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Oxidative Stress Induced Apoptosis by Thiazolidine Compounds: A Unique Antifilarial Approach

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Abstract

The huge disability burden and its great socioeconomic impact has made research on antifilarial therapeutics to be essential. Mechanistic validation of antifilarial activity of already screened thiazolidine compounds showed statistically significant level of oxidative stress in terms of lower GSH and higher carbonyl content of protein in lysates of drug treated human lymphatic filarial parasites as opposed to those of untreated control. Evidence of apoptosis similar to positive control was also recorded in drug treated but not in the untreated control parasites. Therefore, it

might be concluded that oxidative stress mediated apoptosis can be considered as a novel strategy for development of antifilarial therapeutics. Thiazolidine derivative is an important antifilarial lead with unique therapeutic rationale.

Key words: Thiazolidine, Oxidative stress, Apoptosis, Lymphatic filariasis, Drug development.

Introduction

Lymphatic filariasis, a vector borne disease, is caused by filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. Although categorised as neglected tropical disease, 1.23 billion people in 58 countries are living in areas where lymphatic filariasis is transmitted and are at risk of being infected. 120 million people in tropical and subtropical areas of the world are infected with lymphatic filariasis with about 40 million disfigured [1]. Considering epidemiological burden of this incapacitating and disabling disease, WHO has vowed for placing emphasis on development of new and effective drug development under tropical disease research scheme [2].

Currently, diethylcarbamazine (DEC) and ivermectin are frequently used for chemotherapy, but they cause many side effects and are poor macrofilaricidal [3]. Knowledge of exact mechanism of action of sole drug DEC is also lacking. Moreover, although new agents with different functional properties have been getting assessed against filarial parasites from all around world, however they are mostly limited to demonstration of empirical activity rather than providing substantial mechanistic insight [4-7].

In the context of therapeutic rationale, most significant clue might be considered from innate response mediated antimicrobial combating mechanism which by and large depends on oxidative onslaught by macrophages [8]. Interestingly, the almost sole drug DEC is also known to harness and potentiate the similar stratagem for its antifilarial effect [9]. Therefore, targeted oxidative stress can be envisaged to generate desired antifilarial effect. During the recent decades, there has been intense investigation on thiazolidineones, which are otherwise in use as

popular anti-diabetic compounds; many of these derivatives are known to possess interesting therapeutic properties such as anticancer, antiproliferative, antimalarial and antibacterial [10-13].Our previous study with such thiazolidine derivatives showed antifilarial potential with wide and safe therapeutic window (In communication). Moreover, unlike DEC which require involvement of host immune cells for action, synthetic compounds of thiazolidine group were found to be effective against the human lymphatic parasite in *in vitro* condition of cell free system, suggesting direct mode of action on the parasite. Considering the wide spectrum of action and particularly its antiproliferative and anticancer potential prompted us to speculate a possible apoptotic impact. Oxidative stress being one of the powerful known inducer of apoptosis [14] a plausible hypothesis of oxidative stress related apoptosis was postulated for testing.

Towards this end, a representative compound from the in-house library of thiazolidine derivatives, Im10 which showed significant antifilarial effect both on microfilariae (Mf) as well as macrofilariae (adults) in *in vitro* was chosen for exploring any possible involvement of oxidative impact and concomitant apoptosis in the pharmacodynamics.

Materials and methods

Materials

All reagents and chemicals were obtained from commercial sources (Himedia Laboratories Pvt. Ltd, Mumbai and Sigma Aldrich Chemicals Pvt. Ltd, Mumbai). The thiazolidine derivative Im10 was obtained from library of synthesized and purified compounds from our collaborator's laboratory.

Establishment and maintenance of *Brugia malayi* life cycle

The human filarial parasite *B. malayi* life cycle was maintained in jirds (*Meriones unguiculatus*), mastomys (*Mastomys caucha*) using mosquitoes (*Aedes aegypti*) as vectors by

standard methods as described earlier [15]. The use of animals for this study was approved by the Institutional Animal Ethics Committee, which follows the norms of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) in India.

Microfilariae (Mf) were freshly obtained from the peritoneal cavity of the jirds exposed to infective 3rd stage larvae (L_3) 4–5 months back. The microfilariae and adult worms were washed with RPMI 1640 medium (containing 20 μg mL^{-1} gentamycin, 100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) plated on the sterile plastic petri dishes and incubated at 37ºC for 1 h to remove the peritoneal exudate cells of the jirds. The microfilariae were collected from the petri dishes, washed with RPMI 1640 medium and used for *in vitro* experiments.

Determination of inhibitory concentration of Im10

The efficacy of compounds to affect the viability of Mf in *in vitro* was assessed by the extent of parasite motility. A stock solution of 2 mM concentration was made for thiazolidine compound Im10 in DMSO. Further dilutions were made to obtain the desired final concentration in the range of 0.5 μM to 500 μM. The highest concentration of DMSO used along with compound was <1% hence comparable vehicle control was also taken with 1% DMSO. Approximately, 300 Mf in 1000 μl of sterile 0.9% saline were introduced into each vial for every test drug (over a dose range of 0.5 μM to 18 μM) along with above mentioned vehicle control and incubated on shaker incubator at 37ºC for 30 minutes with 150 rpm (Scigenics Biotech, India). After incubation, Mf were washed with RPMI 1640 media and 100 Mf were plated in each well (each individual samples in triplicates) in sterile 24 well culture plates (Nunc, Denmark) containing 1000 μl of RPMI media. The plates were re-incubated at 37ºC for 48 hour in 5% CO_2 incubator (pre-optimized conditions). Mf motility was assessed by microscopy (using Nikon Diaphot, TMD inverted microscope). Each experiment was repeated thrice to check the reproducibility.

Percent inhibition in terms of loss of motility was determined as described earlier [16]. The IC_{100} value (concentration required to achieve complete loss of motility of all parasites) was also calculated [17].

Preparations of Mf lysate.

Fifty thousand Mf were treated with Im10 at its IC_{100} value as described above. Mf treated with DMSO only was used as a control. After 48 h incubation, Mf were collected and washed two times with 0.05M PBS. After washing pelleted Mf were lysed using 100 µl lysis buffer (NaOH + SDS) for 1 h at 37ºC. Supernatant was collected for further assays by centrifugation at 5000 rpm at 4ºC for 5 minutes.

Reduced Glutathione assay by DTNB reagent.

Reduced glutathione content of with or without drug treated Mf lysate was measured using standard method [18]. Briefly, Im10 treated and control Mf were lysed and supernatant collected as described above. Then 400 µl of 0.3 M phosphate buffer and 50 µl DTNB reagent (4 mg DTNB + 100 mg sodium citrate in 10 ml DDW) was added. The resultant chromophore generated was measured spectrophotometrically at 412 nm. The concentration was determined by standard graph using reduced GSH.

Estimation of carbonyl content of protein

Protein carbonylation as a marker of oxidative changes in protein was measured using standard procedure [19]. Briefly, proteins from supernatant of Im10 treated and control Mf lysate were precipitated using 500 µl of 10% trichloroacetic acid (TCA) reagent and centrifuged at 5000 rpm for 5 minutes. The precipitated proteins so obtained were treated with 500 µl of 2, 4 dinitro phenyl hydrazine reagent and incubated at room temperature for 30 minutes with intermittent vigorous mixing at every 15 minutes interval. Again 500 µl TCA (10%) was added to it and centrifuged at 5000 rpm for 5 minutes. After discarding the supernatant, the precipitate was washed twice with 1 ml of ethanol-ethyl acetate (1:1), each time centrifuging out the supernatant, to remove the free DNPH and lipid contaminants. The precipitate was dissolved in 1

ml of protein dissolving solution and incubated at 37ºC water bath for 10 minutes. The color intensity of the supernatant was measured spectrophotometrically at 370 nm against 2 M/l HCl. Carbonyl content was calculated by using molar extinction coefficient ($21x10^3$ $l/mol^{-1}cm^{-1}$).

Acridine orange/ Ethidium bromide (AO/EB) staining for the detection of apoptosis.

Morphological analysis for the evidence of apoptosis was performed with AO/EB dual staining procedure with treated and untreated Mf as per standard protocol [20]. The dye mix consisted of 100 µg/mL AO and 100 µg/mL EB in phosphate-buffered saline. Untreated control as well as Im10 along with staurosporine (20 µM; as positive control for the induction of apoptosis) treated Mf were washed and re-suspended in 25 µL cold phosphate-buffered saline, followed by the addition of 5 µL AO/EB dye mix. Stained Mf were viewed under an epifluorescence microscope (Nikon) with the excitation filter set at 480/30 nm and the barrier filter at 535/40 nm. Tests were carried out in triplicate, counting a minimum of 10 Mf in each observation for detection of differential staining.

Statistical Analysis.

The results were expressed as mean ± SEM for the three separate observations made in each case. For comparison of means of different parameters between the test compound and control, Student's t test was used. P values of <0.05 were considered as significant.

Results and Discussion

After confirmation of antifilarial action, IC_{100} was determined for the said thiazolidine compound for the optimization of the dose for mechanistic study. It was found to be 12 µM which was characterized by complete loss of motility of 100% Mf at that concentration. Further, Mf were treated at this concentration to determine an oxidative parameters (reduced glutathione (GSH) and protein carbonylation level) and evidences of apoptosis in Mf.

The culture supernatants of Im10 treated and non-treated control Mf lysate obtained after

48 h were assessed for reduced GSH and carbonyl content. Reduced glutathione level and protein carbonyl content were displayed in table 1. Significant decrease in GSH level and concomitant significant increase in carbonyl content of protein were recorded indicating oxidative stress.

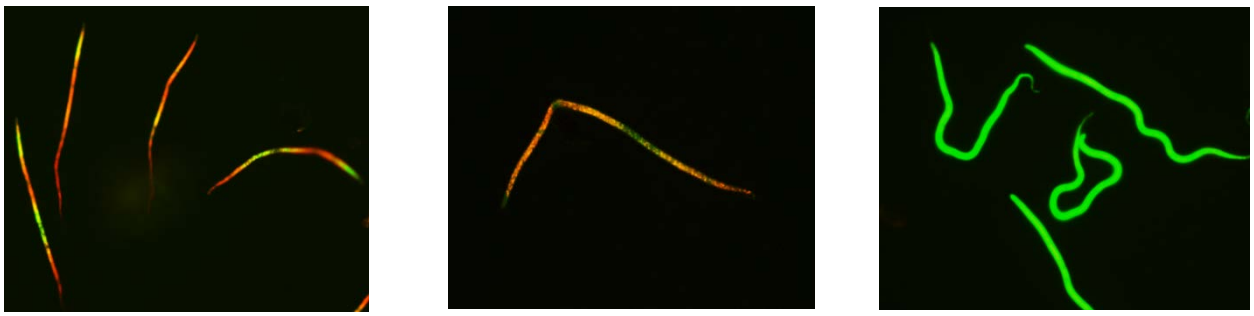
Table1: Reduced glutathione and protein carbonylation level of control and Im10 treated Mf

Sr. No.	Aliquot	Reduced glutathione (nM/mg of protein)	Carbonylation (nM/mg of protein)
1	Control	15.75 ± 0.22	1.05 ± 0.07
2	Im10	13.72 ± 1.03*	1.33 ± 0.03**

After 48h incubation reduced GSH and protein carbonylation level of Mf treated with Im10 was estimated and expressed in mean ± SEM. *$p < 0.05$ when compared with respective control levels.

The AO/EB-stained *B. malayi* mf were observed under the Nikon LABOPHOT epifluorescence microscope and the photographs recorded. After 48 hrs of incubation, untreated Mf remained green due to permeation of AO stain; whereas staurosporine treated Mf, which was used as a standard inducer for apoptosis, showed the presence of orange and green fluorescence characteristic of apoptotic effect. Similarly, Mf treated with Im10 compounds at IC_{100} concentration were found to show orange-yellow colour with EB reflecting loss in cellular integrity due to apoptosis (Fig1).

The present study was designed to illustrate the mechanism of action of thiazolidine compound by which this antifilarial effect is induced. As mentioned above, although widely used with success, quite embarrassingly the actual rationale of DEC is still far from elucidation. The



Im10 treated Mf Staurosporine treated Mf Control Mf

Fig1: AO/EB dual staining of Im10 and staurosporine treated and control (untreated) Mf.

modern pharmacological science demands therapeutics with validated pharmacodynamics. Based on our earlier work, wherein we found potential antifilarial effect of thiazolidine compound with reasonable therapeutic safety, one suitable representative, Im10 which showed lowest IC_{100} value and was effective against both Mf as well as adults (in communication), was selected for further mechanistic studies.

Possible role of oxidative stress has been evaluated as the major contemplated mechanism of the antimicrobial effect for these agents [21]. Such oxidative effect has been further implicated in tumour necrosis factor-α associated apoptotic impact [22]. Possible role of oxidative effect was evidenced by the results of assessment of reduced GSH level and protein carbonylation level. Im10 treated Mf showed markedly less reduced GSH and higher carbonyl content as compared to control (RPMI medium) Mf validating induction of oxidative stress.

Oxidative stress is crucial for cellular signalling [23] and an excess above the threshold is implicated in induction of apoptosis [14]. To test whether Im10 induced oxidative stress triggers such apoptosis, experiment was conducted to detect apoptosis by acridine orange and ethidium bromide differential staining. AO is a vital dye that stains both live and dead cells; EB only stains cells that have lost membrane integrity and stains the nucleus red. Thus live cells have a normal green nucleus due to AO; early apoptotic cells have bright green nucleus with condensed

or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus. Treated parasites but not the untreated control parasites showed significant apoptotic impact. Similar evidence of apoptosis observed with positive control in the form of Mf treated with staurosporine validated the result. Strikingly, the most popular drug, DEC has been shown to have apoptotic impact on this parasite *in vitro* by other workers [24], albeit this effect was not found to result into any demonstrable filaricidal effect. Whereas unlike DEC, our results suggest that Im10 compound has direct oxidative effect on Mf *in vitro* devoid of involvement of any host immune cells and moreover this alone is sufficient enough to set-in direct filaricidal effect through concomitant apoptotic impact.

Conclusion

This small scale work unravels a unique mechanism of novel thiazolidine compounds against filarial pathogen. From this result it can be surmised that targeted oxidative stress, having subtle link with apoptosis can be utilized to formulate a new therapeutic strategy against this so called 'neglected tropical disease'.

Conflict of interest statement

We declare that we have no conflict of interest.

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