ORIGINAL ARTICLE

IN VITRO INACTIVATION OF RAT BRAIN ACETYLCHOLINESTERASE BY DSP-4 AND ITS DERIVATIVES OS-21 AND OS-23 AND PROTECTIVE ACTIVITY OF TACRINE (9-AMINO-1,2,3,4-TETRAHYDROACRIDINE)

Jiří Patočka[1,3], Daniel Jun[1,2], and Kamil Kuča[1,]*

University of Defence, Faculty of Military Health Sciences, Czech Republic: Department of Toxicology[1]; Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Czech Republic: Department of Pharmaceutical Botany and Ecology[2]; Faculty of Health and Social Studies, South Bohemia University, Czech Republic: Department of Radiology and Toxicology[3]

Summary: Tertiary N-haloethylamines are able to cyclize to the corresponding aziridinium ions. The inhibitory activity of the DSP-4 (N-(o-brombenzyl)-N-ethyl-2-chlorethylamine) and its two derivatives OS-21 (N-benzyl-N-ethyl-2-chloroethylamine) and OS-23 (N-fenylethyl-N-ethyl-2-chloroethylamine) was studied toward rat brain acetylcholinesterase (AChE) in vitro. The influence of the THA (tacrine; 9-amino-1,2,3,4-tetrahydroacridine) on AChE inhibition by these substances was also evaluated. The results demonstrated that all of three aziridinium compounds formed in solution caused a time- and concentration-dependent irreversible enzyme inhibition. The association of aziridinium compounds with the AChE was a relatively slow second-order reaction. DSP-4 showed the fastest rate of AChE alkylation, OS-21 had a lowered rate and OS-23 displayed the lowest rate. Pretreatment of the enzyme by THA decreased the rate of alkylation by all three aziridinium compounds by allosteric mechanism.

Key words: *Aziridines; Acetylcholinesterase; Inhibition; Tacrine; DSP-4; N-haloethylamines*

Introduction

Tertiary N-haloethylamines are able to cyclize to the corresponding aziridinium ion according to Fig. 1. (8,18) Aziridinium compounds thus formed are easily attached by nucleophiles resulting in a ring-opening and form a covalent bond by alkylating some groups of biological structures (enzymes, receptors) according to Fig. 2, where A^- is a nucleophilic site of biological structure (17,20).

The positively charged aziridinium ion may be attracted to the anionic site of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) and may act as an active-site-directed irreversible inhibitor of this enzyme (11,14). The aziridinium compounds possibly alkylate the carboxyl group of the anionic site of AChE, causing almost total inhibition.

The purpose of our work was to study the inhibitory activity of the DSP-4 (N-(o-brombenzyl)-N-ethyl-2-chlorethylamine) and of two other derivatives OS-21 (N-benzyl-N-ethyl-2-chloroethylamine) and OS-23 (N-fenylethyl-N-ethyl-2-chloroethylamine) (Fig. 3) toward AChE in vitro. The influence of the THA (tacrine, 9-amino-1,2,3,4-tetrahydroacridine), which bounds to the hydrophobic site at the periphery of the AChE(5,6,11), on AChE inhibition by these aziridines was also studied. Later studies have found that DSP-4 is an important compound for studying noradrener-

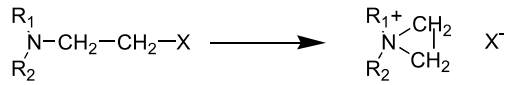

Fig. 1: Cyclization of tertiary N-haloethylamines.

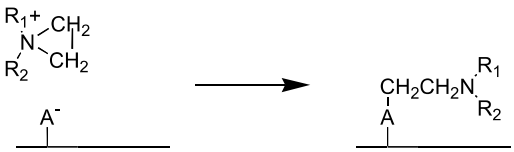

Fig. 2: Alkylation of the nucleophilic site of the biological structures.

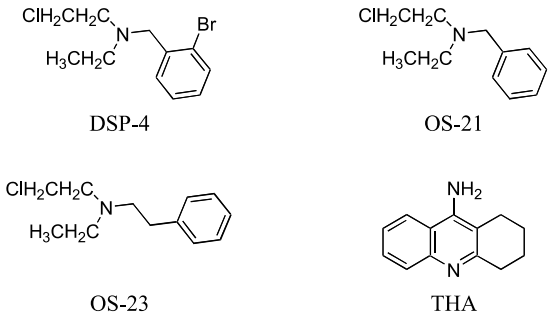

Fig. 3: Structures of the tested compounds.

gic mechanisms in the CNS (Central Nervous System) as well as in the in the periphery (7,16).

Thanks to the fact that adrenergic as well as cholinergic hypofunctions were observed after intracerebral application of DSP-4 to the rat (1), we have studied the influence of this compound on rat brain AChE.

Materials and methods

DSP-4 was prepared as described by Ross et al. (15), compounds OS-21 and OS-23 were prepared using the same synthetic approach. Their chemical structures are shown in Fig. 3. THA was prepared as reported earlier (4). Triton X-100 was a product of Koch-Light Lab. The other chemicals used were purchased from Lachema (Brno, Czech Republic) in analytical purity.

Rat brain AChE was taken as the source of the enzyme. The enzyme was solubilized by 1% (v/v) Triton X-100 by the procedure described earlier (13). The enzymatic activity varied around 0.5 μmol of acetylcholine hydrolyzed per min per ml at 25°C and pH 8.0. The activity of the AChE was measured using the modified titrimetrical method (9,10) with the Radiometer titrator RTS 822 (Radiometer, Denmark) in the pH-stat. Acetylcholine iodide (ACh) was used as the substrate. The acetic acid released was titrated by 0.05 M NaOH and the measurement was carried out at the pH 8.0 and 25 °C. A correction for the spontaneous hydrolysis of the substrate was made.

The solutions of the aziridinium chlorides were prepared immediately before the experiments by dissolving a weighted samples of tertiary N-chloroethylamines in 0.1 M phosphate buffer (pH 7.4). The AChE alkylation reactions were carried out in the same buffer at 25 °C and the reaction was followed by measuring the decrease in the initial rate of the enzymatic AChE hydrolysis. At the appropriate time intervals the samples were taken from the incubation mixture, diluted 20-fold into the enzyme assay medium (0.15 M NaCl) and remaining AChE activities were estimated (v_i). These experiments were also carried out in the presence of THA as a ligand of hydrophobic site of AChE.

The rate of cyclization of the chloralkylamines to quaternary aziridinium derivatives was determined in water-methanol (1:1, v/v) mixture at pH 7.4 by determination of the chloride ion formed by potentiometric titration (15).

The apparent first order rate constants of AChE alkylation by quaternary aziridinium salts (k_{app}) were calculated from the slope $-k_{app}/2.303$ of the plot log 100 (i_f-i) vs. time of exposure duration, where i is the fractional inhibition calculated as $i = 1 - (v_i/v)$ and i_f is the final level of inhibition achieved at 24 hrs (19). The second-order rate constant (k_2) was calculated as $k_2 = k_{app}/c_I$, where c_I is the molar concentration of aziridinium compound. The best fit to the experimental data points was calculated by linear regression analysis. All values of constants are means ± confidence range for P 0.95.

Results

Cyclization rates expressed as half-life periods of the tertiary N-chlorethylamines, $t_{0.5}$, are very quick (Tab. 1) and at 37 °C the formation of aziridinium salts are completed to 5 min.

AChE incubation with 0.001 to 1.0 mM solutions of all three aziridinium compounds results in a progressive and irreversible decrease in the enzyme activity. In spite of the large excess of the aziridinium ion, however, the inhibition was not complete and was dependent on the initial concentration of the aziridinium ion. The kinetic data for the AChE alkylation reaction with the aziridinium salt OS-23 as example are shown in Fig.4. All three aziridinium compounds caused a time- and concentration-dependent inhibition of AChE activity. The addition of THA substantially protected against the loss of AChE activity that occurred in the presence of aziridinium ions alones. In the absence as well as in the presence of THA, the decrease of the enzyme activity followed the first-order kinetic (Fig.4). The rate constants obtained are listed in the Tab. 2, as well as the i_f values.

Tab. 1: Half-life periods of cyclization.

Compound	$t_{1/2}$ (min)
DSP-4	1
OS-21	0.5
OS-23	0.5

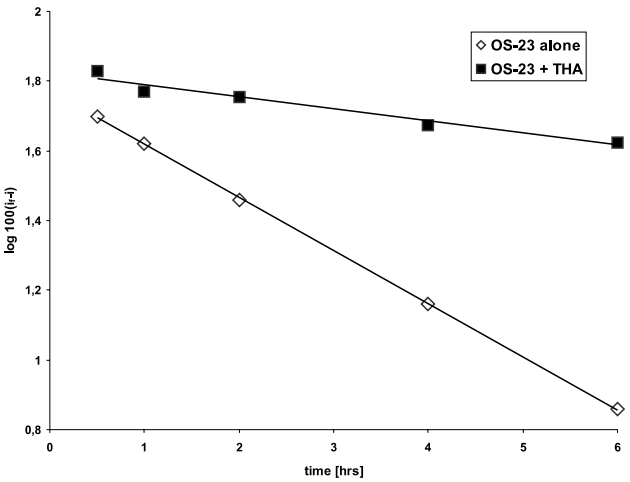

Fig. 4: Kinetics of AChE alkylation with OS-23 alone and in the presence of THA.

A complete analysis of the kinetic mechanism of the alkylation by aziridinium compound and protection by THA was carried out in the case of DSP-4. For this compound, the k_{app} values were measured at different concentrations of both alkylating and protecting ligands (Tab. 2) and after that were plotted as dependence of $1/k_{app}$ on $1/c_I$

Tab. 2: Kinetic constants of alkylation of AChE by quaternary aziridinium compounds alone (I) and in the presence of tacrine (T).

Compound	I (μM)	T (μM)	k_{app} (min^{-1})	k_2 ($M^{-1}min^{-1}$)	i_f
OS-21 alone	100	0	$(1.52\pm0.16)10^{-2}$	152.0±16	0.89
	500	0	$(7.31\pm0.86)10^{-2}$	146.2±17	0.95
	1000	0	$(1.64\pm0.18)10^{-1}$	164.0±18	0.96
OS-21+THA	100	0.5	$(1.73\pm0.22)10^{-3}$	17.3±2.2	0.52
DSP-4 alone	1	0	$(2.50\pm0.21)10^{-3}$	2500±210	0.48
	5	0	$(1.07\pm0.17)10^{-2}$	2140±340	0.85
	10	0	$(1.26\pm0.15)10^{-2}$	1266±144	0.87
	50	0	$(2.09\pm0.16)10^{-2}$	418±32	0.90
	100	0	$(4.53\pm0.32)10^{-2}$	453±32	0.93
DSP-4+THA	10	0.5	$(2.43\pm0.20)10^{-3}$	243±20	0.48
	50	0.5	$(4.63\pm0.26)10^{-3}$	93±5.2	0.49
	100	0.5	$(9.80\pm0.62)10^{-3}$	98±6.2	0.46
OS-23 alone	100	0	$(5.85\pm0.42)10^{-3}$	58.5±4.2	0.76
OS-23+THA	100	0.5	$(1.34\pm0.23)10^{-3}$	13.4±2.8	0.51

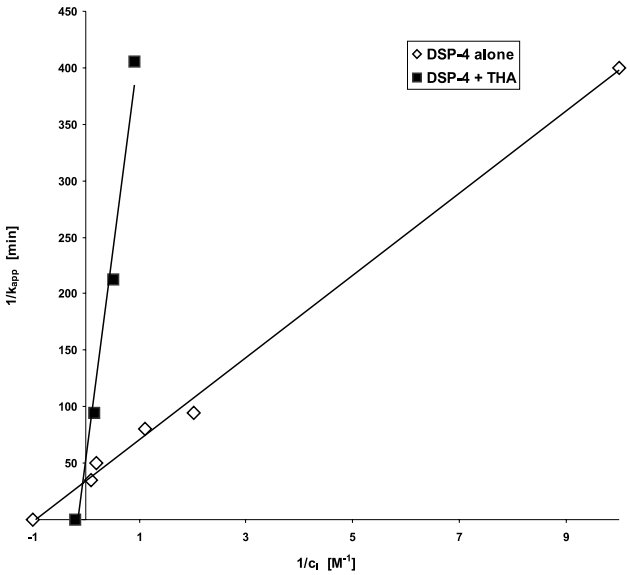

Fig. 5: Dependence of $1/k_{app}$ on $1/c_I$.

Fig. 6: Scheme of the reaction of the enzyme with the alkylating agent.

as shown in Fig. 5. This can be summarized by the reaction scheme in Fig. 6. E is the free enzyme, I is the alkylating agent, EI the reversible enzyme-aziridinium complex, and EI' the alkylated enzyme. The K_D for DSP-4 estimated from these kinetic data is (1.25 ± 0.46) x 10^{-5} $mol^{-1}l^{-1}$ and the k_a is (3.85 ± 1.02) x 10^{-2} min^{-1}. In the presence of THA the K_D is (8.35 ± 2.46) x 10^{-5} $mol^{-1}l^{-1}$ and constant k_a is the same as in the absence of this ligand. It is seen that THA protects the enzyme against DSP-4 in such a way that decreased the affinity of aziridinium compound to the enzyme, whereas the rate of transformation of reversible complex EI to alkylated enzyme EI' is the same. Overall bimolecular rate constant (k_2) calculated as $k_2=k_a/K_D$ were 3080 $mol^{-1}l^{-1}min^{-1}$ in the absence of THA and 461 $mol^{-1}l^{-1}min^{-1}$ in its presence. The ratio of both constants is 6.68.

Discussion

The present results showed that DSP-4 as well as two other derivatives of this compound are able to alkylate the AChE. The aziridines are compounds which resemble the enzyme's natural substrate acetylcholine but contain a respective functional group which can form a covalent adduct with the AChE after the molecule has reached the active site and may be classified as irreversible active-site-directed inhibitors (2). Our study indicates that association of aziridinium compounds with the enzyme is a relatively slow second-order reaction.

From the comparison of the second-order rate constants of alkylation of all three tested compounds, it is clear that DSP-4 inactivates AChE the most quickly. Debromation of DSP-4 (compound OS-21) decreased the rate of AChE alkylation more than 10–fold. Yet more decrease of k_2 value was observed at compound OS-23, which is a homologue of OS-21.

Pretreatment of the AChE by THA, hydrophobic-site-directed reversible inhibitor of the AChE (5,6), decreased the rate of alkylation by all three aziridinium compounds. Our results showed that the ternary complex EIT between the enzyme (E), aziridinium inhibitor (I) and THA (T) is formed. THA binds to the hydrophobic site of AChE while aziridinium compounds binds to the anion site of the enzyme (12). THA protects the enzyme against alkylation by aziridinium compounds by allosteric mechanism. It is probable that also other hydrophobic-site-directed inhibitors of

AChE protect this enzyme against alkylation by aziridinium ions. On the contrary, Palumaa and Järv showed that the presence of alkylboronic acids, which are known to be esteratic-site-directed reversible inhibitors of the AChE (3), accelerated the alkylation of AChE by aziridinium ions.

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Ing. Kamil Kuča,
Faculty of Military Health Sciences,
Department of Toxicology,
Třebešská 1575, 500 01 Hradec Králové,
Czech Republic.
e-mail: kucakam@pmfhk.cz