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Toxicological Effect of Herbicides (Diuron and Bentazon) on Snake Venom and Electric Eel Acetylcholinesterase

Mushtaq Ahmed · Nadia Latif · Rehmat Ali Khan · Akhlaq Ahmad

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Abstract The toxicological effects of the active ingredients of the herbicides diuron and bentazon on the activity of acetylcholinesterase (AChE) of krait (*Bungarus sindanus*) venom and electric eel (*Electrophorus electricus*) were studied. The diuron and entazon caused non-competitive inhibition of AChE from both species. For the venom AChE, the calculated IC50 for diuron and bentazon were found to be 3.25 and 0.14 μM, while for eel AChE, the respective IC50 values were 3.6 and 0.135 μM. In comparison, bentazon was a more potent inhibitor than diuron of AChE from both species. The insecticide lindane did not have any inhibitory effect on AChE activity in either species, even when tested at high concentrations (200–800 μM).

Keywords Snake venom · Acetylcholinesterase · Inhibition · Kinetics · Pesticides

The toxicity of organophosphate (OP) insecticides is mostly related to its inhibitory effect on acetylcholinesterase (AChE), which hydrolyses the neurotransmitter acetylcholine (ACh) in cholinergic synapses of both the central and peripheral nervous system (Ecobicon and Corneau 1973). Inhibition of AChE leads to the accumulation of acetylcholine in synapses resulting in excessive stimulation of cholinergic receptors in the receiving (post-synaptic) neurons (Pope 1999), causing disturbance of numerous body functions and finally death due to respiratory failure (Eddleston et al. 2006).

M. Ahmed (✉) · N. Latif · R. A. Khan · A. Ahmad
Department of Biotechnology, University of Science and Technology, Bannu, KPK, Pakistan
e-mail: mushtaq213@yahoo.com

Acetylcholinesterase (AChE; E.C.3.1.1.7), or true cholinesterase, is a non proteolytic enzyme mainly found in synaptic tissue of muscles, brain, erythrocytes and cholinergic neurons, and plays an essential role in controlling physiological events (Ecobicon and Corneau 1973; Prody et al. 1987; Dave et al. 2000). Frobert et al. (1997) reported that this enzyme is also present in non-synaptic tissue (i.e., snake venom). They reported that the krait (*Bungarus*) genus had the highest amount of AChE (8 mg/g of dry venom), and that the enzyme was very active. We did not find any other source containing a higher amount of AChE, including the electric organ of electric eel.

Agricultural areas and rivers may become contaminated with pesticides after their application to crops.The herbicides diuron (1-(3,4-dichlorophenyl)-3,3-dimethylurea) and bentazon (3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide) are in common use in agriculture practice in Pakistan. A recently published study reported the herbicide glyphosate inhibit AChE activity (Menéndez-Helman et al. 2012).The goal of the present study was to evaluate the toxicological effects of diuron and bentazon on AChE from the krait and electric eel. An organochlorine insecticide, lindane, which does not act by inhibition of AChE, was also studied for comparison with the herbicides.

Materials and Methods

Acetylthiocholine iodide, DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], bovine serum albumin, ethopropazine, electric eel AChE (catalog number C3389) and coomassie brilliant blue R-250 were purchased from Sigma (St. Louis, MO, USA). Diuron (97.5 %), bentazon (97 %) and lindane (85.5 %) pure were purchased from Dr. Ehrenstorfer GmbH.

Venom from live adult krait snakes was squeezed out manually (Siddiqi et al. 1991), mixed up, lyophilized immediately and aliquots were stored at −20°C for further used.

AChE activity was determined following the Ellman procedure (Ellman et al. 1961) with some modification (Rocha et al. 1993). For AChE, the assay mixture (1 ml) contained 4 µg snake venom protein or 0.1 unit of pure electric eel AChE, and 0.2 mM DTNB as chromogen in 62 mM phosphate buffer (pH 7.4). The reaction mixture (except control) was pre-incubated with different concentrations (1–4.05 µM) of diuron and bentazon (1–3 µM) at 37°C for 10 min. Ethopropazine (0.06 mM) was used in the assay in order to inhibit any contamination of butrylcholinesterase (BChE) in snake venom. The reaction was started by addition of different concentrations of substrate (0.05–1 mM). The hydrolysis was monitored by verifying the formation of the thiolate dianion of DTNB at 412 nm at 10 s intervals over 2–3 min, using Hitachi U-2001 spectrophotometer (Tokyo, Japan).

IC50 was calculated according to the Dixon and Webb (Dixon and Webb, 1964) plot using 1/V versus [I], and was confirmed by using a simple plot of percentage activity and inhibition versus concentration of an inhibitor (Ahmed et al. 2007). The kinetics of herbicides with cholinesterase were determined using the Lineweaver–Burk (1934), double reciprocal plot, by plotting 1/V against 1/S analyzed over a range of acetylthiocholine concentrations (0.05–1 mM) in the absence and in the presence of diuron (1.35–4.05 µM), bentazon (0.1–0.3 µM) while insecticides, Lindane was used in the range from 200 to 480 µM.

The data are the means of values derived from three separate experiments. Statistical analysis was performed using one-way ANOVA, which was followed by post hoc analysis (Duncan multiple range test).

Tissue protein was determined by the Coomassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford 1976).

Enzyme parameters were analyzed by two-way ANOVA followed by Tukey–Kramer multiple range tests when appropriate. All data are expressed as mean ± standard deviation, with significance level $p \leq 0.05$.

Results and Discussion

In the present study, the pesticides diuron and bentazon inhibited snake venom (Fig. 1) and electric eel (Fig. 2) AChE in a dose-dependent manner, while lindane had no toxic effect, even when tested at higher concentrations (Figs. 1, 2). We observed that diuron was less toxic than bentazon in terms of AChE inibition for both species.

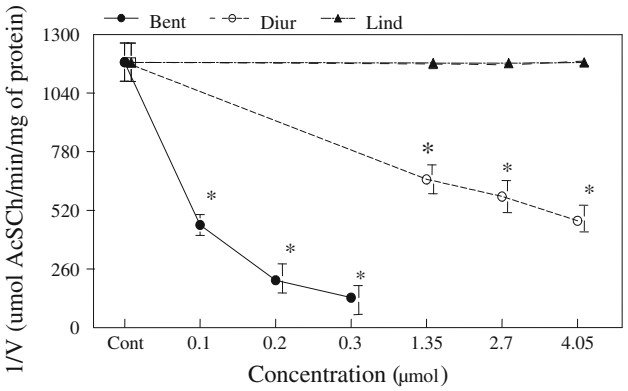

Fig. 1 Inhibition of krait venom AChE activity at a fixed substrate concentration (0.5 mM) in the absence and presence of bentazon, diuron and lindane. Data points represent the means of three different experiments. *Asterisks* (*) represent differences from controls at $p < 0.00017$ for bentazon and $p < 0.005$ for diuron. Lindane showed no effect

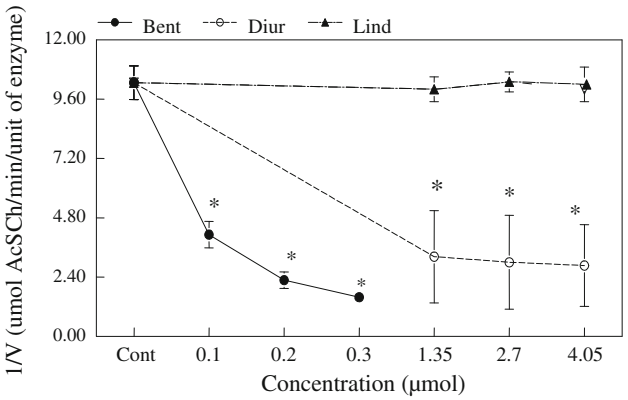

Fig. 2 Inhibition of electric eel AChE at a fixed substrate concentration (0.5 mM) in the absence and presence of bentazon, diuron and lindane. Data points represent the means of three different experiments. *Asterisks* (*) represent differences from controls at $p < 0.005$

AChE was first discovered in the 1930s, and it was found to be one of the most efficient enzymes with a high turnover number (Frobert et al. 1997). In snake venom, it is present in a monomeric form having molecular weight of 74 kDa while in all other sources it is present in a multimeric form (Cousin et al. 1996; Massoulié and Bon 1982). The enzyme has two sites for substrate binding: an acylation or A-site and peripheral or p-site (Rosenberry et al. 2005). Structure–activity relationship (SAR) studies revealed that there are probably two binding domains in the active site: an ionic site containing a glutamate residue that can bind with the cationic head of acetylcholine (ACh) and an esteratic site that contains active serine and histidine residues (Sussman et al. 1991). The hydrolysis of the neurotransmitter ACh occurs at the serine-containing esteratic site, which is functionally coupled to a substrate binding area containing a distinct anionic locus (Barak

et al. 1994; Froede and Wilson 1971; Rosenberry et al. 1975; Quinn et al. 1987). Thus, binding of any other ligand at a p-site will block the entrance of substrate toward the active site. As a result, the rate of hydrolysis of ACh at an active site, or A-site, will decrease. In the present study, the pesticide bentazon may have interacted at the p-site of krait venom (Fig. 3a) and electric eel (Fig. 3b) AChE, causing non-competitive inhibition. This would have resulted in a decreased rate of substrate hydrolysis at the active site.

We clearly observed that both K_m and V_{max} decreased with increased bentazon concentrations (Fig. 3), showing non-competitive inhibition with snake venom and electric eel AChE. The double reciprocal plots showed a similar pattern of inhibition for diuron (Fig. 4). In comparison, the

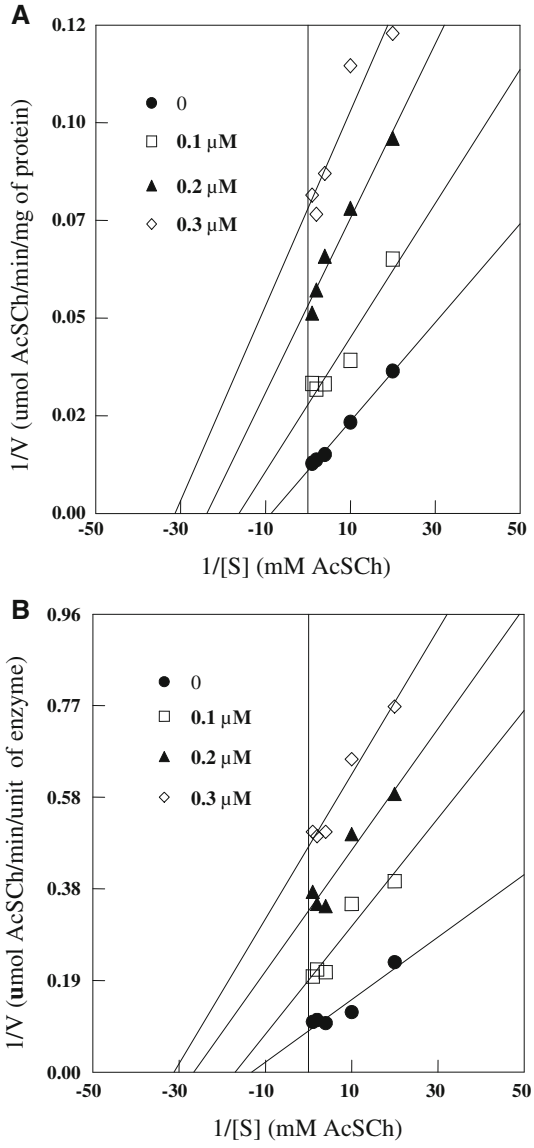

Fig. 3 Lineweaver-Burk double reciprocal plots of krait venom (**a**) and electric eel (**b**) AChE experiments in the absence and presence of bentazon. Data points are the means of three separate experiments

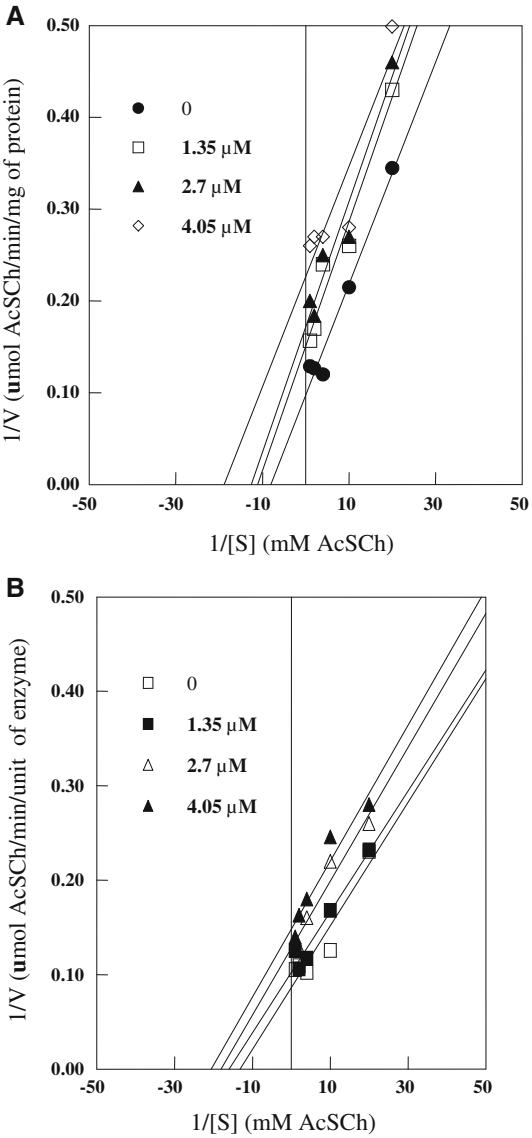

Fig. 4 Lineweaver-Burk plots for krait venom (**a**) and electric eel (**b**) AChE experiments in the absence and presence of diuron. Data points are the means of three separate experiments

commercial herbicide paraquat causes a mixed type inhibition with snake venom AChE (Ahmed et al. 2007). It may be due to the different structures of the pesticides. The K_{mapp} and V_{maxapp} values of snake venom and electric eel AChE decreased in the presence of different concentrations of bentazon and diuron (Tables 1, 2, 3, 4). This is an indication that the type of inhibition is non-competitive. The calculated IC50 values show that diuron has potent inhibitory effect on krait venom and bentazon on electric eel AChE (Table 5).

In conclusion, this study has shown that the two herbicides investigated, diuron and bentazon, inhibited the activity of AChE obtained from krait venom and the electric eel. This finding is important, since AChE

Table 1 Effect of bentazon on K_{mapp} and $V_{maxiapp}$ of krait venom AChE

Bentazon (μM)	K_{mapp} (mM)	% Decrease	$V_{maxiapp}$ (μmol/min per mg)	% Decrease
0	0.124	0	1023	0
0.1	0.095	24	537	48
0.2	0.039	69	224	78
0.3	0.031	75	132	87

Table 2 Effect of bentazon on K_{mapp} and $V_{maxiapp}$ of electric eel AChE

Bentazon (μM)	K_{mapp} (mM)	% Decrease	$V_{maxiapp}$ (μmol/min per unit enzyme)	% Decrease
0	0.075	0	11.6	0
0.1	0.05	33	5	55
0.2	0.037	51	3	75
0.3	0.03	60	2	82

Table 3 Effect of diuron on K_{mapp} and $V_{maxiapp}$ of krait venom AChE

Diuron (μM)	K_{mapp} (mM)	% Decrease	$V_{maxiapp}$ (μmol/min per mg)	% Decrease
0	0.124	0	1,023	0
1.35	0.09	27	662	35
2.7	0.08	35	582	43
4.05	0.053	57	441	57

Table 4 Effect of diuron on K_{mapp} and $V_{maxiapp}$ of electric eel AChE

Diuron (μM)	Km_{app} (mM)	% Decrease	$V_{maxiapp}$ (μmol/min per unit enzyme)	% Decrease
0	0.075	0	11.6	0
1.35	0.06	20	9.7	15.7
2.7	0.055	27	7.8	32.6
4.05	0.048	36	6.7	41.8

Table 5 IC50 values for inhibition of krait venom and electric eel AChE activity in the presence of diuron and bentazon herbicides

	Diuron (μM)	Bentazon (μM)
Krait venom	3.25	0.14
Electric eel	3.6	0.135

inhibition has been generally regarded as a function of OP insecticides.

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