Effect of a Macrofilaricidal Agent on the Bioenergetics of *Acanthocheilonema viteae* as Studied by ^{31}P-NMR and Biochemical Analysis

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Abstract: ^{31}P-NMR has been applied to the study on the energy metabolism of intact rodent filariids *Acanthocheilonema viteae.* Based on chemical shifts and analysis of worm extracts, the phosphorus components included sugar phosphates, inorganic phosphate, glycerophosphoryl choline (GPC) and -ethanolamine (GPE), phosphoenol pyruvate (PEP), nucleotide mono, -di and -tri phosphates, nicotinamide adenine dinucleotide and uridine diphosphate glucose. Effect of adulticidal candidate drug (C. D. R. I. Comp. 82/437, in its clinical phase I trial) on the bioenergetics of *A. viteae* adult filariids was assessed by ^{31}P-NMR and revalidated by metabolic and enzymatic studies. Comp. 82/437 at the active dose of 50 mg/kg, orally $\times$ 5 days, showed maximum effect on day-16 post treatment. ^{31}P-NMR data revealed significantly low amount of GPE (52.2%), GPC (43.5%), ATP (54.8%) and PEP (77.2%) in the treated worms. Biochemically also, ATP and PEP levels in these worms were found to be reduced by 30.9 and 44% respectively. Amongst enzymes the activity of hexokinase rose by 58%. By this enhancement, the enzyme may be able to effectively mediate the entry of extra glucose (48%), into glycolysis. On the other hand, a substantial (30%) decrease in activity seems to make phosphofructokinase a real rate limiting step in the glycolysis. This would ultimately lead to the lower production of ATP. In the energy deprived worm all the metabolic activities will gradually decline and may result in the penultimate death due to drug action. NMR observations and conventional biochemical methods substantiate the findings of one another and direct towards the hitting of bioenergetic machinery of *A. viteae* by macrofilaricidal agent (Comp. 82/437).

Key words: ^{31}P-NMR, *Acanthocheilonema viteae,* Compound 82/437, PEP, ATP, glycolysis.

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INTRODUCTION

Lymphatic filariasis is a major vector-borne parasitic disease of the tropical and subtropical countries, affecting 120 million worldwide (Ottesen and Ramachandran, 1995). The presently available drug, diethylcarbamazine (DEC) is insufficient because of its inadequate effect on adult parasites (Kumaraswami *et al.,* 1988; Fan, 1992). The disease remains unconquered largely due to non-availability of a drug which can kill the adult filariids. There is thus need for generation of better chemical lead for development of an effective macrofilaricide. It is now evident that Nuclear Magnettic Resonance (NMR) spectroscopy is an exquisitely powerful tool for design and development of new drugs (Craik, 1996). The technique is used in visualizing and assessing the drug-parasite interactions, thereby identifying vulnerable targets of filarial parasites for chemotherapeutic attack. In our earlier study (Shukla *et al.,* 1995) we explored the energy metabolism of rodent filariids, *Acanthocheilonema viteae* by ^{31} P-NMR spectroscopy. The bioenergetic status of these filariids identified high energy phosphate molecule phosphoenol pyruvate (PEP) as key metabolite acting as energy reservoir. Adenosine triphosphate (APT) serves as primary currency of cells, fuelling cellular processes from macromolecular synthesis to signal transduction. It is well-known that ATP production is tightly coupled to its consumption. The viability of these filariids depends on their energy utilization and it is probable that cell death, which is related to a breakdown in membrane function and/or inhibition in macromolecular synthesis, is produced by a local decrease of ATP below a critical level. This suggests that a large and unfavourable pertubation in the energy pathways could be responsible for an impaired cellular function (Thompson *et al.,* 1987). Very often, changes in the normal functioning of cells have been shown to be affected due to the alterations in the energy utilization pathways. A detailed understanding of cellular energy metabolism, is therefore vital. NMR spectroscopy is ideally suited for investigating the cellular energy metabolism (Gadian *et al.,* 1979).

In the present study a benzimidazoyl derivative Comp. 82/437 (2,2'-dicarbomethoxylamino 5,5'-dibenzimidazolyl ketone) which was earlier shown to be effective against adult filarial parasites (Abuzar *et al.,* 1986; Fatma *et al.,* 1989) has been used to understand the drug-parasite interactions and the vulnerable targets of parasites' energy metabolism. The results of NMR study was also substantiated by parallel conventional biochemical estimations.

MATERIALS AND METHODS

Host-Parasite Model: The studies were carried out in experimental filarial infection *A. viteae* in rodent host *Mastomys coucha.* Mastomys were infected by injecting subcutaneously 50 infective larvae of *A. viteae* obtained from the infected vector *Ornithodorus moubata* (Worms *et al.,* 1961; Singh *et al.,* 1989). On day 75, the animals showing progressive rise in microfilariae (mf) were selected. These animals were sacrificed and intact, motile adult *A. viteae* parasites were recovered from the subcutaneous tissue for *ex vivo* studies.

Candidate drug used: C. D. R. I. Comp. 82/437, a candidate macrofilaricidal agent, was

used in the present study (Abuzar *et al.,* 1986; Fatma *et al.,* 1989).

Chemicals and enzymes: NADP, glycogen, horse radish peroxidase, hexokinase, glucose-6-phosphate dehydrogenase (G6PDH), aldolase, lactate dehydrogenase (LDH), glycerophosphate dehyrogenase-triose phosphate isomerase (GDH-TPI) and sodium salts of fructose-6-phosphate (F-6-P), phosphoenol pyruvate (PEP), fumarate and adenosine triphosphate (ATP) were obtained from Sigma Chemicals Co., St. Louis, Missouri (U. S. A.). NAD, NADH, ADP, sodium pyruvate, adenosine monophosphate (AMP) and oxalacetate were procured from SISCO Research Laboratories, Bombay, India.

All other chemicals and reagents used were of high purity grade available.

Perfusion method (Thompson *et al.,* 1987; Shukla *et al.,* 1995)

Adult and intact worms of *A. viteae* (55−197 mg) were placed in a 10 mm NMR tube. A spacer was placed at the bottom of the NMR tube so that the parasites reside in the sensitive region of the NMR tube. The apparatus used for maintaining worms during NMR analysis consisted of a fluid reservoir filled with Kreb's Ringer saline, pH 7.4, saturated with 95% N_2/5% CO_2 and an indigenously built assembly working with the phenomenon of gravity and siphon system. The saturated solution was perfused and the perfusion rate was monitored with sterile i. v. set. For quantitative experiments measured amount of methylene diphosphonic acid was dissolved in the buffer medium. The chemical shift of the external reference does not interfere in the region of interest. The worms were perfused in the NMR tube at a rate of 1.5 ml min^{-1}.

NMR analysis (Shukla *et al.,* 1995)

The ^{31}P-NMR spectra were generated at 162 MHz at 22℃ in a Bruker WM 400 MHz spectrometer with a narrow bore (54 mm) and a 9.398 Tesla superconducting magnet. The FID's were collected using inversegated decoupling techniques. The lock was fixed at D_2O field initially by adjusting with D_2O sample and then replaced with the sample. The lock amplitude was raised to the maximum and thereafter the spectra were collected on lock mode. Shimming of the sample was carried out using the ^{1}H FID till the line width of the water signal was below 10 Hz in the non-spinning mode. Two thousand forty eight (2048) transients were acquired to obtain spectra with satisfactory signal to noise ratio for mixed population. A pulse of 45°(22 us), 0.8 s delay and decoupling of 3 Watt was found necessary to maximise signal to noise ratio of all the resonances under non-saturating conditions. In case of mixed population the acquisition time was 45 min. Peak assignments were made on the basis of chemical shifts as reported in the literature (Gadian *et al.,* 1979). The total amount of ATP in the worms was calculated and the level expressed per mg tissue fresh weight. The relative level of adenosine diphosphate (ADP) was estimated by substraction of the peak area for the βATP (peak 12) resonance from the γATP + βADP integral (peak 8), and ATP/ADP ratio calculated as βATP/βADP. Initially six controls were used as standard reference.

Worm extraction

Phosphorus metabolites of *A. viteae* were isolated by the method of Matthews *et al.,* 1985. Extracts were prepared by freezing nearly 81 mg of adult worms in liquid N_2 and

powdering them with a mortar and pestle. The powder was extracted with 1.5 ml of 7% perchloric acid and after centrifugation, the supernate was neutralised to pH 6.5 with 30% KOH. The precipitate was discarded and the final supernate was lyophilised. ^{31}P-NMR spectra of the extract was accumulated by dissolving the lyophilised extract in a solution containing HEPES, 5.9 mg and EDTA, 0.9 mg in 0.4ml of triple distilled water and 0.1 ml of D_2O. The analysis of ^{31}P-NMR experiment was generated at 121 MHz in a DRX 300 MHz FT-NMR spectrometer equipped with a multinuclear 5 mm broad band probe head. Typical experimental conditions were as follows: pulse width, 90°; relaxation delay, 2s; composite pulse decoupling WALTZ-16 and acquisition 35,000 were acquired to obtain a satisfactory spectra. The whole experiment was repeated five times.

Drug treatment: *A. viteae* infected animals (Mastomys) were treated *in vivo* with Comp. 82/437 at the dose level of 50 mg/kg orally for 5 consecutive days in two batches. The parasites were recovered on days 8 and 16 of start of treatment for carrying out ^{31}P-NMR stuides. The whole set of experiment was repeated four times.

Biochemical revalidation was, also, carried out at the dose of 50 mg/kg, p. o. × 5 days and the parasites were recovered on day 16 of start of treatment. The experiment was repeated four times.

Metabolic studies: 20-30 mg intact, motile worms isolated from control and drug treated mastomys were incubated in Hank's balanced salt solution (pH 7.4) containing 0.5% glucose. Exactly after 4 hrs the worms were removed and the medium assayed for glucose (Burleigh *et al.,* 1968) and lactate (Barker *et al.,* 1941) contents. Glucose disappeared from the medium was used as a measure of glucose uptake by the parasites. Similarly lactate content of the medium was a measure of lactate excreted by the parasites.

Fifty-Seventy mg control and drug treated parasites were homogenized (5% w/v) in 0.15 M KCl. The homogenate was centrifuged at 900 g for 10 min and the supernatant was sonicated at 20 K cycles for 4×1 min with successive cooling intervals of 1 min each. The sonicate was centrifuged at 105,000 g for 30 min and the supernate was used for assay of glycolytic enzymes by standard methods as described elsewhere (Mercus *et al.,* 1973). Protein content of the pellet fraction was measured according to Lowry *et al.* (1951) using BSA as a standard. ATP and PEP contents of the worm extract were determined by spectrophotometric method coupled to G6PDH and LDH respectively (Lamprecht and Trautschold, 1963; Shonk and Boxer, 1964; Kornberg, 1955).

RESULTS

In vivo ^{31}P-NMR spectra of live normal *A. viteae*

The *in vivo* ^{31}P-NMR spectrum of live and normal (untreated) *A. viteae* was found to be composed of 12 peaks according to the chemical shift analysis (Fig. 1). The shifts for peaks 8, 9 and 12 were assigned respectively to γATP + βADP, αATP and αADP, and βATP phosphorus resonances bound to Mg. Peak 10 contained NAD + phosphorus resonance of glucose phosphate from uridine diphosphate glucose (UDPG) while that of 11 belonged to

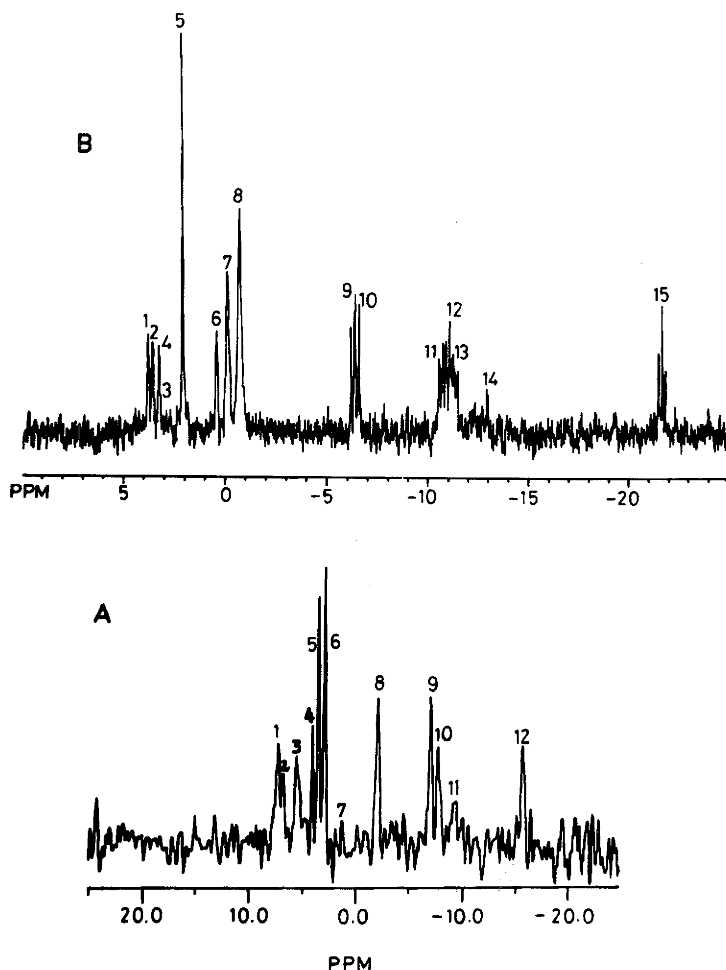

Fig. 1A: Typical ^{31}P-NMR spectra of intact live filarial parasites (*A. viteae*).
Fig. 1B: Typical ^{31}P-NMR spectra of perchlorate extract of *A. viteae* filariids.

phosphorus resonance of uridine phosphate of UDPG. Peak 7, remained unassigned whereas the signals 4, 5 and 6 were assigned to glycerophosphoryl ethanolamine, glycerophosphoryl choline and phosphoenol pyruvate (PEP), respectively. Rest of the other signals (1-3) represented sugar phosphates, phosphoryl choline and inorganic phosphate.

^{31}P-NMR spectra of PCA extract of *A. viteae* adults

Spectra of the PCA extract (Fig. 1B) based on direct chemical analysis indicated that resonances in the downfield monophosphate region arise from primarily glucose-6-P (Peak 1), AMP (Peak 3), fructose-6-phosphate (peak 2), phosphocholine and fructose-1-6-diphosphate (Peak 4) and inorganic phosphate (Peak 5). A resonance of 0.55±0.01 ppm downfield of glycerophopshorylcholine (GPC, Peak 7) in the ^{31}P-NMR spectrum was assigned to glycerophosphoryl ethanolamine (GPE, Peak 6) on the basis of its chemical shift. The most prominent resonance in the spectrum arose from phosphoenol pyruvate (Peak 8). Doublets of

γ-phosphate of ADP (peak 9) and β-phosphate of ADP (peak 10) as well as double doublets of α-phosphates of ATP and ADP (Peak 11 and 12) were also observed in the spectra. $NAD^{+}(H)$ and uridine phosphate of UDPG appeared as peak 13. Peak 14 of glucose phosphate of UDPG and a triplet of β-phosphate of ATP (Peak 15) were also detected. The confirmation of the resonance assignment of PEP (Peak 8) was done by adding trisodium salt of the authentic sample in the PCA extract after the normal ^{31}P-NMR experiment.

The fully resolved spectra of the extract confirmed the assignments of the ^{31}P-NMR spectra of live, normal *A. viteae* filariids.

Evaluation of the effect of candidate drug 82/437 on *A. viteae/mastomys system*

The ^{31}P-NMR spectra of the adult parasites recovered on day 8 post treatment with 82/437 at dose of 50 mg/g (Fig. 2A) exhibited no significant change in comparison to the spectra of normal adult parasites.

The spectra of parasites recovered on day 16 of start of treatment at the same dose, however, showed significant effect (Fig. 2B) i. e. 52.2% loss in GPE, 43.5% loss in GPC and 77.2% loss in PEP and 54.8% loss in the ATP level as well as differentiation of intra and extracellular Pi was easily detectable. There was a total loss of UDPG peak.

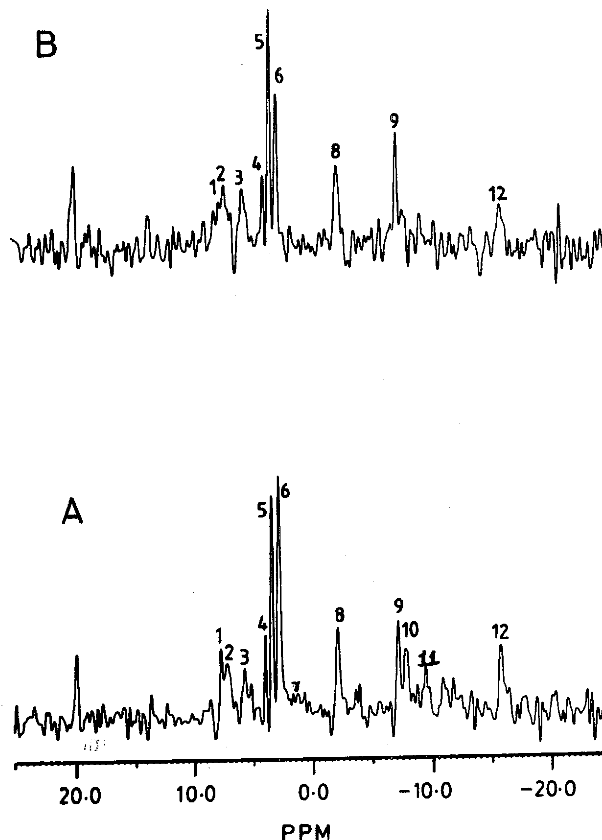

Fig. 2A: ^{31}P-NMR spectra of drug (82/437) treated *A. viteae* 50 mg/kg, p. o. ×5 days, D-8.
Fig. 2B: ^{31}P-NMR spectra of drug (82/437) treated *A. viteae* 50 mg/kg, p. o. ×5 days, D-16.

Biochemical analysis revealed that Comp. 82/437 markedly enhanced the uptake of glucose but had no effect on lactate production (Table 1). The treated worms were also found to possess lower amounts of ATP and PEP (Table 1).

Comp. 82/437 altered activities of all the enzymes studied except that of LDH (Table 2). Amongst these the activities of hexokinase (HK), aldolase, enolase, pyruvate kinase (PK) were enhanced whereas those of phosphoglucose isomerase (PGI) and phosphofructokinase (PFK) were depressed. Statistically the alterations induced by the compound in most of the cases were highly significant.

Table 1: Effect of candidate drug 82/437 on glucose uptake and lactate production, APT and PEP contents *in vitro* using *A. viteae*

Comp.	Parameter	*Conc.* of comp.		% of change with respect to control
		A-Control	B-Treated	
82/437	Glucose uptake (G)[a]	0.60±0.08	0.89±0.16	+48%*
	Lactate production (L)[a]	0.97±0.13	0.92±0.04	−5%
	L/G	1.62	1.03	—
	ATP content[b]	71.69±0.06	49.16±0.06	−30.9%*
	PEP content[b]	68.50±0.71	38.50±0.11	−44%*

[Values expressed as [a]μmole/mg worm/4 hr, [b]pmole/mg protein are mean±SD of 4 experiments]

*P<0.005

Table 2: Activities of carbohydrate metabolizing enzymes in *A. viteae* after treatment with candidate drug 82/437

Enzyme	A-Control	B-82/437 treated	% change with respect to control B
Hexokinase (HK)	104.74±0.43	165.62±0.51	+58%[a]
Phosphoglucose isomerase (PGI)	282.05±0.78	195.53±2.12	−30%[a]
Phosphofructokinase (PFK)	131.17±0.42	90.92±1.06	−30%[a]
Aldolase	86.93±0.28	162.66±0.52	+87%[a]
Enolase	254.56±1.80	379.02±1.27	+48%[a]
Lactate dehydrogenase (LDH)	342.23±1.45	371.13±0.50	+8%[b]
Pyruvate kinase (PK)	97.77±0.48	229.86±1.71	+135%[a]

[Values expressed in nmole/min/mg protein, are mean±SE of 4 experiments]

P values: [a]<0.005, [b]NS (Not significant)

Discussion

Parasitic helminths obtain their nutrients and several essential elements from their hosts, but generate energy on their own. Hence uptake mechanisms and bioenergetic pathways have always been considered logical targets for chemotherapy (Sharma and Srivastava, 1988; Srivastava, 1995). Maki and Yanagisawa (1980a, b) on the basis of detecting high acid phosphatase activity in the cuticle of *Setaria digitata* have expressed the possibility of transcuticular absorption of small molecules in the tissue dwelling parasites. However uptake through this route seems to be limited to the phosphorylated compounds required only in minute amounts. The supply for molecules like glucose required in very high amounts cannot be effectively met by transcuticular absorption (Roy *et al.,* 1988). Hence blocking of cuticular absorption dose not appear to yield fruitful results. From filarial chemotherapy point of view, therefore, evasion of host attack and energy yielding processes seem to be of prime importance. Adult filariids utilize glucose as the principal, if not exclusive source of energy. The energy is generated mostly by glycolysis which yields two molecules of ATP for each molecule of glucose degraded. Nonetheless minor pathways also operate which may become effective under certain conditions (Barrett, 1983).

In our earlier studies comp. 82/437 has been reported to cause oxidative damage to filariids including *A. viteae* through selective inhibition of catalase and glutathione peroxidase (Batra *et al.,* 1990, 1992a, b). In the present study, detrimental effects of this compound *in vivo* on the functionality of glycolysis has been shown to occur by metabolic, enzymatic and NMR studies. According to NMR data, the parasites recovered on day 16 of the treatment with 50 mg/kg dose of compound 82/437 had significantly lower amounts of GPE, GPC, ATP and PEP (Fig. 2B). Biochemical estimation also indicated markedly reduced ATP and PEP levels in these worms (Table 1). Surprisingly, treated *A. viteae* utilized about 1.5 times greater amounts of glucose than the untreated parasites. Lactate production, however, remained unchanged. Consequently lactate/glucose (L/G) ratio dropped from 1.62 to 1.03. At enzymatic level the activity of hexokinase (HK) rose by 58% (Table 2) and hence may effectively mediate the entry of extra glucose (48%, Table 1) into glycolysis. Phosphoglucoseisomerase (PGI) is not rate limiting and therefore an observed decrease in the activity by 30% would not hamper glycolytic rate. Nonetheless, subdued activity of phosphofructokinase (PFK) in the drug treated worms appeared to be the real rate limiting step leading to further slowed down metabolism of glucose and finally causing marked decrease in products like triose phosphates, PEP and lactate. Accordingly the L/G ratio and PEP level showed marked decrease in the drug exposed filariids (Fig. 2B, Table 1). Since ATP through glycolytic pathway is formed at steps falling after PFK, the subdued functioning of this enzyme and therefore of glycolysis would ultimately result in lower generation of ATP. This notion is substantiated both biochemically and by NMR analysis.

In the event of less effective glycolysis, the filariids appear to derive energy by diverting extra amounts of glucose-6-phosphate (formed from glucose) into pentose phosphate pathway. The NADPH generated by the action of G6PDH (Batra *et al.,* 1990) may subse-

quently be utilized in two ways. Firstly, for regeneration of reduced glutathione for providing protection against the drug induced oxidative damage (Batra *et al.,* 1990; 1992a, b). Secondly, for generating ATP through oxidation by site I on the respiratory chain shown to be operative in *A. viteae* (Mendis and Townson, 1985). However, both G6PDH and the respiratory chain operate in this filariid or in general in most of the nematode parasite at extremely low rates. Consequently even this diversion of glucose cannot substantiate for the loss of ATP due to the subdued functioning of glycolysis.

It may therefore be summarized that the adverse effect of Comp. 82/437 on PFK results in the lower production of ATP through glycolysis and oxidative damage (as mentioned earlier). The energy deprived worms die ultimately due to drug action. Thus NMR is a valuable tool in chemotherapy of filariasis for assessing the efficacy of candidate drugs in pipeline as novel drugs.

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