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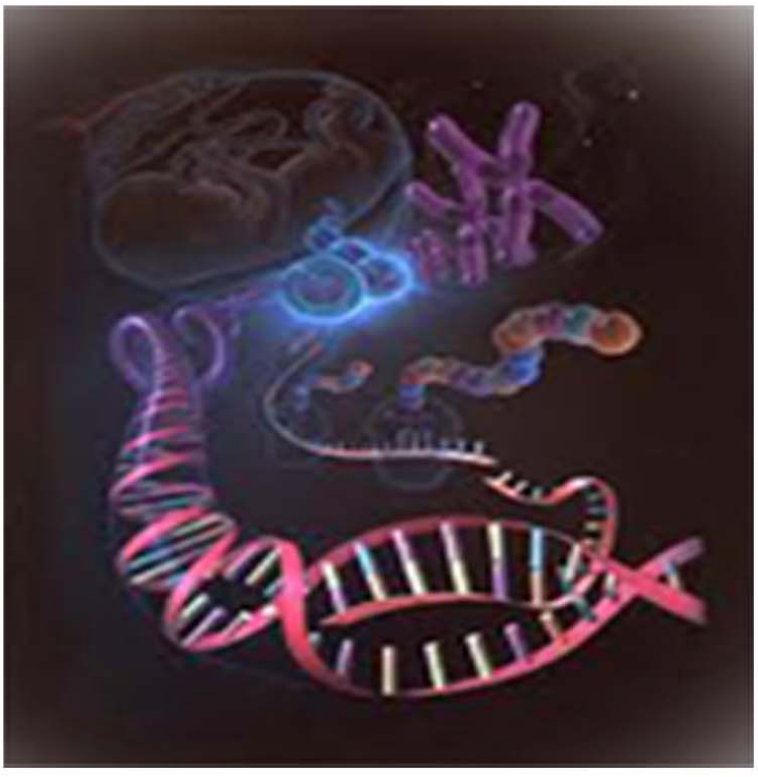

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Research Paper

EFFECT OF INHIBITORS ON THE ACETYLCHOLINESTERASE ENZYME AND LIVE WORMS OF *SETARIA CERVI*

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Filariasis is a major health problem, affecting millions of people in tropical and subtropical regions of the world. Targeting the parasite specific enzyme is not only rational but essential for developing effective control measure against filariasis. In our earlier studies we have identified two isozymic forms of acetylcholinesterase (AchE) from bovine filarial parasites (*Setaria cervi*) different from the host AchE. In the present study, we have studied the effect of enzyme inhibitors (BW284c51, Edrophonium and Eserine) and antifilarial drugs (Diethylcarbemazine and Ivermectin) on parasite acetylcholinesterase and on live adult worms. Out of different inhibitors, only inhibitor BW284c51, strongly inhibited (98%) the enzyme activity of *S. cervi* acetylcholinesterase with an IC_{50} value of 26.87 μM at 0.50 mM substrate concentration. Lineweaver–Burk transformation of the inhibition kinetics data demonstrated that it was a competitive inhibitor of filarial acetylcholinesterase and Ki value was 9.3 μM. However, *in vitro* incubation of live *S. cervi* adult worm with 100 μM concentration of BW284c51 resulted in only 40% inhibition of parasite acetylcholinesterase and 50% reduction of worm motility. These findings suggest that BW284c51 is a potent competitive inhibitor of filarial acetylcholinesterase and AchE can be a potential target for rational screening of antifilarial compounds.

Keywords: Setaria cervi , Acetycholinesterase, BW284c51, ISO-OMPA, Diethylcarbemazine, Ivermectin

INTRODUCTION

Filariasis is a major public health problem in tropical and subtropical countries affecting millions of people worldwide. It has been estimated that globally around 1 billion people are at risk for lymphatic filariasis of which about 473 million people are in India (Agrawal and Sashindran, 2006; Grady *et al*., 2007). The global program to control transmission of lymphatic filariais is through mass drug administration and presently available drugs such as diethylcarbamazine, ivermectin and albendazole

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have certain limitations. Since these drugs are microfilaricidal, treatments with the existing drugs can only reduce circulating microfilariae and eliminate the worm's larval stages, thereby disrupting disease transmission upto some extent but adult worms show longevity hence, longer treatment programs with higher doses may have to be required to kill the adult worms (Liu and Weller, 1996). The sustained use of these drugs increases the threat of drug resistance and the evidence for drug resistance has already been revealed in various veterinary diseases. There are no vaccines available for filarial parasites; the vector control programs are threatened by insecticide resistance and the repertoire of effective drugs is also very limited. The existing agents are effective against some nematode infections, but noted multiple number of limiting concerns. Therefore, presently the requirement for chemotherapy of lymphatic filariasis is identification of non-toxic and novel antifilarial compounds with long term microfilaricidal or macrofilaricidal activity.

Acetylcholinesterase (AchE, EC3.1.1.7), key enzyme of neuromuscular transmission, normally terminates the neurotransmitter action of acetylcholine at synaptic junctions and also involved with other non-enzymic functions like host parasite interaction (Nalivaeva *et al.*, 2001). This enzyme has been found in many species of helminth parasites including filarial parasites and its secretion has been one of the parameters used for evaluating the effectiveness of anthelmintic (Rathaur *et al.*, 1987; Singh *et al.* 2007). AchE has been demonstrated to be a functional protein involved in multifaceted activities such as for drug development, vaccine and diagnostic purposes in helminth parasites like Schistosomes and the cholinesterase inhibitors are of potential interest in anthelmintic chemotherapy (Boyle *et al.*, 1997). Various synthetic (Edrophonium, Neostigime, BW284c51) and natural compounds (Huperzine A and Galantamine) are known to reduce the activity of vertebrate AchE enzymes and act in a variety of ways, however, studies on effect of inhibitor on nematode AchE activity is rather scanty. BW284c51 is a powerful and reversible blocker of nicotinic acetylcholine receptors besides acting as a specific AchE inhibitor (Olivera-Bravo *et al*, 2005). Additionally, its high specificity allows it to discriminate between AchEs from different species, since BW284c51 binding depends on the presence of specific amino-acid residues in the catalytic and peripheral sites of the enzyme (Radic *et al*., 1993; Eichler *et al*., 1994). This inhibitor has the similar binding pattern like decamethonium and Donepezil (E2020) as evidenced from the X-ray crystal structure of *Torpedo californica* AchE complexed with BW284c51 (Felder *et al*, 2002).

Setaria cervi, the bovine filarial parasite, resides in the peritoneal cavity of buffaloes with cosmopolitan distribution. *S. cervi* resembles human bancroftian parasite in having nocturnal periodicity and antigenic similarities (Kaushal *et al.*, 1987) and the easy availability of adult worms makes it more convenient model parasite for conducting preliminary studies related to drug development. We have earlier purified and characterized AchE from adult stage of *Setaria cervi* and have also identified two isoenzymic forms of AchE of *S. cervi* and *Brugia malayi* (human filarial parasite) different from the host enzyme (Singh *et al.*, 2007). In the present study, we have studied the effect of certain AchE inhibitors (BW284c51, Edrophonium and Eserine), BchE inhibitor (ISO-OMPA) and antifilarial agents (Diethylcarbemazine and

Ivermectin) on partially purified *S. cervi* AchE (ScAchE) and on living adult worms.

MATERIALS AND METHODS

Collection of Parasites

Adult motile worms of *Setaria cervi* were collected from the peritoneal folds of freshly slaughtered buffaloes at a local slaughter house and transported to the laboratory in normal saline. The parasites were washed extensively with normal saline before use.

Preparation of Enzyme Extract

A 20% extract of *S. cervi* adult worms was prepared as described elsewhere (Singh *et al*, 2007). Briefly, the adult worms were grind to a fine paste and then extracted with 50 mM phosphate buffer, pH 8.0, containing 0.5% Triton X-100 on ice for 1.5 h with occasional vortexing. The extract was centrifuged at 1,05,000 x g for 60 min and the supernatant obtained was used for the purification of enzyme by Con-A Sepharose affinity chromatography as described elsewhere (Singh *et al.,* 2007). The partially purified ScAchE was used for inhibitor kinetics.

Enzyme Assay

The activity of acetylcholinesterase assayed by the method of Ellman *et al.* (1961) with slight modifications (Singh *et al.*, 2007). Briefly, the reaction mixture in a total volume of 1.0 ml contained 800 μl of 0.05 M phosphate buffer pH 8.0, 50 μL of 40 mM acetylthiocholine iodide and 10-100 μl of enzyme preparation and total volume of the reaction mixture was made 950 μl with 50 mM phosphate buffer. The reaction was started by adding 50 μl of 2 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] and the change in absorbance was recorded at 30 s interval at 412 nm in Spectrophotometer (Labomed, USA). The enzyme unit was expressed as μmoles of substrate hydrolyzed per min.

Measurement of AchE Enzyme Activity in Presence of Inhibitor and Anthelmintic Drugs

The effect of AchE inhibitors 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW248c51), Edrophnium, Eserine, tera-(monoisopropyl) pyrophosphortetramide (ISO-OMPA, a BchE-specific inhibitor) and antifilarial drugs (Diethylcarbamazine and Ivermectin) on the enzyme activity was studied by incubating the enzyme with different concentrations of inhibitors for 10 min at 37 °C and measuring the enzyme activity. The enzyme incubated without inhibitor was used as control. The percent inhibition was calculated from the enzyme activity in presence of inhibitors as compared to control. The AchE activity was measured at different concentrations of BW284c51 (0.5, 12.5, 62.5 μM) and ISO-OMPA (125, 625 μM) at varying substrate concentrations (0.5 to 4.0 mM). The *Ki* and IC_{50} values for the two inhibitors were calculated according to Lineweaver-burk plots (Lineweaver and Burk, 1934) and Cheng and Prusoff Equation (Cheng and Prusoff, 1973).

In order to see the effect of AchE inhibitors and antifilarial compounds on live parasites, 5 actively motile adult worms of *S. cervi* were incubated *in vitro* in 10 ml of sterile Ringer's salt solution containing 100 μM of inhibitors (BW284c51, Edrophonium, Eserin, ISO-OMPA) and antifilarial drugs (Diethylcarbamazine and Ivermectin) at 37 °C. After 6 h of *in vitro* incubation, the experiment was terminated and the worms were washed, parasite extract was prepared and AchE activity was measured as described above. The motility of worms was monitored visually and scored after

6 h of *in vitro* incubation compared to the control.

RESULTS AND DISCUSSION

Effective control and treatment of the filarial infections is still a major problem as none of the available drugs has desired efficacy for killing the long-lived adult stage of these parasites. New drugs that affect new molecular targets are hence required for improving the treatment and control strategies against the adult parasites including embryo, microfilaria, and to replace the currently used drugs when resistance to these drugs become evident (Kaminsky, 2003).

The effect of AchE inhibitors BW248c51, Edrophonium, Eserine and Iso-OMPA was studied on Con-A purified ScAchE as well as on live adult worms of *S. cervi*. The incubation of Con-A purified enzyme with 100 μM concentration of BW284c51, Edrophonium, Eserine resulted in inhibition of 98%, 89%, 87%, respectively. Significant reduction (70%) of enzyme activity was also observed at lower concentration (10 μM) of BW248c51 (data not shown). The ISO-OMPA (BchE inhibitor) did not have any significant inhibitory effect on activity of Con-A purified ScAchE even at 100 μM concentration (Table 1).

We next examined whether these inhibitors and antifilarial agents have any effect on the motility of live parasite and subsequently on AchE activity. The measurement of AchE activity after in vitro incubation of live adult worms with AchE inhibitors resulted in only 25-40% inhibition of AchE activity in live worm treated for 6 h with these compounds (Table 1). The incubation of adult worms in presence of these inhibitors, about 50% reduction in motility of adult worm was observed with BW248c51 & eserine and 25% reduction by edrophonium as given in Table 1.

Table 1: Effect of Inhibitors and Antifilarial Drugs on Live *S. Cervi* Adults and ScAchE Activity

Inhibitor (100 μM)	% Inhibition of ScAchE activity		Motility[c]
	Purified Enzyme[a]	Enzyme extract from worms treated with Inhibitors[b]	
BW284c51	98	40	++
ISO-OMPA	4	2	++++
Edrophonium	89	28	+++
Eserine	87	25	++
DEC	15	9	+++
IVE	41	34	+++
Control	0	0	++++

Note: [a] Con-A purified ScAchE was incubated with different inhibitors (BW284c51, Edrophonium, Eserin, ISO-OMPA) and antifilarial drugs (Diethylcarbamazine, Ivermectin) followed by estimation of AchE activity as described in Materials and Methods.

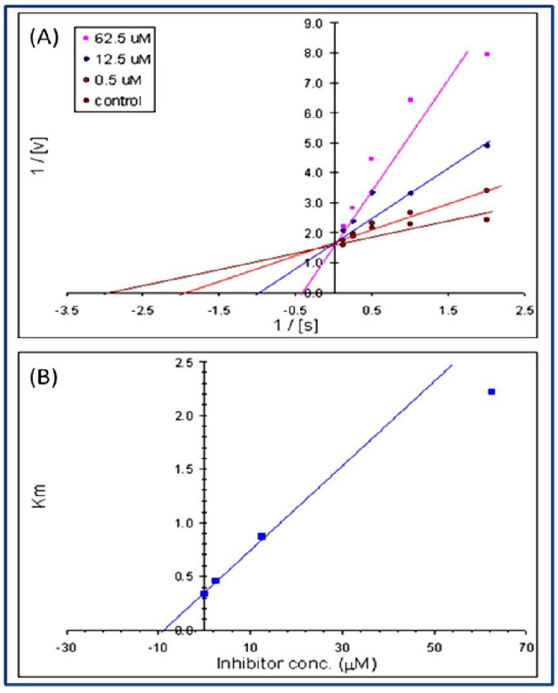
[b] Five S. cervi adult worms of almost equal size were incubated with each of inhibitors (BW284c51, Edrophonium, Eserin, ISO-OMPA) and antifilarial drugs (Diethylcarbamazine, Ivermectin) separately in 10 ml of Ringer's salt solution at 37°C. After 6 h of incubation, the worms were washed with buffer, enzyme extract was prepared and AchE activity estimated in control and treated worm extract.

[c] The adult worms incubated in Ringer's salt solution only served as control. Motility was visually checked after 6 hour of in vitro incubation. The motility assessment scored as + + + + (100%, motility equal to control); + + + (75%), + + (50%) motility compared to control. Concentration of inhibitors and drug: 100 μM

The effect of two antifilarial drugs Diethylcarbamizine and Ivermectin was also studied on live *S. cervi* worm as well as on Con-A purified ScAchE. The *in vitro* incubation of adult worms for 6 h with the drugs (Diethylcarbamizine and Ivermectin) resulted in about 25% loss in the motility but the AchE activity was reduced by 9% and 34% respectively (Table 1). Moreover, these drugs are microfilaricidal and very high concentrations are required to exert any significant effect on adult parasites that is why partially affecting the motility of adult worms. The reason for inefficiency of these drugs to inhibit AchE activity may be these drugs do not target AchE but affect the parasites by altering some other metabolic activities.

The inhibitor kinetics study were attempted to explain how inhibitor act on enzyme and predict its efficacy. The kinetic constants *Km*, V_{max} and *Ki* are critical to understand enzymatic action on controlling metabolism of an organism. We studied enzyme inhibition kinetics of BW284c51 (inhibitor of true AchE) and ISO-OMPA (inhibitor of BchE) on *S. cervi* AchE, as a function of inhibitor concentrations. The effect of substrate concentration on parasitic AchE activity was studied and optimized in our earlier study. BW284c51 is a competitive enzyme inhibitor of AchE and interferes with the binding of substrate to AchE enzyme so as to raise the *Km* value without affecting the *Vmax*. The *Km* and *Ki* values of *S. cervi* AchE (ScAchE) for ATI and inhibitors were determined by the linear lineweaver-burk plot or double reciprocal plot shown in the Figure 1 (*y*-intercept equivalent to $1/V_{max}$ and an *x*-intercept of the graph representing $-1/K_m$, $1/v=1/V_{max}$, the *x*-intercept is an extrapolation of the experimental data taken at positive concentrations) (Kaakar *et al.*, 1999). As competitive inhibition is overcome by increasing substrate concentration with the increasing concentration of BW284c51 the apparent value of *Km* changed but the *Vmax* did not change as shown by the Lineweaver-burk plot in Figure 1. The *Ki* value of 9.3 µM was obtained as calculated from the Lineweaver-burk plot. *Ki* was also calculated by The Cheng Prusoff equations that described the relationship mathematically and yielded a value of 10.75 µM. These kinetic studies clearly indicated that the inhibition of AchE by BW284c51 was competitive, as *Km* and *Vmax* values of uninhibited and inhibited enzyme were different and slops of inhibited and uninhibited AchE were also changed as evident from

Figure 1: (A) Primary Lineweaver–burk plots showing the effect of different concentrations (0, 0.5, 12.5, 62.5 µM) of BW284c51 inhibitor using 0.5, 1, 2, 4 mM ATI concentrations, (B) Corresponding secondary plot used to calculate the inhibition constant (Ki)



lineweaver-burk plots. The *Ki* value of ISO-OMPA was also determined in the same way for the ScAchE and was found to be 250 µM as calculated from the Lineweaver-burk plot and 246.0 µM calculated by the Cheng Prusoff equations (Figure 2).

The half maximal inhibitory concentration (IC_{50} value) has been used to study the inhibition kinetics of an enzymatic reaction and to define the efficacy of an inhibitor/ drug (Caldwell *et al.*, 2012). The IC_{50} value for BW284c51 and ISO-OMPA were calculated at various substrate concentrations (0.5 mM to 4.0 mM) for *S. cervi* AchE as shown in the Table 2. The IC50 values were found to be 26.8, 43.0, 75.3, 140.8 µM for BW284c51 and 738.9, 1477.8, 2709.3, 3172.3 iM for ISO-OMPA at ATI concentrations 0.5, 1.0, 2.0, 4.0 mM, respectively. The IC_{50} values for BW284c51 were very less as compared to ISO-OMPA that indicates the specificity of BW284c51 towards true AchE of *S. cervi* (Table 2).

In conclusion, our studies have demonstrated that BW284c51 strongly inhibited the enzyme activity of Con-A purified ScAchE as compared to other inhibitors and antifilarial drugs. The extent of enzyme inhibition of purified enzyme and the AchE enzyme isolated from treated adult worms was not the same; this might be due to difference in the permeability of parasite cuticular membrane to the inhibitors/antifilarial compounds, but this issue needs further investigation. Our

Figure 2: (A) Primary Lineweaver-burk plots showing the effect of different concentrations (0, 125 and 625 µM) of ISO-OMPA using 0.5, 1, 2, 4 mM ATI concentrations, (B) Corresponding secondary plot used to calculate the inhibition constant (Ki)

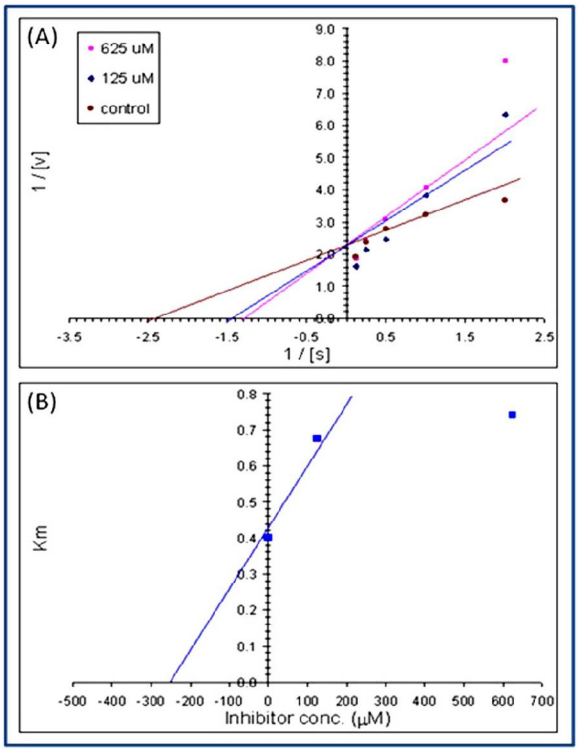


Table 2: IC_{50} values of BW284c51 and ISO-OMPA inhibitors for *S. cervi* AchE

Substrate ATI(mM)	IC50 (µM)	
	BW284c51	ISO-OMPA
0.50	26.8	738.9
1.00	43.0	1477.8
2.00	75.3	2709.3
4.00	140.8	3172.3

Note: The IC50 values of inhibitors were calculated according to Cheng Prusoff equation as described in Materials and Methods.

findings on enzyme inhibition kinetics suggest that filarial AchE can be a rational target for identifying new anti-filarial drug candidates.

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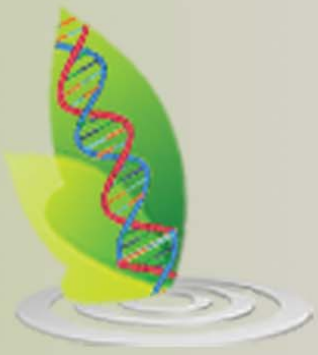

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