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COMPARATIVE RESPONSES OF ACETYLCHOLINESTERASE (AChE) OF RAT BRAIN AND MODEL INVERTEBRATE *Caenorhabditis elegans in vitro*

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ABSTRACT

This *in vitro* study was aimed to compare the kinetic properties of acetyl-cholinesterase (AChE) in two model systems i.e. rat brain homogenate and nematode model *Caenorhabditis elegans.* These models were analyzed with basic kinetic parameters *viz.*, substrate hydrolysis, inhibition pattern, determined Km, V_{max}, IC_{50}, KI and V_{imax} using dichlorvos (DDVP), an organophosphorus compound widely used for insect-pest control in agricultural crops. The rat brain homogenate exhibited K_m, V_{max} and IC_{50} values of 59 μM, 33 nmol min^{-1} mg^{-1} protein and 107.18 ± 3.56 nM, respectively, for rat brain homogenate. *C. elegans* homogenate exhibited K_m, V_{max} and IC_{50} values of 725.41 μM, 112.35 nmol min^{-1} mg^{-1} protein and 45.7 ± 2.68 nM, respectively. The pattern of inhibition was irreversible in both the systems. The study showed that *C. elegans in vitro* system can be used as a model to establish the inhibition pattern of organophosphorus compounds (known inhibitors of AChE) as compared to traditionally used rat brain homogenate system.

Key words: Acetylcholine, acetylcholinesterase, *Caenorhabditis elegans*, organophosphorus insecticides, rat brain homogenate

INTRODUCTION

Cholinesterase enzymes are widely distributed across the animal kingdom. Acetylcholinesterase [AChE] is perhaps the only cholinesterase which has received maximum attention of researchers worldwide due to its role in the regulation of neurotransmission and toxicity of environmental pollutants. In neurotransmission, AChE plays a crucial regulatory role in cholinergic systems by terminating the nerve impulse in a process involving hydrolysis of neurotransmitter, acetylcholine [ACh] (Rand, 2007). AChE is also a functional target of organophosphorus insecticides (OPI), which act as pseudo-substrate to inhibit the enzyme by phosphorylating the serine-OH group of active site (Marcell *et al.*, 1998). The acute toxicity of OPI has been explained on the basis of cholinergic overstimulation resulting from inhibition of AChE.

ACh is the primary excitatory neurotransmitter involved in nematode *Caenorhabditis elegans* motor function, as in higher animals (Rand and Nonet, 1997). Similarly, synaptic degradation of ACh by acetylcholinesterase (AChE) enzyme is conserved in *C. elegans* (Combes *et al*., 2001).

Monitoring of AChE activity in wildlife populations has been proposed as a general method for detecting environmental contamination from OPI (Gagnaire *et al*., 2008). In laboratory studies, detection of residual AChE has extensively been employed as an index of OPI toxicity following *in vivo* exposures. AChE inhibitory potential of dichlorvos and other OPI in *C. elegans* following *in vivo* exposure have been reported (Rajini *et al*., 2008; Jadhav and Rajini, 2009). Similarly, rat brain homogenate system has extensively been used *in vitro* to understand various enzyme inhibition patterns by organophosphates. In this study, we utilized a new, non-target, invertebrate model *C. elegans*, an emerging model system to understand *in vitro* inhibition pattern to organophosphorus compound dichlorvos (Jadhav and Rajini, 2009a,b). Our earlier reports have revealed the expression of heat shock proteins decreased brood size, paralysis and nose contraction on exposure to dichlorovos (Jadhav and Rajini, 2009a,b). Hence, this model system was explored *in vitro* to compare the kinetics model. Also, the study was taken up to understand substrate saturation and inhibition pattern to dichlorvos *in vitro.*

MATERIALS AND METHODS

Acetyl thiocholine iodide (ATCI), BSA (fraction V) and 5,5-dithio-bis-2-nitrobenzoic acid [DTNB] were procured from Sigma Chemical Co., St. Louis, MO, USA while dichlorvos [DDVP; technical grade; 97.6%] was gifted by Hyderabad Chemical Supplies Ltd., India. All other reagents used were of analytical grade. Stock solution [5 Mm] of dichlorvos [DDVP] was prepared in double distilled water and further diluted to obtain working solutions for *in vitro* assays.

Worm culture and preparation of C. elegans homogenate

The nematodes *Caenorhabditis elegans* were cultivated on NGM medium (NaCl 3 g, protease peptone 2.5 g, cholesterol 5 mg, $CaCl_2$ 1 mmol L^{-1}, $MgSO_4$ 1 mol L^{-1}, potassium phosphate 25 mmol L^{-1}, pH 6.0 and agar 17 g L^{-1} on an established lawn of *Escherichia coli* strain OP-50 [Brenner (1974)] and maintained at 20°C. The worms used were larva L4 (3.5 d) obtained from a synchronous culture. The worms were prepared for assays by washing them from the petriplates into a centrifuge tube and allowing them to settle by gravity. The worms were rinsed three times with K-medium (Williams and Dusenbery, 1990) and used for *in vitro* assay. Worms [gravid stage/ 3.5d old] were harvested and homogenized in 50mM tris HCl {pH 7.4}. Homogenates were centrifuged at 10,000 rpm for 10 min and supernatant was stored at –20°C until the assay.

Animal care and preparation of rat brain homogenate

All procedures with animals were conducted strictly in accordance with approved guidelines by the Institute Animal Ethical Committee regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA], Ministry Social Justice and Empowerment, Government of India. During the experiments, maximum care was taken to minimize animal suffering. One rat was sacrificed under mild anesthesia and their brains were excised, washed and placed on ice. Homogenate (10%) was prepared [50 mM tris HCl (pH 7.4)] and supernatant used for *in vitro* assay.

Acetylcholinesterase (AChE) assay

Worm homogenate, prepared with 50 mM tris buffer (pH 7.4), was centrifuged at 10,000 rpm for 10 min. and supernatant separated. After protein estimation the supernatant equivalent to 250 µg protein was used for AChE assay with 1.5 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) and various concentrations of acetyl thiocholine iodide (ATCI). The contents were rapidly mixed and the rate of change in absorbance measured at 10 sec. intervals for 90 sec. spectrophotometerically at

405 nm. Kinetic measurements were recorded and converted to total cholinesterase activity using extinction coefficient value 1.36 $x10^4$ for the colored product, 5-thio-2-nitro-benzoic acid (Ellman *et al*., 1961).

The acetylcholinesterase activities were determined as per the method of Ellman *et al*. (1961) with slight modifications suggested by Zhu and Clark (1994) using various concentrations of substrate, ATCI, ranging from 0.10 to 10 mM. Various kinetic parameters *viz*., Michaelis constant [K_m], maximum velocity [V_{max}], and catalytic efficiency [V_{max}/K_m] were determined for AChE of rat brain and *C. elegans* from the respective Lineweaver-Burk (LB) plot.

Determination of IC_{50}

In vitro inhibition of AChE by dichlorvos was monitored by incubation of both homogenate systems *in vitro* with dichlorvos ranging from 5 to 200 nM. Following incubation of both the homogenate systems for 30 min. at 24°C. AChE activity was assayed as described above and IC_{50} determined by linear regression analysis of percent inhibition v/s dichlorvos concentration.

Kinetics properties of acetylcholinesterase by dichlorvos

Enzyme from the two sources were incubated with IC_{25}, IC_{50}, and IC_{75} concentrations of dichlorvos for 30 min. at 24°C and residual AChE activity measured in presence of increasing concentration of ATCI [0.10 to10 mM]. The K_m and V_{max} was calculated from LB plot. The Michaelis constant [K_m], maximum velocity [V_{max}] and catalytic efficiency [V_{max}/K_m] were determined from LB plot.

Protein estimation

For protein estimation, 10 µL *C. elegans* homogenate was added to 490 µL distilled water and incubated with 2.5 mL alkaline copper sulfate solution for 10 min. at at 24°C. The 250 µL Folin's reagent [1:1 diluted with water] was added. After 30 min. incubation, colour was measured spectrophotometerically at 670 nm. Bovine serum albumin [BSA] was used as a standard.

RESULTS AND DISCUSSION

The K_m, V_{max}, IC_{50}, K_i and $V_{i\ (max)}$ of AChE for both rat brain and *C. elegans* homogenate systems were determined for comparison. The kinetics of AChE in presence of acetyl thiocholine iodide (ATCI) exhibited substrate saturation in both the systems (Fig. 1, Table 1). AChE activities decreased remarkably when ATCI substrate concentration was higher than optimum thereby showing a characteristic phenomenon of substrate inhibition at higher concentrations. Rat brain homogenate AChE showed typical saturation at 2 mM ATCI and in *C. elegans* AChE the saturation was obtained at >5 mM ATCI. Interestingly, kinetic parameters for ATCI substrate, obtained from LB plot which assess substrate affinity to AChE as indicated by K_m values, revealed K_m for rat AChE as 59 µM and for *C. elegans*AChE as 725.41 µM. This showed that affinity of rat brain AChE to ATCI was 12.6-fold higher than *C. elegans* AChE. However, the efficiency of substrate being hydrolyzed by AChE, as indicated by V_{max} values, were 112.35 nmol min^{-1} mg^{-1} protein and 33 nmol min^{-1} mg^{-1} protein for rat brain and *C. elegans* AChE, respectively (Fig. 2, Table 1).

The IC_{50} is the concentration of dichlorvos required to inhibit AChE activity by 50% at room temperature on 30 min. incubation. The IC_{50} for *C. elegans* and rat brain AChE were 45.7±2.68 and 107.18±3.56 nM, respectively (Fig. 3, Table 2).

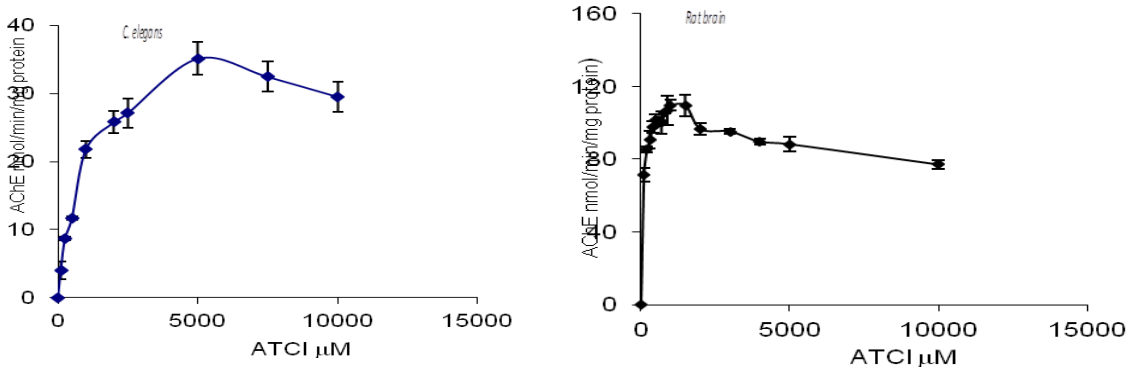

Fig. 1: Effect of substrate concentrations on rat brain and *C. elegans* AChE. Values are the mean of two sets with duplicate at each concentration

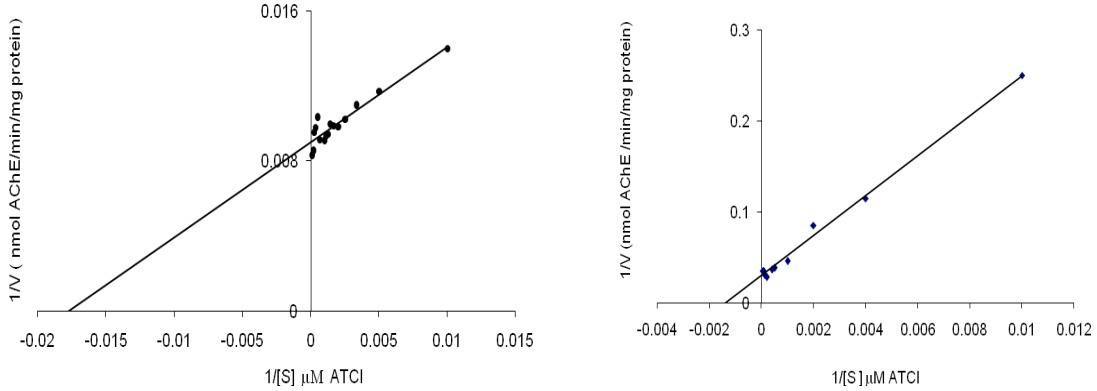

Fig. 2: Lineweaver-Burk plot for AChE activity in *C. elegans* and rat brain

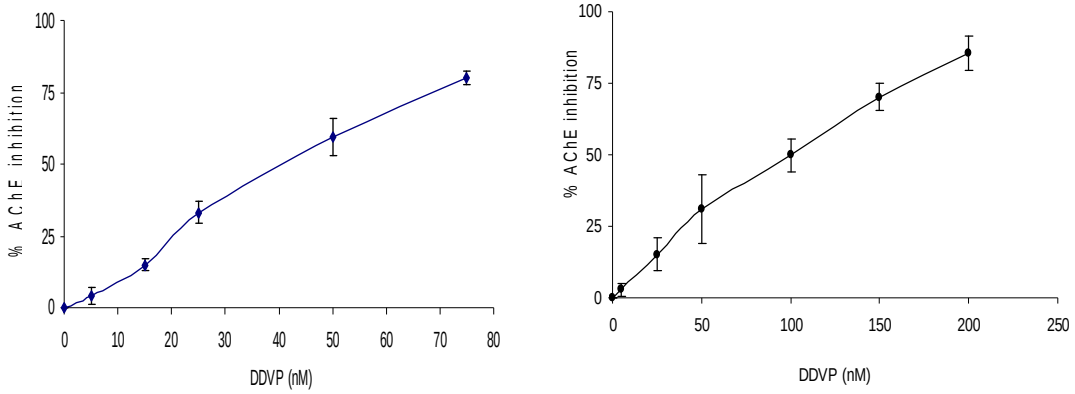

Fig. 3: Inhibition pattern of acetylcholinesterase by dichlorvos (DDVP) in *C. elegans* and rat brain homogenate

Table 1: Inhibitory concentrations of dichlorvos in *C. elegans* and rat brain AChE

Homogenate	Dichlorvos (nM)		
	IC_{25}	IC_{50}	IC_{75}
C. elegans	22.85 ± 1.34	45.7 ± 2.68	68.56 ± 4.02
Rat brain	49.18 ± 2.68	107.18 ±3.56	165.17 ±6.42

Table 2: Kinetic characteristics of *C. elegans* and rat brain AChE in presence of dichlorvos

Dichlorvos	*C. elegans*			Rat brain		
(nM)	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
IC_{25}	789.27 ± 0.56	13.00 ± 0.69	0.016	57.31 ± 0.54	61.72 ± 0.87	1.07
IC_{50}	734.70 ± 0.85	10.10 ± 0.78	0.013	57.48 ± 0.98	42.55 ± 0.65	0.74
IC_{75}	800.27 ± 0.48	6.94 ± 0.65	0.0008	56.83 ± 0.69	33.00 ± 0.58	0.60

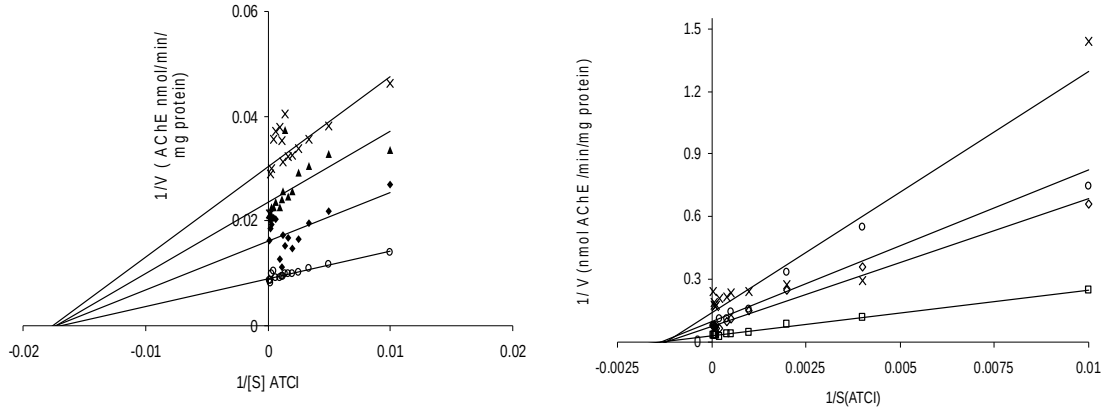

Fig. 4: Inhibition of AChE activity in *C. elegans* and rat brain homogenates with IC_{25}, IC_{50} and IC_{75} concentration of dichlorvos plot of reciprocal of initial velocities v/s reciprocal concentrations of ATCI (left) *C. elegans* (right) rat brain

As determined from L-B plot, the K_m, V_{max} and V_{max}/K_m values of inhibited AChE of rat brain at IC_{50} concentration of dichlorvos were 57.48 µM, 42.55 nmol ATCI hydrolyzed min^{-1} mg^{-1} protein and 0.74 min^{-1} mg^{-1} protein, respectively (Fig. 4, Table 3). For *C. elegans* the K_m, V_{max} and V_{max}/K_m at IC_{50} were 734 µM, 10.10 nmol ATCI hydrolyzed min^{-1} mg^{-1} protein and 0.013 min^{-1} mg^{-1} protein. The increase in velocity was linear with increase in substrate concentration. LB plot revealed the inhibition of AChE in both the system to be of irreversible in nature.

Table 3: Linear relationship of velocity with substrate concentration with significant at 1% level of significance (most significant)**

Dichlorvos	*C. elegans*		Rat brain	
(nM)	Co-efficient B	Standard error	Co-efficient B	Standard error
V_0	11.952**	1.486	0.525**	0.050
V_{25}	60.695**	2.578	0.928**	0.318
V_{50}	72.736**	6.888	1.351**	0.389
V_{75}	115.24**	12.359	1.72**	0.502

DISCUSSION

In present study Ellman *et al.* (1961) assay was used to assess acetylcholinesterase inhibition by dichlorovos. This assay is a standard format to assess the activity by using substrate acetyl thiocholine iodide (Pohonka *et al*., 2011). Franz *et al*. (2012) have compared organophosphorus compound toxicity with that of Oximes *in vitro* and suggested it as accurate measure of acetylecholinesterase activity. Hence, the rate of hydrolysis by both *C. elegans* and rat brain homogenate system showed typical Michaelis-Menten patterns with increasing concentration of ATCI resulting in increased rates followed by the attainment of zero order kinetics.

Acetylcholinesterase activities in both the homogenates exhibited inhibition at higher substrate concentrations. *C. elegans* AChE hydrolyzed ATCI with a K_m of 725 μM and V_{max} of 112.35 ± 33 nmol min^{-1} mg^{-1} protein. Interestingly the K_m of rat brain AChE for ATCI was 59.01 mM and V_{max} 112.35 ± 3.5 nmol min^{-1} mg^{-1} protein. The work of Filho *et al.* (2004) demonstrates considerable variations in K_m values of brain AChEs among different neotropical fishes. Analysis of kinetic parameters of insect and mammalian AChE revealed 3-fold greater affinity of mammalian AChE for ATCI than insect AChE (Ali *et al*., 2003).

A characteristic of true AChE activity is the inhibition of enzyme activity at high substrate concentrations. Of the two AChEs tested, rat brain AChE exhibited inhibition at concentrations of 2 mM ATCI and 5 mM *C. elegans* AChE. Cholinesterases from nematodes are inhibited by the substrate at relatively higher substrate concentrations such as 10 mM for *N. brasiliensis* (Sanderson *et al*., 1967), 10 mM for *M. apri* (Reiner *et al*., 1978) and 20 mM for *S. dentatus* (Rhodes, 1981). Our results of inhibition of *C. elegans* AChE at concentration above 5 mM in comparison that of rat brain at 2 mM are in agreement with these findings.

In present study, regression analysis of per cent inhibition of both AChEs by dichlorovos was 45.7 ± 2.68 and 107.18 ± 8.68 nM as IC_{50} for *C. elegans* and rat brain AChE, respectively. As is the case of variations in the toxicities of OPI which varies from species to species, the relative sensitivities of AChE, from different species and different organs from the same organism differ (Anderson *et al*., 1978). Wang and Murphy (1982) and Singh (1985) suggested that differential sensitivities of AChEs cannot sufficiently be explained on the basis of binding of inhibitors to non-specific proteins by differences in affinity of inhibitor for enzymes active site and rates of phosphorylation has provided most satisfactory explanation (Wang and Murphy, 1982). It is important to note that the K_m of *C. elegans* was much higher (lower affinity for substrate) than rat brain AChE. The finding is in agreement with Filho *et al*. (2004) who demonstrated that neotropical fish brain AChE with higher K_m values were more sensitive to methyl parathion inhibition *in vitro*.

The double reciprocal Lineweaver-Burk plot provides information necessary for identifying the mechanism of enzyme inhibition. The double reciprocal groups for inhibited AChEs of both *C. elegans* homogenate and rat brain homogenate systems revealed similar pattern of inhibition by dichlorvos characterized by concentration related decrease in V_{max} and unaltered K_m values. These changes are hall mark of irreversible inhibition. The present study emphasizes that *C. elegans* homogenate system can be used to understand the inhibition pattern of dichlorvos as similar to rat brain homogenate *in vitro*. As use of rats is restricted by the animal ethical committee, hence *C. elegans* homogenate system could be an alternate system for *in vitro* assessment or screening of acetylcholiesterase inhibitors. Since this organism is out of the purview of animal ethics and ease of maintenance in laboratory and having vast genetic resources available through worm community wormbase (*www.wormbase.org*).

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