materials safe for human and warm-blooded animals actualizes the development of methodology for prediction of potential biological effects of this class compounds at the stage of their syntheses and *in vitro* studies.

Inhibition of serine esterases is a common action mechanism of OPCs.[1] To characterize the efficiency of *in vitro* interaction of OPCs with target esterases, we have proposed earlier the concept of esterase profile, *viz.*, a set of kinetic constants describing the inhibitory activities of compounds against serine esterases of different functional significance:[2,3] acetylcholinesterase (EC 3.1.1.7, AChE), a target for acute cholinergic toxicity; neuropathy target esterase (EC 3.1.1.5, NTE), a target for organophosphate-induced delayed neurotoxicity (OPIDN) (distal neuropathies being manifested after a two—three-week latent period); as well as butyrylcholinesterase (EC 3.1.1.8, BChE) and carboxylesterase (EC 3.1.1.1, CaE) which act as stoichiometric scavengers of OPCs.[4—7]

In addition, inhibition of AChE and BChE in brain in dementias of different origin improves cognitive functions.[8—10] Esterases CaE[11—13] and BChE[14—16] in blood and liver are known to hydrolyze many drugs containing ester, amide, and carbamate groups and inhibitors of these enzymes are modulators of metabolism of these drugs.[17]

Figure 1 shows toxic (italized) and therapeutic effects resulted from inhibition of four serine esterases by organophosphorus compounds.[18,19]

The concept of esterase profile was found to be a convenient approach to the quantitative analysis of structure—inhibitory activity and structure—inhibitory selectivity relationships for anticholinesterase compounds and has allowed studying the structural determinants of inhibitory activity/selectivity in a series of OPCs with different structures,[18,20—22] as well as performing the molecular design of compounds with desired esterase profiles, *viz.*, selective inhibitors of BChE and CaE having no such adverse side effects as delayed neurotoxicity and high acute toxicity.[18,22,23]

The analysis of esterase profile allows revealing both the main potential pharmacological effect of a compound and its possible side and toxic effects. An important parameter for this analysis is the inhibitory selectivity of compound against different target esterases.[3,18,19,22] For example, the relative inhibitory potency of organophosphorus compounds against NTE as compared to AChE ($RIP = k_i(NTE)/k_i(AChE)$) characterizes the neuropathic potential of a compound, *i.e.*, its ability to cause acute cholinergic toxicity compared to OPIDN.[24] Compounds are neuropathic when $RIP > 1$ and exhibit predominantly the cholinergic toxicity when $RIP < 1$.[24—28] The selectivity against BChE *vs.* AChE is an important characteristic

* Dedicated to Academician of the Russian Academy of Sciences N. S. Zefirov on the occasion of his 80th birthday.

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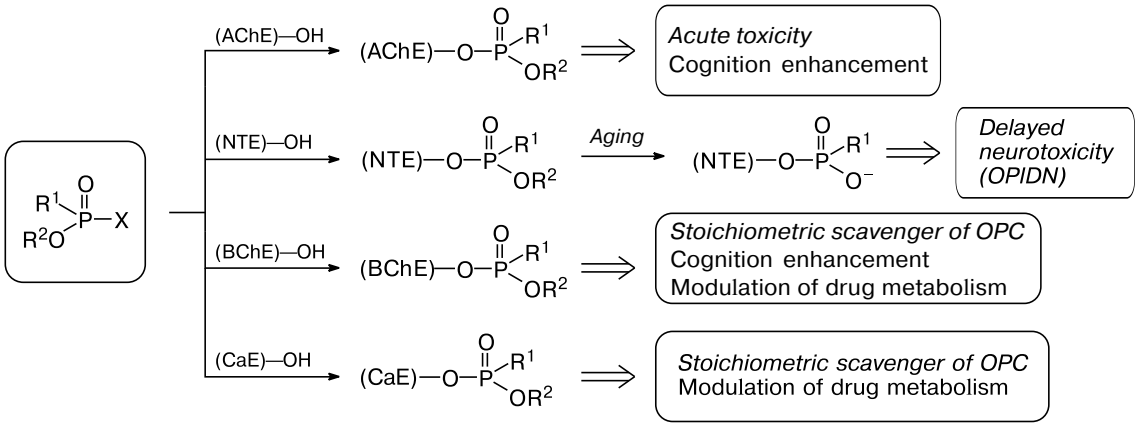
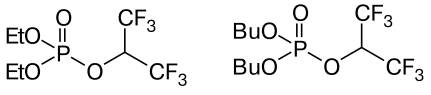


Fig. 1. Toxic (italic) and therapeutic effects of organophosphorus compounds resulting from inhibition of serine esterases. AChE is acetylcholinesterase, NTE is neuropathy target esterase, BChE is butyrylcholinesterase, and CaE is carboxylesterase.

of the pharmacological profile of compound as a potential drug for the treatment of Alzheimer´s disease.[9,29,30] In this case, the fact that compound has the capability of inhibiting CaE can result in adverse drug-drug interactions when applied.[31] However, efficient and selective inhibitors of CaE can be used for the metabolic and pharmacokinetic control of ester-containing drugs.[13,17]

The concept of esterase profile is based on the assumption that the esterase profile of anticholinesterase compound determines a considerable degree its biological effect *in vivo*. The aim of the present work was to verify this hypothesis in experiments in the whole animals. For this purpose, we studied the relationship between the esterase profiles of two OPCs and their inhibitory activities and selectivities against mouse brain esterases *in vitro* in the brain 9S-homogenate preparations and in the experiments on the whole animals 1 h after intraperitoneal injection of compounds.

To solve the designated problem, the representatives of *O*-phosphorylated hexafluoropropan-2-ols studied earlier, *viz.*, *O*,*O*-diethyl-*O*-(1-trifluoromethyl-2,2,2-trifluoroethyl)phosphate (**1**) and *O*,*O*-dibutyl-*O*-(1-trifluoromethyl-2,2,2-trifluoroethyl)phosphate (**2**),[3,32] were chosen as organophosphorus inhibitors. Compounds **1** and **2** possess different hydrophobicities, as well as have different esterase profiles and different acute toxicities.



1 **2**

Experimental

S-Acetylthiocholine iodide (ATCh); *S*-butyrylthiocholine iodide (BTCh); 5,5´-dithiobis-2-nitrobenzoic acid (DTNB), tris(hydroxymethyl)aminomethane hydrochloride (Tris), potassium ferricyanide ($K_3[Fe(CN)_6]$), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), ethopropazine (EPr), 1-naphthyl acetate (1-NA), 4-nitrophenyl acetate (4-NPA), eserine, diethyl 4-nitrophenyl phosphate (paraoxon) were obtained from Sigma-Aldrich (USA). The commercially available 4-aminoantipyrine (4-AAP) (Acros Organics, Belgium) and sodium citrate (Merck, Germany) were used in the present work. Phenyl valerate and mipafox were synthesized in the Institute of Physiologically Active Compounds of the Russian Academy of Sciences (IPAC RAS). *O*-Phosphorylated hexafluoropropan-2-ols **1** and **2** were synthesized according to the earlier described procedures.[3,32]

Inhibitory activities of compounds 1 and 2 against AChE, BChE, CaE, and NTE were determined using the commercially available enzymes (Sigma-Aldrich, USA): AChE from human red blood cells (500 U mg^{-1}, C0663), BChE from horse serum (500 U mg^{-1}, C4290), CaE from porcine liver (150 U mg^{-1}, E2884), and stable lyophilized NTE preparation from chicken brain (LyoNTE) prepared according to the procedure developed in our laboratory and described in detail.[33,34]

The IC_{50} values (the concentration of compound needed to inhibit the enzyme activity by half) were determined after incubation of enzymes for 20 min in the corresponding buffer with studied compounds within the concentration range from 10^{-11} to 10^{-3} mol L^{-1}. Compounds **1** and **2** were dissolved in DMSO whose final concentration in the reaction mixture was not higher than 1 vol%. The residual activities of AChE and BChE were determined by the Ellman method[35] using 1 m*M* ATCh and 1 m*M* BTCh as substrates, respectively, in 0.1 *M* K-Na phosphate buffer (pH 7.5) at 37 °C, the detection wavelength was $\lambda = 412$ nm ($\varepsilon_{412} = 14150$ mol^{-1} cm^{-1}) in the presence of 0.33 m*M* DTNB. The CaE activity was determined using 1 m*M* 4-NPA as a substrate in 0.1 *M* K-Na phosphate buffer (pH 8.0) at 37 °C at $\lambda = 405$ nm ($\varepsilon_{405} = 13300$ mol^{-1} cm^{-1}). The NTE activity was determined as the difference between the paraoxon-resistant (LyoNTE) and (paraoxon + mipafox)-resistant (LyoNTE + mipafox 250 μ*M*, 20 min) esterase activities in accordance with the Johnson differential method.[36] The reaction was performed in 50 m*M* Tris-HCl (pH 8.0) containing 0.2 m*M* EDTA at 37 °C, the substrate was 1.4 m*M* phenyl valerate. The reaction-produced phenol was determined at $\lambda = 510$ nm ($\varepsilon_{510} = 13900$ mol^{-1} cm^{-1}).

Measurements were performed on a Bio-Rad Benchmark Plus microplate spectrophotometer (France) in 96-well plates. The IC_{50} values were calculated using the Origin 6.1 program.

Preparation of the 9S-fraction of mouse brain homogenate. Mouse brains were obtained from 1.5—2-month-old white outbred CD1 mice with a weight of 20—25 g (Pushchino, Russia). The animals were kept and handled in compliance with the requirements of IPAC RAS bioethics committee and "Rules of Good Laboratory Practice" (Order of the Ministry of Healthcare and Social Development of the Russian Federation No. 708n of August 23, 2010). The animals were sacrificed by decapitation under CO_2 anesthesia.

The mouse brain after decapitation was removed on cooling, washed with 0.9% NaCl, dried, weighed, frozen in liquid nitrogen, and stored at −70 °C. Prior to measurements, the brain was thawed slowly on ice, homogenized in a Potter homogenizer (teflon—glass) at 4 °C in working buffer (50 m*M* Tris-HCl, 0.2 m*M* EDTA, pH 8.0) using 5 ml of buffer per 1 g of tissue. The homogenate was centrifuged on a K-24 centrifuge (Germany) for 15 min at 4 °C at 9000 *g*. The resulting 9S-supernatant was aliquoted, frozen in liquid nitrogen, and stored at −70 °C until measurements.

The activities of esterases in the brain preparations were determined on a Gilford-250 spectrophotometer (United Kingdom) in 3 mL cuvettes with an optical path length of 1 cm at 37 °C. The activities of AChE and BChE were determined by the Ellman method[35] as described above. The activity of CaE in the brain preparations was measured under the above-described conditions using 2 m*M* EDTA for inhibition of PON1/arylesterases and 40 μ*M* eserine for inhibition of cholinesterases.

The activity of NTE in the 9S-fraction of mouse brain homogenate was determined by the Johnson differential method[36] with slight modifications[37] as the difference between the rates of phenyl valerate hydrolysis in the samples incubated for 20 min with 50 μ*M* paraoxon (paraoxon-resistant esterase activity) and with a mixture of 50 μ*M* paraoxon and 250 μ*M* mipafox ((paraoxon + mipafox)-resistant esterase activity) as previously described.[25]

The IC_{50} values for inhibition of AChE, BChE, NTE, and CaE by the tested compounds in the mouse brain preparations were determined after 20 min incubation of the tissue preparation in the corresponding buffer with the studied compounds at concentrations from $1 \cdot 10^{-11}$ to $1 \cdot 10^{-3}$ mol L^{-1}. Pooled brain samples were used. The volume of reaction mixture was 3 mL. The residual activities of brain enzymes were determined according to the methods described above. The measurements were performed on a Gilford-250 spectrophotometer (United Kingdom). Each measurement was performed in triplicate. The IC_{50} values were calculated using the Origin 6.1 program.

In animal experiments adult outbred male CD1 albino mice (20—25 g, 1.5—2 months of age) (Pushchino, Russia) were used. In the study of dose-dependent inhibition of AChE, BChE, CaE, and NTE in mice brain, the tested compounds were dissolved in DMSO and injected once intraperitoneally in a volume of ~0.1 mL in 5—10 increasing doses. At least six animals were used for each dose. Control animals received only DMSO. One hour after injection of the tested compounds, the mice were decapitated under CO_2 anesthesia and the brain was extracted. The brain samples were prepared as described above.

Statistical processing and analysis of data were performed using the GraphPad Prism 6.05 program (GraphPad Software, United States). The data are given as means±SEM. Data plotting, regression analysis, and correlations were obtained using the the Origin 6.1 program.

Results and Discussion

Estimation of the esterase profiles of compounds 1 and 2. The inhibitory activities of compounds **1** and **2** were determined toward the standard set of enzymes used for estimation of the esterase profiles of anticholinesterase compounds: AChE from human red blood cells, NTE from chicken brain, BChE from horse serum, and CaE from porcine liver.[3,19,32,38]

Table 1 gives data on the antiesterase activities of compounds **1** and **2** represented by the IC_{50} values obtained after 20 min incubation of the studied compounds with esterases, which characterize the esterase profiles of compounds. The results agree good with the earlier published data.[3,32]

As Table 1 shows, compound **2** is a more efficient inhibitor of all studied esterases compared to compound **1**, both compounds inhibit CaE the most efficiently. The rank order of inhibitory potency for each compound against four esterases is as follows: NTE < AChE < BChE < CaE for compound **1** and AChE < NTE < BChE < CaE for compound **2**.

The differences in the esterase profiles of the studied compounds are demonstrated in Fig. 2.

The inhibitory selectivities (*S*) of the studied compounds toward enzymes E_X and E_Y calculated by the formula $S(E_X/E_Y) = IC_{50}(E_Y)/IC_{50}(E_X)$ and the acute toxicities of the compounds for mice (LD_{50}, mg kg^{-1}) determined after intraperitoneal injection and observation time of 24 h are given in Table 2.

As Table 2 shows, compound **1** has very low neuropathic potential (RIP = 0.07) and moderate acute toxicity (LD_{50} = 200 mg kg^{-1}). At the same time, the hydrophobic compound **2** is a hazardous delayed neuropathic agent (RIP = 6.6), possesses very low acute toxicity (LD_{50} > > 2000 mg kg^{-1}), and exhibits higher selectivity against BChE and CaE compared to AChE.

***In vitro* inhibitory activities and selectivities of compounds 1 and 2 against mouse brain esterases in the 9S-fraction of brain homogenate.** At the first stage, we studied

Table 1. Inhibitory activities of compounds **1** and **2** against the AChE, NTE, BChE, and CaE (the esterase profiles of compounds)

Enzyme	IC_{50}/mol L^{-1}	
	1	**2**
AChE	$(1.93\pm0.16) \cdot 10^{-5}$	$(4.10\pm0.17) \cdot 10^{-7}$
NTE	$(2.67\pm0.13) \cdot 10^{-4}$	$(6.21\pm0.31) \cdot 10^{-8}$
BChE	$(7.70\pm0.49) \cdot 10^{-6}$	$(1.40\pm0.08) \cdot 10^{-8}$
CaE	$(3.39\pm0.23) \cdot 10^{-7}$	$(3.18\pm0.23) \cdot 10^{-9}$

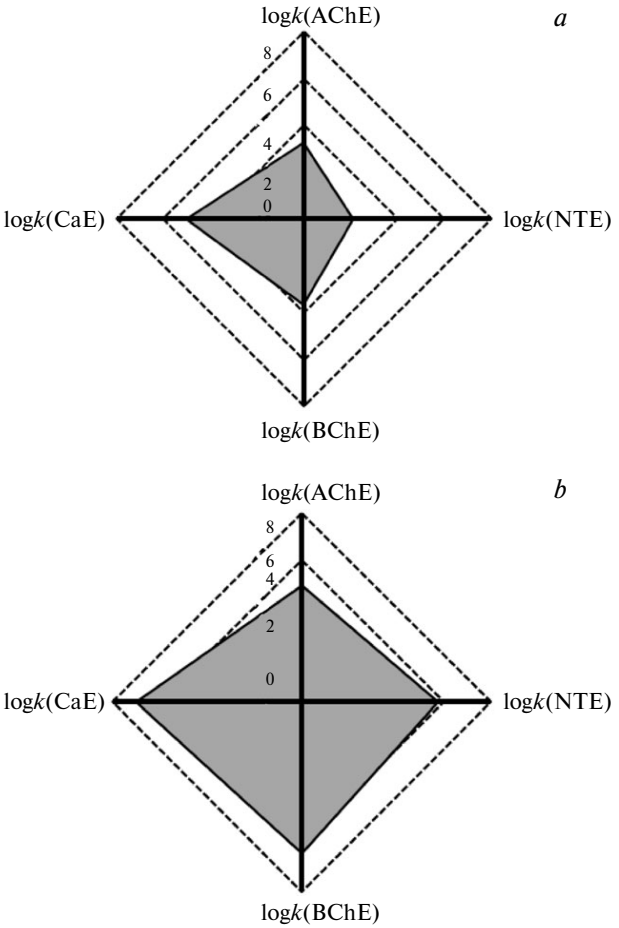


Fig. 2. Esterase profiles of compounds **1** (*a*) and **2** (*b*).

in vitro inhibitory activities and selectivities of compounds **1** and **2** against AChE, NTE, BChE, and CaE in the 9S-fraction of mouse brain homogenate *vs.* the esterase profiles of compounds. The IC_{50} values were determined after 20 min incubation with inhibitors (Table 3) and the inhibitory selectivities $S(E_X/E_Y)$ of compounds **1** and **2** toward brain esterases (Table 4) were calculated.

Table 2. Inhibitory selectivities ($S(E_X/E_Y)$) of compounds **1** and **2** against AChE, NTE, BChE, and CaE and acute toxicities of these compounds

Parameter	**1**	**2**
S(BChE/AChE)	2.5	29.3
S(CaE/AChE)	56.60	129.0
S(BChE/NTE)	34.60	4.4
S(CaE/NTE)	778.60	19.5
RIP*	0.07	6.6
LD_{50}/mg kg^{-1}	200[39,40] (161—239)**	>2000[39,40]

* RIP = *S*(NTE/AChE).** The distribution of resulting LD_{50} values relative to the median is given in parentheses.

Table 3. Inhibitory activities of compounds **1** and **2** against AChE, NTE, BChE, and CaE in the mouse brain preparations*

Ferment	IC_{50}/mol L^{-1}	
	1	**2**
AChE[40]	$(4.80\pm0.24)\cdot10^{-5}$	$(4.87\pm0.22)\cdot10^{-7}$
NTE[40]	$(2.84\pm0.12)\cdot10^{-4}$	$(6.78\pm0.30)\cdot10^{-8}$
BChE	$(1.26\pm0.07)\cdot10^{-5}$	$(2.33\pm0.11)\cdot10^{-8}$
CaE	$(1.81\pm0.13)\cdot10^{-5}$	$(1.52\pm0.16)\cdot10^{-7}$

* The time of esterase incubation with the tested compounds was 20 min.

Table 4. Inhibitory selectivities (*S*) of compounds **1** and **2** against AChE, BChE, CaE, and NTE in the mouse brain preparations

$S(E_X/E_Y)$*	**1**	**2**
S(BChE/AChE)	3.81	21.00
S(CaE/AChE)	2.65	3.20
S(BChE/NTE)	22.50	2.91
S(CaE/NTE)	15.70	0.45
S(NTE/AChE)	0.17[40]	7.30[40]

* $S(E_X/E_Y) = IC_{50}(E_Y)/IC_{50}(E_X)$.

The comparison of data given in Tables 1 and 3 shows that the inhibitory activities of compounds **1** and **2** against the studied mouse brain esterases generally correspond to the esterase profiles of compounds except for CaE, which was found to be less sensitive to both compounds compared to their esterase profiles. The efficiency of esterase inhibition in the brain preparations increases in the order NTE < AChE < CaE < BChE in the case of ethyl derivative **1** and AChE < CaE < NTE < BChE in the case of butyl derivative **2**, *i.e.*, both compounds inhibit CaE to a lesser degree than BChE. The comparative study of the inhibitory activity of compound **2** against CaE from the mouse brain, liver, and blood revealed that, in the same species, the brain enzyme is less sensitive compared to the liver and blood enzymes: $IC_{50} = (1.5\pm0.16)\cdot10^{-7}$ mol L^{-1} (brain CaE), $(4.6\pm0.4)\cdot10^{-8}$ mol L^{-1} (liver CaE), and $(2.4\pm0.13)\cdot10^{-8}$ mol L^{-1} (CaE from the mouse plasma). We assume that different sensitivity of the brain CaE to OPCs compared to the liver and blood enzymes can be due to the presence of different CaE isoforms in these tissues differing in both the expression level and specificity. It is known from the literature that the carboxylesterase expressed in the brain is represented by the CES6 isoform, which is the product of *CES6* gene located in chromosome 16. CES6 is an individual secreted CaE form of mammalian neural tissue;[41] it differs from CES1 (liver CaE), ES1

(mouse plasma CaE),[42] and CES2 (small intenstine CaE) which are the products of corresponding genes located in chromosome 8.[41] Due to the features of brain CaE, we did not include this enzyme in further analysis.

The inhibitory selectivities of compounds **1** and **2** toward the brain AChE, BChE, and NTE (see Table 4) agree good with the values calculated from the esterase profiles of these compounds (see Table 2), which is confirmed by high indicators of correlation between these parameters: $r = 0.997$, $p = 0.0021$, $n = 3$ for compound **1**; and $r = 0.979$, $p = 0.00073$, $n = 3$ for compound **2** (Fig. 3).

Thus, compounds **1** and **2** exhibit *in vitro* effects on the mouse brain preparations, which in general correspond to their esterase profiles with regard to AChE, BChE, and NTE in terms of both inhibitory activity and inhibitory selectivity. The tissue-specific isoform of brain CaE is an exception.

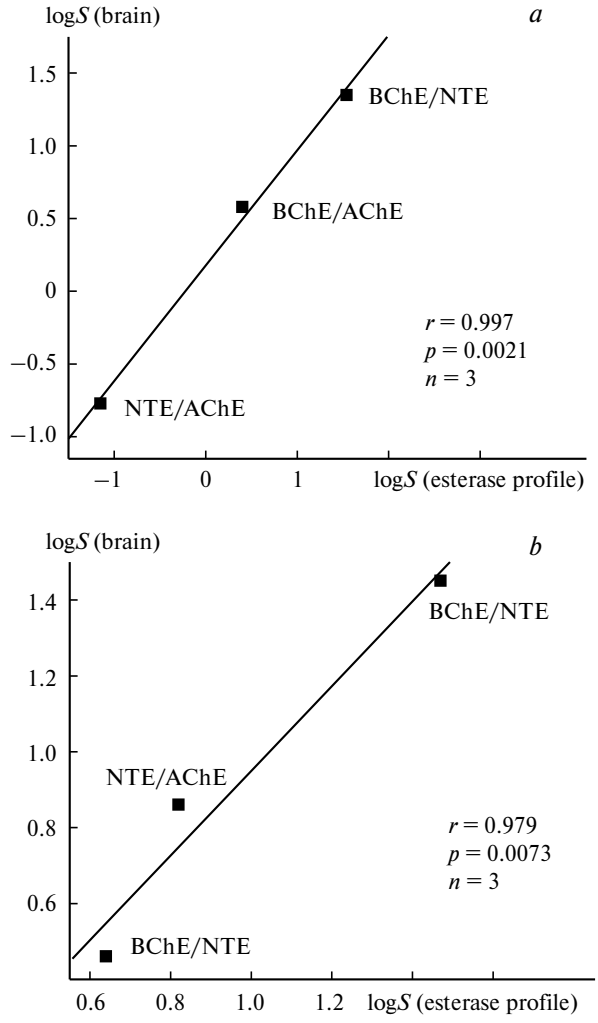


Fig. 3. Correlation between the inhibitory selectivities (S) calculated from the esterase profiles of compounds ($\log S$ (esterase profile)) and the inhibitory selectivities against esterases in the mouse brain samples ($\log S$ (brain)) for compounds **1** (*a*) and **2** (*b*).

Inhibition of the mouse brain esterases upon intraperitoneal injection of compounds 1 and 2. To estimate whether the predictions and conclusions on the biological effects of OPCs made from the analysis of their esterase profiles correspond to their effects in whole organism, we studied changes in the activities of mouse brain AChE, NTE, BChE, and CaE 1 h after single intraperitoneal injection of increased doses of compounds **1** and **2**.

The administered doses of compounds **1** and **2** did not exceed the LD_{50} values: the dose range was 15—200 mg kg^{-1} for compound **1** and 0.5—2000 mg kg^{-1} for compound **2**. The results of the study are shown in Fig. 4. As it is seen from Fig. 4, *a*, compound **1** in the used dose range almost does not inhibit NTE, inhibits weakly AChE and CaE,

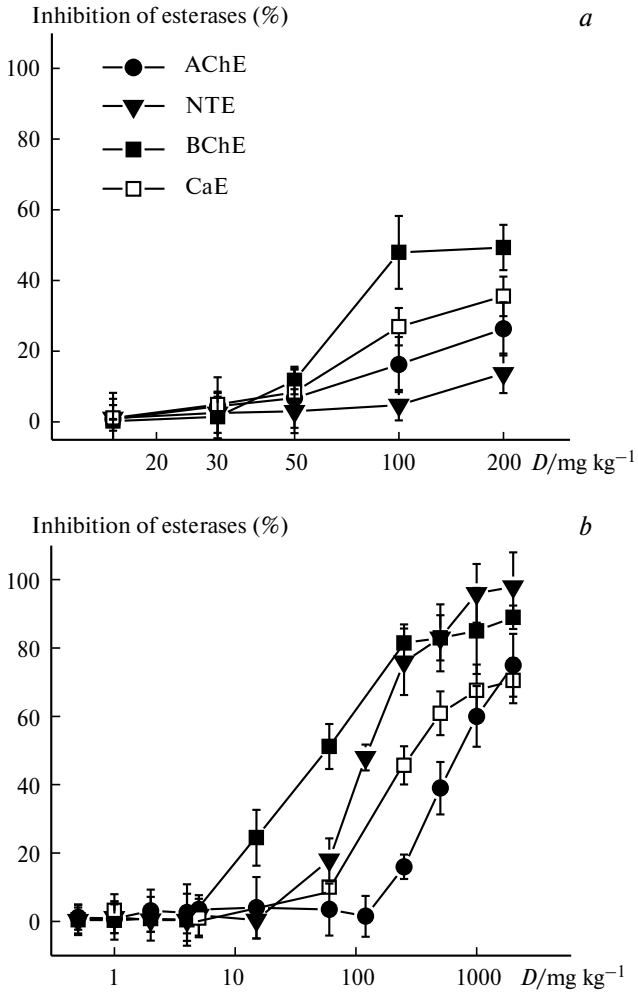


Fig. 4. Inhibition of AChE, NTE, BChE, and CaE in the mouse brain 1 h after intraperitoneal injection of compounds **1** (*a*) and **2** (*b*) in increasing doses (D). The data are shown as percentage inhibition of the corresponding esterases (%) in control animals. The activities of esterases in the brain of control animals, nmol (min mg of protein)$^{-1}$, (mean±SEM) are 69.20±3.54 ($N = 6$) for AChE,[40] 13.44±0.52 ($N = 8$) for NTE,[40] 1.90±0.01($N = 8$) for BChE, and 11.15±0.2 ($N = 8$) for CaE.

and suppresses most strongly the activity of BChE. Compound **2** (Fig. 4, *b*) inhibits all studied esterases in the mouse brain in a dose-dependent manner.

The ED_{50} values, mg kg^{-1} (the dose at which the enzyme activity is reduced by half) were determined from the dose-dependences for compound **2**; for compound **1** the percentage inhibition of each esterase after administration of the highest dose was determined (Table 5).

As Table 5 shows, compound **2** is the most efficient inhibitor of mouse brain esterases compared to compound **1** that corresponds to the esterase profiles of these OPCs (see Table 1). The efficiency of brain AChE, BChE, and NTE inhibition by these compounds also corresponds to their esterase profiles and increases in the order NTE < AChE < < BChE for **1** and in the order AChE < NTE < BChE for **2** (see Table 5). Carboxylesterase is inhibited by both compounds to a lesser degree compared to BChE as in the experiments *in vitro* on the brain preparations. It should be noted that, for inhibition of CaE in the mouse blood, no such deviations in the enzyme sensitivity to the studied compounds were observed and the efficiency of blood esterases inhibition agreed completely with the esterases profiles of the compounds.[43,44]

The values of the inhibitory selectivity $S(E_X/E_Y)$ of compound **2** toward the mouse brain AChE, BChE, and NTE after intraperitoneal injection are given in Table 6. There is a good agreement between the *in vivo* inhibitory selectivity of OPC **2** against the mouse brain AChE, NTE, and BChE (Table 6) and the selectivities calculated from the esterase profile of the compound (see Table 2) that is confirmed by good indicators of correlation between these parameters: $r = 0.998$, $p = 0.00425$, $n = 3$ (Fig. 5). In accordance with the esterase profile, compound **2** in the experiments *in vivo* exhibits selectivity toward inhibition of BChE compared to AChE: ED_{50} = 38.45±5.61 and 498.3±41.2 mg kg^{-1}, respectively (see Table 5), the inhibitory selectivity is S(BChE/AChE) = 13 (see Table 6). The ratio ED_{50}(AChE)/ED_{50}(NTE) = 4.1 (see Table 6) suggests that the low-toxic compound **2** (LD_{50} > 2000 mg kg^{-1}) presents a high neuropathic hazard that agrees completely with the predictions based on the esterase profile (see Table 2).

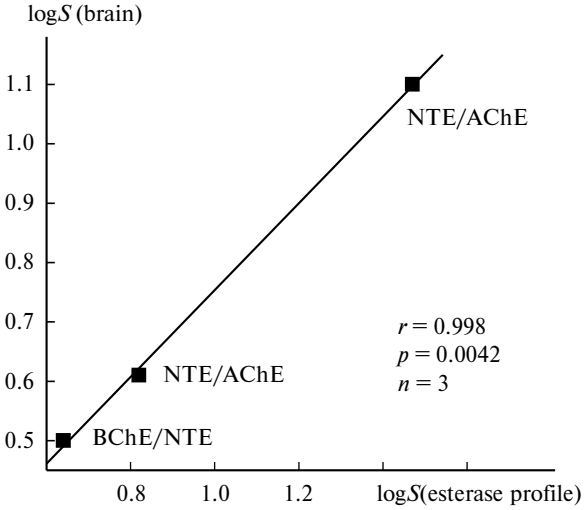


Fig. 5. Correlation between the inhibitory selectivities of compound **2** against brain esterases *in vivo* (log$S$ (brain)) and the corresponding parameters of esterase profile (log$S$ (esterase profile)).

Compound **1** exhibits *in vivo* lower inhibitory activity against mouse brain esterases compared to compound **2** and, at moderate acute cholinergic toxicity, is not neuropathic (see Fig. 3, *a* and Table 5) that also corresponds to its esterase profile.

Thus, the performed study showed the complete agreement between the predictions made from the analysis of esterase profiles of the studied OPCs and their inhibitory effects against AChE, BChE, and NTE in the mouse brain after single intraperitoneal injection and improperiety of such prediction for the brain CaE due to the features of the brain enzyme isoform.

Table 5. Inhibitory activities of compounds **1** and **2** against the mouse brain AChE, NTE, BChE, and CaE 1 h after intraperitoneal injection

Enzyme	**1** (inhibition (%))*	**2** (ED_{50}/mg kg^{-1})
AChE	26	498.3±41.2
NTE	10	121.9±2.7
BChE	50	38.45±5.61
CaE	36	185.3±7.35

* The percentage inhibiton of brain enzymes after injection of compound **1** at the highest dose equal to LD_{50} (200 mg kg^{-1}).

Table 6. Inhibitory selectivity of compound **2** against the mouse brain AChE, NTE, and BChE 1 h after intraperitoneal injection

E_X/E_Y	$S(E_X/E_Y)$*
BChE/AChE	13
BChE/NTE	3.2
NTE/AChE	4.1

*$S(E_X/E_Y) = ED_{50}(E_Y)/ED_{50}(E_X)$.

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