Original Article

Artesunate Effect on Schistosome Thioredoxin Glutathione Reductase and Cytochrome c Peroxidase as New Molecular Targets in *Schistosoma mansoni*-infected Mice

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Abstract

Objective To investigate the possible effect of artesunate (ART) on schistosome thioredoxin glutathione reductase (TGR) and cytochrome c peroxidase (CcP) in *Schistosoma mansoni*-infected mice.

Methods A total of 200 laboratory bred male Swiss albino mice were divided into 4 groups (50 mice in each group). Group I: infected untreated group (Control group) received a vehicle of 1% sodium carbonyl methylcellulose (CMC-Na); Group II: infected then treated with artesunate; Group III: infected then treated with praziquantel, and group IV: infected then treated with artesunate then praziquantel. Adult *S. mansoni* worms were collected by Animal Perfusion Method, tissue egg counted, TGR, and CcP mRNA Expression were estimated of in *S. mansoni* adult worms by semi-quantitative rt-PCR.

Results Semi-quantitative rt-PCR values revealed that treatment with artesunate caused significant decrease in expression of schistosome TGR and CcP in comparison to the untreated group. In contrast, the treatment with praziquantel did not cause significant change in expression of these genes. The results showed more reduction in total worm and female worm count in combined ART-PZQ treated group than in monotherapy treated groups by either ART or PZQ. Moreover, complete disappearance (100%) of tissue eggs was recorded in ART-PZQ treated group with a respective reduction rate of 95.9% and 68.4% in ART- and PZQ-treated groups.

Conclusion The current study elucidated for the first time that anti-schistosomal mechanisms of artesunate is mediated *via* reduction in expression of schistosome TGR and CcP. Linking these findings, addition of artesunate to praziquantel could achieve complete cure outcome in treatment of schistosomiasis.

Key words: Schistosomiasis; Artesunate; Praziquantel; Thioredoxin glutathione reductase; Cytochrome c peroxidase

Biomed Environ Sci, 2013; 26(12): 953-961 doi: 10.3967/bes2013.030 ISSN: 0895-3988

www.besjournal.com (full text) CN: 11-2816/Q

INTRODUCTION

Human schistosomiasis remains one of the most important parasitic diseases in terms of large endemic area with approximately 207 million people worldwide are infected, with an estimated 280 000 deaths annually[1-3] and another 779 million individuals are at risk of infection[4]. In the absence of a readily available vaccine for practical application[5-7],

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Received: October 30, 2012; Accepted: April 13, 2013

chemotherapy is the recommended strategy[3].

Since advent of praziquantel (PZQ) as a new schistosomicidal compound, it became a milestone in the chemotherapeutic control of schistosomiasis. The effectiveness of praziquantel against schistosomes and other helminths is well documented[8], but after 20 years of large-scale use of PZQ, the spectra of drug-resistant parasites are looming and it is time for introducing a new therapeutic approach[9]. Despite that more than 100 million people are currently being treated for schistosomiasis with praziquantel; they are rapidly reinfected and must be retreated on an annual or semiannual basis[10]. Low-cure rates have been recorded in many studies in Africa including Egypt where patients have yielded isolates that are tolerant to higher dosages of PZQ[11-13]. The efforts to expand mass drug administration programs in these endemic countries may accelerate emergence of resistance[14]. Moreover, praziquantel effectiveness could be limited due to its inability to kill schistosomes as early as 2-4 weeks post-infection[15].

Studies of schistosome life cycle have focused on a fact that it can survive for decades in the blood stream of the human host without being severely affected by assault caused by various reactive oxygen species (ROS) because it has a mechanism to degrade ROS[16]. A distinction between host and parasite physiology with respect to detoxification of ROS has led to identification of a novel line of potential schistosomicides. Mammals have two distinct detoxification enzymes, thioredoxin reductase and glutathione reductase (GR), while in schistosomes these catalytic activities are performed by only one molecule, thioredoxin-glutathione reductase[17-18]. Thus TGR has become one of the most appealing drug targets against schistosomiasis[19]. In addition, CcP protects the worms from H_2O_2 generated in the mitochondria of *S. mansoni* and also from exogenous H_2O_2 produced by activated phagocytes from the host. The absence of this enzyme in mammalian cells makes it as a possible interesting target for therapeutic control of schistosomiasis[20].

Anti-schistosomial activity of artemisinin was first reported by Chen et al. in 1980[21]. Based on previous studies either *in vitro*[22], or *in vivo*[23-29]; artesunate (ART) was introduced as a new anti-schistosomal drug. Among several artemisinin derivatives, artesunate is documented to be less toxic especially when given orally owing to its pharmacokinetic properties[30-32]. The water-soluble compound artesunate is safer because it is absorbed and eliminated rapidly, whereas in oil-based derivatives such as artemether or arteether, blood concentrations are sustained throughout the dosing interval[33-34]. Although, their antimalarial effect is basically mediated by heme-dependent cleavage of endoperoxide with subsequent liberation of cytotoxic intermediates and free radicals, but their molecular mechanism of action on schistosomes is not yet fully defined[13,35]. The proposal that anti-schistosomial effect of artesunate could be mediated *via* other mechanisms is supported by observation that artemisinin analogs act against plasmodium and other parasites by a clear difference in concentration ranges and dosage regimen[35]. Also, the conclusion that severe iron-deficiency would not influence the efficacy of artesunate raising non heme-mediated pathway as a possible mechanism[27].

Thus, the present work was designed to determine the possible effect of artesunate on Schistosome TGR and CcP c peroxidase as possible targets for therapeutic control of schistosomiasis.

MATERIALS AND METHODS

Parasite

Laboratory bred *Biomphalaria alexandrina* snails were purchased from the Schistosome Biological Supply Program, Theodore Bilharz Research Institute (Giza, Egypt). According to Lewis et al. [36], the snails were placed in beakers containing dechlorinated water (1 mL/snail) and exposed to direct light at 28 °C for at least 4 h. *Schistosoma mansoni* cercariae shed from the snails were used to infect the experimental animals of the study. The cercarial suspension was adjusted to contain 80-100 cercariae/0.1 mL dechlorinated water.

Animals and Experimental Design

A total of 200 laboratory bred male Swiss albino mice, 6-8 weeks old, weighing 20-25 g were purchased from Theodore Bilharz Research Institute (Giza, Egypt). The experiment was adopted by the Research Ethics Committee (REC), Faculty of Medicine, Tanta University in accordance with EU Directive 2010/63/EU for animal experiments. Mice were housed in appropriate cages and allowed ad libitum for a commercial rodent chow and tap water. Each mouse was infected by subcutaneous

injection of 0.1 mL cercarial suspension as described by Peters and Warren (1969)[37]. They were then divided into four groups, 50 mice each; group I: infected untreated group (Control group) received a vehicle of 1% sodium carbonyl methylcellulose (CMC-Na), group II: infected then treated with artesunate, group III: infected then treated with praziquantel and group IV: infected then treated with artesunate then praziquantel.

Treatment Protocol

Artesunate (Sigma-Aldrich) was dissolved in 1% sodium carbonyl methylcellulose (CMC-Na) and given by oral gavage in a dose of 300 mg/kg at time schedule started at the 14th day post-infection (PI) and repeated once every 2 weeks for 4 consecutive doses. This dosage regimen was selected on the basis of a previous study by Shaohong et al.[27], evaluating different treatment protocols of artesunate in S. mansoni infected mice. Praziquantel (Distocide; 600 mg tablet, E.I.P.I.Co. Pharmaceuticals, Egypt) was dissolved in distilled water and given orally to mice in a single dose of 600 mg/kg at the 42nd day post-infection[38] (Figure 1). The used dose for mice was matched to the Human Equivalent Dose based on body surface area[39]; where Human Equivalent Dose (mg/kg) = Animal dose (mg/kg) x Animal Km / Human Km, where Km values for mouse and human are 3 and 37; respectively with the average adult human body weight is 70 kg. Drugs were administrated orally using one mL syringe equipped with a blunt, 18-gauge needle.

Collection of Adult S. mansoni Worms by Animal Perfusion Method

All mice were sacrificed ten weeks post-infection. Hepatic and portomesenteric vessels were perfused according to Duvall and DeWitt[40] to recover worms. All *S. mansoni* adult worms in the liver and mesenteric veins of the small and large intestines were removed from the perfusion fluid, collected in Petri dish, washed, sexed and counted. Worms were then stored at -80 °C for further estimation of TGR and CcP mRNA expression. The reduction rates of total and female worms were calculated by comparing the mean worm numbers in the treated groups with that of control group.

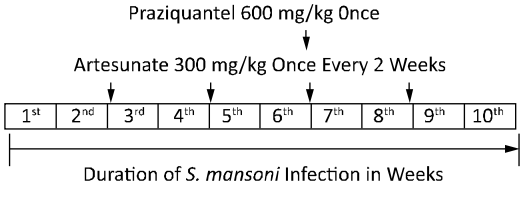

Figure 1. Scheme for the treatment protocol.

Tissue Egg Count

One gram from liver was put in a test tube containing 2 mL of 5% KOH and left overnight at room temperature. The second day, all test tubes were put in the incubator at 37 °C for 6 h. Each test tube was shaken then 0.1 mL of the digest was examined microscopically for counting *S. mansoni* eggs. The average number of ova in 0.1 mL was determined and the number of ova/gram tissue was calculated[41-42]. The reduction rates of tissue egg count were calculated by comparing the mean tissue egg count in the treated groups with that of control group.

Estimation of TGR and CcP mRNA Expression in S. mansoni Adult Worms by Semi-quantitative rt-PCR

S. mansoni adult worms were homogenized in 1 mL Trizol (Invitrogen, USA) and processed for RNA extraction using MagNA Pure compact Nucleic Acid isolation kit I (Roche Diagnostics, GmbH, Mannheim, Germany) following manufacturer's instructions. The yield of total RNA obtained was quantified spectrophotometrically. A total of 1 µg parasite RNA was used to prepare cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's instructions.

A final reaction volume of 20 µL was prepared using Light Cycler-DNA Master SYBR Green I kit (Roche diagnostics, Germany). Each mix is formed of 1 µL each primer (0.5µmol/L), 2 µL Light Cycler DNA Master SYBR Green I (1x), 2.4 µL $MgCl_2$ stock solution (4 mmol/L), 11.6 µL H_2O sterile PCR grade and 2 µL of cDNA template (30 ng/µL). PCR reactions were done in Light Cycler (Roche, Germany). The program included an initial melting phase at 50 °C for 2 min, denaturation at 95 °C for 10 min followed by 45 cycles of amplification (15 s at 95 °C, 1 min at 60 °C). Using the standard curve, GAPDH as an internal control gene, semiquantitation of genes expression was calculated as a ratio between target gene and internal control (Figures 2 and 3). The sequences of the primers are supplied in Table 1.

Statistical Aanalysis

Quantitative values of the measured parameters were expressed as mean±standard

deviation (SD). The data were analyzed by One Way-ANOVA (*F* value) to detect significance in between groups followed by post-hoc analysis to detect difference between each two means, using Statistical Package for Social Sciences (SPSS), version 14.0 for windows. The difference was considered statistically significant when $P<0.05$.

RESULTS

The number of total worms recovered from *S. mansoni*-infected mice 10 weeks post-infection was significantly reduced in ART-treated group (73.4%).

Table 1. Primer Sets for Amplification of TGR, CcP, GAPDH Genes of *S. mansoni* Adult Worms

Genes	Primers (5'-3')
Thioredoxin glutathione reductase (TGR)	F: CTATTTCCGTAGACGTCTGT
	R: AATACAGTTTCCTTCCCGTT
Cytochrome c peroxidase (CcP)	F: TCCTTTATCAATTTGAGAGG
	R: CCAACCATAAACATATGATG
GAPDH	F: GTTTTGGTCGTATCGGGAGA
	R: ATGCGTTAGAAACCACGGAC

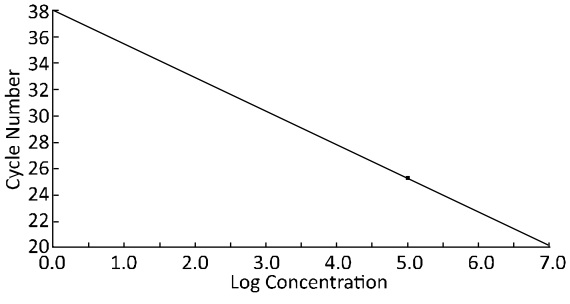

Figure 2. Standard curve for GAPDH.

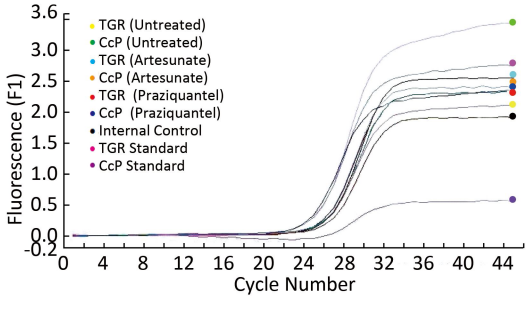

Figure 3. Amplification curve of semi-quantitative rt-PCR presents m-RNA expression of TGR and CcP of *S. mansoni* adult worms (10 weeks post-infection).

The respective total worm burdens reduction in PZQ-treated group was 86.7% and 95.4% in combined ART-PZQ treated group. The reduction rate in female worms in ART-treated group and PZQ-treated group was 74.8% and 88.3%; respectively, whereas 100% reduction rate in female worms was achieved in ART-PZQ treated group (Table 2). Regarding tissue egg count, complete disappearance of tissue eggs was recorded in ART-PZQ treated group (100% reduction rate) with a respective reduction rate of 95.9 % in ART-treated group. Tissue egg count showed only 68.4% reduction in PZQ-treated group (Table 3).

Semi-quantitative rt-PCR values revealed that treatment with artesunate caused significant decrease in expression of schistosome TGR and CcP in comparison to the untreated group. In contrast, the treatment with praziquantel did not cause significant change in expression of these genes (Table 4).

Table 2. Effect of Different Treatment Protocols on Total and Female Worm Burdens in Mice Experimentally Infected with *S. mansoni* Versus Infected Untreated Mice

Groups	Total		Female	
	N (Mean±SD)	%reduction	*N* (Mean±SD)	%reduction
I	24.10±4.01	-	11.10±1.85	-
II	6.40±1.90*	73.4	2.80±1.48*	74.8
III	3.20±1.32$^{*,\#}$	86.7	1.30±0.82$^{*,\wedge}$	88.3
IV	1.10±0.32*	95.4	0*	100

Note. $^{*}P<0.001$ between group II, group III and group IV as compared with group I; $^{\#}P<0.05$ between group III as compared with group II; $^{\wedge}P>0.05$ between group III as compared with group II.

Table 3. Effect of Different Treatment Protocols on Tissue Egg Count in Mice Liver Experimentally Infected with *S. mansoni* versus Infected Untreated Mice (eggs/g liver).

Groups	Tissue Eggs *N* (Mean±SD)	% Reduction
I	2511.50±162.44	-
II	104.20±23.41*	95.9
III	793.30±100.53$^{*,\#}$	68.4
IV	0*	100

Note. $^{*}P<0.001$ between group II, group III, and group IV as compared with group I; $^{\#}P<0.001$ between group III as compared with group II.

Table 4. Semi-quantitative rt-PCR Values of m-RNA Expression of TGR and CcP of *S. mansoni* Adult Worms (10 weeks post-infection)

Groups	TGR (mean±SD)	CcP (mean±SD)
I	14.68±0.35	12.72±0.29
II	1.04±0.17*	2.36±0.34*
III	14.56±0.32#	12.34±0.30#

Note. *P<0.001 between group II and group III as compared with group I; #P>0.05 between group II and group III as compared with group I.

DISCUSSION

Extensive using of praziquantel with concerns about possibility of drug resistance development, unavailability of an applicable vaccine, and absence of a reasonable alternative to praziquantel, those all represent a real challenge in control of schistosomiasis[43]. Artemisinins are new promising anti-schistosomal compounds with well tolerated therapeutic dosage range, but their molecular mechanism of action on schistosomes is still in need to be investigated[13,35]. Living in an aerobic environment, worms must have effective mechanisms to maintain cellular redox balance. Schistosome TGR[19,44-45] and CcP[20] have been reviewed as possible interesting macromolecular targets for therapeutic control of schistosomiasis.

The results of the current study elucidated for the first time that anti-schistosomal mechanisms of artesunate is mediated via reduction in expression of schistosome TGR and CcP. The loss of these two defensive enzymes makes the parasite more vulnerable during its different stages to be assaulted by the host generated reactive oxygen species. Regarding the functional and biochemical differences between the redox metabolism of *S. mansoni* and its human host, TGR was hypothesized to be an essential parasite protein and a potentially important drug target. Characterization of *S. mansoni* TGR revealed its multifunctional oxidoreductase activities with remarkably wide substrate specificity, capable of directly reducing peroxides, selenium-containing compounds, as well as thioredoxin (Trx), oxidized glutathione (GSSG), and reduced glutathione (GSH). These substrate preferences might possibly be exploited for future TGR-directed anti-schistosome drug design[18]. Regarding that schistosome lacks catalase[46], the previous reports suggesting that glutathione peroxidase plays a pivotal role in protection of schistosomes supported by the fact that it shows the highest affinity for phospholipid hydroperoxides, a major product of lipid peroxidation[16,47]. It is evidenced that transcription and translation of various schistosome antioxidant enzyme genes is dependent on developmental regulation, where the early skin and lung stages (3 h and 7 d post-infection, respectively), exhibit the lowest level of specific mRNA while adult mature egg-producing worms (42 d post-infection) exhibit the highest transcript levels protecting them against oxidant killing[48]. It is interest to refer that previous studies showed that artesunate caused morphological changes in *S. mansoni* tegument[49]. The fact that most of antioxidant enzymes are localized in tegument of adult schistosomes, provides an adaptive response against the host cellular response. Their localization in gut epithelium of adult schistosomes protects against reactive oxygen species released from host blood cells[50]. Although CcP, is present in mitochondria in the adult worm tegument and can neutralize hydrogen peroxidase, but it is unlikely to be released from the mitochondria to have a general effect against peroxidation[20]. This could be debated by the fact that energy metabolism of *S. mansoni* is shifted through its life cycle from being aerobic in early stages to be totally anaerobic in adult worms[51]. Thus, the mitochondrial antioxidants could basically have a defensive role since they are not directed to energy production. In regard to notion that the host immune system could have more than one mechanism to eliminate parasites, argument may be raised against role of oxidative stress in parasite killing. Previously, Scott et al.[52] have demonstrated that macrophage cell lines that do not produce a respiratory burst are still able to kill schistosomula when activated with cytokines. This is most likely as a result of formation of nitric oxide through immunoregulatory mechanisms[53-54]. However, recent studies contributed the potential direct cytotoxicity of nitric oxide to production of reactive nitrogen intermediates and peroxynitrite ($ONOO^-$), a toxic oxidant generated when it couples with the superoxide radical[55-56].

Interestingly, the current results showed that ART caused significant reduction in tissue egg reduced by 95.9% versus 68.4% in PZQ-treated group although it caused less reduction in female worm reduced by 74.8% in comparison to 88.3% in PZQ-treated group. This observation indicating that

artesunate impaired the fecundity of adult female worms rendering them sterile rather than affecting their count. The same phenomenon was observed by Botros et al.[28] who found that residual worms recovered became sterile and incapable of laying eggs. Abdul-Ghani et al.[57] reported that artemether, another artemesinin derivative, induced significant reductions in the liver tissue egg load, ranging from 75.2% to 82.6% as well as significant alterations in oogram pattern with cessation of oviposition and increased rates of dead eggs. Araújo et al.[23] explained the reduction in egg load by a fact that artesunate modified the reproductive organs of *S. mansoni* female worms in the form of reduction of ovarian volume and rarefaction of the vitelline follicles. This explanation is supported by findings of Bartley et al.[58] who described that worms became smaller (30%-50% reduction in size) with atrophic testes and ovaries. Most, if not all, remaining worms are males being not responsible for any morbidity and even their persistence in blood might be useful in eliciting concomitant immunity against schistosomiasis[59]. El-Lakkany et al.[60] showed that early treatment of *S. mansoni*-infected mice (28 d post-infection) could protect the host from later damage caused by schistosome eggs. When Shao hong et al.[27] used different treatment protocols of artesunate against experimental *S. mansoni* infection in mice; they found that artesunate not only diminished the number of loaded tissue eggs, but also affects immature worms and seems to kill mature adults. The reduction rate of immature adult *S. mansoni* worms reached a maximum when artesunate is given at the post-lung stage that coincides with their peak growth profiles. The worm reduction rate was further enhanced when additional doses were administered at 8 or 9 weeks post-infection. For *S. mansoni*, drug administration starting 14 or 21 d after infection followed by 3 repeated doses at 2-week intervals provided optimal protection. Araújo et al.[23] and Bartley et al.[58] mentioned that effects on the worms appear to be partly reversible by day 56 post-infection and the surviving worms recovered and restarted ovi-position. Considering the lengthy exposure period in heavy endemic areas, a treatment regimen with long intervals might be practical for field application[27]. So, Utzinger et al.[25] suggested that repeated doses are mandatory for effective prevention of patent schistosomal infections and that use of integrated treatment strategies has considerable potential for schistosomiasis control. It has been established that the schistosomes become most sensitive to PZQ at 6 weeks post-infection timing that corresponded to the period of ovi-position and plateau phase of parasite susceptibility[13,61-62]. The relative lack of efficacy of PZQ against juvenile schistosomes is a potential factor in poor cure rates and treatment failures observed in some patient groups, particularly those living areas suffering very high rates of transmission[63]. Regarding this aspect, it is preferred to use drugs affecting different stages of the schistosome parasites life cycle to achieve radical cure[64]. Artemisinin derivatives are of particular concern because they are more active against early developmental stages of schistosome[13]. This observation is quite interesting per se, because this is exactly the time when praziquantel is ineffective[65-66]. Consequently, a combined treatment with praziquantel together with an artemisinin derivative has been suggested as a strategy for transmission control in endemic area in order to avoid or delay induction of parasite drug resistance, and to prevent recrudescence[25-26,67].

In the present study, treatment protocol with both praziquantel and artesunate provided maximum reduction in total worm count (95.4%) with complete eradication of female worms and tissue egg count (100%) in comparison to the lower reduction rate achieved by monotherapy with either one of them. In view that many researchers have been identified PZQ-resistant isolates of *Schistosoma mansoni* and *S. haematobium*[67-69], a previous study reported that treatment by praziquantel with any of artemesinin derivatives affects the parasites at a different stage, enabling to kill most of the schistosomules and adult worms harboured in the hosts[70]. Doenhoff et al.[13] reviewed that PZQ monotherapy achieves cure rates ranged from 60% up to sometimes 85%-90%, but 100% cures have seldom, if ever, been recorded in an endemic area. Moreover, adult *S. mansoni* parasites that escaped treatment could reside in the mesenteric veins of the human host, where they can survive for up to 30 years[71]. In regard to its anti-schistosomal effect, experimental studies indicated that praziquantel comprises two aspects, first, the direct effect of praziquantel on schistosomes tegument and second, induction of host immune reaction in response to antigens exposed on the disrupted parasite surface[72-74]. Regarding the mechanisms of all such effects, PZQ has been established to cause intense muscular paralysis due to a rapid influx of calcium

ions[75]. Schistosome calcium ion (Ca^{2+}) channels are the only moiety so far identified as the molecular target of PZQ[13,76]. In this context, the present results revealed that praziquantel had no effect on expression of schistosome TGR and CcP. Linking this with the finding that artesunate has been proved to inhibit expression of these targeting enzymes as a new valuable mechanism, confirmed that its addition to praziquantel could achieve complete cure outcome in treatment of schistosomiasis.

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