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In Vitro Acetylcholinesterase Inhibition by Novel OP Compounds in Various Tissues of the Fish *Channa punctatus*

M. F. Rahman, M. Mahboob, P. Grover

Biochemical Toxicology, Biology Division, Indian Institute of Chemical Technology, Hyderabad 500 007, India

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Organophosphorus (OP) compounds are used extensively as agricultural pesticides. But the potential of OP compounds to cause acute effects has posed occupational threats to workers employed in the manufacture and application of pesticide (Rahman et al. 1999). This has necessitated developing new pesticides with more specificity for the target pests and less toxicity to the non-target organisms. Therefore, the Indian Institute of Chemical Technology, Hyderabad has synthesized two novel phosphorothionates and designated them as RPR-II and RPR-V. An Indian patent was obtained for the insecticidal activities of these compounds bearing No.165259 (Rani et al. 1989). In our earlier studies we found that RPR-II and RPR-V were less toxic to the non-target species but effective against target pests and also less neurotoxic when compared with monocrotophos (MCP) (Siddiqui et al. 1993). We have also reported the sub-chronic effect of RPR-II and RPR-V on some biochemical enzymes in rats (Rahman et al. 1997; 1999; 2000). The structures of RPR-II (2-butenoic acid-3-(diethoxy phosphinothioyl)-methyl ester, RPR-V (2-butenoic acid-3-(diethoxy phosphinothioyl)-ethyl ester and MCP (Phosphoric acid dimethyl (1-methyl-3-(methylamino)-3-oxo-n-propenyl ester are shown in Figure 1. The main toxic effect of OP compounds is the inhibition of acetylcholinesterase (AChE). Hence, determination of AChE (EC 3.1.1.7) activity is used in clinical practices and environmental biomonitoring studies. Biomarkers, i.e. biochemical, physiological or histological changes indicate xenobiotic exposure/effect and can constitute screening tools for pollutant exposure. The measurement of AChE activity in fish is well accepted as a method for diagnosing exposure to OP compounds (Wogram et al. 2001) and it was also reported that AChE represents one of the oldest biomarkers in fish (Sturm et al. 2000). OP compounds phosphorylate AChE and inhibit its activity causing accumulation of acetylcholine (ACh) at the nerve synapse, which leads to disruption of the central nervous system and eventually death of the animal. The properties of AChE differ from species to species and also show variations in different tissues of the same species. *In vitro* systems have been suggested as economical and efficient alternatives to animal testing for OP toxicity (Barber et al. 1999).

In the present investigation, *in vitro* studies on AChE were carried out in RBC, brain and liver, of the fish *Channa punctatus* with RPR-II and RPR-V, and the

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Correspondence to: M. F. Rahman

results obtained were compared with MCP. The fifty percent inhibition concentration (IC_{50}) and double reciprocal plots were determined for these compounds. The aim of the present study was to determine the relative toxicity of these three OP compounds as related to the comparative neurotoxic potential and the mechanism of AChE potential in different tissues of fish.

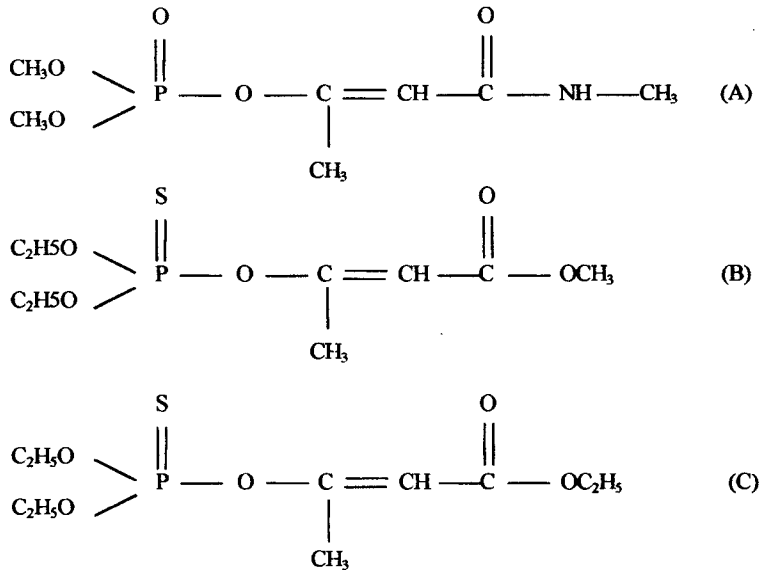

Figure 1. Structure of (A) phosphoric acid dimethyl (1-methyl-3-(methylamino)-3-oxo-n-propenyl)ester or monocrotophos (MCP), (B) 2-butenoic acid-3 (diethoxyphosphino-thioyl)-methyl ester (RPR-II) and (C) 2-butenoic acid-3-(diethoxyphosphinothioyl)- ethyl ester (RPR-V).

MATERIALS AND METHODS

Technical grade MCP was obtained through the courtesy of NOCIL, Mumbai. RPR-II and RPR-V were synthesized conveniently from diethylthio phosphoryl chloride and the respective 1,3 dicarbonyl compounds following the procedure of Jones and Badesha (1981). Tris HCl and quinidine sulphate were purchased from Sigma Chemical Co., USA and all other remaining chemicals were of analytical grade. Live fishes (*Channa punctatus*) weighing 100 – 150 gm were procured from a local market and blood was collected in heparin directly by heart puncture. The fishes were dissected to obtain tissues like brain and liver. They were homogenized in ice cold (0.8 M) sucrose using a Yorco homogenizer. Ten percent (W/V) homogenate was centrifuged at 10,000 g for 10 min at 4°C and the pellet was discarded and supernatant kept at – 80°C in a deep freezer till the completion of the study. Diluted blood (1/25) or the supernatant of brain and liver were used as the enzyme source and AChE activity was assayed following the method of Ellman et al. (1961) as modified by Chambers and Chambers (1989). 4.0 ml total volume of incubating mixture (0.1 M Tris HCl buffer, pH 7.4; 1.0 mM

acetylthiocholine iodide; 0.1 percent quinidine sulphate) and 25 µl of the diluted blood or 10 µl of tissue homogenate were taken. To this, different concentrations of RPR-II, RPR-V (in alcohol) and MCP (in water) were incubated (10 µl) for 15 min with shaking at 37°C. The reaction was then stopped with a mixture of 5,5'-dithiobis (2-nitrobenzoic acid) [DTNB] and sodium dodecyl sulphate (SDS) yielding a final concentration of 0.04 and 0.44 percent respectively. Blood/tissues were collected from six fishes and each assay was run in triplicate for each concentration. The absorbance was read at 412 nm on a Shimadzu double beam Spectrophotometer. Percentage inhibition of AChE at varying concentrations (more than six) of RPR-II/RPR-V/MCP was estimated and the IC_{50} concentration with 50 percent inhibition was determined by a computer program. The double reciprocal plots of the data were constructed according to Lineweaver and Burk (1934). The apparent maximum AChE velocity (Vmax) and apparent Km values were obtained by taking the reciprocal of four different concentrations of substrate (mM) on the x - axis and reciprocal of enzyme activity (µ moles AChE) on the y - axis. Protein was determined as described by Lowry et al (1951) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

This study was conducted to investigate the effect of the newly synthesized OP compounds RPR-II and RPR-V on *in vitro* AChE assays (IC_{50}'s) in RBC, brain and liver of fish and these activities were compared with MCP. The biochemical enzymatic variations are powerful predictive tool in assessment of toxicity (Rahman et al. 1999). AChE is the enzyme that hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses of both invertebrates and vertebrates.

The present study showed that RPR-II, RPR-V and MCP inhibited AChE activity in RBC, brain and liver of the fish. The IC_{50} observed for RBC AChE with RPR-II and RPR-V was greater than 10.00 mM, whereas the IC_{50} for MCP was 0.89 mM (Table 1). Similarly for brain AChE, the IC_{50} observed for RPR-II, RPR-V and MCP were 1.28, 0.27 and 0.13 respectively. The relative potency showed that RPR-II was 10 times less potent, whereas RPR-V was 2 times less potent than MCP (Table 2). For liver AChE, the IC_{50} obtained for RPR-II was 9.70 mM, in case of RPR-V it was >10.00 mM and for MCP the IC_{50} observed was 0.84. Further, the relative potency showed that RPR-II was 12 times less potent than MCP (Table 3).

In vitro studies showed that maximal AChE velocity (Vmax) and Km values decreased with all three OP compounds (Tables 1-3). However, in the case of RBC AChE, the Vmax decreased from 8.33 to 3.70, whereas the Km value decreased from 0.83 to 0.37. Similarly, for brain AChE the Vmax decreased from 38.46 to 12.50 and Km decreased from 16.66 to 0.40. Further, for liver AChE the Vmax decreased from 20 to 1.69 and Km from 2.00 to 0.45 (Tables 1-3). Moreover, for these compounds the maximum inhibition was at 15 minutes. Further, these compounds inhibited this target enzyme in a concentration and a time dependent manner.

Table 1. Alterations in fish RBC AChE activity exposed to RPR-II, RPR-V and MCP.

Compound	IC_{50} (mM)	Vmax	Km
Control	-	8.33	0.83
RPR-II	> 10.00	6.66	0.64
RPR-V	> 10.00	5.00	0.50
MCP	0.89	3.70	0.37

Table 2. Alterations in fish brain AChE activity exposed to RPR-II, RPR-V and MCP.

Compound	IC_{50} (mM)	Relative Potency	Vmax	Km
Control	-	-	38.46	16.66
RPR-II	1.28	0.10	14.28	0.62
RPR-V	0.27	0.48	20.00	0.80
MCP	0.13	1.00	12.50	0.40

Table 3. Alterations in fish liver AChE activity exposed to RPR-II, RPR-V and MCP.

Compound	IC_{50} (mM)	Relative Potency	Vmax	Km
Control	-	-	20.00	2.00
RPR-II	9.70	0.08	3.70	0.57
RPR-V	> 10.00	-	3.12	0.80
MCP	0.84	1.00	1.69	0.45

In our earlier studies with acute doses of RPR-II, RPR-V and MCP we found that these compounds inhibited RBC AChE and various ATPases thereby showing their effect on both synaptic nerve transmission and nerve conduction (Siddiqui et al. 1993). Similarly, it was reported that benthiocarb inhibited *in vitro* fish brain AChE activity in a concentration dependent manner (Babu et al. 1989) while metacid-50 and carbaryl also inhibited *in vitro* brain AChE of *Channa punctatus* (Ghosh and Bhattacharya 1992). It was also reported that chlorfenvinphos, diazinon and carbofuran significantly inhibited *in vitro* and *in vivo* brain AChE in carp (*Cyprinus carpio)* and suggested that carp brain AChE can be a good diagnostic tool for OP and carbamate pollution (Dembele et al. 2000). The results observed in the current study are in agreement with the above reports. Our results indicated that the brain tissue was more sensitive compared to RBC and liver. It was also reported that brain AChE activity is a major target of OP compounds. Its inhibition either directly causes or is an indirect indicator of acute CNS and PNS symptoms (Bakshi et al. 2000). Blood is the only available tissue in humans especially in occupational workers. Hence blood is the tissue of choice and RBC cholinesterase is an excellent indicator of its effect on nerve synapses (Vadekar 1980).

The present study revealed that RPR-II, RPR-V and MCP have inhibited the target enzyme AChE in RBC, brain and liver of fish. MCP was a more potent inhibitor

than RPR-II and RPR-V. However, RPR-II and RPR-V were equally toxic with regard to RBC AChE, whereas with brain AChE, RPR-V was a more potent inhibitor than RPR-II and the reverse trend was observed for liver AChE. This clearly indicated that these compounds showed a structure relationship pattern. MCP has a P=O moiety whereas RPR-II and RPR-V have P=S moieties in their structures. As such MCP was found to be more toxic than RPR-II and RPR-V. The metabolic conversion of thiophosphoryl (P=S) ester to the corresponding phosphoryl (P=O) ester mediated by mixed function oxidation makes them highly potent cholinesterase inhibitors. Similar to RPR-II and RPR-V, parathion also contains a P=S moiety in its structure. It was reported that parathion will be oxidized by monooxygenases in animals and is thereby changed to a derivative containing the P=O group and this resulting analogue will be a more powerful inhibitor of cholinesterase than the original thion phosphate (Hassal 1982). Similarly, Ma et al. (2003) reported methyl paraoxon was 1,000 fold more potent inhibitor of *in vitro* brain AChE in rat than methyl parathion.

AChE is considered as a specific biomarker enzyme for OP and carbamate pesticide exposures, being commonly used to diagnose exposure of natural populations to these chemicals. The results of this *in vitro* study shows that all three compounds decreased Vmax and Km values, indicating un-competative inhibition. Similarly, it was reported that MCP inhibited *in vitro* fish brain by altering Km and Ki (Qadri et al. 1994). It was also reported that MCP inhibited brain AChE in the Nile Tilapia (*Oreochromis niloticus)* fish (Thangnipon et al. 1995).

In conclusion the present study reports the comparative effects of RPR-II, RPR-V and MCP, revealing that RPR-II and RPR-V were less neurotoxic than MCP. Further, the *in vitro* model provides a potential pre-screen for novel chemical entities and compounds. This approach could lead to the reduction in the number of animals used in development of new compounds.

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