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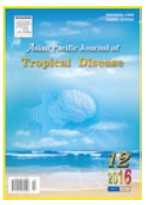

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In vitro screening of BTP-Iso on *Schistosoma mansoni* and its intermediate host *Biomphalaria alexandrina*

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ABSTRACT

Objective: To test the effect of benzimidazole-derived compound (compound BTP-Iso) on cultured adult *Schistosoma mansoni* and clean *Biomphalaria alexandrina* snails.
Methods: Adult schistosomes were incubated at different concentrations of compound BTP-Iso to calculate mortality rate, LC_{90} and LC_{50}, in addition, tegumental changes were recorded using SEM. Clean *Biomphalaria alexandrina* snails were also incubated with compound BTP-Iso at various concentrations to report snail mortality.
Results: Compound BTP-Iso was found to possess potent antischistosomal activities on adult worms but no effect was recorded on snail host. At a concentration of 8 μg/mL, mortality rates were 45.8% and 81.0% after 48 h and 72 h incubation, respectively, while 100% mortality was recorded after 48 h incubation at 20.0 μg/mL and after 72 h incubation at 10.0 μg/mL, with LC_{50} and LC_{90} of 6.1 μg/mL and 9.8 μg/mL, respectively. Morphological changes and tegumental alternation of treated worms suggested loss of the tegument and its vital functions.
Conclusions: This study provided the evidence for the potential antischistosomal effect of compound BTP-Iso and its possible uses as an alternative chemotherapeutic agent for treatment of schistosomiasis.

1. Introduction

The blood-dwelling flatworms of the genus *Schistosoma* are the causative agents of schistosomiasis, a disease afflicting more than 230 million people in 74 countries, most of them in Africa[1]. Five species can affect human, causing a wide spectrum of morbidity, among them *Schistosoma mansoni* (*S. mansoni*) is of interest, as it causes intestinal schistosomiasis which is associated with wide range of chronic pathology including: hepatosplenomegaly, portal hypertension and gastrointestinal varices[2]. To date, there is no vaccine available for schistosomiasis and chemotherapy is the mainstay for controlling the disease. Praziquantel (PZQ), an acetylated quinoline-pyrazine came on the market in early 1980s[3], showed high activity against all forms of human schistosomiasis in a single oral dose. However, PZQ does not kill eggs or schistosomula, therefore repeated doses are needed for treatment of acute schistosomiasis[4]. In addition, appearance of PZQ-resistant strains in laboratory and field is a major concern, which calls for novel antischistosomal agents[5,6]. In parasitic helminthes, muscular activity is essential for worm feeding, host attachment and mating[7]. Moreover, in schistosomes maturation from larva to adult stage is dependent on migration from pulmonary vasculature to portal circulation[8].

Benzimidazoles comprise several compounds such as thiabendazole, albendazole, and flubendazole, all have wide-spectrum activity against various nematodes and cestodes affecting human[9], in addition to triclabendazole, the drug of choice in treatment of fascioliasis. β-Tubulin is the target of benzimidazoles, which upon binding, these compounds interfere with microtubules assembly leading to disruption of spindle formation during cell division and interruption of the movement of subcellular components and metabolites within the cytoplasm[10,11].

The antischistosomal effects of the benzimidazoles have been reported experimentally by several researchers. *In vitro* cultured adult *S. mansoni* showed immediate contraction, tegumental

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destruction and worm death when treated with triclabendazole[12]. In addition, treatment of *S. mansoni*-experimentally infected mice with triclabendazole or flubendazole resulted in significant reductions in worm burden, tissue egg load and amelioration of hepatic granuloma[13-15]. More recent, a novel benzimidazole derivative named BTP-Iso showed promising antischistosomal activity when tested in mice experimentally infected with Egyptian strain of *S. mansoni*. The antischistosomal effects include significant reduction in immature ova, hepatic lesions, and recovered adult worms particularly females[16]. These results highlight the potential antischistosomal activity of compound BTP-Iso making them appropriate alternatives to PZQ.

The present work aimed to test the effect of compound BTP-Iso on cultured adult *S. mansoni*. The efficacy was assessed based on the percentage of dead worms after treatment and morphological changes recorded by SEM. In addition, the molluscicidal effect of compound BTP-Iso was reported on clean *Biomphalaria alexandrina* (*B. alexandrina*) snails.

2. Materials and methods

2.1. Chemicals

Compound BTP-Iso is a novel benzimidazole compound[16], which was designed and synthesized by structural modifications of benzimidazole ring at two-position taking cabendazole and thiabendazole as models to improve the anthelmintic activity. PZQ was purchased from Sigma-Aldrich.

2.2. Parasites and animals

Freshly shed cercariae from infected *Biomphalaria glabrata* snails were used to infect mice (CD1) by subcutaneous injection (400/mice). *S. mansoni* adult worms (Egyptian strain) were obtained 42 days post-infection by portal perfusion. Recovered worms were washed in phosphate buffered saline to remove host blood cells, and then incubated in culture media.

The Schistosome Biological Supply Centre, Theodor Bilharz Research Institute, Giza, Egypt supplied the mice and infected snails used in this study. All experimental procedures involving animals were conducted in accordance to the international valid animal ethics guidelines and approved by the Institutional Research Board (IRB) - Faculty of Medicine- Mansoura University.

2.3. In vitro schistosomicidal activity

The *in vitro* schistosomicidal assay was performed on adult worms, according to Yousif *et al.*[17], with modifications. Adult worms were incubated in RPMI-1640 including L-glutamine, supplemented with 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 20% heat-inactivated fetal bovine serum, 300 IU/mL penicillin, 300 μg/mL streptomycin, and 160 μg/mL gentamycin (all from Gibco) in 24-well culture plates (Costar), each well contained 5 pairs of worms.

Compound BTP-Iso was dissolved in dimethylsulfoxide and added to wells to give various concentrations from 2 to 40 μg/mL. Controls used include worms incubated with media containing 0.8% dimethylsulfoxide in RPMI-1640 (the highest concentration of vehicle used) as negative control, and worms incubated with 10 μg/mL PZQ (reference drug) as positive control. Each assay was performed in duplicate at least three times. Plates were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 for 3 days.

Treated and control worms were examined under a dissecting microscope to record motility, tegument damage, pairing changes and dead worms each 24 h. Treated worms that did not exhibit motility for 2 min were considered dead. Based on the observations, we calculated mortality rate, LC_{50} and LC_{90} of compounds BTP-Iso against adult *S. mansoni in vitro* using SPSS version 16.

2.4. SEM

For ultrastructural analysis of activity of compound BTP-Iso on adult *S. mansoni*, SEM was used. Treated and untreated adult worms were washed with phosphate buffered saline. Glutaraldehyde 2.5% was used to fix samples, which were processed and examined by SEM (Inspect S, FEI Company, Holland).

2.5. Molluscicidal screening

Clean adult *B. alexandrina* snails were used to test the molluscicidal activity of BTP-Iso. The standard method of the World Health Organization recommendations[18] was used. In brief, glass jars each containing 10 snails in 1 L of dechlorinated tap water were used. The snails were tested against different concentrations of compound BTP-Iso, ranges from 5 to 60 mg/L. Snails challenged with the chemical molluscicide (niclosamide) were similarly used as positive controls. For all, each run was performed in duplicate. We maintained the snails in the solution at 25 ℃ + 2 ℃ for 24 h. Then, for recovery, snails were thoroughly washed and transferred for another 24 h to fresh dechlorinated water. Snails run in dechlorinated water only under the same experimental conditions were used as negative control. The snails were examined for viability after the recovery period. Dead snails can be identified by absence of reaction (foot withdrawal) when the snail's foot is stimulated with a blunt wooden probe.

3. Results

3.1. In vitro schistosomicidal activity

Worms exposed to media containing 0.8% of dimethylsulfoxide for 72 h were survived, male and female schistosomes showed normal tegumental appearance and normal motor activity characterized by wavy movement along the body axis, while PZQ-treated worms presented with muscle contraction immediately following PZQ application, most of the worms (65%) died after 24 h and the rest exhibited weak movement. When worms exposed to compound BTP-Iso at serial dilutions (2–40 μg/mL) for 24 h, no apparent effect on worm survival was seen, however worms started to separate, exhibited weak movement and some appears coiled especially males. Incubation for 48 h and 72 h at concentration of 8 μg/mL resulted in worm mortalities of 45.8% and 71%, respectively, with death of all treated worms was seen after 48 h incubation at 20 μg/mL, while at 10 μg/mL all worms died after 72 h (Figure 1A). High mortality rates

have been achieved among male worms of 58.3% and 91.7% at 48 h and 72 h post incubation, respectively (Figure 1B). The LC_{50} and LC_{90} reported after 72 h incubations were 6.1 μg/mL and 9.8 μg/mL, while those of PZQ were 0.21 μg/mL and 0.32 μg/mL, respectively.

3.2. SEM

To confirm the direct effect of compound BTP-Iso on adult *S. mansoni*, treated worms with 10 μg/mL for 72 h were tested

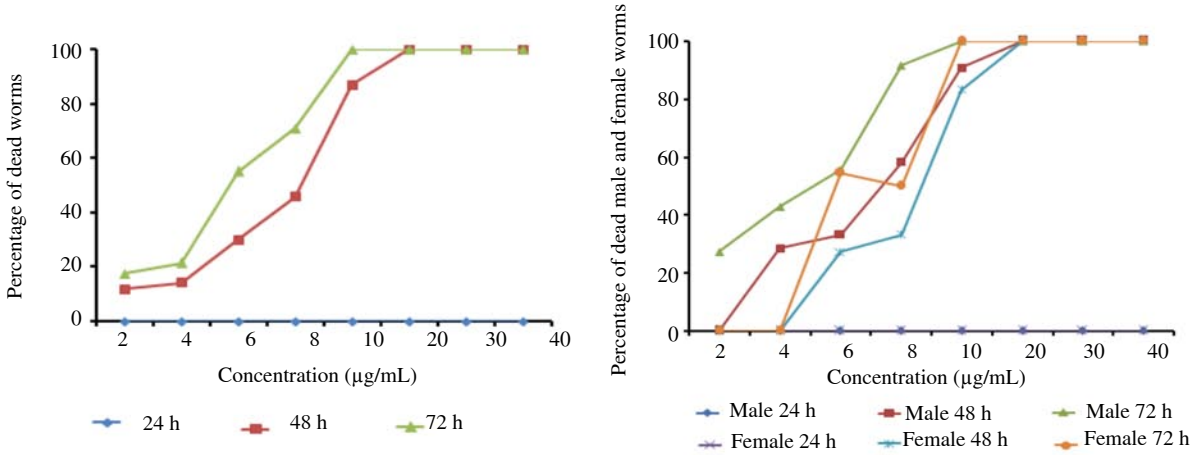

Figure 1. Dose- and time- response curves of adult *Schistosoma mansoni* treated with BTP-Iso at a concentration range 2–40 μg/mL for 72 h.
A: The average percentage of total dead worms; B: The average percentages of dead male and female schistosomes. Death of worms was documented after 2 min of no movement under the dissecting microscope. Note that the intervals on the X-axis are not equal.

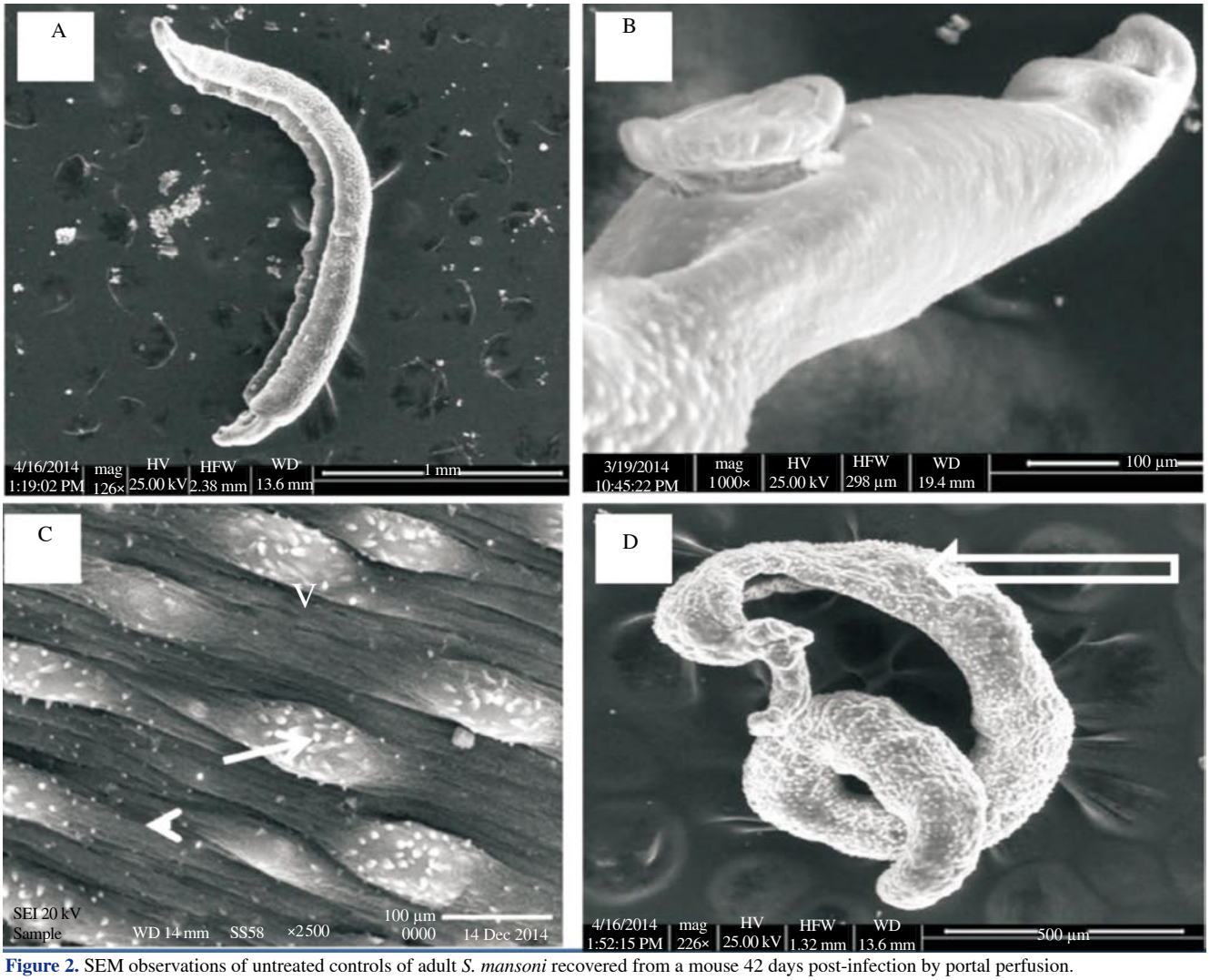

Figure 2. SEM observations of untreated controls of adult *S. mansoni* recovered from a mouse 42 days post-infection by portal perfusion.
A: Male worm, showing no morphological changes; B: Higher magnification of male schistosome showing anterior region with normal oral and ventral suckers and smooth area between them; C: Tegument with tubercles, spines on them (arrow) and parallel-arranged wrinkles (arrow heads); D: Male worm treated with 10 μg/mL PZQ (positive control) showing coiled contracted body, deformity of the anterior end and erosion of the tegument particularly on the dorsal surface (open arrow).

for morphological changes using SEM. Adult male *S. mansoni* untreated with any chemicals (negative control) showed normal morphology as previously reported (Figure 2A), with the anterior part of worms revealed normal morphology of oral and ventral suckers and area between them (Figure 2B). The tegument of untreated worms also showed tubercles with spines (arrow) and parallel-arranged tegumental ridges (arrow heads) between them (Figure 2C). Worms treated with PZQ, showed the coiled contracted appearance with erosion and sloughing of the tegument particularly the dorsal surface (open arrow) (Figure 2D).

When adult worms were incubated with 10 μg/mL of compound BTP-Iso for 72 h, tegument of the male anterior region showed deformity of the oral and ventral suckers, wrinkling and shortening of the area between them, in addition to, disintegration with blebbing, and sloughing was also seen on the inner surface of the oral sucker (Figure 3A). Higher magnification of the tegument of the dorsal surface revealed a shrunken (arrow) and swollen appearance with disappearance of the knobs, spines and the parallel arranged wrinkles with sloughing (open arrow) of several areas (Figure 3B and 3C). Appearance of vesicles (arrow heads) and swollen tegument were recorded (Figure 3D).

3.3. Molluscicidal effect

The experimental study of molluscicidal activity of BTP-Iso on adult *B. alexandrina* snails at many concentrations till 40 ppm showed no effects on the snail host.

4. Discussion

As schistosomiasis is still considered a public health problem and one of the important neglected diseases in many countries, the efforts to find effective drugs for its treatment continue, since PZQ is the only available drug for treatment and controlling of all forms of human schistosomiasis in absence of any available vaccine.

Reports from Africa and South America[19-21] have shown that resistance to PZQ could emerge following its long-term, widespread use and frequent administration. In addition, development of resistant strains and isolates of *S. mansoni* in the laboratory[22] and in the field is a great concern. Hence, the identification of novel alternatives to PZQ is of urgent need.

Many experimental compounds have been designed, produced and tested against adult *S. mansoni* and its larval stages[9] but many of them remain unused until their effects on the biology or the physiology of

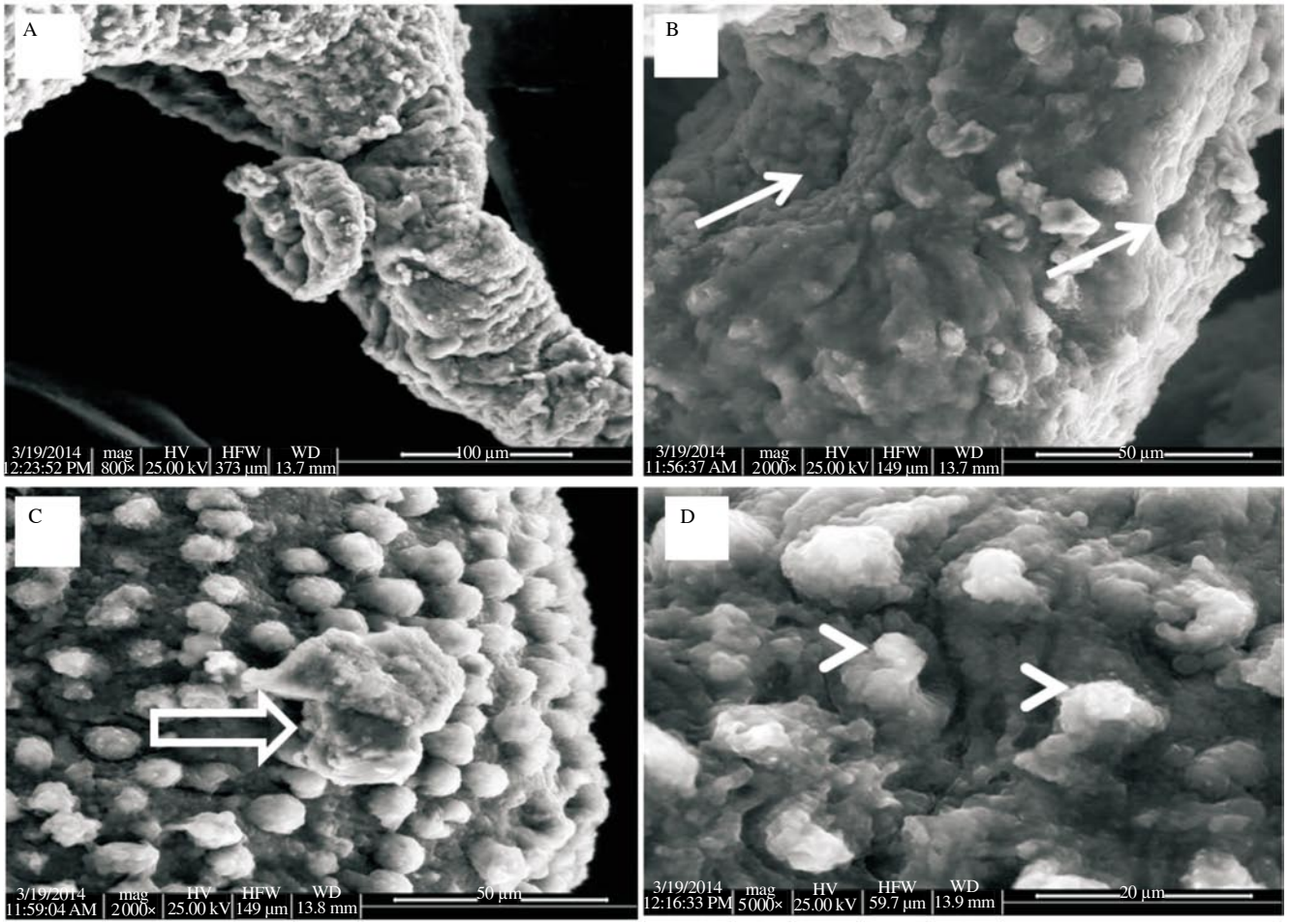

Figure 3. SEM observations of adult *S. mansoni* after *in vitro* incubation with compound BTP-Iso at 10 μg/mL for 72h.
A: Tegument of the male anterior region showing deformity of the oral and ventral suckers, wrinkling and shortening of the area between them, in addition, tegument shows disintegration with blebbing, and sloughing was also seen on the inner surface of the oral sucker; B and C: Higher magnification of the tegument of the dorsal surface has a shrunken (arrow) and swollen appearance with disappearance of the knobs, spines and the parallel arranged wrinkles with sloughing (open arrow); D: Tegument is swollen with appearance of vesicles (arrow heads).

the parasite can be established. It is well known that the application of different approaches to the study of the biology of the parasites has a great influence on drug development.

In this *in vitro* study, we analyzed the activity of a synthetic benzimidazole, known as BTP-Iso, used at the range of 2–40 μg/mL, against adult worms. Our results showed the potent *in vitro* schistosomicidal effects of compound BTP-Iso, although not as potent as PZQ but a bit better than those reported with our previous work using the antimalarial artemisinin-naphthoquine phosphate combination[23].

In this study, motility reduction was the first sign observed upon incubation of the adult worms with the tested compound. It is well known that locomotion is essential for development of schistosomes. During parasite's journey in the host, larvae exhibit several developmental changes to reach maturity. However, immotile larva will not achieve full maturity, therefore they will be unable to produce eggs, which are the main cause of inflammatory granuloma[24].

Herein, we observed greater sensitivity of male worms to the tested compound BTP-Iso than females. The same results were reported previously with PZQ[25,26]. Generally, drugs targeting male schistosomes will indirectly affect female through improper feeding, fertilization and locomotion. These effects will be reflected on ova production, and hence decrease liver pathology and disease transmission.

In our previous work testing the effect of compound BTP-Iso on *S. mansoni* harbored in mice, female worms were more affected than males[16]. This discrepancy between the effect of the drug *in vivo* and *in vitro* might be attributed to the environment in which the parasite is located which will affect the parasite physiology and drug sensitivity. However, the effect of the drug metabolites on worms cannot be ruled out.

In previous studies using triclabendazole, a benzimidazole compound effective against *Fasciola*, it has been reported that its metabolite named triclabendazole sulphoxide is responsible for the fasciolicidal activity not triclabendazole itself[27], through inhibition of protein synthesis in addition to microtubule inhibition[28].

SEM allows observation of the changes in the morphology of the worm tegument making it possible to elucidate the mechanism of action of the tested compound. The main changes induced by treatment of this compound are related to deformity and damage in oral and ventral suckers particularly male worms. Suckers are muscular organs essential for fixing worms in the vascular system and ingestion of blood (the main source of parasite food). Destruction of suckers will affect worm feeding and dislodge worms in the circulation. Additionally, swelling of the tubercles, decrease numbers of spines and blebs formation were common with this compound, besides, peeling and erosion of the tegument particularly in the dorsal region, as reported previously by many researchers when testing the *in vitro* effect of several compounds that have antischistosomal activity such as PZQ[29], oxamniquine[30], artemether[31], miltefosine[32], mefloquine[33] and artemisinin-naphthoquine phosphate[23].

Since the tegument is a vital structure for schistosomes survival, nutrient absorption, lipid metabolism, signaling mechanism and modulation of the host response[34,35]. Therefore, interference of one of these mechanisms could be lethal to the adult worm, in addition, once the tegument is destructed, the drug will penetrate deeper into the worm internal tissues causing widespread disruption and harm to the worm. However, *in vivo*, part of the schistosomicidal activity could be probably dependent on the host immune system, leading to host-mediated immune killing of adult worms[36].

Based on our results, the exact mechanism of action of compound BTP-Iso has not yet been elucidated. However, since this compound is structurally related to benzimidazoles, we speculate that the mode of action could be similar. Generally, benzimidazole compounds are known as microtubule inhibitor, binding of these compounds to β-tubulin will lead to depolymerization of cytoplasmic microtubules and disruption of microtubule-based process in helminthes such as the block in transport of tegumental secretory bodies and inhibition of mitosis in vitelline and spermatogenic cells[10].

Separation of paired worms were detected in incubation with compound PTB-Iso, this effect could be attributed to loss of some tubulin functions. Tubulin is one of the components of the worm musculature. Muscles are present mainly in suckers, lining of alimentary, excretory and reproductive organs and in the gynaecophoric canal to keep intact coupling of males and females[37,38]. Separated female worms are unable to produce eggs, the main pathological mediator in schistosomiasis.

In this work, we observed no molluscicidal effect of compound BTP-Iso on the *S. mansoni* snail host *B. alexandrina*, so higher doses could be tested to get better effect.

In conclusion, the results obtained in the present study confirmed the action of compound BTP-Iso on *S. mansoni* adult worms causing tegumental alternations particularly in male, locomotory changes and worm death, but no detectable molluscicidal effect. The effects of this compound BTP-Iso are a good example to get new compound with better activity through redesigning of an old drug, so it would be worthwhile to test the effect of BTP-Iso on *Schistosoma haematobium in vivo* and *in vitro* with emphasis on immunological interaction with the host. Additionally, the potential synergistic effect of combination of compound BTP-Iso with other antischistosomal agents, especially PZQ has to be evaluated. Finally, testing the curative effect of compound BTP-Iso in experimental animal model, where schistosomiasis and infection with other soil-transmitted nematodes or biologically-related trematodes such as *Fasciola gigantica* co-exist, similar to what is prevalent in various countries in Africa and South America would be a simple way to reduce drug resistance through minimizing the exposure to several therapeutic agents.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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