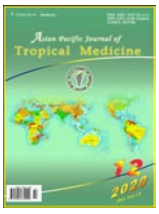

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In vitro efficacy of new synthetic benzimidazole-related compounds against *Schistosoma mansoni* adult worms

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ABSTRACT

Objective: To evaluate the *in vitro* antischistosomal activity of two new synthetic benzimidazole-related compounds: NBTP-OH and NBTP-F.

Methods: *Schistosoma* adult worms were recovered from mice infected with *Schistosoma mansoni* cercaria, washed and then incubated in the culture media with different concentrations of compounds NBTP-OH and NBTP-F up to 72 h. Scanning electron microscopy was conducted to report morphological changes.

Results: Incubation of adult *Schistosoma mansoni* with 10 μg/mL of NBTP-OH for 48 h killed 81.25% of worms. The calculated LC_{50} and LC_{90} 72 h post-incubation were 6.8 μg/mL and 9.8 μg/mL, respectively. Exposure of worms to 10 μg/mL of NBTP-F killed 89.5% of worms after 48 h, mostly males (83.3%), the LC_{50} and LC_{90} after 72 h of incubation were 4.8 μg/mL and 6.9 μg/mL, respectively. Worms incubated for 72 h with these compounds revealed swelling and deformity of oral sucker, disorganization and erosion of the tegument when examined with scanning electron microscopy.

Conclusions: NBTP-OH and NBTP-F possess *in vitro* antischistosomal activities; however, *in vivo* studies should be conducted to examine their antischistosomal effects..

KEYWORDS: *Schistosoma mansoni*; Benzimidazole; Tubulin; Tegument; *In vitro*

1. Introduction

Schistosomiasis caused by helminthes of the genus *Schistosoma* is a chronic debilitating disease afflicting more than 290 million people in 78 countries most of whom live in Africa. The disease annual loss is approximately 4.5 million disability adjusted life years (DALYs), which are expected to increase in the coming days[1]. *Schistosoma (S.) mansoni*, causing intestinal schistosomiasis, is associated with a wide range of chronic pathology including hepatosplenomegaly, portal hypertension and gastrointestinal varices[2].

To date, there is no vaccine available for schistosomiasis and controlling the disease depends solely on praziquantel (PZQ), which interferes with calcium haemostasis, causing spastic paralysis of worms; however, the appearance of PZQ-resistant strains in laboratory and field raised major concerns and called for development of novel antischistosomal drugs[3]. One important rationale to develop a new schistosomicidal agent is to target a protein that interferes with the worm vital function as feeding and locomotion.

Tubulin is a protein composed mainly of two homologous polypeptides α and β, both assembled together to form heterodimer in microtubules, the major component of eukaryotic cytoskeleton. In addition, microtubules play a vital role in structural support, as well as cell division, motility, transport and signaling mechanisms[4].

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In trematodes, the cytoskeleton has vital roles in maintaining the tegumental structure, transportation of tegumental granules from tegumental cells to the syncytium, thus it is directly involved in the synthesis and turnover of the surface membrane and its outer coat[5]. Previous research recorded the presence of tubulin in the tegument cytoskeleton of schistosomes. Within the syncytium, tubulin appeared as vertical lines passing throughout its thickness, in the cell bodies, their cytoplasmic processes, tubercles and parenchymal cells[6]. It is worthy of noting that tubulin is a target of several agents such as anticancers, antigouts, antifungal and antihelmintics[7,8].

β tubulin is the target of benzimidazoles, upon binding, these drugs 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[7].

Benzimidazoles as albendazole, thiabendazole, and flubendazole, have wide-spectrum activity against cestodes and various nematodes affecting human. In addition to triclabendazole, the drug was used in the treatment of fascioliasis[9].

The antischistosomal effects of triclabendazole and flubendazole have been reported experimentally by several researchers[10-13]. More recently, a novel benzimidazole derivative named BTP-Iso showed promising *in vivo* and *in vitro* effects on adult *S. mansoni*[14,15] as significant reductions in immature ova, hepatic lesions, and recovered adult worms particularly females, as well as marked tegumental changes were seen by scanning electron microscopy. These data highlight the potential antischistosomal activity of benzimidazole-derived compounds, making them appropriate alternatives to PZQ.

In this *in vitro* study, we tested the effect of two benzimidazole-derived compounds: NBTP-OH and NBTP-F on adult *S. mansoni*. The efficacy was assessed based on the percentage of dead worms after treatment and morphological changes recorded by scanning electron microscopy.

2. Materials and methods

2.1. Compounds and synthetic procedure

Compounds NBTP-OH: 4-[5-(6-nitro-1H-benzimidazol-2-yl) thiophen-2-yl]phenol and NBTP-F: 2-[5-(4-Fluorophenyl)thiophen-2-yl]-6-nitro-1H-benzimidazole are novel benzimidazole derivatives synthesized as shown in (Figure 1A). Physical and chemical properties of the two compounds are shown in (Table 1). The calculated lipophilicity of NBTP-OH and NBTP-F are 4.9 and 5.7 respectively (Figure 1B).

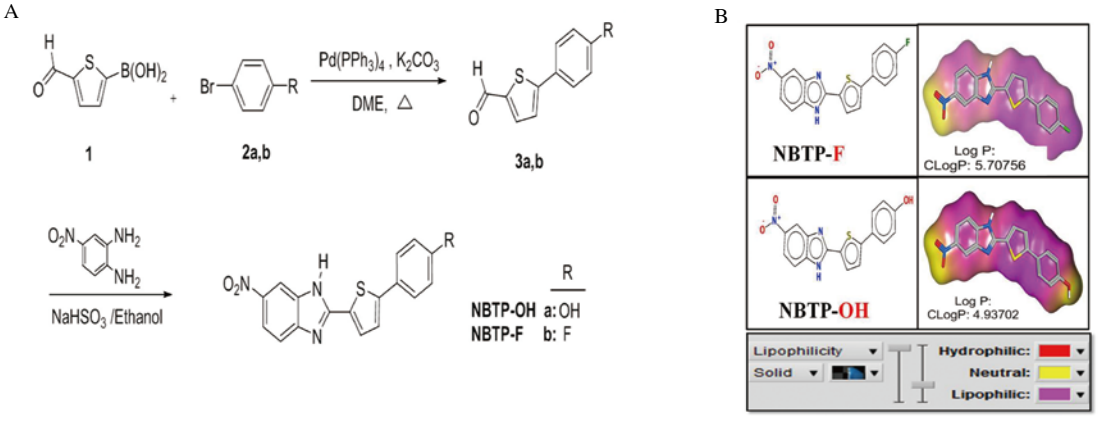
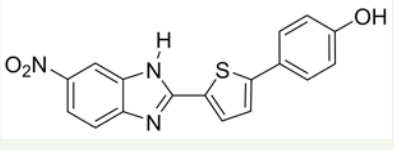
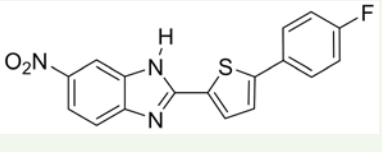


Figure 1. (A) Synthetic pathway of compounds NBTP-OH and NBTP-F; (B) Structure and calculated lipophilicity (clogP) of both compounds.

Table 1. Some physical and chemical properties of NBTP-OH and NBTP-F.

Items	NBTP-OH	NBTP-F
Structure		
Chemical name	4-[5-(6-Nitro-1*H*-benzimidazol-2-yl)thiophen-2-yl]phenol	2-[5-(4-Fluorophenyl)thiophen-2-yl]-6-nitro-1*H*-benzimidazole
Physical characters	Ruddy crystals	Ruddy crystals
Melting point (Mp) ℃	> 300	295-297
Yield (%)	73	71
Chemical formula	$C_{17}H_{11}N_3O_3S$	$C_{17}H_{10}FN_3O_2S$
Molecular weight (g)	337.35	339.34
Lipophilicity (ClogP)	4.937 02	5.707 56 (more lipophilic)
Molarity (mole/L)	337.35 g/1 000 mL	339.34 g / 1 000 mL

For synthesis of the two compounds, we adopted Suzuki-Miyaura reaction[16] and 5-formyl thiophen-2-ylboronic acid (1) was coupled with the 1-bromo-4-hydroxy benzene (2a) to provide 5-(4-hydroxyphenyl) thiophene-2-carbaldehyde (3a)[17]. Likewise, 5-(4-fluorophenyl) thiophene-2-carbaldehyde (3b) was synthesized by Bussolari and Rehborn[18]. In the present work, the appropriate aldehydes 3a and 3b were reacted with 4-nitrobenzene-1,2-diamine in ethanol in the presence of sodium bisulfite to afford 4-[5-(6-nitro-1*H*-benzimidazol-2-yl)thiophen-2-yl]phenol (NBTP-OH) and 2-[5-(4-Fluorophenyl)thiophen-2-yl]-6-nitro-1H-benzimidazole (NBTP-F) respectively. PZQ used in the experiments was purchased from Sigma-Aldrich.

2.2. Parasites and animals

Subcutaneous injection of freshly shed cercariae from infected *Biomphalaria glabrata* snails were used to infect CD1 mice using 400 cercariae/mouse. Adult *S. mansoni* worms (Egyptian strain) were obtained by portal perfusion 42 d post-infection. Recovered worms were washed in phosphate buffered saline (PBS) to remove host blood cells then incubated in the culture media.

Infected snails and mice used in the study were obtained from the Schistosome Biological Supply Centre (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Animals were maintained and experiments were carried out at the SBSC/TBR and Mansoura Faculty of Medicine.

2.3. In vitro schistosomicidal activity

The *in vitro* schistosomicidal assay was performed on adult worms, according to the method of Yousif *et al*.[19] with a few modifications. Roswell Park Memorial Institute medium (RPMI-1640), supplemented with L-glutamine, 10 mM HEPES, 20% heat-inactivated fetal bovine serum (FBS), 300 U/mL penicillin, 300 μg/mL streptomycin, and 160 μg/mL gentamycin (all from Gibco) were used for adult worms incubation in 24-well culture plates (Costar), each well contained 5 pairs of worms. Adult *S. mansoni* were incubated for 2 h in the culture medium to be allowed for adaptation.

Compounds NBTP-OH and NBTP-F were dissolved in dimethylsulfoxide (DMSO) and added to wells to give concentrations from 2 to 40 μg/mL. Controls used include worms incubated with 10 μg/mL PZQ (reference drug) and those incubated with RPMI-1640 only or with 0.8% DMSO (highest concentration of vehicle used). Each assay was performed at least three times in duplicate. Plates were incubated in a humidified atmosphere of 5% CO_2 at 37 ℃ for 3 d.

Before incubation with drug and immediately after incubation, all worms were observed under dissecting microscope. Then, treated and control worms were examined every 24 h under a dissecting microscope to record motility, integument damage, pairing changes and dead worms. Treated worms that did not exhibit motility for two minutes were considered dead. Based on the observations, mortality rates, and the LC_{50} and LC_{90} of each compound against adult *S. mansoni* were calculated using SPSS version 16.

2.4. Scanning electron microscopy

For ultrastructural analysis of the compounds activity on adult *S. mansoni*, scanning electron microscopy was used. Control and treated adult worms were washed with PBS, fixed in 2.5% glutaraldehyde-phosphate buffer for 24 h at 4 ℃, then post fixed in osmium tetroxide buffer (1%) for 1 h. Dehydration of adult worms were done through a graded series of ethanol (from 25% to 100%, for 10 min in each ethanol grade), and critical-point dried through carbon dioxide then worms were embedded in epon resin, coated with gold particles before examination with electron microscopy (Inspect S, FEI Company, Holland).

2.5. Ethics statement

All the study procedures were approved by Mansoura Faculty of Medicine Institutional Research Board (MFM-IRB approval number: R.19.12.701), Mansoura University, Mansoura, Egypt and complied with the international valid animal ethics guidelines.

3. Results

3.1. In vitro schistosomicidal activity

Worms incubated in media containing 0.8% of DMSO for 72 h survived; male and female schistosomes showed normal tegumental appearance and motor activity characterized by wavy movement along the body axis. While PZQ-treated worms presented with muscle contraction immediately and with incubation, all worms died in 24 h.

Treatment of adult schistosomes with different concentrations (2-40 μg/mL) of compound NBTP-OH for 24 h resulted in changing the worm behavior in the form of coiling and slow locomotion. After 48 h exposure to 8 μg/mL, 54.6% of the worms died (Figure 2A), female worms (44.4%) were more affected than males (Figure 2B). With increasing the incubation time for additional 24 h, 63.6% of worms died but mostly males (Figure 2B). At 10 μg/mL, 81.25% of worms died after 48 h of incubation and the remaining worms died over the next 24 h (Figure 2B). A 20 μg/mL of compound

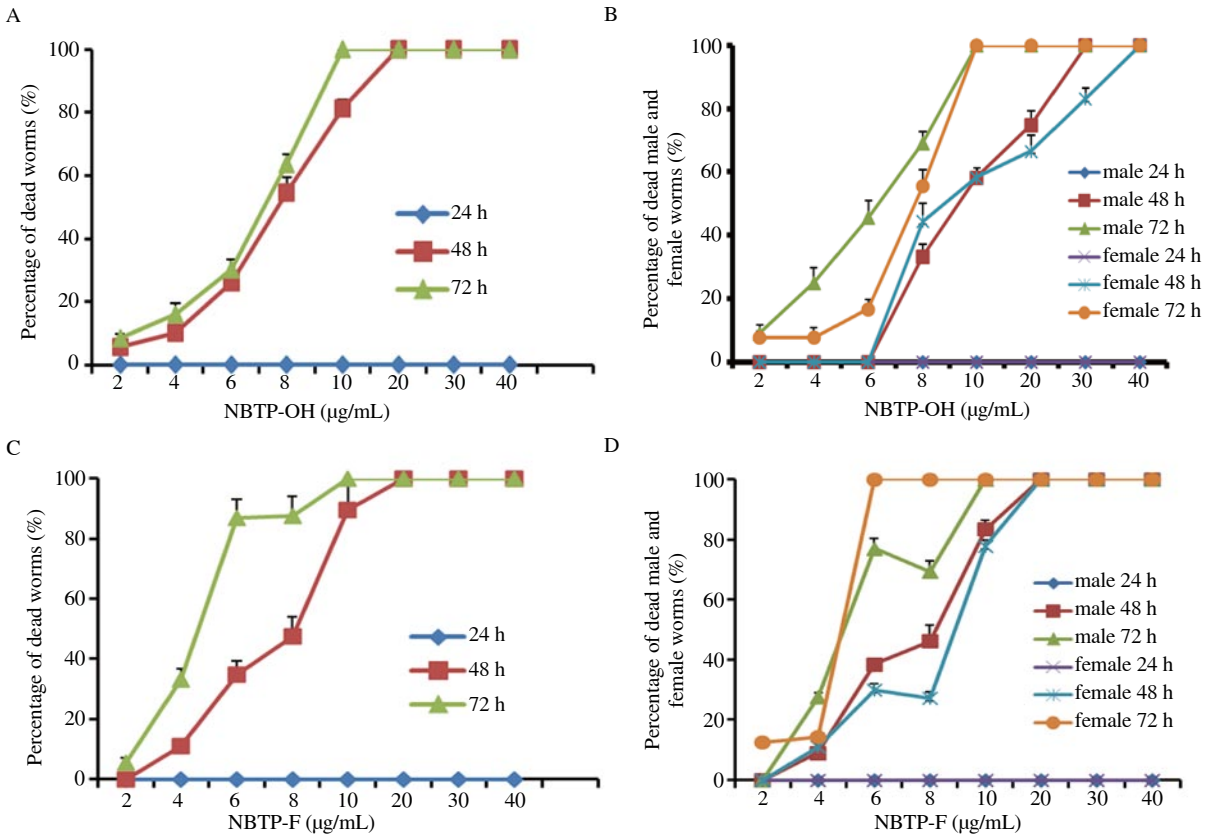


Figure 2. Dose- and time- response curves of adult *Schistosoma mansoni* treated with either NBTP-OH or NBTP-F at concentrations of 2–40 μg/mL for 72 h. (A) The average percentage of total dead worms exposed to NBTP-OH ; (B) The average percentage of dead male and female schistosomes exposed to NBTP-OH. (C) The average percentage of total dead worms exposed to NBTP-F; (D) The average percentage of dead male and female schistosomes exposed to NBTP-F. 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.

NBTP-OH killed all worms after 48 h. The calculated LC_{50} and LC_{90} of compound NBTP-OH after 72 h incubation were 6.8 μg/mL and 9.8 μg/mL, respectively.

Exposure of worms to 8 μg/mL of compound NBTP-F for 48 h resulted in death of 47.5% of worms and the death rate was higher in male than female worms (Figure 2C and 2D), while 10 μg/mL resulted in 89.5% death rate at the same time point (Figure 2C), the mortality rate in male worms was 83.3% (Figure 2D). All worms died after 48 h at 20 μg/mL, and at 10 μg/mL after 72 h incubation (Figure 2D). Moreover, NBTP-F compound LC_{50} and LC_{90} were 4.8 μg/mL and 6.9 μg/mL, respectively, while PZQ LC_{50} and LC_{90} were 0.2 μg/mL and 0.3 μg/mL, respectively. NBTP-F showed a more potent schistosomicidal activity than NBTP-OH against *S. mansoni* worms, although not as potent as PZQ as detected from LC_{50} and LC_{90}.

Separation of paired worms was detected after 24 h incubation with both compounds. The percentages of separated worms were 46.7% and 86.7% for NBTP-OH and NBTP-F, respectively.

3.2. Scanning electron microscopy results

To report the possible morphological changes caused by compounds NBTP-OH and NBTP-F, adult *S. mansoni* worms treated with DMSO or 8 μg/mL of each compound for 48 h were examined using SEM. Worms treated with compound NBTP-OH revealed deformity of the oral sucker and anterior part of male worms, shrunken body with swellings and furrows (Figure 3A). Deformity of the dorsal aspect of the worm and gynaecophoric canal with presence of blebs, erosions and vesicles, as well as swelling of tubercles and ridges with peeling of the tegument (arrow) and few or even absence of spines were also seen (Figure 3B, 3C and 3D).

Male worms exposed to compound NBTP-F showed swollen, shrunken and deformed oral sucker and anterior part of the body (Figure 3E). Moreover, erosion, disorganization, blebs and fissures along with swelling and fusion of tegumental folds, in addition to fusion and collapse of tubercles, and deformity of the gynaecophoric canal were recorded (Figure 3F, 3G and 3H).

Adult male exposed to culture media only has normal surface membrane with no apparent destruction and normal body structure (Figure 4A), while DMSO-treated worms, the tegument is intact showing many tubercles with spines (Figure 4B).

Unfortunately, we did not examine PZQ-treated worms as very few worms were recovered from PZQ-treated mice and nearly all of them were destructed and/or lost during preparation for scanning electron microscopy examination.

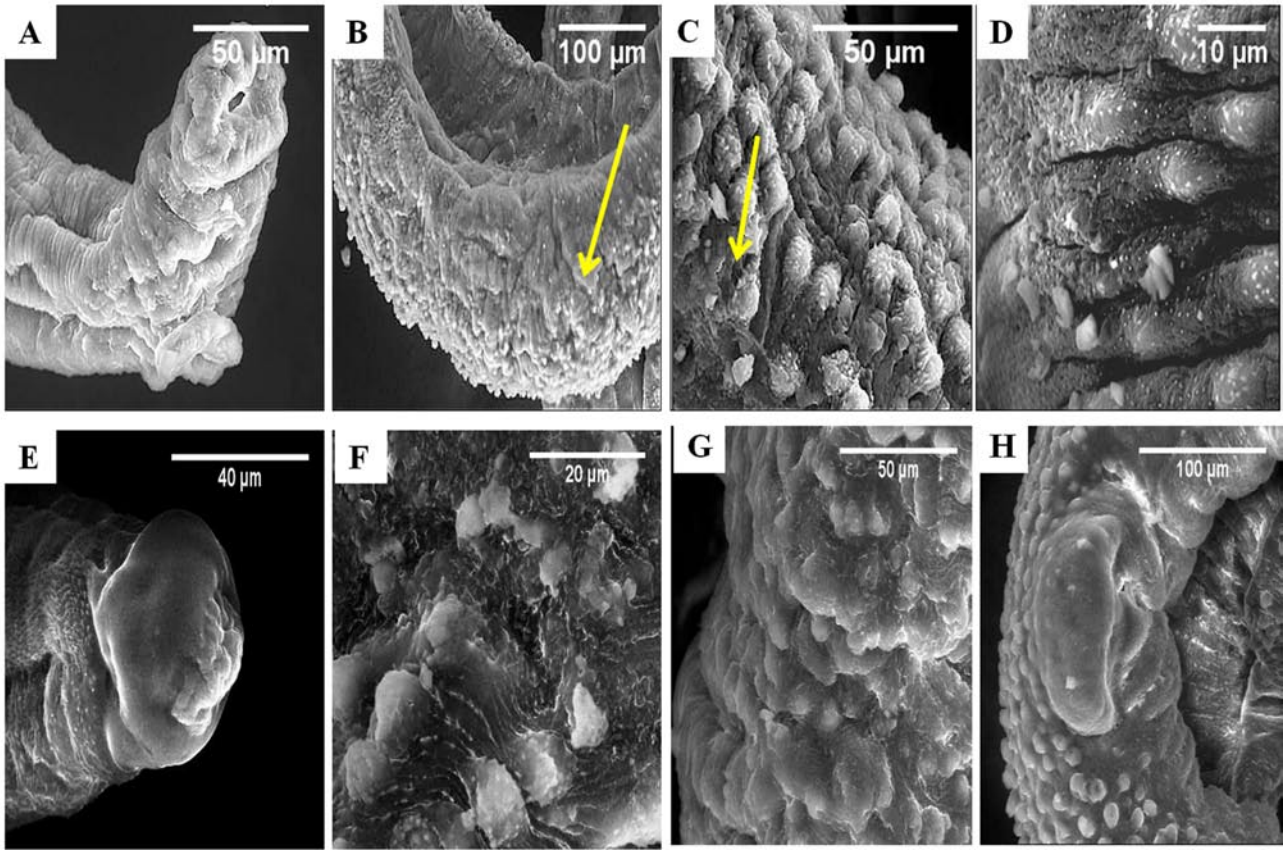


Figure 3. Worms exposed to NBTP-OH. (A) Deformity of oral sucker and the anterior part of male, shrunken body with swelling. (B) Deformity of the dorsal aspect and gynaecophoric canal with erosions. (C, D) Swelling of tubercles and tegument erosions (arrow) with few spines on the tubercles. (E) Worms exposed to NBTP-F showed deformed male oral sucker and anterior body part. (F) Erosion, disorganization, blebs and fissures of tegument. (G) Swelling of tegumental folds and collapse of tubercles. (H) Disorganization of male tegument and deformity of the gynaecophoric canal.

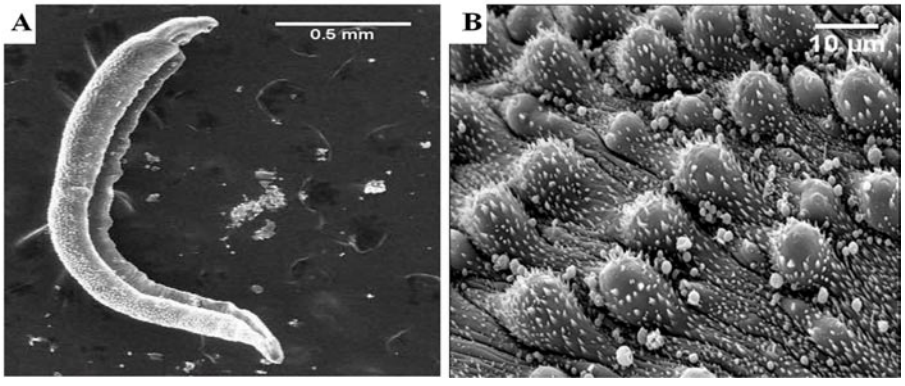


Figure 4. (A) Adult male exposed to media only has normal surface membrane with no apparent destruction and normal body structure. (B) DMSO-treated worms, the tegument is intact showing many tubercles with spines. NO PZQ-treated worms were examined as very few worms were recovered and nearly all lost and/or destructed during preparation for examination by scanning electron microscopy.

4. Discussion

Schistosomiasis is a major neglected public health disease that comes second after malaria in many tropical and subtropical countries. To date, the cornerstone for control of human schistosomiasis depends on PZQ; therefore, it is urgent to identify new alternatives.

Globally, many studies have scientifically assessed numerous natural and synthetic compounds on *S. mansoni* worm[14,20-22], however, a lot of them still remain unused, pending their efficacy on the parasite biology is confirmed. It is realized that the study of parasite biology is fundamental to potential drug discovery and development.

In this *in vitro* study, we assessed the effectiveness of 2-40 µg/mL of two structurally-related synthetic benzimidazoles compounds: NBTP-OH, and NBTP-F, on *S. mansoni* adult worms. The results clearly recorded the evidence for their promising schistosomicidal effects. NBTP-F showed a more potent schistosomicidal activity than NBTP-OH against *S. mansoni* worms, although not as potent as PZQ as detected from LC_{50} and LC_{90}.

In this work, we observed that male worms were more sensitive to the tested compounds especially 48 h post-treatment. Male schistosomes were more susceptible than females as described in some *in vitro* studies[21,23]. Others reported that females were more susceptible[20,24], whereas other researchers did not record any differences between male and female worms sensitivity to the tested drugs[25]. The greater sensitivity of male worms to both compounds in spite of their thick tegument in comparison to females might be due to a sex-specific interference of the drug with the target or presence of different targets in males compared to females[26].

The thick tegument is a major target for antischistosomal agents because of its essential roles for schistosomes. In order to test the effect of these new agents on the tegument and surface membrane of the worm, we used scanning electron microscopy. The main change induced by treatment with the two compounds was destruction of the tegument.

Upon disruption of the tegumental coat, the drug can deeply penetrate into the internal structures to bind another target as muscles of the parasite or causing disturbance in the normal physiological and biochemical process of the worm. Similar results have been previously reported by many researchers when testing the *in vitro* effect of several compounds on schistosomes such as PZQ, oxamniquine, artemether, mefloquine, miltefosine and artemisinin-based combination therapies[14,22,27,28].

In a previous study, we tested the compound BTP-Iso, another new synthetic benzimidazole compound[15], which showed a lethal effect nearly similar to compound NBTP-OH, although it is more lipophilic. The *in vitro* schistosomicidal activity of the three compounds against *S. mansoni* adult worms ranked as NBTP-F, followed by BTP-Iso, and then NBTP-OH.

Results obtained from the two compounds tested in this study go with the calculated lipophilicity as compound NBTP-F, which is more lipophilic, was found more effective. Lipophilic agents are more able to penetrate the membranes including tegument. The tegument of schistosomes is a normal plasma membrane overlain by a membrane-like secretion known as the membranocalyx[29].

Herein, the precise mechanism of action of these compounds has not yet been clarified. But we can hypothesize that these benzimidazole compounds can interfere with tegument structure and function (based on scanning electron microscopy results) through penetration of membranocalyx to interact with tubulin in syncytium, cell bodies and the subtegumental structures including microtubule-lined cytoplasmic channels, which are responsible of transporting secretory inclusions synthesized in the cell bodies to the tegument to form the secreted membranocalyx[29].

The tegument is a known essential structure for schistosomes subsistence, nutritional support, lipid metabolism, motility, proliferation, and host immune evasion[22,30]. Therefore, interference of one of these mechanisms could be lethal to the adult worm.

Also, deformity and damage in both oral and ventral suckers, particularly in male worms were observed with exposure to both compounds. If this effect is the same *in vivo*, both compounds will affect worm nutrition and subsequent interference of worm maturation and female oviposition besides elimination of the worm from the circulation[31].

Another possible mechanism could be considered as the tested compounds are structurally-related to benzimidazoles, thus the mode of action could be alike. Benzimidazoles are well-known microtubule inhibitors; upon their binding to β-tubulin, they cause depolymerization of cytoplasmic microtubules, instability of the muscular skeleton and disturbance of microtubule-based process in helminthic parasites in the suppression of mitosis in vitelline and spermatogenic cells[7].

Separation of paired worms was detected after incubation with compound NBTP-OH, but was observed more with NBTP-F. This effect could be due to loss of some tubulin functions, as tubulin is one of the components of the worm musculature. Muscles are located in the gynaecophoric canal to retain mating between males and females worms. Separated female schistosomes are incapable of eggs production, the key factor in schistosomiasis sequelae.

Generally, *in vitro* studies do not cover anthelmintic activities of the tested compounds, particularly with respect to pharmacological and immunological interaction with the host; however, they provide the first evidence of anthelmintic activity and insight into the mode of action of these agents[32]. Docking studies and molecular dynamics simulation predicted NBTPs bind tubulin with high stability (unpublished data). Therefore, it would be worthwhile to test for this hypothesis; also it is interesting to investigate "based on modeling results" whether these compounds could exert such lethal effect *in vivo* on different *Schistosoma* species and on other trematodes and nematodes as well.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Authors' contributions

AT initiated the idea, participated in the experimental work and wrote the manuscript. SB, MT and MA conduced the experimental work, read and approved the final manuscript. BM prepared the two tested compounds, read and approved the final manuscript. FS and SNB participated in the experimental work, revised and edited the manuscript.

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