Mitochondria as a potential target for the development of prophylactic and therapeutic drugs against *Schistosoma mansoni* infection

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Running title: *S. mansoni* mitochondrion as potential drug target

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ABSTRACT

Emergence of parasites resistant to praziquantel, the only therapeutic agent, and its ineffectiveness as a prophylactic agent (inactive against the migratory/juvenile *Schistosoma mansoni*), makes the development of new antischistosomal drugs urgent. The parasite's mitochondrion is an attractive target for drug development because this organelle is essential for survival throughout the parasite's life cycle. We investigated the effects of 116 compounds against *Schistosoma mansoni* cercariae motility that have been reported to affect mitochondria-related processes in other organisms. Next, eight compounds plus two controls (mefloquine and praziquantel) were selected and assayed against motility of schistosomula (*in vitro*) and adults (*ex vivo*). Prophylactic and therapeutic assays were performed using infected mouse models. Inhibition of oxygen consumption rate (OCR) was assayed using Seahorse XFe24 Analyzer. All selected compounds showed excellent prophylactic activity, reducing the worm burden in the lungs to less than 15% that obtained in the vehicle control. Notably, ascofuranone showed the highest activity with a 98% reduction of the worm burden, suggesting the potential for development of ascofuranone as a prophylactic agent. The worm burden of infected mice with *S. mansoni* at the adult stage was reduced by more than 50% in mice treated with mefloquine, nitazoxanide, amiodarone, ascofuranone, pyrvinium pamoate, or plumbagin. Moreover, adult mitochondrial OCR was severely inhibited by ascofuranone, atovaquone, and nitazoxanide, while pyrvinium pamoate inhibited both mitochondrial and non-mitochondrial OCRs. These results demonstrate that the mitochondria of *S. mansoni* are feasible target for drug development.

Keywords: schistosomiasis, mitochondria, electron transport chain, fumarate respiration, *in vivo* model, drug development

Introduction

Schistosomiasis, a disease caused primarily by *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium* (1), results in approximately 280,000 deaths per year, making schistosomiasis the second-most-devastating parasitic disease after malaria (2, 3). In contrast to malaria, little effort has been spent on the development of new drugs against schistosomiasis, making the disease one of the 20 neglected tropical diseases, as designated by the World Health Organization (WHO) (4). In acute schistosomiasis, the cercaria, the larval stage of the parasite, actively penetrates mammalian skin and transforms into a distinct juvenile stage named the schistosomulum, which then invades the blood vessel and migrates sequentially through the lungs, heart, and portal vein, subsequently maturing into female and male adults. After mating, the worm pair migrates to the mesenteric veins where the female lays eggs, causing chronic schistosomiasis (5). The eggs are passed into the stool and to the environment, hatching into miracidia, which in turn infect snails, transforming into sporocysts, daughter sporocysts, and then cercariae (6). Finally, cercariae leave the snails and infect mammals, completing the schistosome's life cycle.

Praziquantel (PZQ) is the only drug available for treatment of schistosomiasis (7). However, PZQ does not confer protection against infection (i.e., prophylaxis) and does not completely kill adult parasites (8). Although the mechanism of action of PZQ is not well understood (9), parasites resistant to PZQ can be induced experimentally in infected mice (10), and reduced susceptibility have been reported to occur in various endemic areas (11). Given these shortfalls

of PZQ, the development of new drugs for the treatment and prevention of schistosomiasis are needed.

Given the complexity of the helminths life cycle, these parasites have evolved efficient mechanisms for the smooth transitions among environments of varying hosts and free-living stages (12), where their mitochondria are known to play key roles (13, 14). Under normoxic environment (egg and larval stages) these parasites employ a classical oxygen-dependent electron transport chain (ETC) composed of nicotinamide adenine dinucleotide (NADH) dehydrogenase (complex I), succinate:quinone reductase (complex II), quinol:cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV), and a high redox potential quinone (ubiquinone, E_m = + 110 mV) (15), similar to that found in the mammalian host. Complex I and II receive electrons from NADH and succinate, respectively, transferring the electrons to ubiquinone and then (consecutively) to complex III and complex IV via cytochrome *c*. Complexes I, III, and IV pump protons into the intermembrane space, generating an electrochemical gradient used by complex V for adenosine triphosphate (ATP) synthesis (oxidative phosphorylation). However, once parasites mature into adults in the small intestine (a hypoxic environment), the parasite employs fumarate respiration, a pathway that is composed by complex I, a low-redox-potential quinone (rhodoquinone, E_m = - 63 mV), and the reverse reaction of complex II (quinol:fumarate reductase) (16).

The most prominent advantage of fumarate respiration is the ability to produce ATP by oxidative phosphorylation independently of oxygen availability. In *S. mansoni*, fumarate respiration has been reported in the adult and sporocyst stages, while rhodoquinone-10 has

been identified in all life cycle stages (17), suggesting that fumarate respiration occurs in all stages. Although adult stage parasite lives in the mesenteric veins, where the oxygen saturation is about 60-75% (18), only 2% of the total oxygen is available in its dissolved form (19). In addition, mesenteric veins carry approximately 300 µM of hydrogen sulfide (20), a toxic gas produced by gut flora and a potent inhibitor of oxygen respiration (21), thus, suggesting that fumarate respiration is active in such environment.

Drug development targeting the mitochondrial respiratory chain has been explored (22). The anthelminthic pyrvinium pamoate has been shown to inhibit fumarate respiration by the adult stage of *A. suum* (23). Moreover, several antifungal agents such as siccanin, flutolanil, and fluopyran target complex II (24, 25). Atovaquone, an antimalarial drug, potently inhibits complex III from *Plasmodium falciparum* (26). Most recently, bedaquiline, a Food and Drug Administration-approved antitubercular drug, has been reported to be a complex V inhibitor (27) and also a mild uncoupler (28). Despite these pieces of evidence representing the proof-of-concept that ETC enzymes constitute a valuable target space for the development of new drugs to combat infectious diseases, little information is available about the impact caused by disruption of mitochondria-related processes in prophylaxis and treatment of *S. mansoni* infection. In the present study, we investigated the *in vitro, ex vivo*, and *in vivo* antischistosomal activities of several compounds reported to inhibit mitochondria-related processes and demonstrated the potential use of these compounds for prevention and treatment of *S. mansoni* infection.

Results

120 ***Motility assay***

121 *Cercariae*

122 The motility of *S. mansoni* cercariae was assessed in the presence of a panel of 116
123 compounds (listed in Table S1) known or thought to target mitochondrial function. After 41
124 hours of exposure, 48 compounds showed motility score 2.0 or less; of these, 37 compounds
125 showed complete inhibition of cercariae motility (with scores of 0.0) and another 11 compounds
126 showed mean scores of 0.1 – 2.0 (Table S1).

127 As complex I inhibitors, we tested rotenone (29), pyrvinium pamoate (30), fenpyroximate
128 (31), and derivatives of aurachin C and D (32) (Table S1). Rotenone, pyrvinium pamoate, and
129 aurachin derivatives AC-0-12 and AD-9-1 completely inhibited motility of the cercariae (score
130 0.0) after 41 hours of exposure. However, fenpyroximate was less active at this concentration
131 than the other tested compounds, given that motile cercariae still were observed after 41 hours
132 of exposure (mean score 1.3) (Fig. 1).

133 Tested complex II inhibitors included atpenin A5 (33), ferulenol and its derivatives (34),
134 flutolanil and its derivatives (24), 2-heptyl-4-hydroxyquinoline n-oxide (HQNO) (35) and siccanin
135 (36) (Table S1). Ferulenol showed complete inhibition of cercariae motility after 18 hours of
136 incubation; however, none of the tested ferulenol derivatives showed inhibition even after 41
137 hours. Flutolanil showed limited inhibition (mean score 2.7) after 41 hours; on the other hand,
138 flusulfamide, a flutolanil derivatives, inhibited motility of cercariae to a mean score of 0.0 after
139 18 hours. Interestingly, siccanin, an inhibitor of fungal complex II, showed a high mean

inhibition of score 1.3 within 1 hour of exposure; however, cercariae motility recovered upon prolonged incubation with this compound (Table S1).

The complex III inhibitors licochalcone A (37), atovaquone/ascofuranone, and their derivatives (38, 39) showed mean inhibition scores of 0.0 at 41 hours (Table S1). In addition to atovaquone, two of its derivatives, plumbagin and 511-12 (2 - hydroxy - 3 - [(2E,6E) - 3,7,11 - trimethyldodeca - 2,6,10 - trien - 1 - yl] - 1,4 - dihydronaphthalene - 1,4 - dione) showed excellent anti-cercarial activity, with mean scores at 41 hours of 0.0 and 0.3, respectively. Out of 32 variants of ascofuranone, 26 derivatives showed mean inhibition scores below 1.3 after 41 hours (Table S1).

Amongst the anthelmintics tested in this study, ivermectin (40) showed complete inhibition of cercarial motility after 18 hours (mean score 0.0), while nitazoxanide (41) reduced cercarial motility to a mean score of 1.7 after 41 hours (Table S1). (Note that pyrvinium pamoate, which is also an anthelmintic, is listed as a complex I inhibitor in Table S1.) Five anti-malarials were tested in this study; only mefloquine inhibited cercarial motility with mean score of 0.0 after 18 hours (Table S1). Among compounds with anti-trypanosomal activity tested in this study, only amiodarone (42) showed complete inhibition of cercariae motility (mean score of 0.0) after 18 hours (Table S1 and Fig. 1).

Evaluation of compounds reported to affect mitochondria-related processes in other organisms resulted in the demonstration that α-, β-, and γ-mangostin (43), along with gambogic acid (44), and shikonin (45), showed complete inhibition of cercarial motility after 18 hours

(mean scores 0.0). 3-Nonylphenol (46) also affected cercariae motility, though with lower potency (mean score 1.3) than the compounds mentioned above.

Cercariae treated with any of the remaining compounds or with 1% (*v/v*) DMSO survived 41 hours of exposure.

Schistosomula

No difference could be observed in the motility of schistosomula after 48 hours incubation with DMSO, PZQ, ascofuranone, fenpyroximate, or flusulfamide (Fig. 2). Complete inhibition of schistosomula motility was observed after incubation for 8 hours with atovaquone; 24 hours with amiodarone, nitazoxanide, mefloquine, or plumbagin; and 48 hours with pyrvinium pamoate (Fig. 2).

Adults

Upon exposure to selected compounds, the pair of *S. mansoni* adults began to separate and the effect on fecundity could not be addressed in this study. Therefore, the effect of each compound was evaluated individually for each male and female. Although amiodarone inhibited the motility of adult *S. mansoni*, inhibition after 20 hours of incubation was not complete, providing mean motility scores of 0.3 and 1.0 for the male and female, respectively (Fig. 3b and 3d). The remaining compounds completely inhibited the motility of male *S. mansoni* after 20 hours of incubation (Fig. 3a and 3b). In the case of females, similar results were obtained after 20 hours of incubation with PZQ, nitazoxanide, mefloquine, pyrvinium pamoate, plumbagin, ascofuranone, and flusulfamide (Fig. 3c and 3d). However, atovaquone and fenpyroximate were not effective against females of *S. mansoni* (Fig. 3c).

In vivo studies

Prophylaxis

Consistent with previous reports (47, 48), PZQ did not protect mice against *S. mansoni* infection, with animals exhibiting a worm burden of 89.6% ($p > 0.05$) relative to the negative control treatment (vehicle) (Fig. 4a). In contrast, the worm burden was significantly suppressed ($p < 0.05$) following prophylaxis with each of the selected compounds, such that hosts exhibited worm burdens ranging between 1.9 and 15.0% (Fig. 4a). Among the selected compounds, ascofuranone, plumbagin, and pyrvinium pamoate exhibited the strongest prophylactic activity, with worm burden reduced to 1.9%, 2.3%, and 2.9%, respectively ($p < 0.05$) compared to mefloquine (14.1%; positive control) and DMSO (100%; negative control) (Fig. 4a). Furthermore, the worm burdens of mice treated with fenpyroximate, atovaquone, flusulfamide, amiodarone, or nitazoxanide also were reduced, in these cases to 8.7%, 10.8%, 12.8%, 14.7%, or 15% (respectively) relative to vehicle (Fig. 4a).

Therapy

Under the conditions tested in this study, no worms were recovered from the mice treated with PZQ (Fig. 4b). In the groups of mice treated with fenpyroximate, atovaquone, or flusulfamide, the worm burdens were 72.0%, 69.9%, and 86.6%, respectively (Fig. 4b). A reduction of worm burden below 50% was achieved in mice treated with ascofuranone (45.0%), plumbagin (18.3%), pyrvinium pamoate (23.6%), amiodarone (29.2%), nitazoxanide (23.0%) and mefloquine (19.9%; $p > 0.05$) (Fig. 4b).

Oxygen consumption assay

Under all the tested conditions, the OCR was increased upon addition of respiratory substrates (Fig. 5 and Fig. S1). After addition of a mitochondrial uncoupler (FCCP) further increases in the OCR were observed (Fig. 5 and Fig. S1). A significant reduction in OCR was observed after addition of nitazoxanide, atovaquone, ascofuranone, pyrvinium pamoate, mefloquine, amiodarone, or fenpyroximate (Fig. 5 and Fig. S1). Decrease of the OCR to baseline levels (mitochondrial OCR) was achieved following addition of atovaquone to reactions containing nitazoxanide, atovaquone, ascofuranone, mefloquine, flusulfamide, amiodarone, or PZQ (Fig. 5 and Fig. S1). Reactions containing plumbagin or plumbagin plus atovaquone showed no change in OCR, which remained at the same level as that observed in the presence of FCCP (Fig. 5 and Fig. S1). In the case of pyrvinium pamoate, both mitochondrial and non-mitochondrial OCRs were completely inhibited (Fig. 5 and Fig. S1).

Discussion

In contrast to mammalian respiration, which is strictly aerobic, depending on the life cycle stage, helminths are able to perform aerobic (oxygen) and anaerobic (fumarate) respiration (16, 49). Interestingly, fumarate respiration also has been detected in isolated mitochondria from protozoan parasites such as *P. falciparum* (50) and *Eimeria tenella* (24), suggesting the use of fumarate respiration among evolutionarily unrelated parasitic organisms. It is important to note that development from *S. mansoni* cercariae to the adult stage is characterized by a gradual transition from a normoxic to a hypoxic environment, with changes in energy metabolism according to available carbon sources. We hypothesized that, given these transitions in setting, disruption of mitochondria-related processes could be detrimental for *S. mansoni* development

223 and survival, a weakness that might be exploited to prevent infection as well as to treat
224 established infection.

225 Whilst the effects of ascofuranone, flusulfamide, fenpyroximate and amiodarone on the
226 various life cycle stages of *S. mansoni* are reported here for the first time, 4 out of the 8
227 selected compounds (atovaquone, pyrvinium pamoate, plumbagin and nitazoxanide) have been
228 previously reported to be active against adult stage *in vivo* (atovaquone and nitazoxanide), *ex*
229 *vivo*-adult (nitazoxanide and plumbagin), *in vitro*-schistosomula (pyrvinium pamoate,
230 nitazoxanide and plumbagin), and cercariae (nitazoxanide and plumbagin) as summarized in
231 Table S2. For better comparison, all the 10 compounds (including the controls) were evaluated
232 in this study.

233 Most compounds active against cercariae are thought to be inhibitors of complex I, II, and/or
234 III, suggesting that *S. mansoni* depends on an active aerobic respiratory chain to survive.
235 Interestingly, the majority of the anti-cercarial compounds identified in the present work are
236 molecules that target complex III, and include ascofuranone, atovaquone, and their derivatives
237 (Table S1). These results suggest that modifications to meroterpenoid (51) and naphtoquinone
238 (52) scaffolds may provide candidates with excellent anti-cercarial activity.

239 In contrast to cercariae, schistosomula were insensitive to PZQ and to inhibitors of complex I
240 (fenpyroximate) (53), complex II (flusulfamide) (54), and complex III (ascofuranone) (55); were
241 less sensitive to a complex I + II inhibitor (pyrvinium pamoate) (23); but were sensitive to a
242 complex II + III inhibitor (atovaquone) (39) (Fig. 2). These findings suggest that simultaneous
243 inhibition of oxygen and fumarate respirations is required to cause lethality in this stage,

indicating that mechanically transformed schistosomula depend on at least one of this pair of respiratory strategies for survival. Moreover, mefloquine (56), amiodarone (57), and nitazoxanide (58) were active against schistosomula, probably because of these compounds' ability to induce the depletion of intracellular ATP levels (Fig. 2). Plumbagin's efficacy against schistosomula may be attributable to its ability to compete for electrons with respiratory quinones (rhodoquinones and ubiquinones), resulting in the generation of semiquinone radicals and reactive oxygen species (ROS) (59) (Fig. 2).

It previously has been reported that male schistosomes have a higher mitochondrial respiration rate than do females, while the non-mitochondrial respiration rate is higher in females than in males (60). These observations highlight the stronger dependence of males than females on the mitochondrial respiratory chain (60), consistent with the results obtained in the present study. Interestingly, pyrvinium pamoate and ascofuranone, which have been reported to inhibit fumarate respiration in parasitic helminths (23, 61). completely inhibited parasite motility within 20 hours of incubation (Fig. 3), suggesting that both females and males employ active fumarate respiration.

From the selected compounds, nitazoxanide (62), atovaquone (39, 63-65), ascofuranone (66), mefloquine (65, 67) and praziquantel (65, 67-70) have been reported to be effective for treatment of parasitic infection models by oral administration. Since the oral administration by previous reports were not standardized, and pyrvinium pamoate has no absorption by oral route (71), for better comparison, the administration of all selected compounds either as prophylactics or therapeutics of *S. mansoni* infection, was done intraperitoneally.

PZQ does not inhibit the motility of schistosomula *in vitro* and as expected, this compound failed to prevent the *S. mansoni* infection in the present study (Fig. 4a) (8). In contrast to the insensitivity to several compounds of schistosomula transformed *in vitro* (mechanically) (Fig. 2), schistosomula transformed *in vivo* (subcutaneously) were susceptible to all the selected compounds (Fig. 4b), suggesting differential dependency on mitochondrial respiration between *in vitro*- and *in vivo*-transformed schistosomula. Our results support previous results indicating that subcutaneously transformed schistosomula show higher rates of mitochondrial metabolism than do mechanically transformed schistosomula (72).

Although the worm burden was nominally decreased in mice treated with flusulfamide, fenpyroximate, or atovaquone, the effect was not significant (p value > 0.05); similar results (apart from flusulfamide) were obtained using *ex vivo* females (Fig. 4b). Possible explanations are that (i) flusulfamide is a weak inhibitor of complex II (having a reported IC_{50} of 76.5 μM in *A. suum*) (54) and (ii) at the dose of 5 mg/kg body weight, the plasma concentration of this compound did not reach a level sufficient to kill the parasites (73). We previously have shown that ascofuranone (61) and pyrvinium pamoate (23) inhibit helminth fumarate respiration. Given the significant (p value < 0.05) reductions in worm burdens (to 45% and 23.6% of vehicle control, respectively) in mice treated with ascofuranone or pyrvinium pamoate (Fig. 4b), these results suggest that the *S. mansoni* adult stage depends on fumarate respiration. Under our experimental conditions, plumbagin showed the strongest reduction in the worm burden (to 18.3% of vehicle control), a result that may be attributable to the generation of ROS (59, 74), as discussed above. Mefloquine, nitazoxanide, and amiodarone reduced the worm burden to 19.9-29.2% of the vehicle control (Fig. 4b); however, the mechanisms of action of these compounds

287 are not completely understood, though these molecules have been suggested to affect
288 mitochondrial membrane potential or ROS generation (75-78). Collectively, our results reinforce
289 the notion that the *S. mansoni* adult stage relies on active mitochondria, making the related
290 pathways feasible as drug targets.

291 The significant reduction in OCR observed with atovaquone, ascofuranone, pyrvinium
292 pamoate, mefloquine, amiodarone, and fenpyroximate (Fig. 5 and Fig. S1) indicates that these
293 compounds are, in fact, *S. mansoni* respiratory chain inhibitors. Unexpectedly, nitazoxanide,
294 which has been reported to act as a mild uncoupler and thereby enhance OCR (77, 78),
295 inhibited the OCR of adult pairs to the same degree as did ascofuranone and atovaquone.
296 Based on this finding, it is tempting to speculate that the nitazoxanide may be inhibiting
297 complex III, an effect that may be surpassing the compound's mild uncoupling effect, thereby
298 causing the observed decrease in OCR. However, additional studies will be needed to verify
299 these results and this hypothesis. Interestingly, pyrvinium pamoate completely inhibited both
300 mitochondrial and non-mitochondrial OCR (Fig. 5), suggesting that this compound might have
301 other targets related to non-mitochondrial respiration. Moreover, plumbagin did not reduce the
302 OCR but instead maintained the OCR at a level similar to that seen with FCCP; in the presence
303 of plumbagin, OCR was insensitive to the effect of atovaquone (Fig. 5). This result supports the
304 hypothesis that plumbagin acts as an electron acceptor for complex I or II while bypassing
305 complex III, thereby maintaining the OCR at a high level.

306 In this study, we demonstrated for the first time (to our knowledge) that inhibitors of
307 mitochondria-related processes have potential for use in chemoprophylaxis and merit further

development. Although the molecular target (complexes I-IV) of these compounds could not be identified, we show that amongst the mitochondria-related processes, mitochondrial respiration is severely inhibited and potentially the target pathway. These compounds, especially those of the ascofuranone and the FDA-approved antimalarial drug atovaquone classes, have great advantages over PZQ, given their high efficacy in reducing the worm burden in the lungs. Thus, these compounds have the potential to meet the requirements of at least two target product profiles from four proposed by Caffrey (79). We postulate that such chemicals could be used in combination with PZQ for the control and elimination of schistosomiasis. In conclusion, the mitochondrion of *S. mansoni* is a good drug target space; the results obtained in the present study provide starting points for the development of new drugs for the prevention and treatment of schistosomiasis.

Materials and Methods

Ethical statement

Mouse experiments were approved by Nagasaki University's Animal Research Committee (No. 1506181240); animals were handled per the relevant protocols of Japanese law specified in the Humane Treatment and Management of Animals (Law No. 105, dated 19 October 1973 and subsequently revised as of 2 June 2006).

Compounds

This study tested a collection of 116 compounds (Table S1). The panel included compounds that have been reported to inhibit mitochondria-related processes such as ETC, cellular respiration, and membrane potential; classical antiparasitic agents; and a small number of

329 molecules used for treatment of human disease. This panel (of our laboratory compound
330 library) previously has been described as part of a separate study conducted in our laboratory
331 (80). Stock solutions of the compounds were available at 1 mM and assayed at 10 µM, in order
332 to maintain the final concentration of dimethyl sulfoxide (DMSO) not more than 1%, against *S.*
333 *mansoni in vitro* and *ex vivo* as has been reported (81).

334 ***Maintenance of S. mansoni parasites***

335 A Puerto Rican strain of *S. mansoni* was maintained essentially as described previously (82).

336 ***Motility assays***

337 The motility and viability assay have been widely used to screen small subset of compounds. It
338 can also be easily adapted to laboratories because of its low cost and was used for first
339 screening in this study (83). Cercariae was evaluated microscopically by comparing parasites
340 in the presence of 10 µM of the compounds to those in wells containing vehicle (DMSO) at
341 three different time points (≤1 hour, 18 hours, and 41 hours) as has been reported (84). Motility
342 was scored using a 5-point scale, as described previously (4 = normal motility; 3 = reduced
343 motility; 2 = uncoordinated minimal motility, 1 = severe reduction in motility; 0 = total absence of
344 mobility) (60).

345 Schistosomula were obtained through mechanical transformation from cercariae and purified
346 using Percoll gradient as described previously (85). Schistosomula were transferred (at
347 approximately 30 per well) into 96-well plates containing RPMI medium supplemented with 5%
348 (*v/v*) fetal bovine serum (FBS), 10 mM glutamine, and penicillin-streptomycin (10 U/mL-10
349 µg/mL, respectively), and the plates were incubated overnight at 37 °C in a CO_2 incubator. On

the following day, a subset of 8 compounds (selected according to their activity against cercariae, as well as their commercial availability and in amounts sufficient for *in vivo* experiments) was selected, including atovaquone, nitazoxanide, flusulfamide, fenpyroximate, plumbagin, amiodarone, mefloquine (Tokyo Chemical Industry Co., Ltd), pyrvinium pamoate, (MP Biomedicals, LLC), and ascofuranone (Institute of Mitochondrial Sciences, Inc.). Compounds of this panel of 8, as well as mefloquine (67, 81, 86) and PZQ (the positive controls), were added at a final concentration of 10 µM (and 1% (*v/v*) DMSO) to the individual wells of the schistosomula-containing plates; each compound was tested in triplicate. The motility of the schistosomula was scored (as described above) at 4 time points (≤ 1, 8, 24, and 48 hours).

Five-week-old female ICR mice (Japan SLC Inc.) were kept in the environmentally controlled animal facility from Nagasaki University (25^{o}C, 70% humidity, 12 hours of light and dark cycle) with availability of water and food. Mice were kept for a week to acclimatize before treatment and/or infection. Thirty-five mice were infected with approximately 150 cercariae per animal. After 8 weeks, mice were euthanized, and adult worms were recovered through perfusion of the hepatic portal system and mesenteric veins (85). Schistosomes were washed using RPMI supplemented with 5% (*v/v*) FBS plus penicillin-streptomycin (10 U/mL-10 µg/mL) and incubated overnight at 37 °C in a CO_2 incubator. Adult schistosomes (10 pairs/well) were transferred to 24-well plates containing selected compounds at 10 µM; each compound was tested in triplicate. Motility was evaluated microscopically and scored as described above.

In vivo prophylactic assay

Because there are no validated drugs neither vaccines for prophylaxis of schistosomiasis, we tested whether or not the selected compounds have potential prophylactic activity. Groups of 6 ICR mice (maintained as described above) each were used to test the effectiveness of selected compounds: atovaquone, 100 mg/kg; nitazoxanide, 50 mg/kg; ascofuranone, 100 mg/kg; flusulfamide, 5 mg/kg; fenpyroximate, 2 mg/kg; plumbagin, 2 mg/kg; pyrvinium pamoate, 2 mg/kg; amiodarone, 50 mg/kg; mefloquine, 100 mg/kg; PZQ, 100 mg/kg; and vehicle (1 × phosphate-buffered saline (PBS) containing 3% (*v/v*) ethanol and 7% (*v/v*) Tween 80). The compounds were administered intraperitoneally (on the left side of the abdomen) one day before infection (Day -1). The respective compounds were administered again on Day 0, and 3 hours later the mice were infected subcutaneously (on the right side of the abdomen) with approximately 500 *S. mansoni* cercariae/mouse. Administration of the respective compounds was repeated once daily for 2 additional days post-infection (Days 1 and 2) (i.e., for a total of 4 doses). Mefloquine (67, 81, 86) and vehicle containing 1% (*v/v*) DMSO was used as positive and negative control, respectively. Six days post-infection, mice were euthanized, lungs were collected, and schistosomula were recovered (85, 87) and counted. The worm burden was calculated as described previously using the following formula (68):

$$\text{Worm burden}\ (\%) = \frac{(NW_{\text{neg}} - NW_{\text{tre}})}{NW_{\text{neg}}} \times 100$$

where NW_{neg} and NW_{tre} represent the mean numbers of worms in the negative control and treated groups, respectively.

In vivo therapeutic assay

ICR mice were infected, as described above, with approximately 150 cercariae/mouse. At week 6, mice were treated with selected compounds by 4 days of once-daily intraperitoneal injection using the same dosage as for the prophylaxis assay. PZQ and mefloquine were used as positive controls (67, 81, 86) and vehicle as negative control. At 14 days after the final dose administration, worms were collected through perfusion, mesenteric veins examined to count for any trapped adult worms (88), and the worm burden was calculated as described above.

Determination of oxygen consumption rates

The oxygen consumption rate (OCR) was determined using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies) as described previously (89, 90). A pair of adults was placed in each well, an Islet Capture Screen was inserted into the wells, and the plate was loaded into the XFe24 analyzer. OCR was determined at 37 °C in the presence of 15 mM glucose, 1 mM pyruvate, and 5 mM L-glutamine (Port A) as respiratory substrates; 10 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; Port B) as uncoupler. Reproducible detection of OCR changes in short period of time (25 min) were achieved using 50 µM of each of the selected compounds (Port C). To completely quench mitochondrial OCR, atovaquone was added at 50 µM (Port D). The experiments were performed using a programmed protocol consisting of 2 min mixing, 3 min waiting, and 3 min measuring time per cycle for five cycles between injections.

Data analysis

All data were analyzed using Prism version 8 (GraphPad). The t-test were performed on all groups and p value < 0.05 was considered significant.

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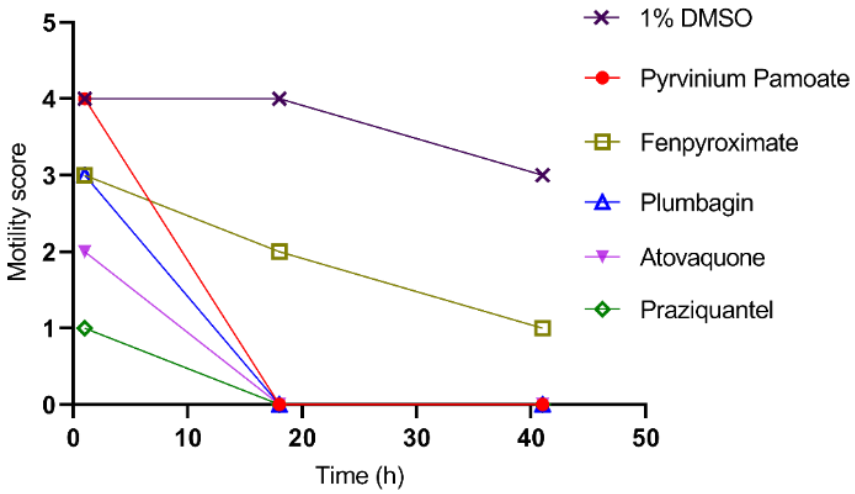
(a)



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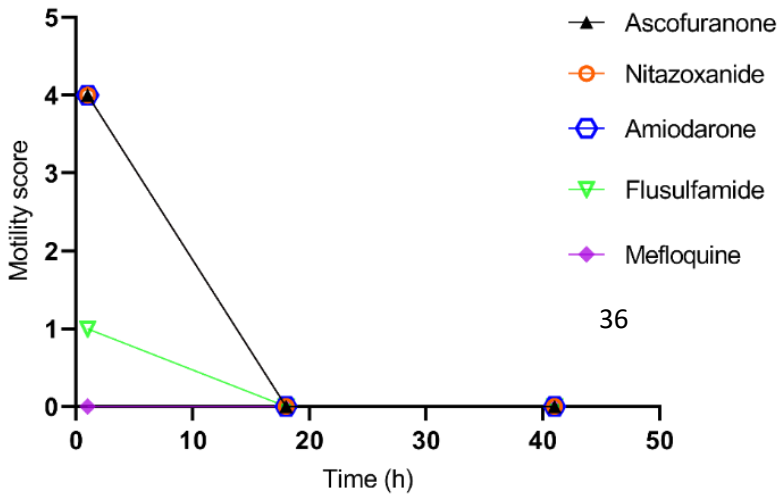
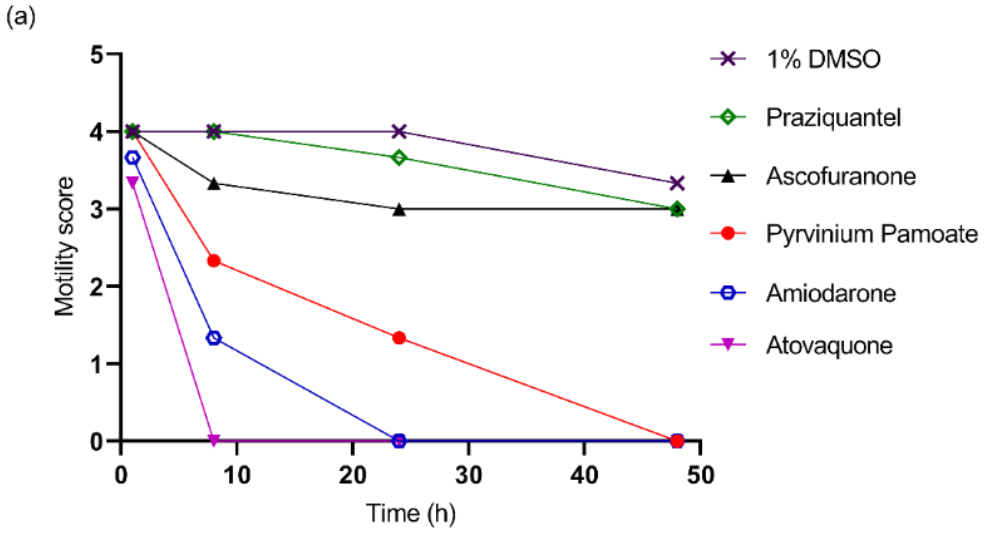
724 **FIG 1** Effects of selected compounds on the relative motility of *S. mansoni* cercariae. Cercarial

(b)



motility was assessed in the presence of (**a**) pyrvinium pamoate, fenpyroximate, plumbagin, atavaquone, praziquantel (PZQ), and dimethyl sulfoxide (DMSO; vehicle), and (**b**) ascofuranone, nitazoxanide, amiodarone, flusulfamide, and mefloquine. Each compound was assayed at final concentration of 10 μM and 1% DMSO. Motility was evaluated microscopically in triplicate at each of three different time points (1, 18, and 41 h); the results are plotted as mean motility scores (ranging from 0 to 4) as described in the Materials and Methods section.



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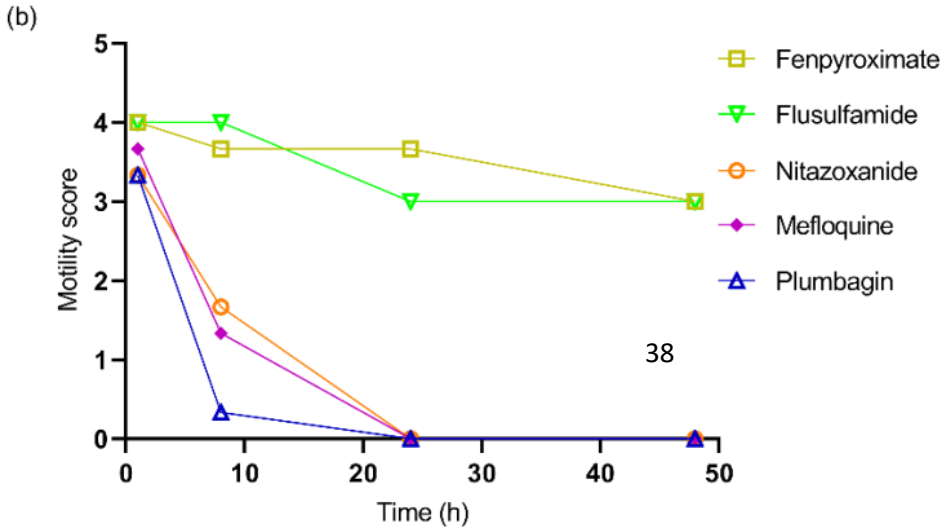
732

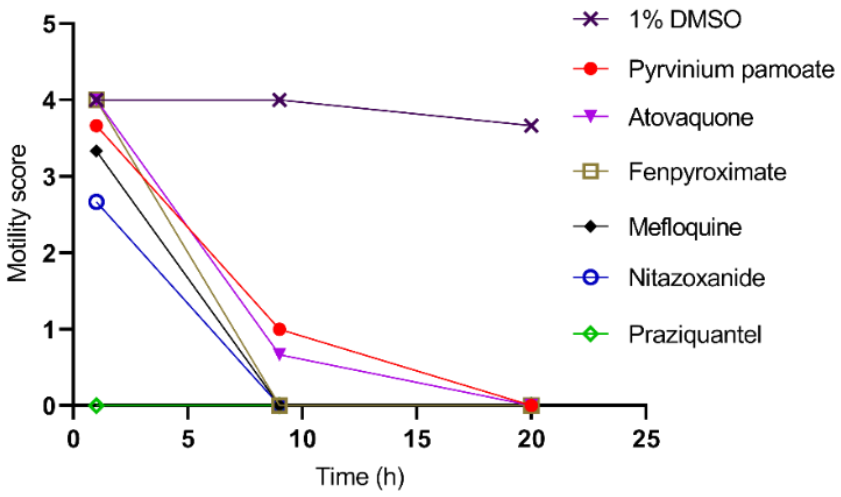
733

734 **FIG 2** Effects of selected compounds on the relative motility of *S. mansoni* schistosomula.
735 Schistosomula motility was assessed in the presence of (**a**) praziquantel (PZQ), ascofuranone,
736 pyrvinium pamoate, amiodarone, atovaquone, and dimethyl sulfoxide (DMSO; vehicle), and (**b**)
737 fenpyroximate, flusulfamide, nitazoxanide, mefloquine, and plumbagin. Each compound was



assayed at final concentration of 10 µM and 1% DMSO. Motility was evaluated microscopically in triplicate at each of three different time points (1, 8, 24, and 48 h); the results are plotted as mean motility scores (ranging from 0 to 4) as described in the Materials and Methods section.

(a)



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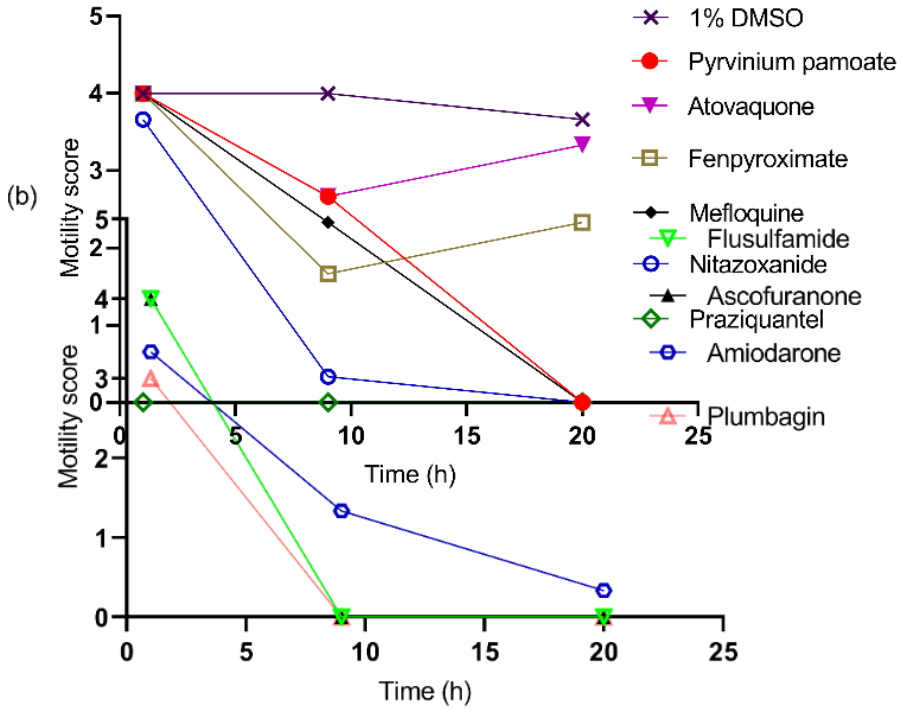
758

(c)

(b)



759

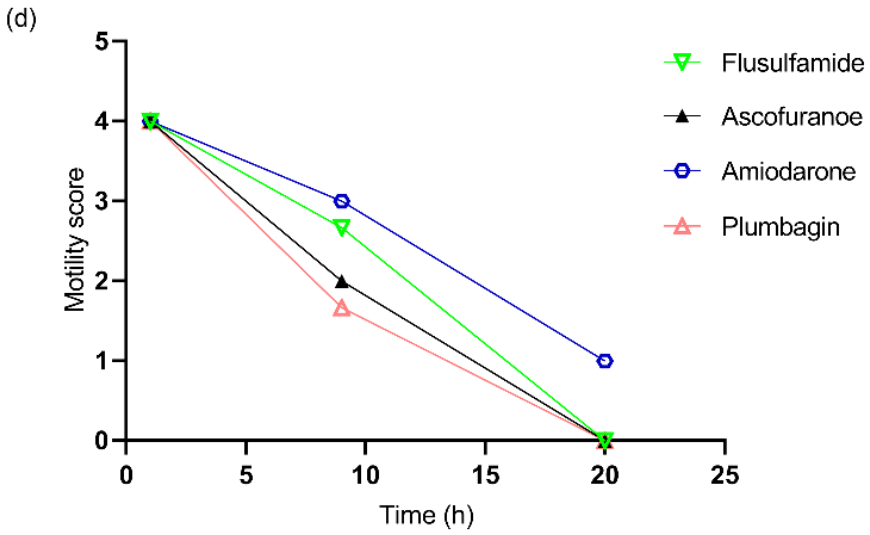
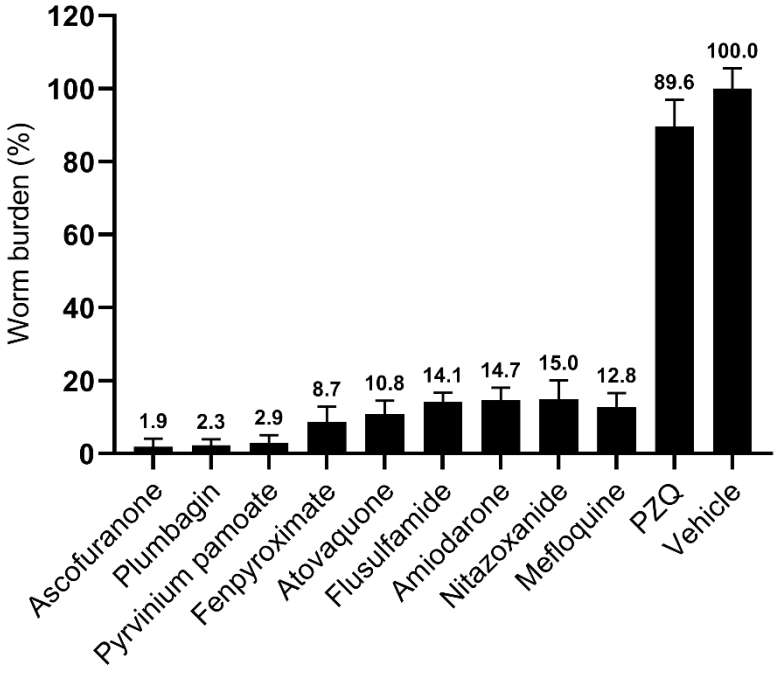


FIG 3 Effects of selected compounds on the relative motility of male and female adult *S. mansoni*. The mean motility score (ranging from 0 to 4) of (**a, b**) male and (**c, d**) female adults in the presence of (**a, c**) dimethyl sulfoxide (DMSO), pyrvinium pamoate, atovaquone, fenpyroximate, mefloquine, nitazoxanide and praziquantel (PZQ), and **(b, d)** flusulfamide, ascofuranone, amiodarone, and plumbagin. Each compound was assayed at final concentration of 10 µM and 1% DMSO. Motility was evaluated microscopically in triplicate at each of three different time points (1, 9, and 20 hours); the results are plotted as mean motility scores (ranging from 0 to 4) as described in the Materials and Methods section.

773

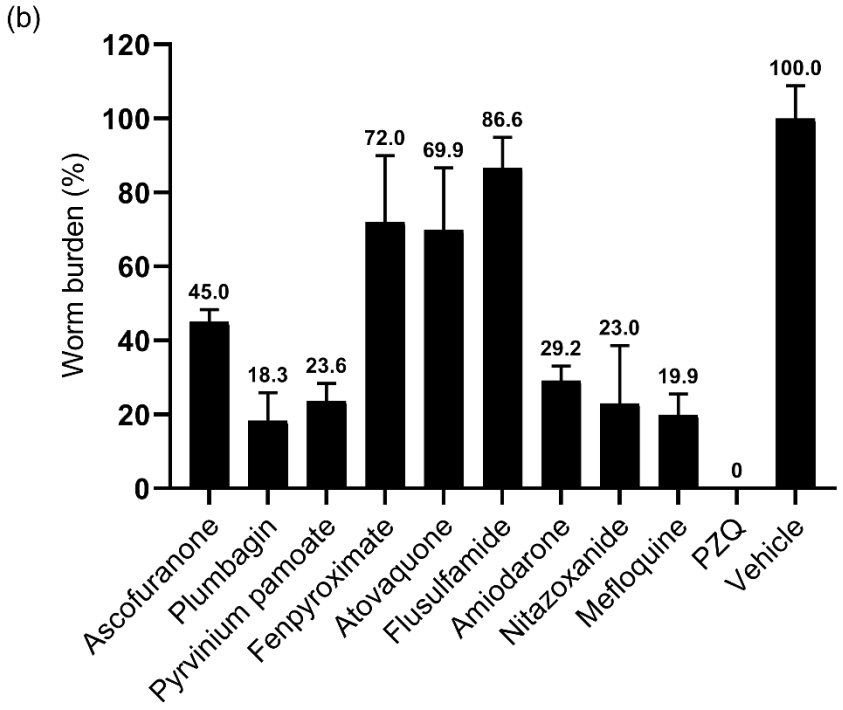
774

(a)


Worm burden (%)
120
100
80
60
40
20
0
1.9
2.3
2.9
8.7
10.8
14.1
14.7
15.0
12.8
89.6
100.0
Ascofuranone
Plumbagin
Pyrvinium pamoate
Fenpyroximate
Atovaquone
Flusulfamide
Amiodarone
Nitazoxanide
Mefloquine
PZQ
Vehicle

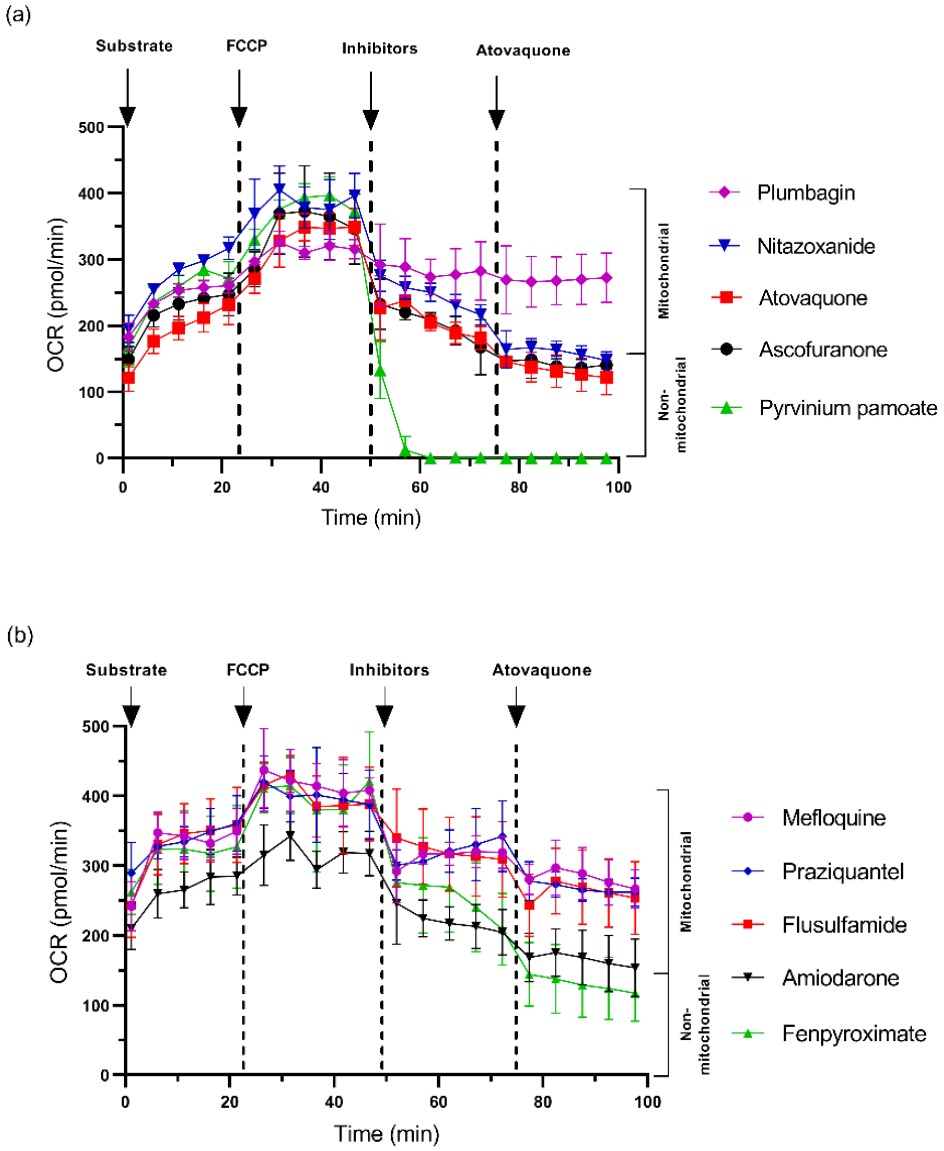
775

FIG 4 Prophylactic and therapeutic activity of selected compounds on *S. mansoni* infection. Mice (n = 6/group) were treated with selected compounds at dosages of ascofuranone (100 mg/kg), plumbagin (2 mg/kg), pyrvinium pamoate (2 mg/kg), fenpyroximate (2 mg/kg), atovaquone (100 mg/kg), flusulfamide (5 mg/kg), amiodarone (50 mg/kg), nitazoxanide (50 mg/kg), mefloquine (100 mg/kg), praziquantel (PZQ; 100 mg/kg), or vehicle (containing 1% DMSO). Animals were dosed by four days of once-daily intraperitoneal injection at the indicated dosage, starting one day prior to infection. Animals were euthanized 7 days after infection; schistosomula then were recovered from lungs of each mouse, counted, and used to calculate the worm burden. (**a**). Mice (n = 6/group) at week 6 post-infection were treated intraperitoneally



with selected compounds for 4 days at dosage mentioned above. Mice were sacrificed 14 days

after the last treatment and adult parasites were recovered and counted. The worm burden was calculated as mentioned in Materials and Methods section. For both panels, data are presented as mean and standard deviation of values normalized to those in vehicle-treated animals (defined as 100%).



792

793 **FIG 5** Oxygen consumption rates (OCR) of adult *S. mansoni* pair in the presence of selected
794 compounds. The OCR was determined in the presence of 25 mM glucose, 1 mM pyruvate, and
795 5 mM L-glutamine (substrates). The first reading after addition of substrates was set as
796 baseline. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was added to a final
797 concentration of 10 µM to induce maximum respiration. Effects on the OCR were evaluated

following addition of (**a**) ascofuranone, plumbagin, nitazoxanide, pyrvinium pamoate, or atovaquone, or (**b**) mefloquine, praziquantel (PZQ), flusulfamide, amiodarone, or fenpyroximate to final concentrations of 50 µM each. Atovaquone then was added to completely inhibit mitochondrial OCR. The OCR resistant to atovaquone was defined as non-mitochondrial respiration. Means and standard deviations are shown for each time point of OCR measured in triplicate.

Table S1 Activity of screened compounds on *Schistosoma mansoni* cercariae motility and proposed targets.

				Mean Motility Score (0-4)[a]		
Target	Compound[b]			Hours		
	No.	Name		≤1	18	41
Complex I	1	**Rotenone**		1.0	0.0	0.0
	2	**Pyrvinium pamoate**		4.0	0.0	0.0
	3	Pamoic acid		4.0	4.0	4.0
	4	**Fenpyroximate**		3.7	2.0	1.3
	5	Aurachin C derivative	AC-0-10	4.0	4.0	4.0
	6		AC-0-11	4.0	3.7	3.0
	7		**AC-0-12**	4.0	1.3	0.0
	8	Aurachin D derivative	AD-0-11	4.0	4.0	4.0
	9		AD-1-10	2.7	3.0	3.7
	10		**AD-9-1**	1.7	2.0	0.0
Complex II	11	Atpenin A5		4.0	4.0	4.0
	12	**Ferulenol**		3.0	0.0	0.0
	13	Ferulenol derivative	Decursinol angelate	3.7	4.0	4.0
	14		Decursin	4.0	4.0	4.0
	15		5-MeO-coumarin	3.7	4.0	4.0
	16		Auraptene	4.0	4.0	4.0
	17	Flutolanil		4.0	4.0	2.7
	18	Flutolanil derivative	**Flusulfamide**	1.0	0.0	0.0
	19		Fluopyran	4.0	4.0	4.0
	20		2-Aminobenzanilide	3.7	4.0	4.0
	21		2-nitro-N-phenylbenzanilide	3.7	4.0	4.0
	22		Ethyl-2-(trifluorometyl)-benzoate	3.7	4.0	4.0
	23		Methyl-2-(trifluoromethyl)-benzoate	3.7	4	4.0

	24		Methyl-2-iodo-benzoate	3.7	4.0	4.0
	25		Trifluorobenzanilide	4.0	4.0	4.0
	26		2-Iodoacetophenol	4.0	4.0	4.0
	27		Methyl-2-iodobenzamide	4.0	4.0	4.0
	28		Fluopyran	4.0	4.0	4.0
	29		Isopyrazan	3.3	4.0	3.3
	30		Mepronil	3.7	4.0	4.0
	31		Mepenil	3.3	4.0	4.0
	32		2-(trifluoromethyl) benzanilide	4.0	4.0	4.0
	33		Tecloftalam	3.3	4.0	3.7
	34		Salicylanilide	2.7	3.0	4.0
	35		Benodanil	3.7	4.0	4.0
	36		Bixafen	1.7	4.0	3.7
	37		Carboxin	4.0	4.0	4.0
	38	2-heptyl-4-hydroxyquinoline n-oxide		4.0	4.0	4.0
	39	**Siccanin**		1.3	3.3	2.0
Complex III	40	Antimycin A		3.3	3.3	2.7
	41	Azoxystrobin		3.7	4.0	4.0
	42	Myxothiazol		4.0	4.0	4.0
	43	**Licochalcone A**		3.0	0.0	0.0
	44	**Atovaquone**		2.0	0.3	0.0
	45	Atovaquone derivative	Lapachol	3.7	4.0	4.0
	46		Lawsone	4.0	4.0	3.3
	47		**Plumbagin**	3.0	0.0	0.0
	48		**511-12**	4.0	0.7	0.3
	49	**Ascofuranone**		4.0	0.0	0.0
	50	Ascofuranone derivative	**Ascochlorin**	4.0	1.7	0.0
	51		±Acetyl-ascofuranone	4.0	4.0	3.3
	52		**±Rac-ascofuranone**	4.0	1.0	0.3
	53		**±Desmetyl-**	4.0	0.0	0.3

				ascofuranone			
		54		**Colletochlorin B**	0.0	0.0	0.0
		55		**Tetrahydro-ascofuranone**	1.0	0.0	0.0
		56		**K-5-9**	0.0	0.0	0.0
		57		K-6-9	4.0	4.0	2.3
		58		**172-11-OPiv**	1.0	0.0	0.0
		59		**173**	1.0	0.0	0.0
		60		**175-12-OPiv**	0.0	0.0	0.0
		61		**193-11-OPiv**	4.0	0.0	0.0
		62		**200-10**	0.0	0.0	0.0
		63		215-9-OH	2.0	4.0	4.0
		64		215-18-Anthra	1.0	4.0	4.0
		65		**216**	1.0	0.0	0.0
		66		**217**	1.0	0.0	0.0
		67		231-9-OMe	3.0	4.0	2.3
		68		**234-12-OPiv**	1.3	0.0	0.0
		69		236-12-O-Tetrahydrofuran	3.3	4.0	4.0
		70		**250**	4.0	0.0	0.0
		71		**264-8**	0.7	0.0	0.0
		72		**264-11-OPiv**	1.3	0.0	0.0
		73		**271-12**	2.7	0.0	0.0
		74		274-9	3.7	4.0	3.3
		75		**275-10-COOMe**	4.0	0.0	1.3
		76		**275-11-COOMe**	4.0	1.7	0.3
		77		**276-9**	4.0	3.7	2.0
		78		277-9-OH	3.0	4.0	2.7
		79		**277-11-OAc**	4.0	0.3	1.3
		80		**280-12**	0.7	0.0	0.0
		81		**281-12**	4.0	0.0	0.0
		82		**287-12-OCOiPr**	2.7	0.0	0.0
DHODH[c]		83	Brequinar		4.0	4.0	4.0
VKOR[d]		84	Warfarin		4.0	4.0	4.0
Antiparasitic	Helminth	85	**Nitazoxanide**		4.0	1.7	1.7
		86	Zoxamide		3.3	4.0	2.3

		87	Tizoxanide	4.0	4.0	3.7
		88	**Ivermectin**	4.0	0.0	0.0
		89	Morantel	4.0	4.0	4.0
		90	Oxantel pamoate	4.0	4.0	4.0
		91	Pyrantel pamoate	4.0	4.0	4.0
	Malaria	92	Artemisinin	4.0	4.0	4.0
		93	Chloroquine	2.3	4.0	4.0
		94	Proguanil	4.0	4.0	4.0
		95	**Mefloquine**	0.7	0.0	0.0
		96	Doxorubicin	4.0	4.0	4.0
	Trypanosomatid	97	**Amiodarone**	4.0	0.0	0.0
		98	Mycophenolic acid	4.0	4.0	4.0
		99	O-Desmethyl-mycophenolic acid	4.0	4.0	4.0
		100	Nifurtimox	4.0	4.0	4.0
Mitochondria		101	Lycoline	4.0	4.0	4.0
		102	Tasquinimod	4.0	4.0	4.0
		103	KU-55933	4.0	4.0	4.0
		104	3.4.5.6-Tetrahydroxanthone	4.0	4.0	4.0
		105	Mangiferin	3.7	4.0	4.0
		106	**α-Mangostin**	1.7	0.0	0.0
		107	**β-Mangostin**	4.0	0.0	0.0
		108	**γ-Mangostin**	3.7	0.0	0.0
		109	**Gambogic acid**	2.7	0.0	0.0
		110	Catechin	4.0	4.0	4.0
		111	Berberine	4.0	4.0	4.0
		112	Quercetin	4.0	4.0	4.0
		113	Resveratrol	4.0	3.3	4.0
		114	**3-Nonylphenol**	2.3	1.3	1.3
		115	**Shikonin**	3.3	0.0	0.0
		116	Toltrazuril	3.7	4.0	4.0

[a]Relative motility of *S. mansoni* cercariae was scored on a scale of 0–4 (4 = normal motility; 3 = reduced motility; 2 = uncoordinated minimal motility, 1 = severe reduction to motility; 0 = total absence of mobility), as reported previously (60). The compounds were tested in triplicate and scores were averaged (mean) for every time point.

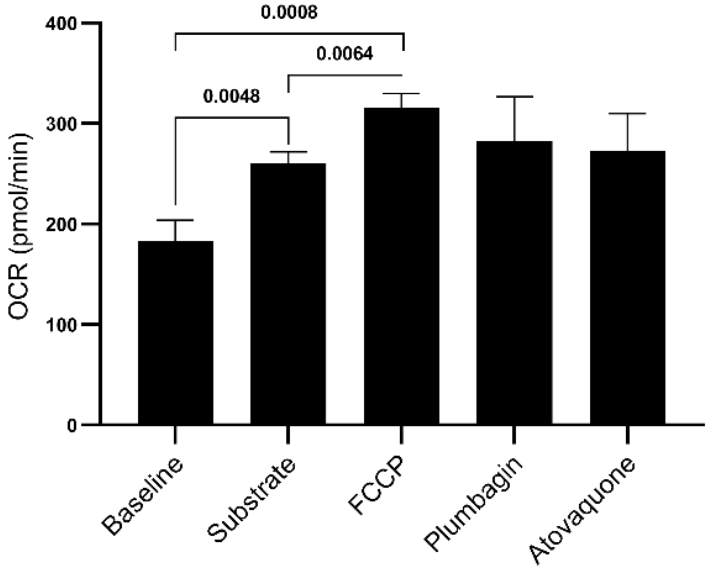
[b]Compounds in bold showed scores below 2 after 41 hours and were considered hits.

[c]DHODH - dihydroorotate dehydrogenase

[d]VKOR - vitamin K epoxide reductase

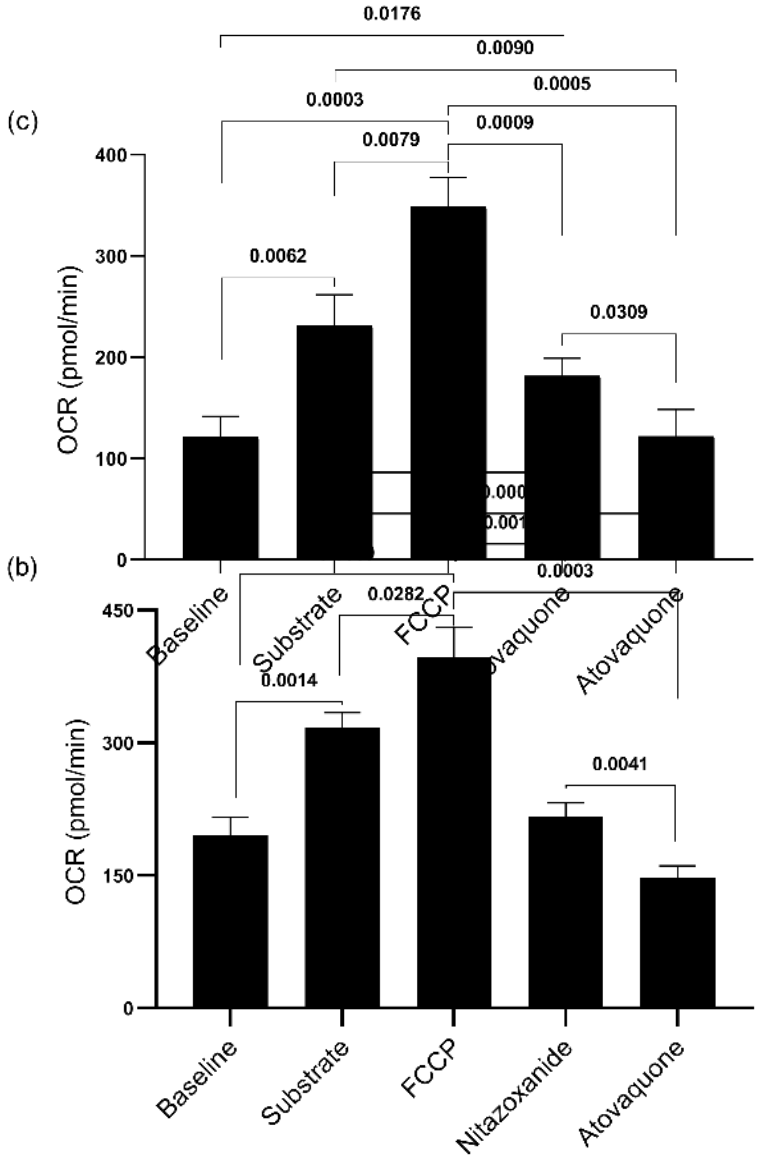
821

(a)


0.0008
0.0064
0.0048
400
300
200
100
0
OCR (pmol/min)
Baseline
Substrate
FCCP
Plumbagin
Atovaquone

822

823

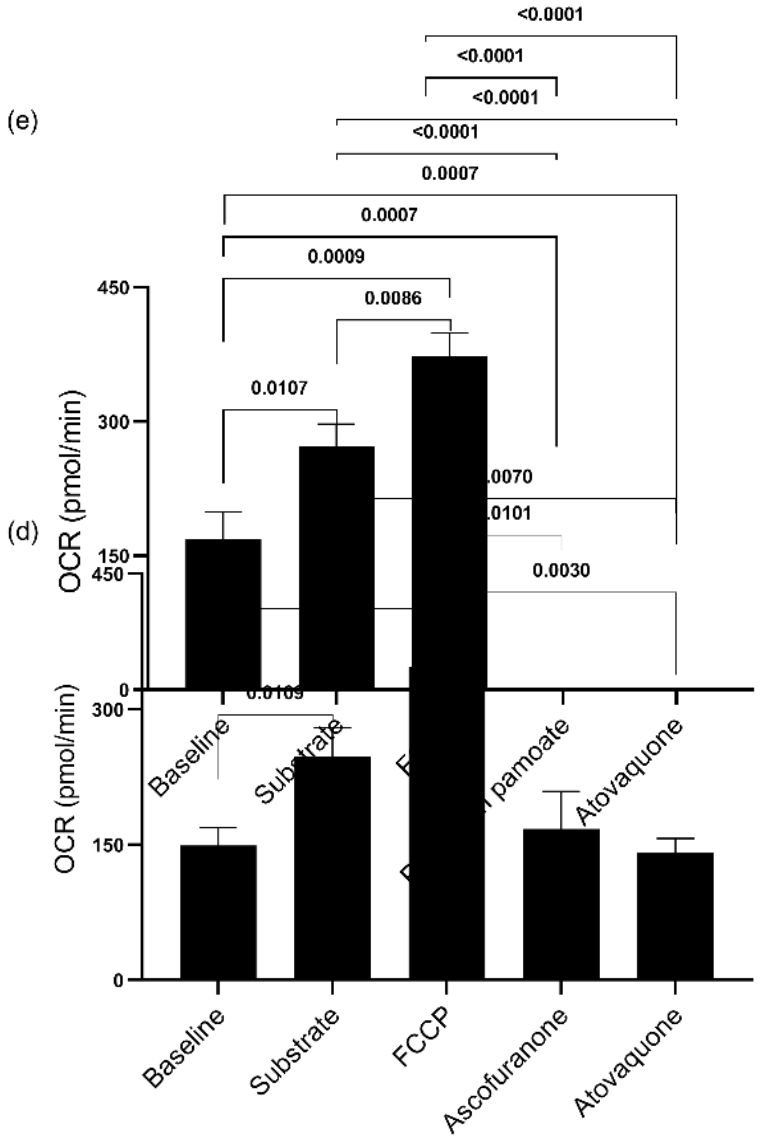

(c)
0.0176
0.0090
0.0005
0.0003
0.0009
0.0079
0.0062
0.0309
OCR (pmol/min)
400
300
200
100
0
(b)
0.0003
0.0282
0.0014
0.0041
OCR (pmol/min)
450
300
150
0
Baseline
Substrate
FCCP
Nitazoxanide
Atovaquone

824

825

826

827



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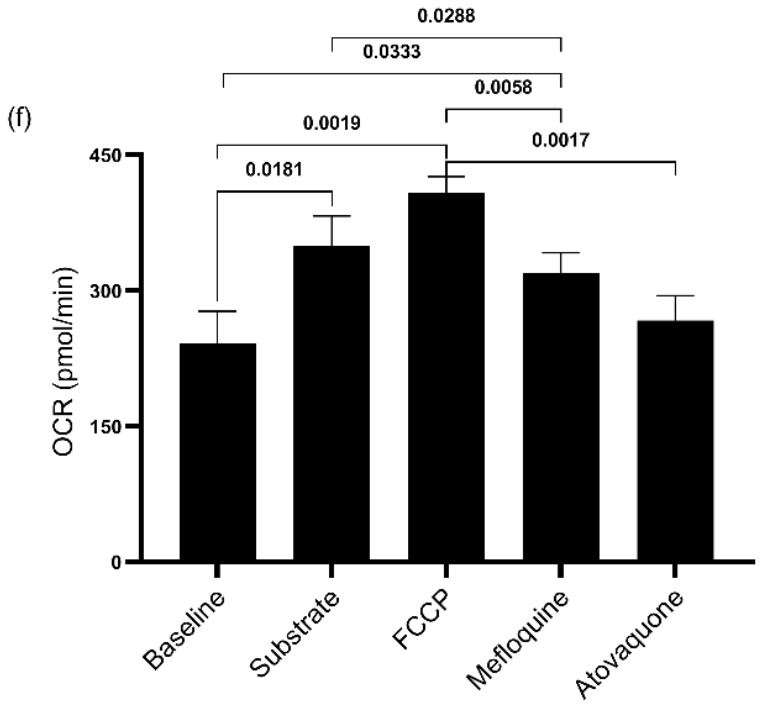
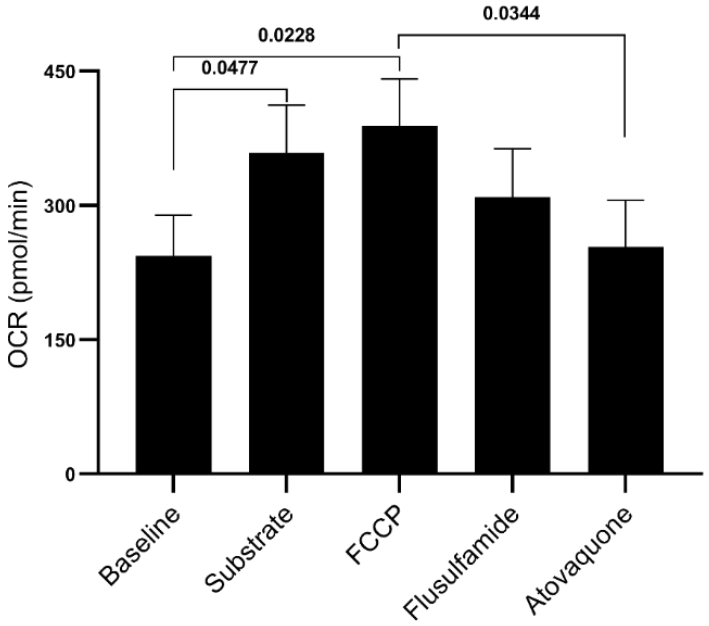
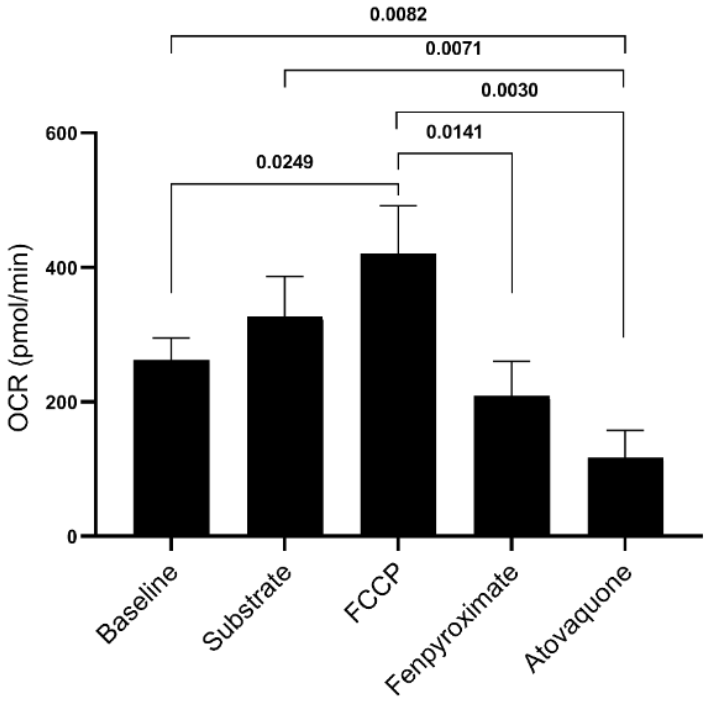

(f)
0.0288
0.0333
0.0058
0.0019
0.0017
0.0181
450
300
150
0
OCR (pmol/min)
Baseline
Substrate
FCCP
Mefloquine
Atovaquone

(g)

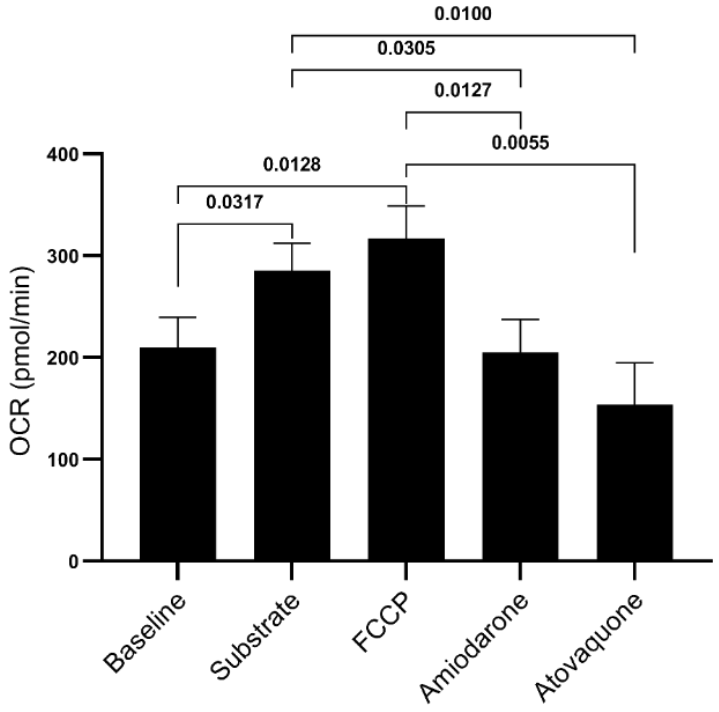

0.0228
0.0344
0.0477
450
300
150
0
OCR (pmol/min)
Baseline
Substrate
FCCP
Flusulfamide
Atovaquone

837

(h)


0.0082
0.0071
0.0030
0.0141
0.0249
600
400
200
0
OCR (pmol/min)
Baseline
Substrate
FCCP
Fenpyroximate
Atovaquone

(i)


0.0100
0.0305
0.0127
0.0055
0.0128
0.0317
400
300
200
100
0
OCR (pmol/min)
Baseline
Substrate
FCCP
Amiodarone
Atovaquone

838

839

(j)

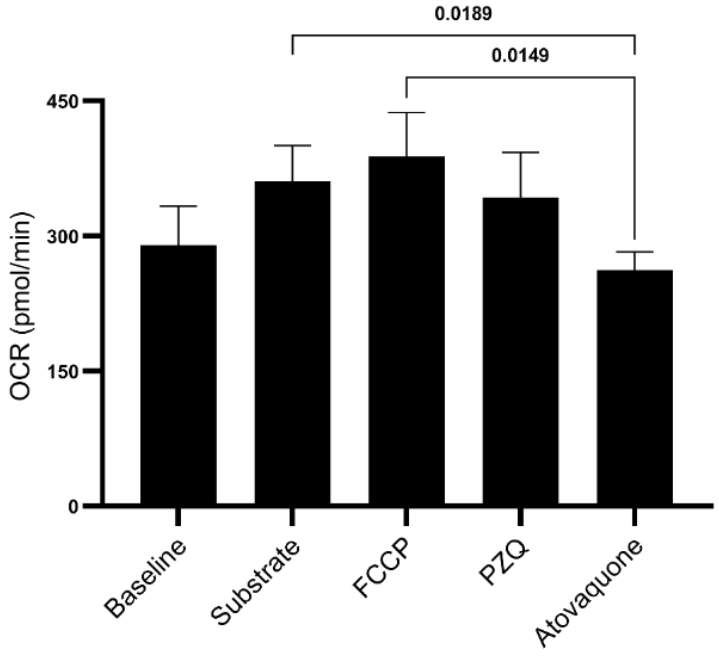

0.0189
0.0149
450
300
150
0
OCR (pmol/min)
Baseline
Substrate
FCCP
PZQ
Atovaquone

FIG S1 Analysis of effects of selected compounds on oxygen consumption rates (OCR). For each condition, the first reading immediately after addition of substrate was set as the baseline. The mean (n = 3) of the last reading after each injection was plotted as substrate. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), compound, and atovaquone with error bars representing standard deviation (SD). Only significant changes (p value < 0.05) in OCR, as analyzed by two-tailed non-paired Student's t-test, are shown.

860 **Table S2** Summary of previous reports and new findings obtained in this study on the effects of selected
861 compounds against various life cycle stages from Schistosome parasites.

	Effects of different compounds at stages of Schistosoma lifecycle				
	In vitro		*Ex vivo*	*In vivo*	
Compound	Cercariae	Schistosomula	Adult	Prophylactic (juvenile schistosome)	Treatment (Adult)
Mefloquine	-Immobilized cercariae after 1 hr at 5 ug/mL (91) -Completely immobilized at 10 µM within 1 hr[a]	-10 µM killed schistosomula within 72 hrs (70, 92) -Killed schistosomula immediately at 75 ug/ml (67) -Completely immobilized at 10 µM within 24 hrs[a]	-Adults were dead after 1 hr of incubation at 100 µg/ml (67) -Completely immobilized adult at 50 mg/mL (91, 92) -Completely immobilized male and female *S.*	-83.9% reduction of worm burden at 100 mg/kg (65) -Worm burden reduction by 87.2%[a]	-400 mg/kg single dose to mice gave a 92% worm burden reduction in mice (67). -77.3% worm reduction at 400 mg/kg (65) -Worm burden reduction by 80.1%[a]

			mansoni at 10 µM within 9 and 20 hrs respectively[a]		
Nitazoxanide	-Immobilized cercarial motility within 1 hr at 10 ug/ml (41) -Completely immobilized at 10 µM within 19 hrs[a]	-Rapid paralysis and tegumental disruption at 10 ug/ml (41) -Completely immobilized at 10 µM within 24 hrs in this study	-Rapid shrinkage and curling at 10 ug/ml (41) -Completely immobilized male and female *S. mansoni* at 10 µM within 9 and 20 hrs respectively[a]	-Worm burden reduction by 85%[a]	-After 7 days of treatment with 100 mg/kg, caused minor tegumental alterations of male worms (62) -Reduced worm burden by 64.9% (93) -Worm burden reduction by 77%[a]
Pyrvinium pamoate	-Completely immobilized at 10 µM within 19 hrs[a]	-10 µM killed schistosomula within 72 hrs (70) -Completely immobilized at 10 µM	-Killed parasite at 33.33 µM within 24 hrs (70) -Completely immobilized male	-Worm burden reduction by 97.1%[a]	-Worm burden reduction by 76.4%[a]

		within 48 hrs[a]	and female *S. mansoni* at 10 µM within 20 hrs[a]		
Plumbagin	-Showed separation of head and tail of cercariae at 10 µM (94) -Completely immobilized at 10 µM within 19 hrs[a]	-Impair viability of schistosomula at 10 µM (95) -Completely immobilized at 10 µM within 24 hrs[a]	-1 µg/ml killed the parasite after 24 hrs (96) -10 µM killed the parasites in 48 hrs (94) -Completely immobilized male and female *S. mansoni* at 10 µM within 9 and 20 hrs respectively[a]	-Worm burden reduction by 97.7%[a]	-Reduced worm burden by 79% at 4 mg/kg/day for 3 consecutive days (69) -Worm burden reduction by 81.7%[a]
Praziquantel	-Immobilized cercariae at 5	-5 µg/mL was ineffective against 14-	-Completely immobilized adult at	-100 mg/kg for 5 days praziquantel	-100 mg/kg induced 79% worm burden

	µg/mL (91) -Completely immobilized at 10 µM within 19 hrs[a]	day old schistosomula (92) -No effect at 10 µM (95) -No effects at 10 µM within 48 hrs[a]	50 µg/mL (91) -Completely immobilized male and female *S. mansoni* at 10 µM within 1 hr[a]	induced a worm burden reduction of 20% (91) -24% worm burden reduction at 200 mg/kg (91) -Worm burden reduction by 10.4%[a]	reduction (91) -Worm burden by 99.3% at single dose of 250 mg/kg (84) -Worm burden reduction by 100%[a]
Atovaquone	-Completely immobilized at 10 µM within 19 hrs[a]	-Completely immobilized at 10 µM within 8 hrs[a]	-Completely immobilized male *S. mansoni* at 10 µM within 20 hrs and less effective to females[a]	-Worm burden reduction by 89.2%[a]	-Ineffective at 100 mg/kg (65) -Worm burden reduction by 30.1%[a]
Ascofuranone	-Completely immobilized	-Less effective at 10 µM within 48 hrs[a]	-Completely immobilized male	-Worm burden reduction by	-Worm burden reduction by 55%[a]

	at 10 µM within 19 hrs[a]		and female *S. mansoni* at 10 µM within 9 and 20 hrs respectively[a]	98.1%[a]	
Flusulfamide	-Completely immobilized at 10 µM within 19 hrs[a]	-Less effective at 10 µM within 48 hrs[a]	-Completely immobilized male and female *S. mansoni* at 10 µM within 9 and 20 hrs respectively[a]	-Worm burden reduction by 85.9%[a]	-Worm burden reduction by 13.4%[a]
Fenpyroximate	-Completely immobilized at 10 µM within 19 hrs[a]	-Less effective at 10 µM within 48 hrs[a]	-Completely immobilized male and female *S. mansoni* at 10 µM within 9 and 20 hrs respectively[a]	-Worm burden reduction by 91.3%[a]	-Worm burden reduction by 28%[a]
Amiodarone	-Completely	-Completely	-Did not completely	-Worm burden	-Worm burden reduction

	immobilized at 10 µM within 19 hrs[a]	immobilized at 10 µM within 24 hrs[a]	immobilized male and female *S. mansoni* at 10 µM within 20 hrs[a]	reduction by 85.3%[a]	by 70.8%[a]

[a]Results obtained in this study

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