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EXPERIMENTAL THERAPEUTICS

In Vitro and *In Vivo* Studies of Spironolactone as an Antischistosomal Drug Capable of Clinical Repurposing

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ABSTRACT Schistosomiasis is a parasitic flatworm disease that infects over 200 million people worldwide, especially in poor communities. Treatment and control of the disease rely on just one drug, praziquantel. Since funding for drug development for poverty-associated diseases is very limited, drug repurposing is a promising strategy. In this study, from a screening of 13 marketed diuretics, we identified that spironolactone, a potassium-sparing diuretic, had potent antischistosomal effects on *Schistosoma mansoni in vitro* and *in vivo* in a murine model of schistosomiasis. *In vitro*, spironolactone at low concentrations ($<10\ \mu$M) is able to alter worm motor activity and the morphology of adult schistosomes, leading to parasitic death. *In vivo*, oral treatment with spironolactone at a single dose (400 mg/kg) or daily for five consecutive days (100 mg/kg/day) in mice harboring either patent or prepatent infections significantly reduced worm burden, egg production, and hepato- and splenomegaly ($P < 0.05$ to $P < 0.001$). Taken together, with the safety profile of spironolactone, supported by its potential to affect schistosomes, these results indicate that spironolactone could be a potential treatment for schistosomiasis and make it promising for repurposing.

KEYWORDS *Schistosoma*, antischistosomal compound, schistosomiasis, schitosomicidal activity, spironolactone

Schistosomiasis, a parasitic disease caused by an intravascular trematode of the genus *Schistosoma*, remains a serious disease and public health problem in the developing world. It affects almost 240 million people worldwide, and more than 700 million people live in areas where this organism is endemic. The infection is prevalent in tropical and subtropical areas, in poor communities without potable water and adequate sanitation. *Schistosoma mansoni*, which causes intestinal schistosomiasis, accounts for around 80 million infections in Africa, the Near East, and South America (1). The disease is caused primarily by parasite eggs in the tissues. In advanced cases, a chronic inflammatory response to *Schistosoma* eggs results in periportal fibrosis, which is frequently associated with ascites and other signs of portal hypertension (2).

With no vaccine, the strategy for schistosomiasis control aims to prevent morbidity through regular treatment with praziquantel, which is currently the only recommended drug for schistosome infections. Although effective against all species of human schistosomes, praziquantel has significant limitations, e.g., incomplete efficacy profile, poor pharmacokinetics, and unpalatable taste, that undermine efforts to eliminate disease (3). In addition, due to its large-scale administration, concerns about drug resistance are increasing (4). Estimates show that at least 206.4 million people required

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TABLE 1 *In vitro* activity of diuretics against *S. mansoni* adult worm

Drug	Class	*S. mansoni* LC_{50} (μM)
Bendroflumethiazide	Thiazide diuretics	>50
Chlorothiazide	Thiazide diuretics	>50
Chlorthalidone	Thiazide diuretics	>50
Hydrochlorothiazide	Thiazide diuretics	>50
Indapamide	Thiazide diuretics	>50
Metolazone	Thiazide diuretics	>50
Bumetanide	Loop diuretics	>50
Ethacrynic acid	Loop diuretics	>50
Furosemide	Loop diuretics	>50
Torsemide	Loop diuretics	>50
Amiloride	Potassium-sparing diuretics	>50
Spironolactone	Potassium-sparing diuretics	7.2 (5.4–10.8)[a]
Triamterene	Potassium-sparing diuretics	>50

[a]The 95% confidence level is indicated in parentheses.

preventive treatment for schistosomiasis in 2016 (5). Reports of praziquantel resistance, both in the field and experimentally induced (4), further highlight the urgent need for new antischistosomal agents (6).

Drug development is a long process that can take decades, and since funding for drug development for poverty-associated diseases is very limited, drug repurposing is a promising strategy. Indeed, drug repurposing has emerged as a tool that can minimize the costs and risks associated with drug development programs. In recent years, drug repurposing has accounted for approximately 30% of the newly approved U.S. Food and Drug Administration drugs, with most of these molecules discovered by phenotypic drug screening (7). Several drugs have been used successfully for chemoprophylaxis and treatment of infectious diseases. Miltefosine, amphotericin B, chloroquine, and quinine are examples of successfully repositioned drugs (8). For these reasons, research into the discovery of new antischistosomal drugs is increasing, with drug repositioning being an attractive resource for beginning such a process (9, 10).

Diuretics are the most commonly prescribed class of drugs for relief from fluid congestion, especially in the patient with heart failure, kidney failure, and cirrhosis of the liver (11). These agents are generally well tolerated, safe, and inexpensive. Diuretic doses are typically higher in the case of congestion relief and can generally be scaled back in the chronic treatment phase (12). Due to their safety and the possibility of their oral administration, and also considering the importance of drug repositioning for schistosomiasis, we evaluated here the antischistosomal properties of the most commonly marketed diuretic drugs. Adopting a phenotypic drug screening strategy, these drugs were first tested *in vitro* against *S. mansoni ex vivo*. From these screenings, we identified spironolactone, a potassium-sparing diuretic, which effectively kills adult parasites. Also, using different drug concentrations, microscopy studies revealed that spironolactone altered the motility and morphology of the schistosomes, demonstrating the potential role of this diuretic as an antischistosomal drug capable of clinical repurposing. Finally, spironolactone was tested *in vivo* using a patent and a prepatent *S. mansoni* mouse model to characterize the full spectrum of activity of this drug.

RESULTS

***In vitro* studies.** For *in vitro* studies, thirteen drugs marketed in the different classes of diuretics were tested against adult schistosomes *ex vivo* and 50% lethal dose (LC_{50}) values were determined. In addition, cultures were monitored with an emphasis on changes in worm motor activity and scanning electron microscopy studies were used to evaluate tegumental damage in schistosomes.

Spironolactone, but not other diuretic drugs, exhibited *in vitro* antischistosomal activity. Thirteen drugs were tested *in vitro* against *S. mansoni* adult worms. Results of the LC_{50} value for each tested drug, separated by class, are summarized in Table 1. Twelve drugs did not show activity at the highest concentration tested (50 μM) for schistosomes. Only one compound (spironolactone), which displayed activity at

50 μM, was further studied and LC_{50} value of 7.2 μM determined after 72 h. A series of three independent experiments revealed that the anthelmintic activity of spironolactone is concentration dependent. For example, at concentrations of 50 and 12.5 μM, the times to reach full mortality for spironolactone were 24 and 72 h, respectively.

Spironolactone altered the motility and morphology of the schistosomes. In comparison to control parasites, light microcopy analysis revealed that schistosomes incubated with spironolactone showed accelerated motor activity, including contractions, and associated with a reduction in body length in a concentration-dependent effect (Fig. 1a to d).

In addition, scanning electron microscopy revealed profound alterations in the morphology of helminths after treatment with spironolactone, with rupture of the tegument along the whole dorsal body surface on all worms examined (Fig. 1g and h). The male worms treated with spironolactone showed changes in the tubercles and loss of the spines on the surface (Fig. 1e and g). In female worms, tegumental damage consisted of swelling and erosion of the surface (Fig. 1f and h).

***In vivo* studies.** On the basis of their *in vitro* activity against adult schistosomes *ex vivo*, spironolactone was administered orally using a single dose (400 mg/kg) or once daily for five consecutive days (100 mg/kg/day) to mice harboring either adult (patent infection) or juvenile (prepatent infection) stages of *S. mansoni*. In all treatments, parasites were quantified after perfusion of mice, and egg development stages (oogram) and fecal egg load were determined. Liver and spleen weights were also measured in order to assess hepato- and splenomegaly.

Oral treatment with spironolactone in mice harboring patent infections significantly reduced worm burden, egg production, and hepato- and splenomegaly. In mice harboring *S. mansoni* adults (patent infections), we observed significant reductions in worm burdens, egg production, and hepato- and splenomegaly in all experimental treatments compared to the control *S. mansoni*-infected mice.

In oral treatment with a single oral dose, spironolactone achieved total and female worm burden reductions of 73.8% ($P < 0.001$) and 69.7% ($P < 0.001$), respectively (Fig. 2a). With respect to egg burdens, a single oral dose of spironolactone led to a reduction of 73.4% ($P < 0.001$) in the number of immature eggs (Fig. 2b), whereas analysis in fecal samples revealed a reduction of 76.8% ($P < 0.001$) of eggs (Fig. 2c). Under the same drug regimen, the protective effect of spironolactone was also found to lead to a significant reduction of hepato- and-splenomegaly ($P < 0.05$ to $P < 0.01$), as measured by weight, compared to control infected mice (Fig. 2d).

In the experiments where spironolactone was administered daily for 5 days to mice infected with adult *S. mansoni*, a decrease of 65.9% ($P < 0.001$) and 63.7% ($P < 0.001$) in total worm and female burdens, respectively, was observed (Fig. 2a). This drug regimen also resulted in a reduction of 75.6% ($P < 0.001$) in the number of immature eggs (Fig. 2b). Likewise, spironolactone reduced the number of eggs in feces by 70.1% ($P < 0.001$) (Fig. 2c). With respect to pathology, treatment daily with spironolactone significantly decreased liver and spleen weights ($P < 0.05$ to $P < 0.001$) relative to the untreated controls (Fig. 2d).

Oral treatment with spironolactone in mice harboring prepatent infections significantly reduced worm burden, egg production, and hepato- and splenomegaly. Oral treatment with spironolactone using a single dose (400 mg/kg) or for 5 days (100 mg/kg/day) to mice infected with juvenile *S. mansoni* showed moderate but significant reductions in worm burden and egg production relative to control infected mice.

A single spironolactone oral dose led to a significant reductions of 51.7% ($P < 0.05$) and 54.4% ($P < 0.05$) in total and female worm burdens, respectively (Fig. 3a). In oogram pattern, the frequency of immature eggs was reduced by 48.7% ($P < 0.01$), and a 52.8% ($P < 0.01$) decrease in the number of eggs in feces was found with spironolactone given at the same dose regimen (Fig. 3b and c). A single oral dose of drug also

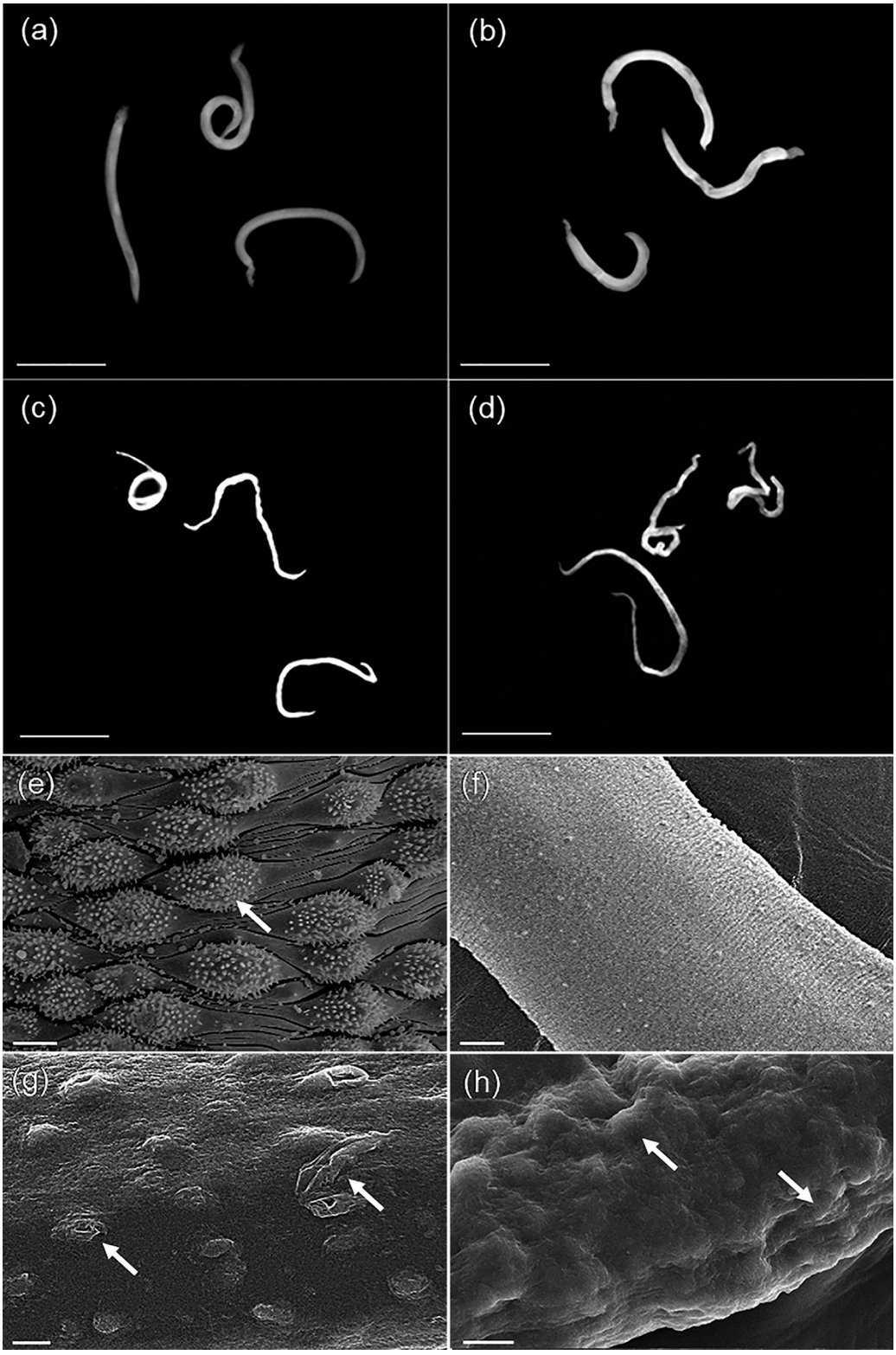

FIG 1 Microscopy images of *S. mansoni* treated with spironolactone for 72 h. (a to d) Light microscopy images show the gross morphological appearance of adult *S. mansoni* organisms after *in vitro* exposure to different spironolactone concentrations. (a) Control; (b) 5 μM spironolactone; (c) 10 μM spironolactone; (d) 20 μM spironolactone. (e to h) Scanning electron microscopy images of dorsal mid part of adult *S. mansoni*. (e) Male worm control showing tubercles and spines on the surface. (f) Female worm control. (g) 20 μM spironolactone. The dorsal tegumental surface shows shortening and collapse of the tubercles with loss of the spines on the surface. (h) 20 μM spironolactone. The dorsal tegumental surface shows swelling and shortening. Images were captured using a Leica Microsystems EZ4E microscope (scale bars, 5 mm [a to d]) and a JEOL SM-6460LV scanning electron microscope (scale bars, 10 μm [e to h]).

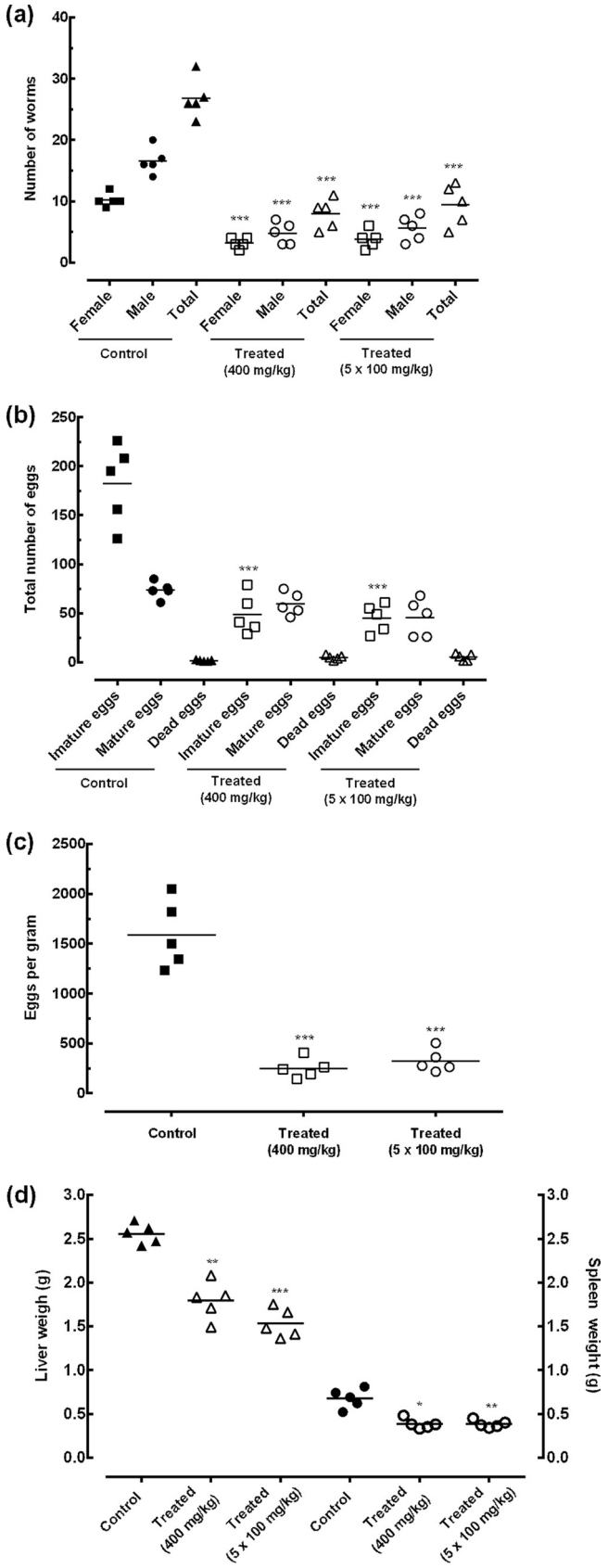

FIG 2 Effect of spironolactone on male and female worm burdens, egg burdens, and organ pathology in mice infected with adult *S. mansoni* (patent infection). Spironolactone was administered orally 42 days

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decreased the liver and spleen weights ($P < 0.05$) relative to the untreated controls (Fig. 3d).

Daily doses of spironolactone for five consecutive days achieved total and female worm burden reductions of 47.4% ($P < 0.05$) and 51.6% ($P < 0.05$), respectively (Fig. 3a). The same drug regimen reduced by 41.2% ($P < 0.01$) and 50.1% ($P < 0.01$) the numbers of immature eggs in the wall of the intestine (oogram) and in feces, respectively (Fig. 3b and c). Daily administration of spironolactone showed marked reductions in weights of the spleens and livers ($P < 0.05$ to $P < 0.01$) relative to control infected mice (Fig. 3c).

DISCUSSION

Despite the public health importance of schistosomiasis, the treatment and control of schistosomiasis has long relied on a single drug, praziquantel, and there is no commercial interest in developing new pharmaceutical compounds for a disease caused by helminths. Consequently, it is imperative to identify other drugs that could be used for treatment of schistosomiasis. Considering that the development of new drugs is a lengthy and costly endeavor, drug repositioning could be a viable alternative. In particular, diuretics are generally well tolerated, safe, and inexpensiv and have been often used in patients with schistosomiasis, especially in the severe form of the disease, but their antiparasitic action has never been described. In this study, we identified spironolactone, a potassium-sparing diuretic, as a potential drug for schistosomiasis chemotherapy after screening in a small approved diuretic drug library. The spironolactone had significant antischistosomal properties in murine models infected with *S. mansoni* when given at both patent and prepatent infections. Furthermore, we have shown that spironolactone caused morphological alterations in the tegument of adult parasites.

The *in vitro* assay demonstrated that schistosomes incubated with spironolactone showed alterations in their motor activity and an increase in their mortality in a concentration-dependent manner. The activity of spironolactone below 10 μM (<4 μg/ml) surpasses criteria established by the World Health Organization for potential compounds for schistosomiasis (13). Moreover, the active anthelmintic concentration obtained for spironolactone is lower than that obtained for other promising known drugs, such as artemether, artesunate, chlorambucil, mefloquine, and miltefosine (6).

Furthermore, given the importance of the schistosomes' tegument as a target for drugs (14), we also evaluated the surface of worms exposed to spironolactone. Scanning electron microscopy images indicated that tegumental structure was altered in both male and female *S. mansoni* organisms and intensified progressively when the concentrations of spironolactone increased. These morphological alterations were similar to those reported in studies with other anthelmintic compounds (15, 16). Likewise, it is known that praziquantel exhibits potent *in vitro* effects against schistosomes, and it causes extensive tegumental alterations in a concentration-dependent manner (17, 18).

In the murine model of schistosomiasis, two therapeutic regimens were adopted during periods of patent and prepatent infections: (i) mice were orally treated with spironolactone at a single dose of 400 mg/kg and (ii) spironolactone at a dose of 100 mg/kg/day for 5 days. It should be emphasized that a single oral dose of 400 mg/kg is the pattern chosen for experimental schistosomiasis (6). Also, based on the pharmacokinetic properties of spironolactone, which is rapidly metabolized and has a short half-life, we also chose administration of the drug in daily doses. Furthermore, spironolactone is water soluble, orally bioavailable (60% to 90%) and, at the therapeutic dose used here, neither toxic nor mutagenic (19).

FIG 2 Legend (Continued)
after infection at the doses indicated. (a) Worm burden, stratified by sex. (b) Egg development stages (oogram). (c) Stool egg load. (d) Organ pathology, as measured by liver (triangle) and spleen (circle) weights. Points represent data from individual mice that were either infected and treated with spironolactone or that were infected and left treated (control) mice. Horizontal bars represent median values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to untreated groups).

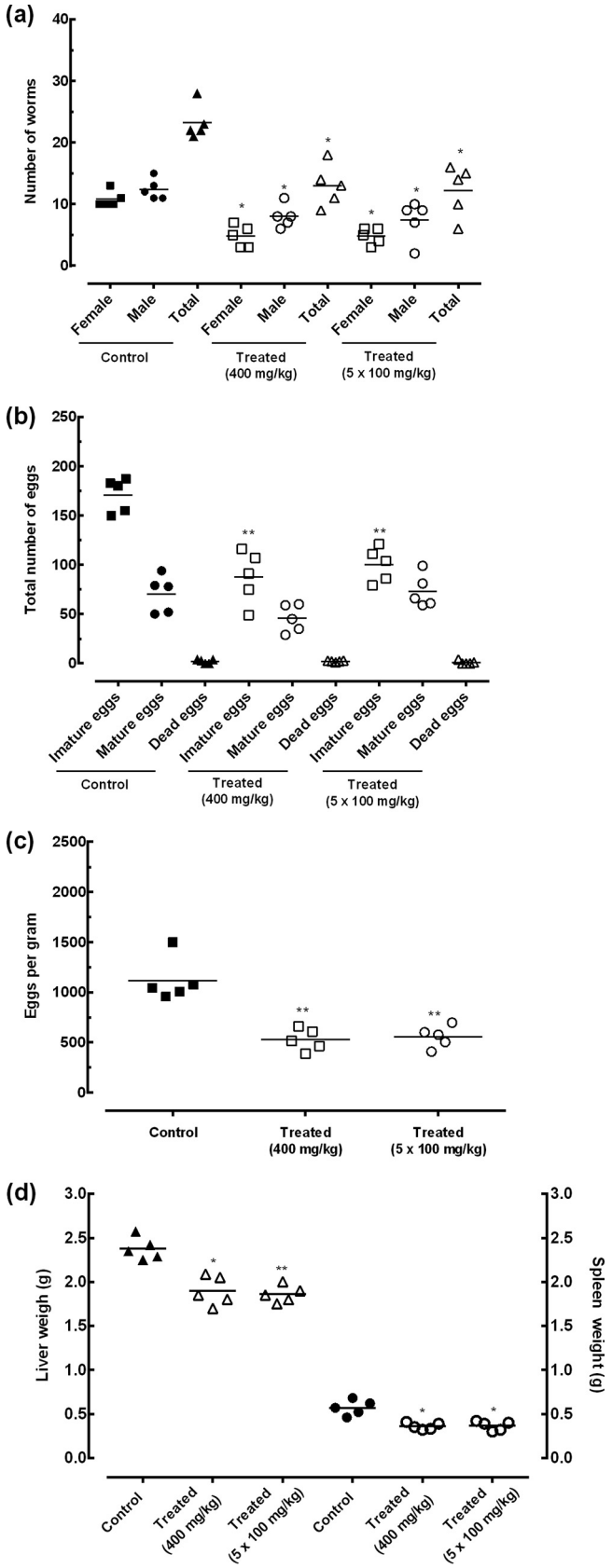

FIG 3 Effect of spironolactone on male and female worm burdens, egg burdens, and organ pathology in mice infected with juvenile *S. mansoni* (prepatent infection). Spironolactone was administered orally 21 days after infection at the doses indicated. (a) Worm burden, stratified by sex. (b) Egg development

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Interestingly, spironolactone demonstrated various parasitological efficacies and amelioration of hepatic and splenic pathology. Comparatively, adult schistosomes appeared more sensitive to spironolactone (patent infection) than juvenile stages (prepatent infection). Indeed, a high total worm burden reduction was achieved with spironolactone at a single dose of 400 mg/kg or 100 mg/kg/day for 5 days in patent infections, whereas a moderate total worm burden reduction was observed in prepatent infections. Unlike praziquantel, which is characterized by low activity against juvenile schistosomes, producing only a 25 to 30% reduction in worm burden (20), our results show that spironolactone has significant activity against the juvenile stage of the parasite. In contrast, the antischistosomal effect of spironolactone is weaker compared to praziquantel, which is known to reduce >90% of the worm burden (20).

The assessment of therapeutic efficacy in *S. mansoni*-infected mice was also based on the technique of quantitative and qualitative oograms and the Kato-Katz method for quantitative fecal examination, as well as organ pathology (as measured by weight). In all treatments, a significant reduction in immature eggs (oogram pattern) and eggs in feces was seen. This finding could be attributed to a high reduction in the worm burden as a result of treatment with spironolactone and/or inhibition of oviposition by adult helminths. A significant reduction in eggs collected from the intestine (oogram) or feces has been described with other antischistosomal compounds (21–23). In addition, the pathology normally associated with the deposition of schistosome eggs in the spleen and liver was clearly ameliorated, mainly when spironolactone was administered for 5 days. This result could be attributed to reduction in the worm burden and oviposition, as well as the pharmacological properties of spironolactone to treat edematous conditions (24).

Taken together, similar to other antischistosomal agents (6), the exact mechanism by which spironolactone exerts its effect on *S. mansoni* is still not clear. Spironolactone is the prototypic mineralocorticoid receptor antagonist, acting primarily through competitive binding of receptors at the aldosterone. Structurally, it is a synthetic derivative of progesterone, which also acts as an inhibitor of androgen production. Although schistosome species do not appear to have androgen receptors (25), it has been demonstrated that adrenal hormones affect the survival and oviposition of schistosomes both *in vitro* and *in vivo*, whose mechanism binds to the classical nuclear receptor and inhibits glucose metabolism (26). In addition, a large proportion of current anthelmintic drugs, including praziquantel, target ion channels of the parasite's neuromuscular system (27, 28). The fact that spironolactone induces concentration-dependent changes in schistosome motility and length also raises the possibility that this potassium-sparing diuretic may act on the neuromuscular system of *S. mansoni*. Considering these aspects, further studies are needed to evaluate the probable mechanism(s) of action.

In addition to the clinical significance of drug repositioning, the spironolactone has the advantages of water solubility, short half-life, low side effects, and safety compared to the praziquantel, the only drug available for the treatment of schistosomiasis. Moreover, as previous mentioned, spironolactone has significant activity against both adult and juvenile schistosomes stages. Praziquantel targets the adult worm, with minor activity against juvenile stages, and retreatment is necessary to kill those parasites that have matured.

In conclusion, from a screening of 13 diuretics, we identified that spironolactone had potent antischistosomal effects on *S. mansoni in vitro* and *in vivo* in a murine model of schistosomiasis. *In vitro*, spironolactone is able to alter worm motor activity and the morphology of adult schistosomes, leading to parasitic death. *In vivo*, oral treatment

FIG 3 Legend (Continued)
stages (oogram). (c) Stool egg load. (d) Organ pathology, as measured by liver (triangle) and spleen (circle) weights. Points represent data from individual mice that were infected and treated with spironolactone or that were infected and left treated (control) mice. Horizontal bars represent median values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to untreated groups).

with spironolactone at a single dose or daily to mice harboring patent and prepatent infections significantly reduced worm burden, egg production, and hepato- and splenomegaly. Taken together with the safety profile of spironolactone, supported by its potential to affect schistosomes, these results indicate that spironolactone could be a potential treatment for schistosomiasis, alone or in combination with praziquantel, and make it promising for repurposing.

MATERIALS AND METHODS

Reagents and drugs. All diuretic drugs (amiloride, bendroflumethiazide, bumetanide, chlorothiazide, chlorthalidone, ethacrynic acid, furosemide, hydrochlorothiazide, indapamide, metolazone, spironolactone, and torsemide, triamterene) were purchased from Sigma-Aldrich (St. Louis, MO). Praziquantel was purchased from Merck (São Paulo, SP, Brazil). Roswell Park Memorial Institute (RPMI 1640) culture medium containing phenol red and L-glutamine, M199 medium, inactivated fetal bovine serum (FBS), penicillin G/streptomycin sulfate, and HEPES buffer were obtained from Vitrocell (Campinas, SP, Brazil). Dimethyl sulfoxide (DMSO) and glutaraldehyde solution were obtained from Sigma-Aldrich.

Maintenance of *S. mansoni* life cycle. Female Swiss mice (3 weeks old; weight, ~14 g) were purchased from Anilab (São Paulo, Brazil). All animals were maintained under controlled conditions (temperature, ~22°C; humidity, ~50%; 12/12-h light/dark cycle; free access to rodent diet and water). For maintenance of parasite life cycle, mice were infected with *S. mansoni* (BH strain) by subcutaneous injection of ~120 cercariae. Cercariae were harvested from infected intermediate host snails *Biomphalaria glabrata* by exposure to light for 3 h according to standard procedures from our laboratory (21, 29).

***In vitro* antischistosomal assay.** Adult *S. mansoni* of both sexes were collected by dissecting the intestinal veins of mice euthanized 7 weeks postinfection. All the adult worms recovered were incubated in RPMI medium supplemented with 10% FBS containing antibiotics at 37°C in a 5% CO_2 atmosphere. In preparation for *in vitro* biological assays, all compounds were solubilized in DMSO (30).

For *in vitro* antischistosomal assay, one pair of worms was transferred to each well of a 24-well culture plate (tissue culture plastics; TPP, St. Louis, MO) containing RPMI medium supplemented with FCS and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) at 37°C in a 5% CO_2 atmosphere as previously described (31, 32). The *S. mansoni* cultures were then incubated with each drug at final concentration of 50 μM. Parasites were then kept for 72 h, and the effects of compounds were assessed microscopically, with an emphasis on changes in worm motor activity, morphological changes, and mortality rate. Schistosomes were also incubated in the presence of the highest DMSO concentration (0.5%) as a negative control and 5 μM praziquantel as a positive control.

Next, compounds presenting antischistosomal activity at 50 μM were characterized further. Therefore, additional concentrations of selected drugs were tested (25, 12.5, 6.25, and 3.12 μM). The half-maximum lethal concentrations (i.e., the LC_{50}s) for active compounds were calculated with GraphPad Prism software using sigmoid dose-response curves and the 95% confidence intervals as previously described (33, 34). Each concentration was tested in triplicate, and experiments were performed at least three times.

***In vivo* studies in a mouse model of schistosomiasis.** *In vivo* antischistosomal assay was performed in a mouse model of schistosomiasis according to the operating procedures for experimental chemotherapy for antischistosomal drug (35, 36). Three-week-old Swiss female mice were used for *in vivo* drug efficacy studies. The animals were kept with constant access to food and water. In all treatments, mice were subcutaneously injected with a suspension containing 80 *S. mansoni* cercariae.

Spironolactone was dissolved in saline and tested at a 400-mg/kg single dose or a 100-mg/kg daily for five consecutive days. Drug was administered 21 days (prepatent infection) or 42 days (patent infection) postinfection by oral gavage to groups of five mice. *S. mansoni*-infected control were given a corresponding amount of saline on the same timetable. All mice were euthanized with CO_2 and dissected at 56 days postinfection. The worms were then collected, separated by sex, and counted. Assessment of therapeutic efficacy was also based on the technique of quantitative and qualitative oograms using a fragment (10 mm) of the ascending colon (37), as well as the Kato-Katz method for quantitative feces examination (38). The compound-treated group and control group were compared using a parametric Dunnett's multiple-comparison test, where statistical significance was set to $P < 0.05$ in GraphPad Prism software (39).

Microscopy studies. During *in vitro* studies, schistosomes were monitored microscopically using a Leica Microsystems EZ4E (Wetzlar, Germany). For scanning electron microscopy, control and treated adult parasites were fixed for at least 3 h in 2.5% glutaraldehyde (Sigma) at room temperature. Experimental protocols for scanning electron microscopy were published previously (22, 23). Samples were metalized with gold using a Desk II sputter coater (Denton Vacuum LLC, Moorestown, NJ) and then observed using a JEOL SM-6460LV high-resolution scanning electron microscopy (JEOL, Ltd., Tokyo, Japan).

Ethics. Animals were subjected to experimental protocols (authorization 31/2017) approved by the Brazilian Ministry of Science, Technology, Innovation, and Communication, and experiments were conducted according to the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Brazilian law.

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