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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

In vitro schistosomicidal activity of tamoxifen and its effectiveness in a murine model of schistosomiasis at a single dose

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Abstract

Schistosomiasis is a neglected tropical disease affecting 220 million people worldwide. Praziquantel has proven to be effective against this parasitic disease, though there are increasing concerns regarding tolerance/resistance that calls for new drugs. Repurposing already existing and well-known drugs has been a desirable approach since it reduces time, costs, and ethical concerns. The anti-cancer drug tamoxifen (TAM) has been used worldwide for several decades to treat and prevent breast cancer. Previous reports stated that TAM affects *Schistosoma* hormonal physiology; however, no controlled schistosomicidal in vivo assays have been conducted. In this work, we evaluated the effect of TAM on female and male *Schistosoma mansoni* morphology, motility, and egg production. We further assessed worm survival and egg production in *S. mansoni*-infected mice. TAM induced morphological alterations in male and female parasites, as well as in eggs in vitro. Furthermore, in our in vivo experiments, one single dose of intraperitoneal TAM citrate reduced the total worm burden by 73% and led to a decrease in the amount of eggs in feces and low percentages of immature eggs in the small intestine wall. Eggs obtained from TAM citrate-treated mice were reduced in size and presented hyper-vacuolated structures. Our results suggest that TAM may be repurposed as a therapeutic alternative against *S. mansoni* infections.

Keywords Drug repurposing · Egg · Experimental treatment · *Schistosoma mansoni* · Tamoxifen

Introduction

Schistosoma mansoni is a trematode parasite found in South America, sub-Saharan Africa, Sudan, Egypt, and parts of the Middle East (WHO 2016). While infecting humans, adult schistosomes that live in the bloodstream are not able to cause significant pathology; however, eggs produced after female fertilization can trigger relevant immunopathological responses. This phenomenon is explained by the intense granulomatous reaction observed around eggs in the liver and small intestine, which accounts for almost all of schistosomiasis clinical symptoms (Burke et al. 2009). Oral administration of praziquantel (PZQ) remains as the first choice for schistosomiasis treatment as it is a well-tolerated, low cost, and effective drug. Despite its clinical safety, tolerance and resistance to PZQ have now been recognized for different *Schistosoma* species, including *S. mansoni* strains both in vitro and in vivo. These findings raise the scientific community concern, justifying the need for new drugs research and development (Lamberton et al. 2010; Mwangi et al. 2014; Pinto-Almeida et al. 2016).

Repurposing anti-cancer and antibacterial drugs against several helminthic infections has been trialed, due to reduced costs and ethical reasons (Cowan and Keiser 2015; Panic et al. 2014). One of these drugs is tamoxifen (TAM), an anti-cancer drug widely used to treat and prevent breast cancer as it works as a selective estrogen receptor modulator (Bhattacharya et al. 2017). TAM proved to be efficient in reducing parasite burden of *Taenia crassiceps*-infected mice (Terrazas et al. 1994) and against *Echinoccocus granulosus* infections by impairing

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hydatid development and the establishment of hydatidosis in mice (Nicolao et al. 2014). A limited number of studies have reported the effect of TAM against *S. mansoni*, focusing on its interference with the parasite hormonal physiology (Giannini et al. 1995a, b). There is no available data regarding TAM schistosomicidal activity in controlled in vitro and in vivo assays. Therefore, in the present work, we describe the in vitro effects of this anti-cancer drug on female and male *S. mansoni* morphology, motility, and egg production. Also, *S. mansoni*-infected mice were treated with TAM for further evaluation of worm survival and egg production.

Materials and methods

Animals and parasites

S. mansoni BH strain (Belo Horizonte, MG, Brazil) was used in all experiments. The parasite life cycle was maintained in *Biomphalaria glabrata* freshwater snails in our laboratory at the Department of Animal Biology, Biology Institute, UNICAMP. Swiss/SPF female mice weighing ~ 20 g (4 weeks of age) were used as definitive hosts, being infected through tail exposure to a suspension containing 70 cercariae (Olivier and Stirewalt 1952). The Ethics Commission approved all experiments using rodents (CEUA/UNICAMP, protocol no. 2170-1).

Drug preparation

Tamoxifen (TAM) and TAM citrate (cTAM) were purchased from Sigma-Aldrich® (St. Louis, MO, USA) and dissolved in 0.1% dimethylsulfoxide (DMSO) and sterile normal saline solution (0.9% NaCl in water), respectively.

Schistosomicidal in vitro assays

S. mansoni worms were collected 8 weeks after infection, through the hepatic portal system and mesenteric vein perfusion as previously described (Pellegrino and Siqueira 1956). Worm couples (male/female pairs) were carefully washed in RPMI-1640 medium (Nutricell®) supplemented with 0.05 g/L streptomycin, 10,000 UI/ml penicillin, 0.3 g/L L-glutamine, 2.0 g/L D-glucose, 2.0 g/L $NaHCO_3$, and 5.958 g/L HEPES. Sequentially, one couple was transferred to each well of a 24-well culture plate (TPP, St. Louis, MO, USA) containing RPMI-1640 medium and incubated at 37 °C, 5% CO_2 atmosphere (de Oliveira et al. 2014). TAM and cTAM were tested at 2.5, 5, 15, or 45 μM. Schistosomes incubated with 0.1% DMSO was used as vehicle control groups. All experiments were performed in five replicates. Parasites were monitored for 72 h using an inverted optical microscope (DM-500, Leica®). Schistosomicidal effect was assessed with emphasis on the following parameters: morphology, parasite mating, motility, oviposition, and mortality (de Oliveira et al. 2017b). Images of adults and eggs at the bottom of 24-well culture plates were obtained for each treatment condition using a camera adapted to DM-500, Leica® inverted optical microscope.

In vivo assays

Balb/C female mice weighing ~ 20 g were infected with 70 *S. mansoni* cercariae by tail immersion as previously described. Eight weeks after infection, animals were allocated to four groups ($n = 5$ mice/group) and treated as follows:

- Group (I): a single dose of 100 mg/kg cTAM by gavage
- Group (II): a single dose of 100 mg/kg cTAM by intraperitoneal injection
- Group (III and IV): received 300 μL PBS solution orally and intraperitoneally, respectively

Animals were euthanized 2 weeks after treatment and parasites were recovered from the hepatic portal system through perfusion and quantified. Worm burden of treated mice was compared with infected and PBS-injected animals. Worm reduction percentages were calculated as described by Delgado et al. (1992). Oogram investigation was performed as recommended by Pellegrino et al. (1962). According to Pellegrino and colleagues, considering that the eggs are the main causative agents of schistosomiasis' pathogenicity, the oogram is a valuable method when choosing a new drug against the disease, as it is an indicative of interruption in females' oviposition. Then, small intestines were collected, opened longitudinally, washed in PBS, and compressed between two microscopy slides. Eggs retained in the small intestinal wall were classified as immature, mature, and dead, and the percentage of the different egg developmental stages (oogram pattern) was recorded (de Oliveira et al. 2017a; Pellegrino et al. 1962). At least 100 eggs were evaluated per animal. Kato-Katz method was used for quantitative fecal examination (Katz et al. 1972). Fecal samples were collected individually from all animals and examined for further comparison with the oogram findings. Eggs were counted under a light microscope (Leica® DMI-500) after 24 h. Images of eggs were obtained and individual areas (A; in μm^2) were measured using the software *Leica*® LAS EZ4 HD, according to the formula: $A = 1/2a \times 1/2b \times \pi$; where a = major radius and b = minor radius of the ellipse. Statistical analysis was performed with GraphPad Prism® v5.0a. Results were compared using the Mann-Whitney test for pairwise comparisons. Differences in parasite burdens and histological scoring were considered significant if $p < 0.05$.

Results

TAM is active against *Schistosoma mansoni* in vitro in a time- and dose-dependent manner

S. mansoni couples were maintained in 24-well plates for 72 h with increasing concentrations of TAM. As shown in Fig. 1 a, incubation of adult worms with 45 μM TAM led to approximately 50% of worm mortality. cTAM, instead, proved to be more effective not only at 45 μM, but also at lower concentrations in short intervals of incubation (Fig. 1b). At 15 and 45 μM, cTAM killed 40 and 80% of adult worms after 48 h, respectively. After 72 h, mortality rates were around 20% for 2.5 and 5 μM, and resulted in 100% for 15 and 45 μM cTAM (Fig. 1).

Female worms exposed to TAM and cTAM showed esophageal alterations, i.e., bulb-shaped esophagus (Fig. 2a, d) and the presence of eggs with vacuolated content (Fig. 2b, e). Eggs were severally malformed with internal content overflow and abnormally elongated lateral spine (Fig. 2c, f). Male parasites suckers showed no movements and, upon touching, they were found completely stiff.

Oral and intraperitoneal administration of cTAM, at one single dose, reduces worm burden and the total number of *S. mansoni* eggs

One single dose of 100 mg/kg cTAM significantly reduced the total worm burden in *S. mansoni*-infected mice, either through oral (54%) or intraperitoneal (73%) administration. Furthermore, there was a decrease in the number of couples found for both treatments, as well as a significant reduction in the number of recovered males (Table 1).

Treatment led to a significant reduction in the number of eggs in feces when administrated both orally (~ 88%) and by the intraperitoneal route (~ 80%). A significant decrease in the number of immature eggs followed by an increase in the percentage of mature eggs was also detected in the intestinal tissues oogram pattern for treated animals (Table 2). Vacuolated cells were found in fecal and intestinal tissue eggs as well as dead miracidia. Eggs present in the stool of mice treated with cTAM intraperitoneally showed a significant decrease in area size when compared with the control group (Fig. 3).

Discussion

Several studies reported the effectiveness of repurposing already marketed drugs against helminthiasis (Panic et al. 2014). Some broad-spectrum drugs with well-established clinical use have been tested against different pathogens in an attempt of reposition them has therapeutic alternatives. An example of such drugs is tamoxifen (TAM), used over four decades for breast cancer (Kennecke et al. 2006; Panic et al. 2014; Radmacher and Simon 2000). TAM belongs to the selective estrogen receptor modulators family (SERMs), along with other drugs such as toremifen and raloxifen, since it presents an agonist or antagonist activity on estrogen receptors, depending on the target tissue. Its usage is mainly restricted against breast cancer in postmenopausal women, even though clinical studies attest its long-term efficiency in comparison to aromatase inhibitors (Aihara et al. 2014). Although TAM-estrogen receptor interaction is well recognized, we still do not understand its entire mechanism of action. However, it is known that TAM exhibits other molecular effects that are independent from its interaction with the estrogen receptor, such as interference with signaling proteins (e.g., caspases and calmodulin) (Altan et al. 1999; Lavie et al. 1997; Mandlekar and Kong 2001; Zhang et al. 1994).

TAM's activity against protozoan parasites is well known against intra- and extracellular forms of trypanosomatids, as well as in experimental infections in BALB/c mice and golden hamsters (Eissa et al. 2011; Miguel et al. 2010, 2007, 2008, 2009). Additionally, Vargas-Villavicencio et al. (2007) described the cysticide activity of TAM against *Taenia crassiceps* in vitro and also showed its protective activity against murine cysticercosis. Escobedo et al. (2013) showed that golden hamsters treated every other day with TAM (1 mg/kg for 15 days) presented 70% reduction in the

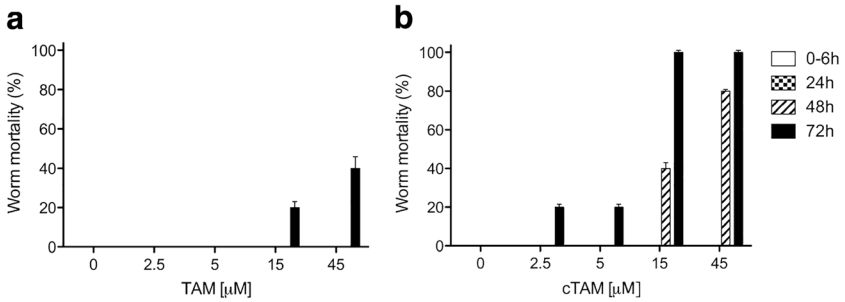

Fig. 1 Percentages of worm mortality quantified after 6, 24, 48, and 72 h of incubation with TAM (**a**) and cTAM (**b**). All experiments were performed in five replicates, being each replicate represented by one couple of *Schistosoma mansoni* incubated at 37 °C, 5% CO_2 atmosphere in RPMI-1640 medium. "0", *x*-axis: control groups of worms maintained in medium for up to 72 h in the presence of 0.1% DMSO (**a**) or not (**b**)

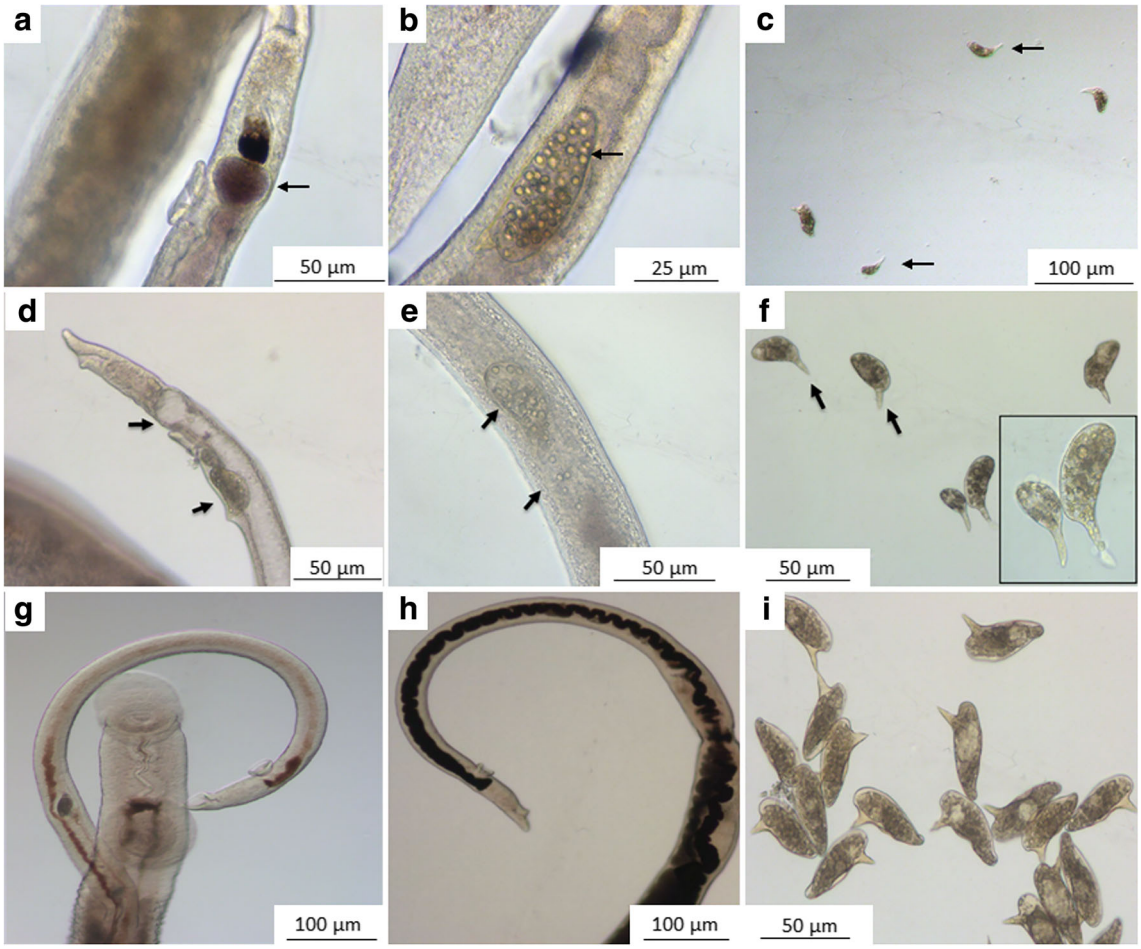

Fig. 2 Cultured-*S. mansoni* worms and eggs exposed to 15 μM TAM (**a**–**c**) or cTAM (**D-F**). **a**, **b**, **c** Parasites after 24-h incubation. Arrows point to internal structure alterations. **a, d** Female worm presenting esophageal alterations (arrow, bulb-shaped esophagus). **b**, **e** Internal malformed eggs with vacuolated content (arrow). **c**, **f** Malformed eggs (laid by females in vitro) showing content overflow. Arrow points to the aberrant lateral spines (detailed image in the **box**). **g**, **h**, **i** Untreated control group; adult parasites showing normal internal structure morphology, as well as normal eggs laid by females

intestinal fixation of *T. solium*. It has been demonstrated that TAM inhibits *E. granulosus* protoscoleces and metacestodes survival as well (Nicolao et al. 2014).

Given TAM's anti-plathyhelmintic potential, we aimed to investigate its activity against trematoda parasites, such as *S. mansoni*. Few data regarding TAM's activity against

Table 1 Worm recovery from the hepatic portal system and mesenteric intestinal veins of mice experimentally infected with *S. mansoni* and treated with PBS (control group) or one single dose of 100 mg/kg cTAM

Administration route	Treatment	Worm burden (mean ± SD)				
		Couples	Males	Females	Total	Total % reduction
Intraperitoneal	Control	8.5 ± 3	5.4 ± 3.1	3.9 ± 1.4	26.3 ± 5.9	–
	cTAM	2.5 ± 1*	1.3 ± 0.9***	0.7 ± 0.7	7 ± 2.3*	73%
Oral	Control	12.2 ± 3.4	5.8 ± 1.9	1.8 ± 0.7	32 ± 6.4	–
	cTAM	6.7 ± 1.9**	0.9 ± 1****	0.5 ± 0.6	14.7 ± 3.5*	54%

*Significant reduction when compared with control group, $p < 0.05$

*$p < 0.001$

**$p = 0.0012$

***$p = 0.021$

****$p = 0.0051$

Table 2 Development stage of eggs retained in the intestinal tissues (oogram pattern); fecal egg count (EPG, number of eggs per gram of feces); and fecal egg count reduction (%) of mice experimentally infected with *S. mansoni* and treated with PBS (control group) or one single dose of 100 mg/kg cTAM

Administration route	Treatment	Oogram pattern			EPG	
		Immature eggs (%)	Mature eggs (%)	Dead eggs (%)	Total	% Reduction
Intraperitoneal	Control	68.2 ± 4.6	26.2 ± 4.1	5.8 ± 1.7	1084 ± 322	–
	cTAM	18.1 ± 3.3*	70.5 ± 2*	7.2 ± 2.1	214 ± 53*	80%
Oral	Control	77.6 ± 6	18.4 ± 5.1	4 ± 1.7	1521 ± 339	–
	cTAM	13 ± 6.9*	66 ± 9.6*	13.6 ± 4.5	181 ± 91*	88%

*Significant reduction when compared with control group, $p < 0.0001$

S. mansoni can be found, and most of it evaluates the physiologic aspects regarding the parasite sexual maturity. Giannini et al. (1995b) described the role of estrogen receptors during *S. mansoni*'s egg development, observing lower maturation levels in females and eggs with morphologic changes. Our results are in accordance with the previously described, as in in vitro assays, the drug caused morphological alterations in eggs and adults. In vitro, both TAM and cTAM were capable of inducing worm mortality, particularly cTAM that led to 100% mortality rate at 15 and 45 μM after 72 h. Perhaps, TAM in its salt form was more active due to its more effective solubilization. In fact, cTAM is the commercial form of the drug orally taken by patients treating breast cancer. In 2015, Cowan and Keiser (2015) showed that cTAM, at 5.7 μM, reduced 50% of *S. mansoni* survival.

In *S. mansoni*-infected mice, TAM and cTAM caused significant reduction in the total worm burden after administrated orally (54%) or intraperitoneally (73%) after one single oral dose. In previous studies obtained by our group (Frezza et al. 2015), a single dose of PZQ (100 mg/kg) led to total worm burden reduction of 27%. There was also a significant reduction in the number of eggs in feces for cTAM-treated groups as well as a decrease in the percentage of immature eggs and increase of mature eggs in intestinal tissues. It is worth mentioning that eggs in the intestinal wall and in feces presented dead miracidia and vacuolated atypical internal structures, similar to those found in in vitro assays. Our results suggest that TAM affected the parasite reproductive system, inhibiting the development of *S. mansoni* eggs and interrupting or reducing oviposition of the female worms. It is also important to notice that cTAM administration led to a significant decrease in egg size. The decrease in the number of fecal eggs as well as morphological alterations observed are extremely relevant in terms of schitosomiasis controlling and prevention. In this scenario, *S. mansoni* eggs play a key role in terms of parasite life cycle maintenance and schistosomiasis pathogenesis, as eggs retention in the host tissues and organs are capable of triggering inflammatory responses resulting granuloma formation (Gryseels et al. 2001).

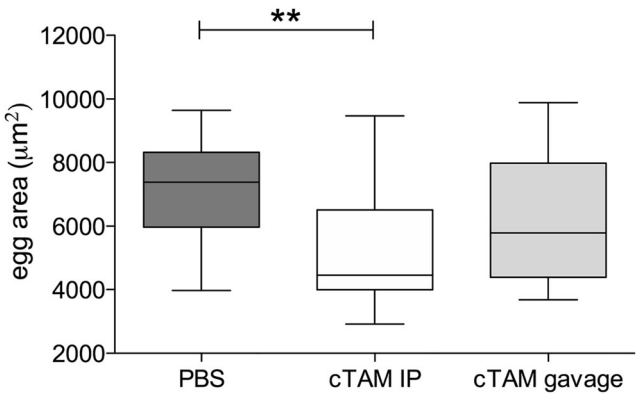

Fig. 3 Egg area quantified of at least 20 egg images recorded from intestinal oograms of *S. mansoni*-infected mice treated with 100 mg/kg cTAM intraperitoneally (cTAM IP) or by the oral route (cTAM gavage). "PBS", control group. $^{**}p = 0.0015$

Usually, breast cancer treatment with TAM is long term (~ 5 years) and, although TAM is well tolerated and has few side effects, thromboembolic events and endometrium proliferation are associated with prolonged use (Miguel et al. 2008). The dose choice in our study was based on previous studies that showed TAM's safety for this animal model (DeGregorio et al. 1987; Miguel et al. 2009; Rotheneichner et al. 2017). No death and changes in weight, hair loss, or bristling fur were noticed for cTAM-treated mice. Our results were obtained after treatment with a single dose, further reducing the risk of potential side effects and showing the therapeutic potential of TAM against *S. mansoni*.

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Authors' contributions Designed the study: R.N.O., S.M.A., and D.C.M.; performed the experiments: R.N.O., S.A.P.C., K.M.V., and D.C.M; analyzed the data: R.N.O., T.M., S.M.A., and D.C.M.; wrote the paper: T.M., S.M.A., and D.C.M.

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Compliance with ethical standards

The Ethics Commission approved all experiments using rodents (CEUA/UNICAMP, protocol no. 2170-1).

Conflict of interest The authors declare that they have no conflict of interest.

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