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ORIGINAL ARTICLE

Ivermectin efficacy against *Biomphalaria*, intermediate host snail vectors of Schistosomiasis

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The impact of ivermectin on adult snails of the genus *Biomphalaria* (*B. glabrata*, *B. tenagophila* and *B. straminea*), *B. glabrata* infected with *Schistosoma mansoni*, snail egg-masses cercariae and miracidia, as well as on guppy fish (*Poecilia reticulata*) was examined and evaluated. *Biomphalaria* snails, egg-masses, parasite stages and guppies were all exposed to different concentrations of ivermectin for 24 h, followed by regular observations of mortality. The calculated lethal doses of ivermectin were around an LD_{50} of 0.03 μg ml^{-1}, and an LD_{90} of 0.3 μg ml^{-1} for the three species of snails. Specimens of *B. glabrata* actually shedding parasite cercariae all died when exposed to ivermectin at a concentration of a mere 0.01 μg ml^{-1}. Ivermectin B_{1a}, the major (80%) component of commercially available ivermectin, proved to be inactive, and it was the minor (20%) component, ivermectin B_{1b}, which caused snail death. Snail egg-masses were not affected, even at the highest concentration of 100 μg ml^{-1}. With respect to *S. mansoni* parasite stages, 0.2 μg ml^{-1} ivermectin killed 50% of cercariae and miracidia within five minutes, rising to 90% after 30 min. Mortality of guppy fish within 24 h of exposure to ivermectin at concentrations of 0.5 μg ml^{-1} and 0.01 μg ml^{-1}, were 100% and 30%, respectively. The concentration of 0.01 μg ml^{-1} that killed *Schistosoma mansoni*-infected snails only caused 30% mortality in guppy fish. Ivermectin can be considered a promising molluscicide, especially as it is more potent against infected snails than uninfected ones, although it has no impact on egg-masses. Ivermectin and its derivatives could be explored in the search for a new agent to help control schistosomiasis transmission.
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INTRODUCTION

Schistosomiasis is a widespread tropical parasitic disease caused by infection with trematode worms of the genus *Schistosoma*. People become infected when larval forms (cercaria) of the parasite emerge from infected freshwater snails and penetrate human skin that comes into contact with infested water. Some 258 million people worldwide were treated in 2014 and, in terms of the detrimental impact on human health, schistosomiasis ranks second behind malaria. A continuous cycle of infection and re-infection occurs where sanitation is unsatisfactory or absent, where hygiene is poor and where exposure to infective water sources is unavoidable.[1]

The life cycle of one disease-causing parasite (*Schistosoma mansoni*) requires transmission through an intermediate host, notably susceptible snail species of the genus *Biomphalaria*. In Brazil, three species are involved in transmission of cercaria: *B. glabrata, B. tenagophila* and *B. straminea, with B. glabrata* being recognized as the main vector throughout South America. Early-stage worm miracidia infect the freshwater snails, eventually developing into cercariae, which exit the snails into water to penetrate human skin. Several steps are necessary to control schistosomiasis transmission, including curative and preventive chemotherapy, sanitary education, provision of basic sanitation and action to combat snails involved in the transmission cycle. Due to a variety of factors, including cost-effectiveness, attempts to control snails is now recommended only in special cases and as a complementary feature, for example, when there is a localized outbreak of acute cases or when high rates persist even with periodic drug treatment of the population at risk.[2]

Niclosamide (Bayluscide), a compound recommended by the World Health Organization (WHO), is the most effective molluscicide for killing intermediate host snails, as it causes the least amount of damage to the environment and to human health in comparison with other inorganic or synthetic molluscicides. However, it has several disadvantages: it is comparatively expensive, deployment costs are also high due to the need for repeated applications, it can cause environmental damage and its impact is only temporary.[2] There is also a possibility of snails developing resistance to niclosamide, all of which illustrates the need for a better alternative, one that is less expensive, more effective, biodegradable, environmentally safe, easy to apply and that is widely available.[3,4]

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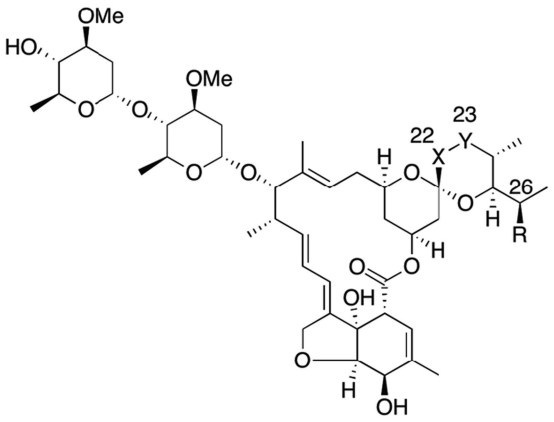

Ivermectin : mixture of dihydro derivatives of

B_{1a} (**1**) [R = Et; X–Y = H_2C–CH_2]

B_{1b} (**2**) [R = Me; X–Y = H_2C–CH_2]

Avermectin B_{1a} (**3**) [R = Et; X–Y = ⟿]

Avermectin B_{1b} (**4**) [R = Me; X–Y = ⟿]

Figure 1 Structure of ivermectin: mixture of dihydro derivatives of ivermectin B_{1a} (**1**) and B_{1b} (**2**) and avermectin B_{1a} (**3**) and B_{1b} (**4**).

Commercially available generic ivermectin (IVM) is an approximate 4:1 mixture of ivermectin B_{1a} (**1**) and B_{1b} (**2**), which are 22, 23-dihydro derivatives of avermectin B_{1a} (**3**) and B_{1b} (**4**), respectively (Figure 1).[5] Discovered in 1975 and marketed in 1981, ivermectin has been shown to be an extraordinary antiparasitic drug, active against a broad spectrum of organisms and widely used in veterinary medicine in 60 countries.[5–10] The compound is traditionally used to combat verminoses,[6] but is also effective against most species of mites, ticks, insect larvae, and some species of head lice. It is commonly deployed as the primary parasiticide in a variety of livestock and pets and, since 1987, it has also been used for the treatment of parasitic infections in humans. Indeed, some 300 million people are taking ivermectin annually to combat two major tropical diseases, onchocerciasis and lymphatic filariasis.

In 1987, when IVM was recognized as an ideal drug to help combat onchocerciasis, it was immediately donated for treatment of the disease, under the brand name Mectizan. Soon thereafter, researchers reported that IVM showed potent molluscicidal activity against *B. glabrata*.[11] In 2005, a Research Collaboration was established between Brazil's Oswaldo Cruz Foundation (Fiocruz) and Japan's Kitasato Institute, where the origins of the avermectins and ivermectin lie. An initial joint project was to examine the impact of ivermectin and related compounds on *Biomphalaria* snails. We report herein the bioactivity of the compounds against three species of *Biomphalaria* species, including specimens of *B. glabrata* experimentally infected with *S. mansoni* (LE strain) parasites, with snails shedding cercariae, eggs from *B. glabrata,* as well as cercariae and miracidia of *S. mansoni.* We also report the impact of ivermectin on guppy fish (*Poecilia reticulata*).

MATERIALS AND METHODS

Molluscs and parasites

The snails used in this work were as follows: *B. glabrata* snails (originally collected from the Barreiro region, in Belo Horizonte, Minas Gerais (MG), Brazil) measuring 8–18 mm diameter; *B. tenagophila*, (descendants from specimens collected in Cabo Frio, Rio de Janeiro, Brazil) measuring 8–10 mm diameter, and *B. straminea* (originally from Justinopolis, MG, Brazil) measuring 4–6 mm diameter. All snails were reared at the Mollusc Room Lobato Paraense, Research Center René Rachou/FIOCRUZ, Belo Horizonte, Brazil. The snails were kept under laboratory conditions (room temperature 24–26 °C and natural lighting) in polyethylene aquaria containing about 40 liters of water dechlorinated by means of activated charcoal filter. Ten specimens were randomly selected and used for each of the experimental groups, and similarly for the control groups. Each experimental procedure was carried out in duplicate, with all experiments being undertaken according to the guidelines of the World Health Organization.[12]

Egg-masses from *B. glabrata* were collected using uncolored polyethylene strips placed on the water surface of the aquaria. The strips where snails had deposited eggs were collected, observed under a stereomicroscope and sample masses were cut out and selected according to the following criteria: intact membranes and about 150 normal, viable eggs for each concentration with eggs in the blastula stage (i.e. 5 to 15 h after the first egg cleavage).[13] After exposure to the test compound, a record was made of the number of dead embryos detected at each observation point, allowing a relatively easy counting of the number of eggs, the stage of embryonic development, and accurate and rapid determination of dead embryos.

Specimens of *B. glabrata* with a mean weight of 600 mg, were experimentally infected with *S. mansoni* (LE strain) and those shedding cercariae were exposed to different concentrations of IVM (0.06–0.001 μg ml^{-1}) for 24 h, and followed up daily for 96 h to observe mortality.

S. mansoni cercariae and miracidia were collected and counted under a stereomicroscope (100 cercariae or miracidia for each concentration). These were placed in Petri dishes, together with the IVM solution to be tested, at concentrations of 0.1, 0.2, 0.3 or 0.4 μg ml^{-1}. In the controls, cercariae and miracidia were placed in dechlorinated water. Observations of mortality were made under a stereomicroscope at 5, 15, 30 and 60 min.

Specimens of guppy fish (*P. reticulata*), purchased at the Central Market in Belo Horizonte, after a 7-day period of adaptation in aquaria in the Mollusc Room, were exposed to IVM concentrations of 1.0, 0.5 and 0.1 μg ml^{-1} (10 specimens/concentration), simultaneously alongside an untreated control group.

All experiments were performed in duplicate. The values listed in this report are the mean of the duplicate experiments.

Chemicals

The ivermectin (IVM) used was a Brazilian generic product, Neoquímica. Suspensions were prepared starting from 50 mg of the chemical diluted into 500 ml of dechlorinated water (100 μg ml^{-1} suspension). All other concentrations were prepared via dilution of this parent solution. IVM was used at different concentrations in the experimental groups, varying between 100 and 0.001 μg ml^{-1}, following on from pilot experiments performed with IVM at concentrations of 100, 50 and 10 μg ml^{-1}.

Two specific separate analog compounds, ivermectin B_{1a} (**1**) and B_{1b} (**2**), were prepared in Kitasato University from avermectin B_{1a} (**3**) and B_{1b} (**4**) (both obtained from cultured *Streptomyces avermectinius*[14]) by chemoselective hydrogenolysis.[15]

Avermectins 3 and 4 were separated by HPLC on a Pegasil ODS SP100 column (20 i.d. × 250 mm; Senshu Scientific, Tokyo, Japan) and elution with 80% CH_3CN plus 0.05% formic acid at 8.0 ml min^{-1}, monitoring at UV 254 nm. The peaks at retention time of around 20 min for 4 and 25 min for 3 were collected, respectively.

Ivermectins 1 and 2 and avermectins 3 and 4 were diluted in dechlorinated water and tested at differing concentrations.

Experimental procedure

The specimens of snails and fish were kept in 250 ml glass beakers throughout the experiment, and the egg-masses in Petri dishes with 50 ml dechlorinated water. The experimental groups remained in contact with ivermectin diluted in dechlorinated water for 24 h. After this period, the organisms were removed, washed in running dechlorinated water and maintained in dechlorinated water for 4 days after exposure. The water was changed daily, while any dead snails, fish and embryos were removed and numbers recorded. The remaining living snails were fed fresh lettuce, and the fish with commercial feed (ProFish). The

egg-masses were observed under a stereomicroscope, with all surviving embryos remaining under observation until eclosion.

Mortality

Retraction of the snails into their shells or hemolymph release was the criteria used to determine snail death. Temperature and pH of the various tested concentrations for adult snails, as well as of egg-masses and fish, were measured at the beginning and end of each test. Temperatures ranged between 25 and 26 °C and pH ranged from 6.1 to 7.0.

Calculation of the lethal concentrations, LD_{50} and LD_{90}, for snails infected or not infected, using the program Graph-Pad Prism 4.0 was carried out according to the mortality rates obtained after a 96-hour period. The experiments has been repeat twice, LD_{50} and LD_{90} of each experiment were calculated by simple linear regression and Studentś *t*-test. The significance level was determined considering $P \leqslant 0.05$ as the confidence level.

RESULTS

Commercial ivermectin (IVM) killed 100% of *B. glabrata* snails within the first 24 h after initial exposure, at a concentration of 100 μg ml^{-1}, and after 48 h at 50 and 10 μg ml^{-1} concentrations. With reference to these specific concentrations, there was no apparent impact on *B. glabrata* egg masses, all intact eggs hatching normally until the end of the experiment.

As can be seen in Table 1, at a concentration of 1.0 μg ml^{-1}, all *B. glabrata* died within 48 h, whereas *B. tenagophila* and *B. straminea* were less affected, showing 100 and 90% mortality after 96 h, respectively. At a concentration of 0.5 μg ml^{-1}, all *B. glabrata* were found to be dead within 72 h, while 90% of *B. tenagophila* and 80% of *B. straminea* were dead after 96 h. At a concentration of 0.3 μg ml^{-1}, a mortality rate of 90% after a period of 96 h was detected for both *B. glabrata* and *B. tenagophila*, and 70% for *B. straminea*. The mortality difference among the three species was not significant ($P = 0.14$) (Table 1). The calculated lethal doses were: $LD_{50} = 0.03$ μg ml^{-1} and $LD_{90} = 0.3$ μg ml^{-1} for *B. glabrata* and *B. tenagophila*, respectively, plus $LD_{50} = 0.13$ μg ml^{-1} and $LD_{90} = 1.0$ μg ml^{-1} for *B. straminea*.

Table 1 Molluscicidal activity of commercial ivermectin (a mixture of ivermectin B_{1a} (1) and B_{1b} (2)) against *B. glabrata*, *B. tenagophila* and *B. straminea*

		Mortality (%)[b]			
		Time (h)			
Species[a]	Concentration (μg ml^{-1})	24	48	72	96
B. glabrata	1.0	60	100		
	0.5	20	90	100	
	0.3	0	80	90	90
	Control	0	0	0	0
B. tenagophila	1.0	10	60	90	100
	0.5	0	70	90	90
	0.3	0	60	80	90
	Control	0	0	0	0
B. straminea	1.0	20	70	80	90
	0.5	0	70	80	80
	0.3	0	40	60	70
	Control	0	0	0	0

[a]Diameter of the specimens used in this work. *B. glabrata*: 8–10 mm; *B. tenagophila*: 8–10 mm; *B. straminea*: 4–6 mm.
[b]The results are the mean of experiments performed in duplicate.

As can be seen in Table 2, snails infected with *S. mansoni* parasites exhibited 100% mortality within 24 h at an IVM concentration of 0.3 μg ml^{-1}. At 0.03 μg ml^{-1}, complete mortality was seen after 72 h post-exposure. After 48 h at a concentration of 0.01 μg ml^{-1}, mortality was 100%, the figure being 70% at a concentration of 0.005 μg ml^{-1} and 30% at 0.001 μg ml^{-1}. The IVM tested and found to be effective appeared to be significantly more potent against infected snails than against uninfected molluscs. The LD_{50} of IVM for infected snails was 0.002 μg ml^{-1} and 0.006 μg ml^{-1} for the LD_{90} value, whereas the figures for uninfected snails were an $LD_{50} = 0.03$ μg ml^{-1} and $LD_{90} = 0.3$ μg ml^{-1}, respectively (Table 3). Potentiation of molluscicidal activity shown against *B. glabrata* infected with *S. mansoni* compared with uninfected snails was statistically significant.

Ivermectin B_{1a} (**1**), the major component of IVM, was inactive at a concentration of 0.3 μg ml^{-1}, whereas in the case of the minor component, ivermectin B_{1b} (**2**), the same concentration produced 100% mortality of snails, although it did not show statistical significance when comparing the results presented by infected and uninfected snails at the concentrations tested. The related natural products, avermectin B_{1a} (**3**) and avermectin B_{1b} (**4**) demonstrated no molluscicidal activity at a concentration of 0.3 μg ml^{-1} (Table 3).

The efficacy of IVM against *S. mansoni* parasites was also evaluated, with 50% of miracidia and cercariae dying within 5 min of first contact with the chemical at a concentration of 0.2 μg ml^{-1}. After 30 min of exposure, mortality was around 90%, rising to 100% after 60 min (data not shown)

The piscicidal activity of ivermectin was examined and, with respect to guppy fish (*P. reticulata*), 100% of specimens died within the first 24-h following exposure to concentrations of 1.0 and 0.5 μg ml^{-1}, while 30% of fish died within 48-hour after exposure to a concentration of 0.01 μg ml^{-1}, with values of 0.03 and 0.1 μg ml^{-1} for the LD_{50} and LD_{90}, respectively (Table 4).

DISCUSSION

The WHO recommends niclosamide as the standard molluscicide to combat schistosomiasis intermediate host snails. A concentration of 1.0 μg ml^{-1} causes 100% mortality in *B. glabrata* within 8 h[13] and a concentration of 0.14 μg ml^{-1} causes 100% mortality for egg-masses after 24-h of contact,[16] while the lethal concentration for fish is 0.2 μg ml^{-1}.[17] Schistosome miracidia are killed by niclosamide at a concentration of 0.3 μg ml^{-1} in minutes, and 0.1 μg ml^{-1} if the exposure is longer,[18] while cercariae are affected by concentrations of 0.1–0.2 μg ml^{-1}. However, niclosamide is far from an ideal molluscicide due to its various adverse effects and there is an urgent need for safe and effective alternatives.

Matha and Weiser were the first to report the molluscicidal activity of ivermectin, under laboratory conditions, against *B. glabrata*. They found that snails were highly susceptible to ivermectin in low concentrations: $LC_{50} = 0.03$ μg ml^{-1}, $LC_{90} = 0.042$ μg ml^{-1}, and $LC_{100} = 0.055$ μg ml^{-1} 12 to 24 h after treatment.[11] They concluded that ivermectin was 60 to 120 times more toxic to *B. glabrata* than trifenmorph or niclosamide. Research in Africa also concluded that ivermectin killed various intermediate host snails, including *B. pfeifferi*, with an LC_{50} value of 0.71 μg ml^{-1}. Moreover, sub-lethal concentrations (0.01 μg ml^{-1}) led to considerable decreases in the number of eggs laid by the exposed snails.[19] Our study confirms that ivermectin is an effective molluscicide, killing *B. glabrata, B. tenagophila* and *B. straminea*, with LD_{90} of 0.3 μg ml^{-1} for the first two species, and 1.0 μg ml^{-1} for *B. straminea*. *B. straminea* is less susceptible to *S. mansoni* infection than *B. glabrata* end *B. tenagophila* in Brazil. Compatibility Index assessments using three strains of *S. mansoni*

Table 2 Molluscicidal activity on *B. glabrata* snails[a] of commercial ivermectin (IVM)—a mixture of ivermectin B_{1a} (1) and B_{1b} (2), 1 and 2 alone, avermectin B_{1a} (3) and B_{1b} (4)

		Mortality (%)[b]							
		Snails without infection				Snails shedding cercariae			
		After exposure (h)				After exposure (h)			
Compound	Concentration ($\mu g\ ml^{-1}$)	24	48	72	96	24	48	72	96
IVM	0.3	80	100			100			
	0.03	0	10	40	70	0	50	100	
	0.01	10	20	40	40	50	100		
	0.005	10	10	30	30	50	70	70	70
	0.001	0	10	20	20	30	30	30	30
1	0.3	0	0	0	0	0	0	0	0
2	0.03	20	100			40	100		
3	0.3	0	0	0	0	0	0	0	0
4	0.3	0	0	0	0	0	0	0	0
Control	—	0	0	0	0	0	0	0	10

[a]Snails diameter: 10–18 mm.
[b]Mean of experiments performed in duplicate.

Table 3 LD_{50} and LD_{90} of commercial ivermectin—a mixture of ivermectin B_{1a} (1) and B_{1b} (2), and 2 alone against *B. glabrata* snails

	Ivermectin		Ivermectin B_{1b} (2)	
Organism	LD_{50}	LD_{90}	LD_{50}	LD_{90}
Snails without infection	0.03	0.3	0.007	0.009
Snails shedding cercariae	0.002	0.006	0.001	0.008

Table 4 Piscicidal activity on guppy fish (*Poecilia reticulata*) of commercially available ivermectin

	Mortality (%)[a]			
	Time after exposure (h)			
Concentration ($\mu g\ ml^{-1}$)	24	48	72	96
1.0	100			
0.5	100			
0.1	70	70	80	90
0.01	0	30	30	30
Control	0	0	0	0

[a]The results are the mean of experiments performed in duplicate.

(LE, SJ and AL) demonstrating that *B. straminea* displays by far the lowest levels of infection.[20]

We found ivermectin to be less potent than previously reported, *B. glabrata* appearing 5-fold less susceptible to ivermectin in comparison with the data presented by Matha and Weiser. It is therefore important to emphasize our finding that only one of the two constituents of IVM appears to have molluscicidal properties. Ivermectin B_{1b} (**2**) kills snails, while ivermectin B_{1a} (**1**) does not demonstrate such activity. The two chemicals are extremely similar, the only difference being that ivermectin B_{1a} has an ethyl group at the C-26 position whereas ivermectin B_{1b} has a methyl group at the C-26 position. Thus the presence of the methyl group at the C-26 position in 2 which appears to be crucial to confer the molluscicidal property. The compound 'ivermectin' is routinely recognized as being a mixture containing 80% of 1 and 20% of 2. However, there is significant variance, with several authorities using slightly different specifications, the UN Food and Agriculture Organization (FAO) defining ivermectin as containing 'no less than 80%' of 1 and 'no more than 20%' of 2 (http://www.fao.org/fileadmin/user_upload/vetdrug/docs/41-3-ivermectin.pdf) while Merck, the original manufacturer of ivermectin, defined it as being composed of 'at least 80%' of 1 and 'not more than 20%' of 2.[21] Such possible minute variations, which could lead to differing amounts of the molluscicidal minor component 2 in commercially available products, might account for differences in the potency of IVM tested against snails by different researchers.

IVM did not act on egg masses from *B. glabrata*, even at the highest concentration used (that is, 100 $\mu g\ ml^{-1}$). This low susceptibility of egg masses to chemical attack has already been reported.[22–24] The high degree of resistance to molluscicides shown by parasite egg masses is probably due to the high molecular weights of these substances, which prevents them from penetrating the gelatinous membrane of the egg masses.[25] However, IVM did prove to be piscicidal, producing 100% mortality at a concentration of 0.5 $\mu g\ ml^{-1}$ when tested on guppy fish, with an LD_{90} value of 0.1 $\mu g\ ml^{-1}$.

One very important and highly significant finding to emerge from this work is that *B. glabrata* snails that were shedding *S. mansoni* cercariae were more susceptible to IVM, with 100% mortality at a concentration of 0.01 $\mu g\ ml^{-1}$. It is notable that this concentration, which is fatal for infected snails, kills only 30% of fish. In relation to LD_{90}, specimens of *B. glabrata* shedding cercariae died when a concentration much lower than that required to kill the uninfected specimens was used. A similar finding has previously been reported, *Bulinus truncatus* snails infected with *S. haematobium,* at the elimination phase of cercariae or in the pre-patent stage, being more susceptible to niclosamide than those uninfected.[26] The presence of S. mansoni infection in the snail induced a devastating effect on the several organs, specially on the hepatopancreas gland. This organ is the main responsible for the snail metabolism. It is possible that this is the reason for the higher mortality of infected snails.

In conclusion, this study confirmed that ivermectin is an effective molluscicide, killing adult *B. glabrata* snails at a concentration lower than that seen with the commonly used niclosamide. Furthermore, it is even more active against snails infected with *S. mansoni* parasites. The work also confirmed that the actual bioactive component is ivermectin B_{1b}, the minor component of the commercially available chemical. Probably the dose used was too high to allow selectivity action between infected or normal snails. Although ivermectin showed no impact on egg-masses, it proved to display moderate piscicidal activity at concentrations lethal for infected snails.

It is well-known that ivermectin can be toxic to non-target invertebrates, such as insects, that it remains active in the environment for a comparatively long time, and that repeated applications of the product in an aquatic environment needs much more in-depth research to ascertain its true impact on non-target aquatic organisms. However, taking into account the low dose required to kill 100% of shedding cercariae snails (0.01 $\mu g\ ml^{-1}$), our results support the belief that there is significant scope to explore the use of ivermectin and its derivatives, particularly ivermectin B_{1b}, for the future development of

a safe, effective and much needed tool to help with global efforts to control the transmission of schistosomiasis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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