Insights into the mode of action of 1,2,6,7-tetraoxaspiro [7.11] nonadecane (N-89) against adult Schistosoma mansoni worms

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ABSTRACT

Control of morbidity associated with schistosomiasis via chemotherapy largely relies on the drug praziquantel. Repeated therapy with praziquantel has created concerns about the possible selection of resistant worms and necessitated the search for novel drugs to treat schistosomiasis. Here, a murine model was infected with Schistosoma mansoni and treated with oral 1,2,6,7-tetraoxaspiro [7.11] nonadecane (N-89), which caused a significant reduction in fecundity and egg burden and reduced morbidity when administered at 5-weeks post-infection.

The analysis showed that the mode of action occurred through the ingestion of activated N-89 by the worms, and that there was no direct external effect on the S. mansoni worms. Ultrastructural analysis of the treated worms showed disruptions in the gut lumen and the presence of large volumes of material, suggestive of undigested blood meals or red blood cells. In addition, there were reduced vitelline cells in female worms and damage to sub-tegmental musculature in male worms. Eggs recovered from the treated mice showed both damage to the eggs and the production of immature eggs. Expression of mRNA responsible for gut and digestive function and egg production was also significantly affected by N-89 treatment, whereas control genes for musculature showed no significant changes.

Thus, N-89 drastically affected the total digestive function and egg production of S. mansoni worms. Physiological processes requiring heme uptake such as egg production and eggshell formation were subsequently affected, suggesting that the compound could be a possible therapeutic drug candidate for schistosomiasis control.

1. Introduction

Schistosomiasis, caused by the parasitic Platyhelminthes of the class trematoda, is an infectious disease of global significance due to its endemicity and large number of infected persons [1]. It is second only to malaria with respect to global parasitic morbidity, mortality burden, and socioeconomic importance [2], and an estimated 780 million persons are at risk of schistosomiasis infection worldwide [3]. Currently, > 240 million people are infected and require treatment, of which 141 million are school-aged children [4]. The majority of human intestinal schistosomiasis infections are caused by Schistosoma mansoni, a species found predominantly in Africa and South America [5].

Egg production is important for the completion of the life cycle of schistosomes and enables pathogenesis in vertebrate hosts. In the case of S. mansoni infections, adult worms reside in the mesenteric veins where they copulate and produce eggs. The eggs attach to the wall of the lumen, penetrate into the lumen, and the majority are expelled via the stool to continue their complex life cycle. The eggs produced by the females which are not excreted in the stool move through the blood stream and become trapped in the spleen and liver tissues, inducing granulomatous inflammations and fibrosis, leading to hepatospleno-megaly [6]. The density of eggs in the tissues, rather than adult worm...
numbers, is the major cause of morbidity from *S. mansoni* infection [7].

Currently, the main strategy to control schistosomiasis, adopted by the World Health Organization, is the regular use of the approved drug praziquantel (PZQ) [8]. PZQ is known to kill only adult *Schistosoma* worms, resulting in lower cure rates in areas with high endemicity [9]. PZQ causes both physiological changes, such as rapid Ca²⁺ ion uptake near and on the surface of the worms, causing severe injuries [11]. Although the evidence of drug resistance to PZQ in Egypt where there was a reduced susceptibility of *Schistosoma* to PZQ in field treatments [15]. In Senegal, PZQ drug resistance became a concern in 1991 when an initial trial in Senegal reported low cure rates and high re-infection rates [16]. Reduced susceptibility to PZQ among certain naturally occurring Kenyan isolates of *S. mansoni* has also been reported [17]. As such, it is generally accepted that new drugs are urgently required for schistosomiasis control.

In previous studies, 1,2,6,7-tetraoxaspiro[7.11]nonadecane (N-89), a synthetic compound with a simple endoperoxide structure, was shown to have anti-schistosomal effects on both larval and adult stages of *S. mansoni* [18]. In schistosomules, N-89 causes the dysfunction and disruption of lysosome-like organelles [19], whereas in adult worms effects such as reduced fecundity and reduced egg production have been reported [18].

Therefore, in this study, we attempted to understand how N-89 affects *S. mansoni* egg production and fecundity. We used electron microscopy to study the ultrastructural damages caused to the adult worms and the eggs.

2. Materials and methods

2.1. Ethics statement

All animal care and experimental protocols were conducted following the guidelines of the Committee of Animal Ethics, Tokyo Medical and Dental University, under the registry numbers 0170268A and 01610296A. All aspects related to mouse husbandry were carried out under strict guidelines to assure careful and consistent handling of the animals.

2.2. Parasites and animals

The Puerto Rican strain of *S. mansoni* maintained in our laboratory by routine passage through mice and the snail hosts * Biomphalaria glabrata* was used for the study. Five-week old female BALB/c mice weighing 18–20 g purchased from CLEA (Tokyo, Japan) were also used. Animals were provided food and water *ad libitum*.

2.3. Treatment of *S. mansoni* infected mice with N-89

*S. mansoni* cercariae were obtained from *B. glabrata* after 6–7 weeks of exposure of miracidia to snails and were used to infect the mice. Each mouse was infected with 180 cercariae using a standard method in which mice were percutaneously exposed via the tail to cercariae for 1 h at 25 °C, as previously described [20]. At five weeks post-infection, 5 infected mice were treated with 300 mg/kg body weight of N-89 dissolved in olive oil twice daily for two consecutive days via oral gavage. Another group of five infected mice was treated with olive oil only to serve as controls. All mice were killed 2 weeks post treatment. All experiments were conducted in three biological replicates.

### Table 1: Schistosomicidal effects of N-89 *in vivo*.

<table>
<thead>
<tr>
<th>Factor</th>
<th>N-89</th>
<th>Olive oil control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number of eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestine ± SD</td>
<td>602 ± 364.41</td>
<td>2226 ± 1559</td>
</tr>
<tr>
<td>Reduction (%)</td>
<td>72.9 ±</td>
<td></td>
</tr>
<tr>
<td>Liver ± SD</td>
<td>3238.39 ± 1494.64</td>
<td>26,862.1 ± 2634.</td>
</tr>
<tr>
<td>Reduction (%)</td>
<td>87.9 ±</td>
<td></td>
</tr>
<tr>
<td>Fecundity (%)</td>
<td>314.7 ± 8</td>
<td>1598.24</td>
</tr>
<tr>
<td>Mean worm burden</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ± SD</td>
<td>27.4 ± 8.02</td>
<td>39.4 ± 3.05</td>
</tr>
<tr>
<td>Males ± SD</td>
<td>11.2 ± 4.21</td>
<td>21.2 ± 11.36</td>
</tr>
<tr>
<td>Females ± SD</td>
<td>16.2 ± 4.6</td>
<td>18.2 ± 2.95</td>
</tr>
<tr>
<td>Worm reduction ratio (%)</td>
<td>30.45</td>
<td></td>
</tr>
<tr>
<td>Mean weight of spleen</td>
<td>0.34 ± 0.06</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>Mean weight of liver</td>
<td>1.65 ± 0.06</td>
<td>1.99 ± 0.15</td>
</tr>
</tbody>
</table>

Bold indicates *p* value < 0.01 (CI:95%; *α* = 0.05) Statistical significance was calculated by a *t*-test between the control group & treatment group. *Fecundity ratio was calculated as the total number of eggs recovered from mice divided by the number of female worms.*

### 2.4. Worm recovery and worm burden estimation

Recovery of *S. mansoni* worms from control and treated mice 2 weeks after treatment with N-89 was carried out by portal perfusion, as described previously [21] with slight modifications. Briefly, a 21-gauge needle was connected to a dispenser containing RPMI-1640 medium adjusted with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS). Worms were immediately separated into male and female sexes, counted, and processed for further analysis within 1 h of euthanasia of mice.

### 2.5. Egg burden estimation

The liver and intestines (small and large) of each mouse were collected separately after hepatic portal perfusion. Samples were washed with 1× phosphate-buffered saline (PBS), weighed, and chopped into small pieces. They were subsequently digested in a cocktail of collagenase (0.5 mg/mL), actinase (1 mg/mL), and gentamycin (50 mg/mL) in 2× PBS for 4 h at 147 rpm and 37 °C, after which the mixture was centrifuged at 2000 g for 5 min and the supernatant decanted. Fresh solution was then added, and the mixture was shaken for a further 4 h under same conditions. Digested tissues were successively filtered through 10-μm and 3-μm pore filter meshes, after which filtered eggs were washed three times with 2× PBS and centrifuged at 1500 rpm. The total number of eggs were counted under an Olympus light microscope (Model SZX-ILLD100). The collected eggs were then processed appropriately for identification of ultra-morphological changes.

### 2.6. Examination of retrieved worms and eggs for ultra-morphological changes

Scanning electron microscopy (SEM) was used to examine the retrieved worms and eggs and to compare the extent of damage on the surface of N-89-treated worms and their eggs with that of the non-N-89 (olive oil)-treated worms and eggs. First, the worms and eggs were fixed in 2.5% (w/v) glutaraldehyde in PBS for 24 h at 4 °C. After fixation, samples were washed 3 times in 1× PBS, pH 7.5, and stored in the same
buffer at 4 °C until use. Thereafter, samples were post-fixed with 1% (w/v) osmium tetroxide (OsO₄) in 0.1 M phosphate buffer (PB; pH 7.2) for 2 h at 4 °C. The specimens were then dehydrated with increasing concentrations of ethanol and washed twice in 3-methylbutyl acetate for 15 min each time. Samples were then dried with liquid CO₂ in a Hitachi HCP-2 critical point dryer machine (Hitachi, Japan). Treated specimens were mounted on aluminum microscopy stubs and coated with platinum and palladium particles using a Hitachi E102 ion-sputtering apparatus (Hitachi, Tokyo, Japan). Five male and five female worms from each mouse per group were then observed and photographed using a Hitachi S-4500 SEM (Hitachi High Technologies, Tokyo, Japan).

2.7. Ultra-structural analysis via transmission electron microscopy (TEM)

*S. mansoni* worms (separated into sex) obtained from treated and control mice, as described in section 2.4, were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) at 4 °C immediately after perfusion for 24 h. Samples were then successively immersed in 1% OsO₄ in 0.1 M PB for 2 h, dehydrated in a graded series of ethanol for 15 min each, and embedded in epoxy resin (Oken Epok 812; Oken-shoji, Tokyo, Japan). Ultra-thin silver-gold sections were cut with a diamond knife under a Riechert-Niessie Ultra-cut microscope and transferred to copper grids. The sections were then stained with uranyl acetate and lead citrate and observed with a H-7700 TEM (Hitachi High Technologies, Tokyo, Japan) attached to a XR-81 digital camera (Advanced Microscopy Techniques, USA). Five male and five female worms from each mouse per group were then observed for adequate replication.

2.8. Gene expression analysis of select *S. mansoni* genes

Total RNA was isolated from the five male and five female worms collected from each mouse using Trizol reagent (Invitrogen, USA). All RNA samples were purified using the RNeasy mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. The samples were quantified using a Quantus Fluorometer (Promega).

Complementary DNA (cDNA) was obtained from 1 μg RNA using a Revertra Ace® reverse transcriptase kit (Toyobo Life science, Japan) in a 20 μL reaction mixture using random hexamer oligonucleotides as primers. Primers were designed using the Universal Probe Library Assay Design Center (Roche). A list of all primers used, commercially synthesized by Hokkaido Systems Science (Hokkaido Japan), is shown in Supplementary Table 1. Quantitative real-time PCR (qRT-PCR) analysis
was performed using a SYBR Fast Universal qPCR kit (Kapa Bio systems, USA). Assays, primer sets, and reporter probes were customized, and reagents were purchased from Life Technologies (Carlsbad, USA). cDNA (10 ng) was used for qRT-PCR analysis using the Light Cycler 480 (Roche). As an endogenous control, we used the housekeeping α-tubulin and β-actin genes to compare the relative expression of select genes after N-89 treatment. Melting point analyses were performed to distinguish between the specific amplification product and unspecific primer–dimer formation following each qRT-PCR analysis. All samples were run in triplicate and underwent 40 amplification cycles using the Light Cycler 480 (Roche). The ΔΔCt method was used for relative quantification [22]. Results are representative of three independent experiments.
Fig. 4. Transmission electron micrograph of vitelline organ of adult *S. mansoni* female worms obtained from mice treated at 5 weeks with or without N-89. Bars 1 μm. a shows TEM images of N-89 treated worms. There appeared to be a local fusion in some of the vitelline balls while others remained intact or divided into small fragments. The black arrow indicates a vitelline ball with its vitelline cells disrupted or immature. The fat cells however showed no signs of damage. b shows the TEM image of control worms. Observation of the mid-segment of the control worms showed a regular arrangement of vitelline cells in a circular format. The vitelline cells are indicated in black arrowheads.

Fig. 5. Transmission electron micrograph of the anterior dorsal segment adult *S. mansoni* female worms obtained from mice treated at 5 weeks with or without N-89. Bars 1 μm. a shows TEM images of N-89 treated worms. Black arrowheads show the extensive lysis of tissues inside the sub-tegumental layer. b shows control worms. The arrows depict the presence of lateral spines with a regular morphology. The tegumental and sub-tegumental layers were intact with no evidence of lysis or damage. There was regular arrangement of the longitudinal and circular muscles with no evidence of destruction.
2.9. Statistical analysis

Statistical comparisons were performed using Student’s *t*-test between treated and untreated groups. Differences were considered significant at $p < 0.05$ at a 95% confidence interval.

3. Results

3.1. Ultrastructural morphological damage to *S. mansoni* adult worms treated with N-89

To examine the damage caused by N-89 on *S. mansoni* adult worms, we treated mice 5 weeks post-infection, which were killed 2 weeks post-
Table 2
Effect of N-89 on the egg maturity and hatching potential of S. mansoni.

<table>
<thead>
<tr>
<th>Factor</th>
<th>N-89 treated</th>
<th>Olive Oil control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic examination of eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matured eggs ± SD</td>
<td>2304 ± 346.21</td>
<td>26,179.2 ± 125.6</td>
</tr>
<tr>
<td>Immature eggs</td>
<td>1536 ± 451.2</td>
<td>2908.8 ± 224.5</td>
</tr>
<tr>
<td>% of matured eggs in total egg count</td>
<td>59.7</td>
<td>90.05⁎⁎</td>
</tr>
<tr>
<td>Hatching potential of the eggs into miracidia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hatched (per 1000 eggs)</td>
<td>150.15 ± 152.9</td>
<td>520.08 ± 23.54</td>
</tr>
<tr>
<td>Unhatched (per 1000 eggs)</td>
<td>840.84 ± 224.4</td>
<td>470.91 ± 167.2</td>
</tr>
<tr>
<td>Hatching Reduction Rate (%)</td>
<td>71.2⁎⁎</td>
<td></td>
</tr>
</tbody>
</table>

⁎ Indicates p value < 0.01 (CI: 95%; α = 0.05) Statistical significance was calculated by a t-test between the control group & treatment group.

⁎⁎ Hatching reduction rate was calculated using the formula: (C – T/ C) × 100, where C equals mean hatched eggs from infected control mice and T equals the mean hatched eggs from treated mice.

Table 3
List of selected genes and their biological functions.

<table>
<thead>
<tr>
<th>Target</th>
<th>Gene ID</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobinase (legumain)</td>
<td>Sm075800</td>
<td>Hemoglobinase is localized in the gut plays a pivotal role in digestion of the fluke's blood meal.</td>
<td>El Menawy et al., 1990</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>Sm085180</td>
<td>Cathepsin B is known to be active in the gut of schistosomes and proposed to be as key enzymes that the parasites use in hemoglobin degradation.</td>
<td>Sajid et al., 2003</td>
</tr>
<tr>
<td>Cathepsin S</td>
<td>Sm139240</td>
<td>Cathepsin S is a member of cystine proteases that’s proposed to play critical roles in the digestion of host blood tissues and hemoglobin, in reproduction and surface tegument biogenesis</td>
<td>Buro et al., 2014</td>
</tr>
<tr>
<td>Eggshell precursor protein</td>
<td>Sm000430</td>
<td>Egg precursor protein is an egg-shell precursor protein</td>
<td>Buro et al., 2003</td>
</tr>
<tr>
<td>P14</td>
<td>Sm131110</td>
<td>P14 is an egg-shell precursor protein</td>
<td>Buro et al., 2014</td>
</tr>
<tr>
<td>SmTYR1</td>
<td>Sm050270</td>
<td>Schistosoma mansoni tyrosinase is responsible for cross-linking of eggshell proteins is considered to be essential for helminth eggshell sclerotization, and final egg synthesis</td>
<td>Fitzpatrick et al., 2007</td>
</tr>
<tr>
<td>Titin</td>
<td>Sm105020</td>
<td>Titin is involved in muscle activity</td>
<td>Benian et al., 1999</td>
</tr>
<tr>
<td>Paramyosin</td>
<td>Sm129550</td>
<td>Paramyosin is involved in muscle activity</td>
<td>Gobert, 2005</td>
</tr>
<tr>
<td>HSP 70</td>
<td>Sm106930</td>
<td>HSP70 was chosen due to its known roles in stress response and signal transduction processes</td>
<td>Nollen et al., 2002</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Sm013040.1</td>
<td>It is the lead (or apical) protease that initiate the cascade of proteolytic digestion of human hemoglobin in S. mansoni</td>
<td>Aragon et al., 2008</td>
</tr>
<tr>
<td>Saposin-Like Protein-1</td>
<td>Sm000260</td>
<td>Acts in the esophagus following ingestion of blood</td>
<td>Morales et al., 2008</td>
</tr>
</tbody>
</table>

⁎ Full list of references for this table are listed in Supplementary References.
Fig. 8. Comparative qRT-PCR analyses of selected genes in S. mansoni male and female worms treated with or without N-89 at 5 weeks' post-infection. The mRNA level of each gene was determined by real-time PCR. Relative expression was calculated as the log$_2$ ratio target/control gene. Values are given as the means ± S.D. (N = 5), and *** indicates p value < 0.001. In total, eight (8) genes showed significant transcription changes in female S. mansoni worms. Cathepsin B (Smp.085180), Cathepsin S (Smp.139240), Haemoglobinase (Smp.075800), Eggshell precursor protein (Smp.000430), P-14 (Smp.131110), Cathepsin D (Smp.013040.1), Saposin-like Protein-1(Smp.000260) and SmTYR1 (Smp.050270) showed relative decrease in in relative gene expressions. Statistical analysis between control and treated groups were performed using Student's t-test at a 95% confidence interval and level of significance set at 0.05. *** indicates p value < 0.001.
After N-89 treatment. These genes were selected based on their roles in gut and digestive function, egg production, and worm musculature (used as a control). The complete list of the selected genes and their biological functions are presented in Table 3. The results of the qRT-PCR analysis showed that cathepsin B (Smp_085180), cathepsin S (Smp_139240) and hemoglobinase (legumain) (Smp_075800), showed a 4-fold decrease in relative gene expression compared to the control. Cathepsin B showed a 2-fold decrease in relative gene expression compared to the relative gene expression of worms from control mice. Cathepsin D (Smp_013040.1) and saposin like protein-1(Smp_000260) showed a 3-fold decrease in relative gene expression compared to the relative gene expression of worms from control mice (p < 0.001) (Fig. 8). In addition, genes responsible for egg production in S. mansoni, such as egg-shell precursor protein (Smp_000430), p-14 (Smp_131110), and SmTYR1 (Smp_050270) showed a 6-fold, 18-fold, and 11-fold decrease in relative gene expression, respectively (Fig. 8). In sum, the mRNA expression of these proteins supported our results that showed digestive function and egg production were significantly affected by N-89. Control genes such as heat shock protein (HSP) 70 (Smp_106930), para-myosin (Smp_129550), and titin (Smp_105020), responsible for muscle activity and musculature showed no mRNA expression changes after N-89 treatment. This supports the observations of the SEM analysis, which showed no significant damage to the musculature and structural integrity of S. mansoni worms.

4. Discussion

The present study sought to examine the mode of action of N-89 as a schistosomicidal agent. The main drug for schistosomiasis control, PZQ, has been shown to directly affect the structural integrity of adult worms [26]. In contrast, N-89 primarily acts inside the S. mansoni worm after being ingested and does not affect the structural integrity of the worms. N-89 has a peroxide bridge incorporated within its structure [27] that can be cleaved and activated in the presence of a catalyst to produce reactive oxygen species (ROS). Previously in malaria studies, we have shown that Fe²⁺ acts as a catalyst to cleave the endoperoxide bridge of N-89 and the catalytic cleavage is essential for the killing of the malaria parasite [28]. Schistosomes ingest large amount of host red blood cells (RBCs) [29,30]. Catabolism of host erythrocytes by schistosomes generate large quantities of heme, which contains the catalytic iron (Fe⁴⁺). From this, we hypothesize that the main mode of action of N-89 is via this catalytic cleavage of the peroxide bridge by Fe⁴⁺, resulting in the production of reactive oxygen species, which then acts directly inside the worm. This was evidenced by presence of undigested blood in treated parasites and the absence of hemozoin. Furthermore, both gut-related genes and enzymes responsible for egg production showed impaired expression due to death of the cells such as leucocytes and esophageal gland-cell bodies of S. mansoni [31]. In addition, activated N-89 in the worms can act inside the worm by directly causing injuries to the internal structures such as the vitelline cells in the female worms and the sub-tegumental structure in the male worms.

Nutrient uptake occurs in the gastrodermis (the primitive intestinal epithelium of the digestive system of these parasites) and rampant hemoglobinolysis occurs across this area [32]. We therefore suggest that; first, N-89 treatment causes an inhibition of nutrient absorption by the parasite. This is due to the fact that RBCs are not easily absorbed across the gastrodermis in the lumen of the parasite after N-89 causes disruptions in the gastrodermal wall. Additionally, activated N-89 may directly affect egg structure and integrity, which was evidenced by pores and structural deficits in the eggs collected from N-89-treated mice. This impaired nutrient uptake by the worms led to the production of inviable and immature eggs, since egg production is directly related to nutrient uptake in these worms [33]. From this, we conclude that N-89 impairs egg production by two possible alternative routes. First, the ability of activated N-89 to prevent the parasites from ingesting and digesting host RBCs leads to nutrient deficiencies, which result in the production of immature eggs. In addition, activated N-89 may have the ability to directly cause damage to S. mansoni eggs.

Praziquantel, which has been the drug of choice for schistosomiasis chemotherapy, is fraught with challenges, especially the concern about drug resistance. The advantage of N-89 as a potential drug candidate for schistosomiasis includes the relatively low toxicity against mammalian cell lines [28] and the use of a different mode of action, which is dependent on activation in the host by a physiological catalyst (Fe⁴⁺). Oxamniquine, a pro-drug that used to be a drug of choice in schistosomiasis was discontinued because of factors such as high cost, the limited range of efficacy to only S. mansoni and the emergence of drug resistance. In the case of Oxamniquine, activation of the drug was via a bio-activation process using one specific enzyme i.e. the sulfotransferase [34,35]. Therefore, mutation in the sulfotransferase enzyme led to drug resistance [36]. In contrast, for N-89, the activation process is via a physiological catalyst (Fe⁴⁺) produced by heme. Thus, drug resistance against N-89 will not occur in similar mechanism as that of Oxamniquine. In addition, the issue of drug resistance may not be profound in N-89 treatments, as the drug seems to have a wide range of targets, which will not develop drug resistance mutations simultaneously. Furthermore, combination therapy with N-89 and PZQ may have synergistic abilities to kill the parasites via separate modes of action and achieve high cure rates in areas of high endemicity.

5. Conclusion

Our findings confirm the broad negative effects of 1,2,6,7-tetra-oxaspiro [7,11] nonadecane against S. mansoni worm physiology and egg production. Coupled with its ability to kill schistosomula, we suggest that N-89 should be further investigated in field isolates as a potential drug for schistosomiasis control.

Conflict of interests

Authors declare no conflict of interest.

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Appendix A. Supplementary data

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