Parasitology Research (2019) 118:505–516
https://doi.org/10.1007/s00436-018-6188-9

HELMINTHOLOGY - ORIGINAL PAPER

In vitro and in vivo evaluation of six artemisinin derivatives against *Schistosoma mansoni*

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Received: 8 August 2018 / Accepted: 19 December 2018 / Published online: 8 January 2019

Abstract

Schistosomiasis is a tropical neglected disease whose socioeconomic impact is surpassed only by malaria. Until recently, praziquantel (PZQ) has been the only available drug, raising concerns that tolerant/resistant strains may appear. Since the discovery of the schistosomicidal potential of artemisinin (ART), new derivatives have been produced and evaluated. In this work, we evaluated the activity of ART derivatives against *Schistosoma mansoni*, both in vitro and in vivo. In the in vitro assay, worm survival, oviposition, and morphological alterations were evaluated. Further analysis of morphological alterations and membrane integrity was conducted using scanning electron microscopy and a cell-permeable, benzimidazole dye (Hoescht 33258) that binds to the minor groove of double stranded DNA. For the in vivo assay, artesunic acid (AcART) and dihydroartemisinin acetate (AcDQHS) were selected, since they showed the best in vitro results. Infected mice treated 21, 45, or 60 days post-infection (dpi), with a concentration of 100 mg/kg of either AcART or AcDQHS, showed a significant worm reduction (particularly in females), fewer eggs eliminated in feces, and a decrease of immature eggs in the intestinal tissues. Our results indicate that AcART and AcDQHS have some schistosomicidal activity against juvenile and adult stages of *S. mansoni*.

Keywords *Schistosoma mansoni* · Artemisinin derivatives · Artesunic acid · Dihydroartemisinin acetate

Introduction

Schistosomiasis is an important tropical disease with high socioeconomic impact that affects over 249 million people worldwide (WHO 2016). Currently, the only drug available against this disease is praziquantel (PZQ) (Cioli et al. 2014); however, this drug is only effective against adult worms, compromising treatment programs in endemic areas, where the population may be infected with more than one parasite stage (Thetiot-Laurent et al. 2013). Considering the fact that mass therapy has been the main control method of schistosomiasis for the last three decades, it is not surprising that there are an increasing number of treatment failure reports due to tolerance/resistance. Therefore, there is a rising need for new therapeutic alternatives (Cioli et al. 2014; Doenhoff et al. 2008; Pinto-Almeida et al. 2015).

Developing new drugs is an expensive and long process, therefore, synthesizing derivatives from compounds that have already shown therapeutic efficiency, is an interesting possibility. Artemisinin (ART) is a sesquiterpene lactone with an endoperoxide group, essential to its activity (Ansari et al. 2013). ART is well tolerated by patients, having low toxicity, and it has shown to efficient against all *Schistosoma* species, including juvenile stages (Frezza et al. 2013; Utzinger et al. 2007), however, since it is a low hydrophilic-lipophilic drug, it has low absorption and bioavailability, therefore there is a need to develop new semi-synthetic derivatives (Ansari et al. 2010; Utzinger et al. 2001).

Section Editor: Christoph G. Grevelding

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-018-6188-9) contains supplementary material, which is available to authorized users.

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Considering the schistosomicidal potential of ART derivatives (ARTs), in this work we tested schistosomicidal potential of six derivatives. Dihydroartemisinin acetate (AcDQHS), 10-DQHS glutaric acid (AcGL), 10-DQHS adipidic acid (AcAD), and artesunic acid methyl ester (EAcART) are derivatives produced by semisynthesis (using dihydroartemisinin (DQHS) as an intermediary) and their anti-*Schistosoma* activity, to our knowledge, had not been previously tested. We also decided to include some previously tested compounds (DQHS and artesunic acid (AcART)) to allow a direct comparison between untested and previously tested derivatives, since different parasite strains may have different responses to drug treatment (Allegretti et al. 2012; Frezza et al. 2013; Yoshioka et al. 2002).

Material and methods

Drugs, derivatives synthesis, and PVP incorporation

PZQ was acquired from Merck® (São Paulo, SP, Brazil). ART was extracted from *Artemisia annua* L. leaves, cultivated in the Multidisciplinary Center for Biological Chemical and Agricultural (CPQBA) (Campinas, SP, Brazil) experimental field (Rodrigues et al. 2006).

ARTs (Fig. 1) were acquired from the Organic and Pharmaceutical Chemistry Division, CPQBA/Unicamp.

To improve solubility, PZQ, ART, and ARTs were incorporated in polyvinylpyrrolidone (PVP K30) in the proportion 1:4 (*w*/*w*, compound:PVP).

Parasites and animals

A Sergipe (SE) strain of *S. mansoni* was used in all experiments. This strain is kept in *Biomphalaria glabrata* snails and in Swiss-SPF female mice (*Mus musculus*) weighing approximately 20 g, infected with 70 cercariae using the tail immersion technique (Olivier and Stirewalt 1952). Adult parasites were recovered through liver perfusion after 60 days of infection (Pellegrino and Siqueira 1956) and used in the in vitro assays. For the in vivo assays, we used 30-days-old Balb/c mice, weighing approximately 20 g, infected as previously described. All animals were used in accordance with the recommendations of the Ethics Commission for the Use of Animals (CEUA/UNICAMP, protocol no. 3248-1).

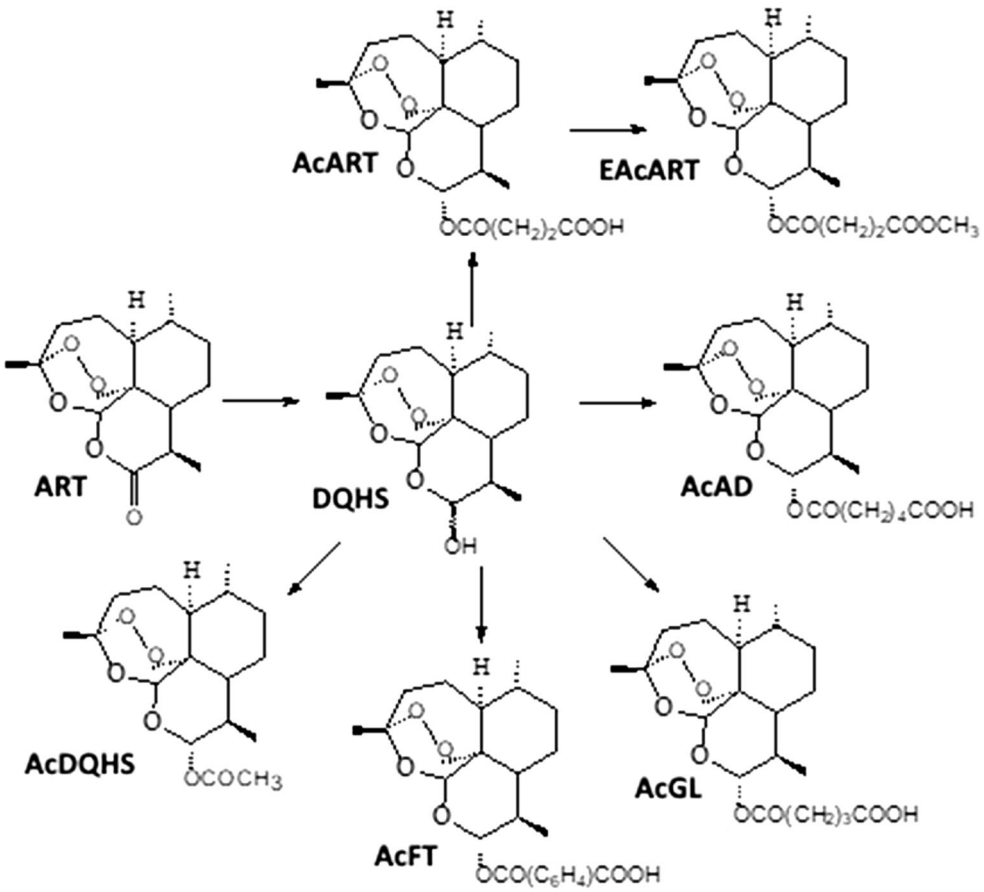

Fig. 1 Artemisinin derivatives synthesis using DQHS as intermediary. ART, artemisinin; DQHS, dihydroartemisinin; AcART, artesunic acid; AcGL, 10-DQHS glutaric acid; AcDQHS, dihydroartemisinin acetate; EAcART, artesunic acid methyl ester; AcAD, 10-DQHS adipidic acid

Table 1 Experimental design: in vivo assay. AcART, artesunic acid; AcDQHS, dihydroartemisinin acetate

Group	Compound/ drug	Dosage*	Time of treatment (dpi)	Number of mice
I	AcART	40 mg/kg	21	8
II			45	8
III			60	8
IV		100 mg/kg	21	8
V			45	8
VI			60	8
VII	AcDQHS	40 mg/kg	21	8
VIII			45	8
IX			60	8
X		100 mg/kg	21	8
XI			45	8
XII			60	8
DC I	PZQ	40 mg/kg	21	8
DC II			45	8
DC III			60	8
DC IV		150 mg/kg	21	8
DC V			45	8
DC VI			60	8
DC VII		300 mg/kg	21	8
DC VIII			45	8
DC IX			60	8
CNTRL I	PBS	0.3 ml	21	8
CNTRL II			45	8
CNTRL III			60	8

*All drugs/compounds were administered in a single dose through gavage

DC, drug control; CNTRL, untreated control

In vitro assay

PZQ, ART, and ARTs were solubilized in 2% phosphate buffered saline (PBS) solution. Nine groups were established (see Fig. 1 for compounds): Group I, parasites exposed to ART; Group II, parasites exposed to DQHS; Group III, parasites exposed to AcART; Group IV, parasites exposed to AcGL; Group V, parasites exposed to AcAD; Group VI, parasites exposed to EAcART; Group VII, parasites exposed to AcDQHS; Group VIII, parasites exposed to PZQ (drug control group); Group IX, parasites exposed to PVP without drug and 2% PBS (untreated control group). After perfusion, the collected parasites were washed with RPMI 1640 medium (Nutricell®) and one couple was added in each well of a 24-well plate (TTP®) with 2 ml of RPMI 1640 medium (0.05 g/L streptomycin, 10.000 UI/ml penicillin, 0.3 g/L L-glutamine, 2.0 g/L D-glucose, 2.0 g/L $NaHCO_3$ and 5958 g/L Hepes) and incubated in a 5% CO_2 environment at 37 °C. Drugs were tested at 25, 50, 100, and 200 μg/mL, with 5 replicates each. Parasites were observed under an inverted microscope (Leica®) DMI-500 at 24, 48, and 72 h and evaluated regarding survival (worms that did not show any movement after observing for 2 min were considered dead), oviposition (total number of eggs per well), and morphological changes (de Oliveira et al. 2017b).

Scanning electron microscopy

Parasite worms were exposed to a lethal concentration of 200 μg/mL of the ARTs that showed in vitro activity, namely: AcART (24 h incubation), DQHS (24 h incubation), AcAD (48 h incubation), AcGL (72 h incubation), AcDQHS (24 h incubation). Plates were incubated in a 5% CO_2 environment at 37 °C. Incubation time was selected in accordance with the amount of time needed to reach 100% mortality in the in vitro assay. An untreated control group was also used, as well as, a drug control group where worms were exposed to 25 μg/mL of PZQ and incubated for 72 h in RPMI medium. After drug incubation, all worms were washed in sodium cacodylate buffer (pH 7.2) and fixed in 2.5% glutaraldehyde (pH 7.4) for 24 h, after which, they were fixated in 1% osmium

Table 2 In vitro mortality after exposing five *S. mansoni* couples to different concentrations of dihydroartemisinin (DQHS), artesunic acid (AcART), dihydroartemisinin acetate (AcDQHS), or praziquantel (PZQ)

Dosage (μg/mL)	Time-points (h)	% Mortality							
		DQHS		AcART		AcDQHS		PZQ	
		(m)	(f)	(m)	(f)	(m)	(f)	(m)	(f)
0	24	0	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0	0
	72	0	0	0	0	0	0	0	0
25	24	0	0	0	0	0	0	0	0
	48	0	20	0	20	0	0	0	0
	72	0	20	0	20	40[a]	20	0	0
50	24	0	100[a,b]	0	100[a,b]	0	20	80[a,b]	0[b]
	48	20	100[a,b]	20	100[a,b]	100[a]	80[a]	80[a,b]	0[b]
	72	60[a]	100[a]	60[a]	100[a]	100[a]	100[a]	80[a,b]	0[b]
100	24	100[a]	100[a]	100[a]	100[a]	20	80[a,b]	80[a,b]	0[b]
	48	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	80[a,b]	0[b]
	72	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	80[a,b]	20
200	24	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	80[a,b]	0[b]
	48	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a,b]	0[b]
	72	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a,b]	0[b]

[a] Significant difference compared with untreated control group (two-way ANOVA, $p < 0.05$)

[b] Significant difference between males and females (two-way ANOVA, $p < 0.05$)

m, male ($n = 5$); f, female ($n = 5$)

tetroxide for 1 h. The parasites were then dehydrated in increasing ethanol concentrations (50 to 100%), dried in a critical point chamber, covered with a thin gold layer using Sputter Coater and photographed using an electron scanning microscope (Jeol-JSM-820) (de Oliveira et al. 2017b).

Membrane integrity evaluation using Hoescht 33258 probe

Hoescht 33258 probe (Life Technologies®), was used to evaluate *S. mansoni* membrane integrity after treatment with 200 μg/mL of either AcART or AcDQHS for 2 h. Two males and two females were transferred to a 24-well culture plate containing 2 mL of RPMI 1640 medium supplemented with 5% fetal bovine serum, 100 μg/mL of streptomycin/penicillin (Sigma-Aldrich®). Plates were then incubated at 37 °C in a 5% CO_2 environment for 2 h and washed 5 times with 2 mL of RPMI-1640 containing 0.3% bovine serum albumin (BSA) to remove excess drug. Afterwards, the parasites were incubated for 15 min in RPMI 1640 medium with 10 μL of Hoescht probe at 37 °C in a 5% CO_2 environment and then washed five times with 2 mL of RPMI-1640 containing 0.3% BSA to remove excess probe. Parasites were transferred to slides demarcated with vaseline and changes in membrane integrity were observed through fluorescence microscopy (Zeiss Axion Imager-A.2 filter DAPI, excitation/maximal emission

Table 3 In vitro effect of different concentrations of ARTs on *S. mansoni* females oviposition

Concentration (μg/mL)	Number of eggs (mean ± SE)						
	DQHS	AcART	AcGL	AcAD	EAcART	AcDQHS	PZQ
25	0.4 ± 0.4[a]	5 ± 5[a]	15 ± 15[a,b]	5.2 ± 5[a,b]	6 ± 5[a]	1 ± 0.7[a]	0 ± 0[a]
50	0.8 ± 0.8[a]	0.4 ± 0.2[a]	0.2 ± 0.2[a]	8.6 ± 5.3[a]	11.2 ± 5.9[a]	0 ± 0[a]	0 ± 0[a]
100	0 ± 0[a]	0 ± 0[a]	0 ± 0[a,b]	0.6 ± 0.4[a,b]	16.2 ± 16[a]	0 ± 0[a]	0 ± 0[a]
200	0 ± 0[a]	0 ± 0[a]	0.2 ± 0.2[a]	0.4 ± 0.2[a]	0.2 ± 0.2[a]	0 ± 0[a]	0 ± 0[a]
CTRL	100 ± 16.25						

[a] Significant difference compared with control group (two-way ANOVA, $p < 0.05$)

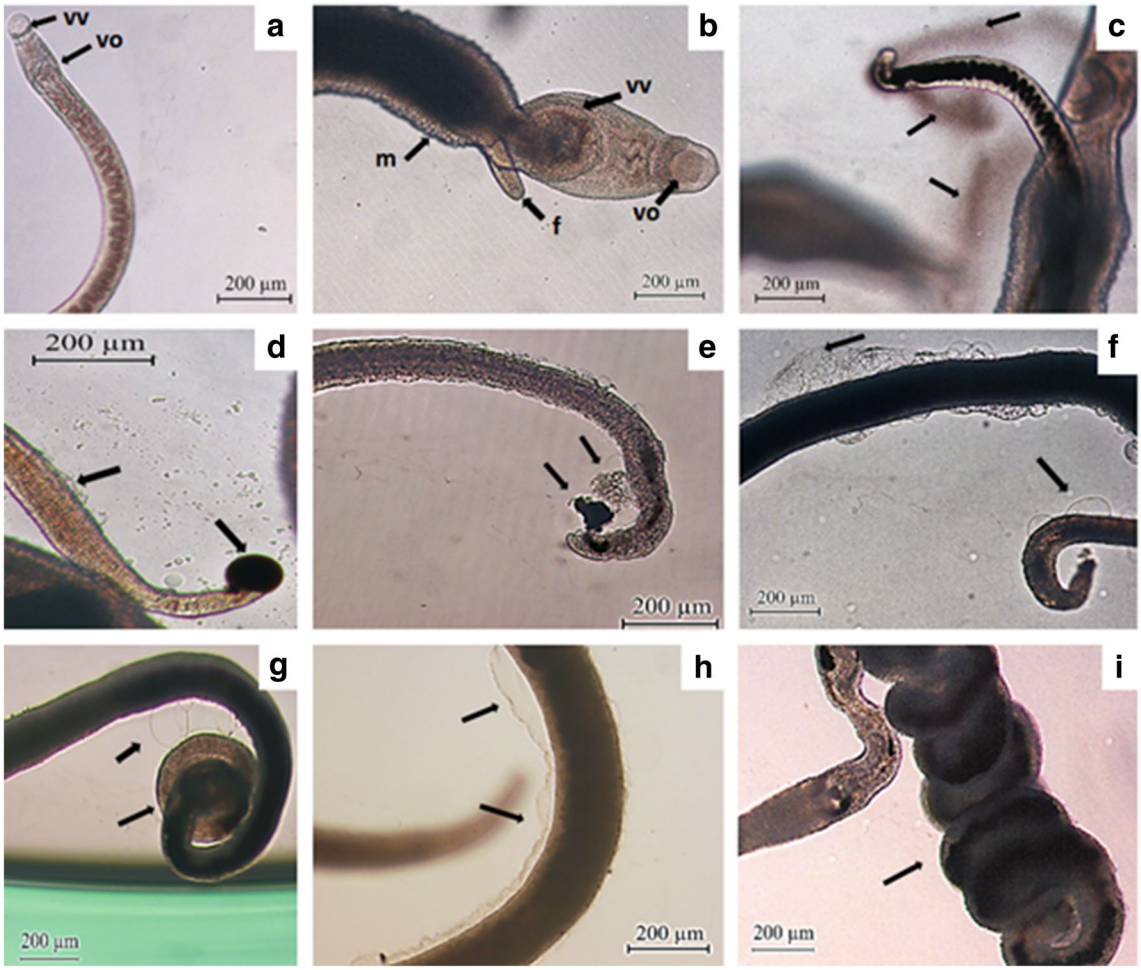

Fig. 2 Observed alterations after in vitro exposure to ARTs and PZQ. (**a**–**b**) untreated control group, vo, oral sucker; vv, acetabular sucker; m, male; f, female. (**c**) Intense regurgitation (black arrows) by female worm after 4 h of exposure to 200 μg/mL of AcAD. (**d**) Oral sucker destruction and formation of blisters in the tegument (black arrows) of a female worm after 24 h of exposure to 50 μg/mL of AcDQHS. (**e**) Tegument peeling and adhesion of regurgitated material to female oral sucker (black arrows) after 72 h of exposure to 50 μg/mL of AcGL. (**f**) Female tegument detachment (black arrows) after 72 h of exposure to 100 μg/mL of EAcART. (**g**) Blisters formation and twisting (black arrows) of the female anterior portion after 72 h of exposure to 25 μg/mL of AcART. (**h**) Tegument detachment (black arrows) after 48 h of exposure to 100 μg/mL of AcDQHS. (I) Intense contraction (black arrow) after 6 h of exposure to 100 μg/mL of PZQ

of Hoechst 33258 a 352/455 nm). The pharmacological control worms were incubated for 15 min in 2 μL/mL of PZQ and the untreated control worms were kept in RPMI-1640 medium only with 10 μL of Hoescht probe (de Oliveira et al. 2017b). All the experiments were performed in triplicate.

In vivo assay

The two artemisinin derivatives with the best in vitro results (AcART and AcDQHS) were selected for the in vivo assay. Two dosages were tested 40 mg/kg and 100 mg/kg single dose and animals ($n = 8$) were treated through gavage at either 21 days post infection (dpi) (to assess the effects on juvenile worms), 45 dpi or 60 dpi (to assess the effects on adult worms) (Table 1). A drug control group, where animals were treated with either 40, 150, or 300 mg/kg PZQ, 21, 45, or 60 dpi, and an untreated control group, where animals received 0.3 mL of PBS. Animals treated 21 dpi were euthanized 25 days after treatment and animals treated 45 or 60 dpi were euthanized 15 days after treatment. Adult worms were recovered from the hepatic portal system and mesenteric veins trough perfusion. The number of males, females, and couples was recorded and worm reduction calculated. Feces were collected individually from each mouse before they were euthanized and the number of eggs per gram of feces (EPG) was assessed through kato-katz technique. A section of the small intestine was collected and 100 eggs were observed. The development stage of the eggs (immature, mature and dead) was recorded (oogram pattern) following the criteria proposed by Pellegrino et al. (1962).

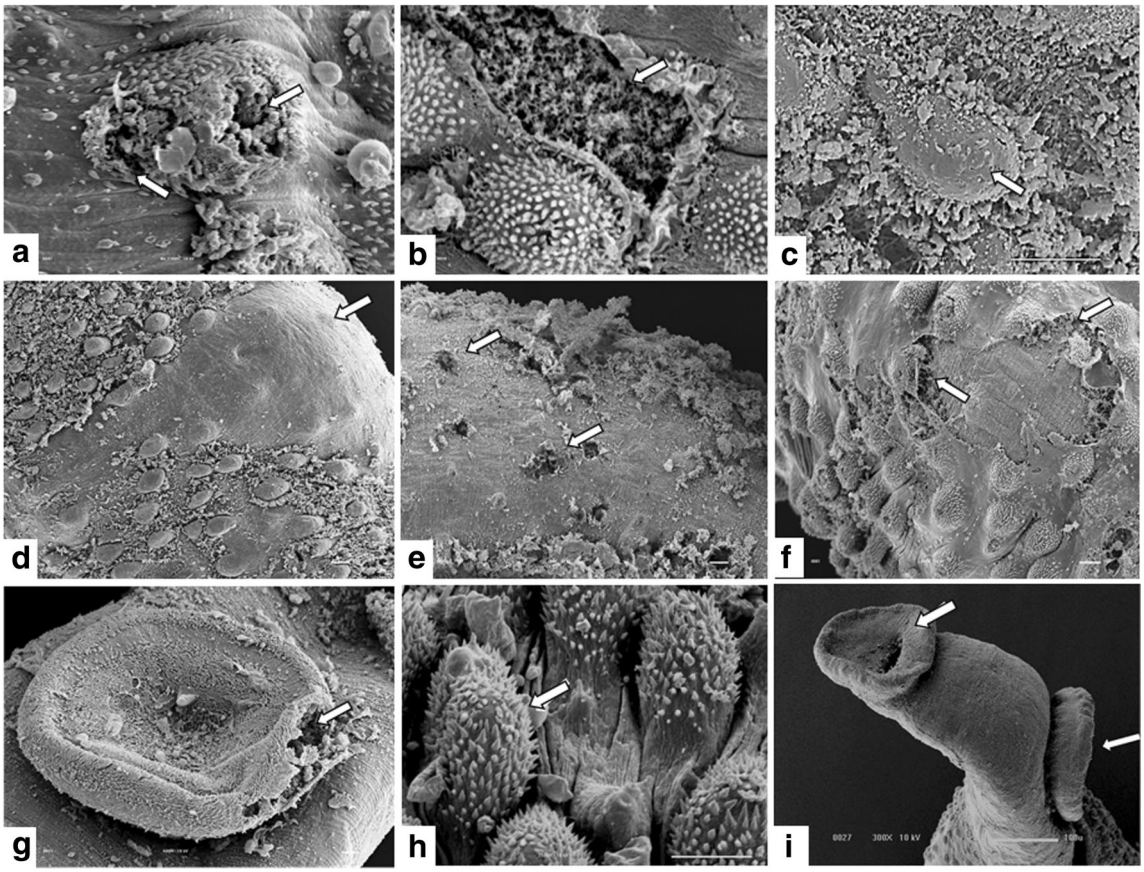

Fig. 3 *S. mansoni* male parasites. Morphologic changes in the tegument and suckers observed through SEM after exposure to ARTs. (**a**) Worms exposed to DQHS showing tubercle destruction (white arrows). (**b**) Worms exposed to AcART, showing tegument destruction (white arrow). (**c**) Worms exposed to AcGL, showing spicule loss (white arrow). (**d**) Worms exposed to AcAD, showing swelling (white arrow) and spicule loss. (**e**) Worms exposed to AcDQHS, showing tegument peeling and perforations (white arrows). (**f**) Worms exposed to PZQ, showing tubercle destruction and tegument peeling (white arrows). (**g**) Worms exposed to AcAD, with acetabular sucker destruction (white arrow). (**h**–**i**) Untreated control worms, showing a healthy tegument, tubercles covered with spicules (**h**, white arrow) and intact oral and acetabular suckers (**i**, white arrows). Images scale bars: (**a**) 3300 × 10 kv, 10 μm; (**b**) 3500 × 10 kv, 10 μm; (**c**) 3300 × 10 kv, 10 μm; (**d**) 600 × 10 kv, 10 μm; (**e**) 700 × 10 kv, 10 μm; (**f**) 800 × 10 kv, 10 μm; (**g**) 600 × 10 kv, 10 μm; (**h**) 2500 × 10 kv, 10 μm; (**i**) 300 × 10 kv, 10 μm

Cytotoxic analysis

A human keratinocytes cell line (HaCaT) was used for cytotoxic analysis. Cells were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 100 μg/mL of streptomycin/penicillin (Sigma-Aldrich®). One hundred microliters of medium were added to each well of a 96-well plate and cells were exposed to 0; 0.25; 2.5; 25 or 250 μg/mL of AcART or AcDQHS. The experiment was performed in triplicate and plates were incubated for 48 h in a 5% CO_2 environment at 37 °C. After incubation, cells were fixated with 50% tricloroacetic acid (TCA) and stained with 0.4% sulforhodamine B and 1% acetic acid. Absorbance was measured in a spectrophotometer at 540 nm (VersaMax, Molecular Devices®). Cellular proliferation was determined and the growth inhibition was calculated using the software ORIGIN 8.0 (OriginLb Corporation) (Denny et al. 2008; Monks et al. 1991; Shoemaker 2006).

Statistical analysis

Statistical analysis was performed using SAS 9.1 software (SAS Institute Inc., Carry, NC, EUA). Analysis of variance (ANOVA) and Tukey's range test to compare the average response between treated and control groups ($p < 0.05$).

Results

ARTs in vitro activity—mortality and tegument damage

Artemisinin and derivatives were evaluated in vitro for mortality and oviposition. Treated groups showed statistical differences after 72 h for all parameters (Two-way ANOVA, $p < 0.05$) when compared with the control group. ARTs did not show significant mortality difference between male and female parasites. When exposed to PZQ, male mortality was

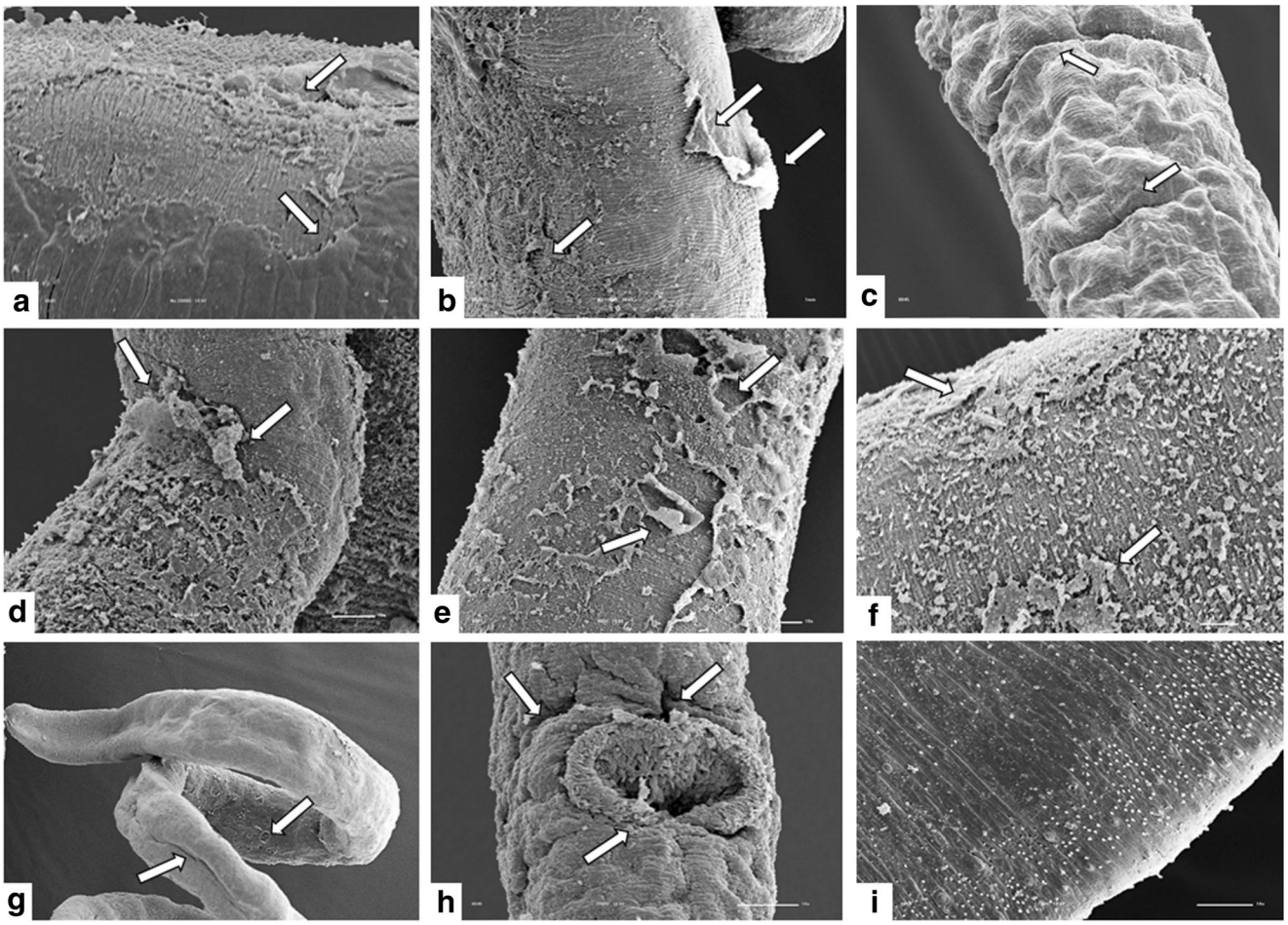

Fig. 4 *S. mansoni* female parasites. Morphologic changes in the tegument and suckers observed through SEM after exposure to ARTs. (**a**) Tegument peeling (white arrows) after exposure to DQHS. (**b**) Tegument peeling (white arrows) after exposure to AcART. (**c**) Tegument wrinkle and peeling (white arrows) after exposure to AcDQHS. (**d**) Tegument peeling (white arrows) after exposure to AcGL. (**e**) Tegument peeling (white arrows) after exposure to AcAD. (**f**) Tegument peeling (white arrows) after exposure to AcDQHS. (**g**) Tegument peeling and wrinkle (white arrows) after exposure to PZQ. (**h**) Acetabular sucker retraction, tegument perforation, and wrinkle (white arrows) after exposure to AcAD. (**i**) Untreated control worms showing intact tegument. Images scale bar: (**a**) 2000 × 10 kv, 10 μm; (**b**) 1000 × 10 kv, 10 μm; (**c**) 1000 × 10 kv, 10 μm; (**d**) 1400 × 10 kv, 10 μm; (**e**) 800 × 10 kv, 10 μm; (**f**) 1200 × 10 kv, 10μm; (**g**) 140 × 10 kv, 10 μm; (**h**) 2000 × 10 kv, 10 μm; (**i**) 1700 × 10 kv, 10 μm

higher than female. Worm mortality was proportional to drug concentration and exposure time (Table 2).

DQHS, AcART caused 100% worm mortality at 100 μg/mL after exposure for 24 h, while AcDQHS, at the same concentration, caused 100% worm mortality after 48 h of drug exposure. AcGL and AcAD caused 100% mortality at 200 μg/mL after 48 h of exposure. ART and EAcART did not cause mortality at any tested concentration. PZQ caused 100% mortality in males after exposure to 200 μg/mL for 48 h and 0% mortality in females (Table 2). With the exception of ART, all derivatives caused a significant reduction of oviposition when compared with the control group (Table 3).

Optical microscopy also revealed important morphological changes, such as adhesion of regurgitated material to the oral sucker (Fig. 2D, E), bubble formation (Fig. 2F, G), tegument peeling and destruction (Fig. 2D, E, F, H), contraction of the anterior portion of females (Fig. 2G) and intense regurgitation (Fig. 2C). These morphological changes were found in all groups treated with ARTs. Groups treated with ART and untreated group did not show morphological alterations. The drug control group, treated with PZQ, only revealed intense body contraction (Fig. 2I).

Assessment of morphologic damage through scanning electron microscopy

Scanning electron microscopy (SEM) was used to evaluate tegument damage caused by ART and derivatives (Figs. 3 and 4). Male worm tubercles suffered severe damage and lost their spines (Fig. 3A, C, D), it was also observed swelling (Fig. 3E), perforations (Fig. 3F) and damage to the acetabular suckers (Fig. 3G). Both male and female worms showed large

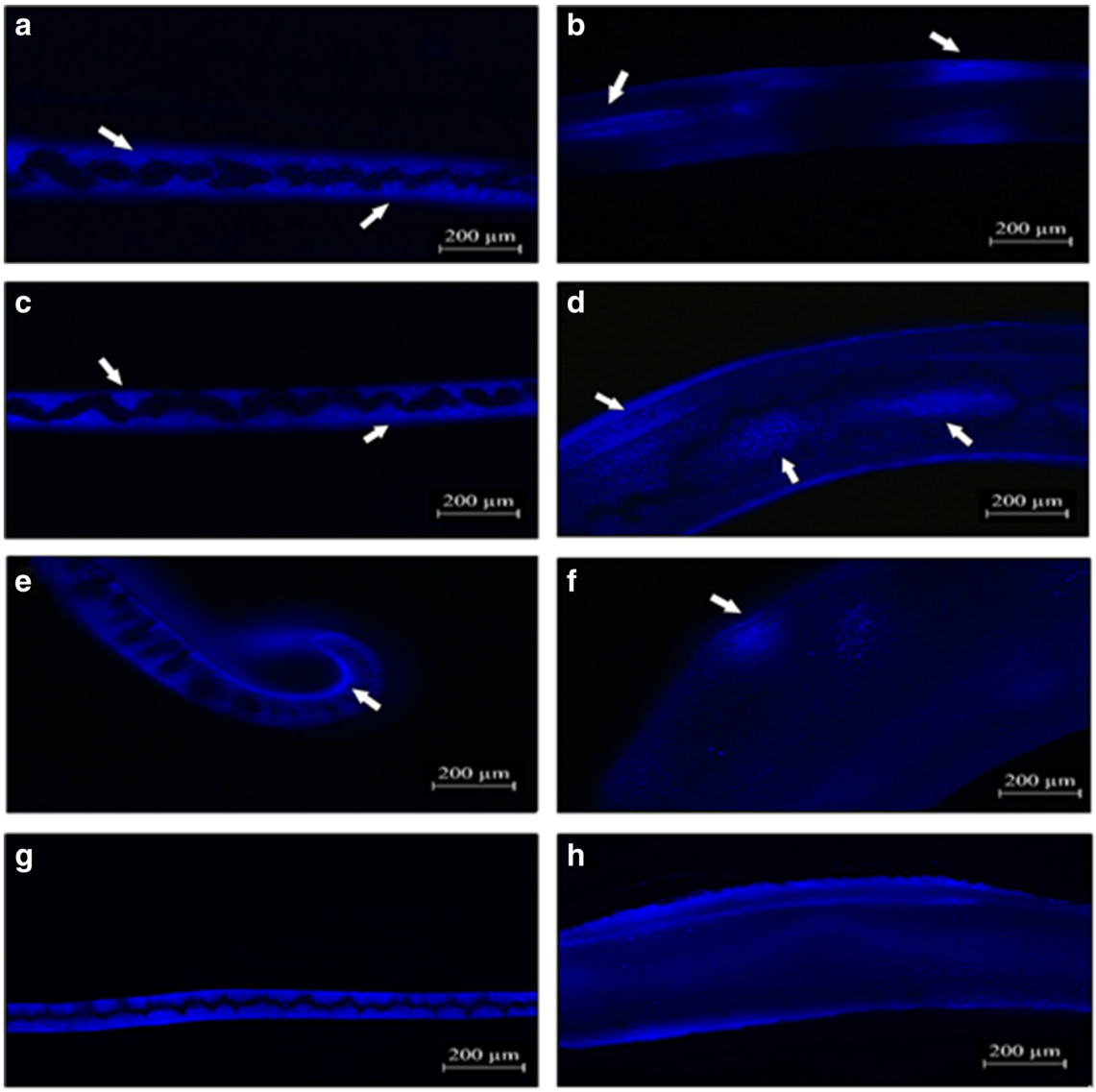

Fig. 5 Female (**a**, **c**, **e**, **g**) and male (**b**, **d**, **f**, **h**) *S. mansoni* worms membrane integrity evaluation after exposure to AcART, AcDQHS, and PZQ using Hoescht 33258 probe. Arrows point to regions where membrane integrity was altered. (**a** and **b**) Worms exposed to 200 μg/mL of AcART for 2 h. (**c** and **d**) Worms exposed to 200 μg/mL of AcDQHS for 2 h. (**e** and **f**) Worms exposed to 5 μg/mL of PZQ for 15 min. (**g** and **h**) Untreated control worms showing an intact membrane

areas of tegument destruction (Fig. 3B, F; Fig. 4A) and peeling (Fig. 3E, F; Fig. 4A–G). Female parasites showed wrinkles (Fig. 4C, G, H), tegument detachment (Fig. 4B, D, E), and oral sucker retraction (Fig. 4H). Untreated control worms kept the tegument and suckers whole (Fig. 3H, I; Fig. 4I).

Alterations in membrane integrity

Membrane integrity was evaluated for AcART, AcDQHS (the derivatives that showed the best in vitro results) and PZQ (drug control). Hoescht 33258 probe showed that worms exposed to both ARTs and PZQ had areas of higher fluorescence in their membrane, indicating tegument damage (Fig. 5).

ARTs in vivo activity—worm reduction, fecal egg reduction, and oogram analysis

Animals treated 21 dpi with 100 mg/kg of either AcART or AcDQHS showed higher worm burden reductions than animals treated with 300 mg/kg of PZQ (Table 4). The same concentration of AcART and AcDQHS administered 45 or 60 dpi also caused a significant worm reduction (Tables 5 and 6).

Kato-katz analysis showed that treatment with 100 mg/kg of AcART 21, 45, or 60 dpi caused an EPG reduction of 100%, 99%, or 99%, respectively. Treatment with 100 mg/kg of AcDQHS 21, 45, or 60

Table 4 Hepatic portal system worm recovery from mice experimentally infected with *S. mansoni* and treated with artesunic acid (AcART), dihydroartemisinin acetate (AcDQHS), and praziquantel (PZQ) on the 21st dpi

Treatment (mg/kg)	Worm recovery (mean ± SD)				Total worm burden reduction (%)
	Couples	Males	Females	Total	
CTRL (0)	8.1 ± 2.1	4.3 ± 2.7	3.9 ± 2.3	16.3 ± 2.3	–
AcART (40)	3.1 ± 1.8[a]	8.6 ± 3.1[a,b]	2.6 ± 1.6[b]	14.3 ± 3.3	28
AcART (100)	0.6 ± 0.7[a]	1.0 ± 1.2[a]	1.2 ± 1.3[a]	2.8 ± 0.3[a]	86
AcDQHS (40)	5.1 ± 2.2[a]	5.2 ± 2.8[b]	3.9 ± 3.0[b]	14.2 ± 0.7	21
AcDQHS (100)	2.9 ± 1.8[a]	1.2 ± 1.0[a]	1.5 ± 1.5[a]	5.6 ± 0.9[a]	67
PZQ (40)	7.6 ± 3.3	3.2 ± 2.2	3.4 ± 1.8	14.2 ± 2.5	10
PZQ (150)	7.0 ± 3.1	5.0 ± 1.6[b]	3.4 ± 1.6[b]	15.4 ± 1.8	8
PZQ (300)	7.0 ± 3.1	4.1 ± 3.2[b]	1.9 ± 1.5[a,b]	13.0 ± 2.6[a]	18

Number of animals/group = 8. CTRL, infected and untreated control group

[a] Significant difference compared with control group (two-way ANOVA, $p < 0.05$)

[b] Significant difference between males and females (two-way anova, $p < 0.05$)

dpi caused an EPG reduction of 99%, 92%, or 79%, respectively (Table 7).

Oogram analysis showed that animals treated with 100 mg/kg of AcART or AcDQHS 21 dpi had a larger number of mature and dead eggs retained in the intestinal tissues (72% and 69.9%, respectively), while the untreated control and drug control group (150 mg/kg concentration) had a greater number of immature eggs (78.1% and 85.5%, respectively) (Table 7). In animals treated 45 dpi with 100 mg/kg of AcART or AcDQHS, 80% and 68.6% (respectively) of retained eggs in the intestinal tissues were mature or dead, while in animals treated with 150 mg/kg of PZQ, 85.9% were mature or dead, while in the untreated control group, immature eggs were the most abundant (52%) (Table 8). In animals treated 60 dpi with 100 mg/kg of AcART and AcDQHS the number of mature and immature eggs was 85% and 84%, respectively, numbers similar to treatment with 150 mg/kg of PZQ (83.7%), while in the untreated control group immature eggs prevail (58.9%) (Table 9).

Cytotoxic assay

Both AcART and AcDQHS showed low toxicity, since cellular growth was only inhibited when exposed to concentrations above 250 μg/mL for 48 h (Supplemental data Fig. S1).

Discussion

In this study, we tested several ARTs against juvenile and adult *S. mansoni* parasites. According to Katz (2008), when researching new drugs, in vitro assays are essential, since they allow an initial selection of substances with

Table 5 Hepatic portal system worm recovery from mice experimentally infected with *S. mansoni* and treated with artesunic acid (AcART), dihydroartemisinin acetate (AcDQHS) and praziquantel (PZQ) on the 45th dpi

Treatment (mg/kg)	Worm recovery (mean ± SD)				Total worm burden reduction (%)
	Couples	Males	Females	Total	
CTRL (0)	8.0 ± 2.4	7.5 ± 3.7	3.3 ± 3.0	18.8 ± 2.6	–
AcART (40)	5.5 ± 1.6[a]	6.5 ± 2.8[a,b]	3.1 ± 1.7[b]	15.1 ± 1.7[a]	26
AcART (100)	2.2 ± 1.0[a]	3.6 ± 1.9[a,b]	0.5 ± 1.1[a,b]	6.3 ± 1.6[a]	69
AcDQHS (40)	7.8 ± 1.7	4.7 ± 1.2[a,b]	1.2 ± 2.1[a,b]	13.7 ± 3.3[a]	21
AcDQHS (100)	2.5 ± 1.4[a]	4.9 ± 3.0[a,b]	1.1 ± 1.2[a,b]	8.5 ± 1.9[a]	61
PZQ (40)	3.8 ± 1.0[a]	5.5 ± 3.1[a,b]	1.6 ± 0.4[a,b]	10.9 ± 2.0[a]	48
PZQ (150)	3.8 ± 1.0[a]	5.1 ± 2.5[a,b]	0.8 ± 0.7[a,b]	9.7 ± 2.2[a]	52
PZQ (300)	2.4 ± 1.1[a]	2.0 ± 1.8[a,b]	0.6 ± 0.8[a,b]	5.0 ± 0.9[a]	72

Number of animals/group = 8. CTRL, infected and untreated control group

[a] Significant difference compared with control group (two-way ANOVA, $p < 0.05$)

[b] Significant difference between males and females (two-way anova, $p < 0.05$)

Table 6 Hepatic portal system worm recovery from mice experimentally infected with *S. mansoni* and treated with artesunic acid (AcART), dihydroartemisinin acetate (AcDQHS), and praziquantel (PZQ) on the 60th dpi

Treatment (mg/kg)	Worm recovery (mean ± SD)				Total worm burden reduction (%)
	Couples	Males	Females	Total	
CTRL (0)	6.3 ± 4.3	3.9 ± 3.7	1.1 ± 1.3	11.3 ± 2.6	–
AcART (40)	4.9 ± 2.8[a]	4.7 ± 3.5[b]	0.4 ± 0.5[a,b]	10.0 ± 2.5	15
AcART (100)	2.0 ± 1.6[a]	0.9 ± 1.0[a]	0.5 ± 0.7[a]	3.4 ± 0.8[a]	69
AcDQHS (40)	4.7 ± 1.5[a]	5.1 ± 1.7[b]	0.7 ± 1.2[a,b]	10.5 ± 2.4	12
AcDQHS (100)	2.0 ± 2.1[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	2.0 ± 1.2[a]	77
PZQ (40)	5.0 ± 2.1[a]	4.0 ± 2.4[b]	1.5 ± 1.2[b]	10.5 ± 1.8	11
PZQ (150)	4.4 ± 1.9[a]	2.4 ± 1.9[a,b]	0.9 ± 1.0[b]	7.7 ± 1.8[a]	31
PZQ (300)	2.4 ± 1.9[a]	0.5 ± 0.7[a]	0.4 ± 0.7[a]	3.3 ± 1.1[a]	70

Number of animals/group = 8. CTRL, infected and untreated control group

[a] Significant difference compared with control group (two-way ANOVA, $p < 0.05$)

[b] Significant difference between males and females (two-way anova, $p < 0.05$)

Table 7 Reduction in number of eggs present in feces of animals treated 21 dpi, compared with the untreated control group and egg development stage of eggs retained in the intestinal tissues

Treatment (mg/kg)	Number of eggs/g feces		Eggs development stage in the intestine (%)		
	Mean ± SD	% Reduction	Immature	Mature	Dead
CTRL (0)	2489.4 ± 863.5	–	78.1	22	0
AcART (40)	333.6 ± 224.9[a]	87	69.5[a]	20.5	10[a]
AcART (100)	0.0 ± 0.0[a]	100	28[a]	52.9[a]	19.1[a]
AcDQHS (40)	17.9 ± 7.9[a]	99	61.4[a]	31.1[a]	7.5[a]
AcDQHS (100)	9.4 ± 9.9[a]	99	30.1[a]	42.2[a]	27.7[a]
PZQ (40)	972.6 ± 536.4[a]	61	72[a]	28[a]	0
PZQ (150)	112.0 ± 12.0[a]	64	85.5[a]	14[a]	0.5
PZQ (300)	498.2 ± 331.3[a]	80	66.9[a]	32.1[a]	1

[a] Significant difference compared with control group (two-way ANOVA, $p < 0.05$)

CTRL, untreated control group; AcART, artesunic acid; AcDQHS, dihydroartemisinin acetate; PZQ, praziquantel

schistosomicidal activity. With the exception of ART and EAcART, all derivatives affected *S. mansoni* survival in vitro. Among them, AcART and AcDQHS were responsible for the best results, both reaching 100% mortality at either 50 or 100 μg/mL, which lead us to select these two compounds for the in vivo assay.

Table 8 Reduction in number of eggs present in feces of animals treated 45 dpi, compared with the untreated control group and egg development stage of eggs retained in the intestinal tissues

Treatment (mg/kg)	Number of eggs/g feces		Eggs development stage in the intestine (%)		
	Mean ± SD	% Reduction	Immature	Mature	Dead
CTRL (0)	1358.9 ± 661.8	–	52	48	0
AcART (40)	1176.5 ± 677.4	13	24.8[a]	58.5[a]	16.7[a]
AcART (100)	8.9 ± 25.1[a]	99	20[a]	41.5[a]	38.5[a]
AcDQHS (40)	1431.3 ± 753.3	12	23.4[a]	56.3[a]	20.3[a]
AcDQHS (100)	94.5 ± 63.5[a]	92	31.4[a]	27.6[a]	41[a]
PZQ (40)	245.5 ± 253.1[a]	82	56.1	34.8[a]	9.1[a]
PZQ (150)	62.5 ± 96.3[a]	95	14.1[a]	33[a]	52.9[a]
PZQ (300)	111.6 ± 76.7[a]	92	30.1[a]	24.9[a]	45[a]

[a] Significant difference compared with control group (two-way ANOVA, $p < 0.05$)

CTRL, untreated control group; AcART, artesunic acid; AcDQHS, dihydroartemisinin acetate; PZQ, praziquantel

Table 9 Reduction in number of eggs present in feces of animals treated 60 dpi, compared with the untreated control group and egg development stage of eggs retained in the intestinal tissues

Treatment (mg/kg)	Number of eggs/g feces		Eggs development stage in the intestine (%)		
	Mean ± SD	% reduction	Immature	Mature	Dead
CTRL (0)	2489.0 ± 616.7	–	58.9	41	0.1
AcART (40)	1141.6 ± 739.3[a]	54[a]	28.6[a]	56[a]	15.4[a]
AcART (100)	14.0 ± 24.2[a]	99[a]	15[a]	42	43[a]
AcDQHS (40)	805.6 ± 568.7[a]	68[a]	24.5[a]	44.1	31.4[a]
AcDQHS (100)	190.0 ± 255.0[a]	79[a]	16[a]	15.8[a]	68.2[a]
PZQ (40)	324.0 ± 288.5[a]	87[a]	38[a]	18.4[a]	43.6[a]
PZQ (150)	921.0 ± 315.0[a]	63[a]	16.3[a]	16.6[a]	67.1[a]
PZQ (300)	75.0 ± 27.3[a]	97[a]	4.3[a]	11[a]	84.7[a]

[a] Significant difference compared with control group (two-way ANOVA, $p < 0.05$)

CTRL, untreated control group; AcART, artesunic acid; AcDQHS, dihydroartemisinin acetate; PZQ, praziquantel

Changes in tegument integrity (revealed using Hoescht 33,258 probe), along with the severe morphological damage such has tegument peeling and destruction observed trough optical microscopy and SEM are extremely relevant since the parasite tegument is the main route for nutrient absorption, metabolite excretion and protection against the host immune system. Also, damage found in the oral and ventral sucker would interfere with the adhesion to the blood vessels as well as in the nutrition process (Xiao et al. 2000).

In the in vivo assay, treatment with 100 mg/kg of AcART and AcDQHS, 21 dpi caused a significantly higher total worm reduction (86% and 67%, respectively) when compared with the groups treated with 300 mg/kg of PZQ (18% worm reduction), while treatment 45 and 60 dpi showed no significant difference. These results show that not only AcART and AcDQHS needed lower concentrations than PZQ to have similar efficiency against adult parasites, but they also appear to be more efficient against *S. mansoni* immature forms, which is extremely important since in endemic areas the possibility of being infected with more than a single maturity stage of the parasite (de Oliveira et al. 2017a).

After treatment with either AcART or AcDQHS (21, 45 or 60 dpi), we also found a significant reduction in the number of eggs eliminated in feces. Along with this reduction, if we consider the oogram pattern (namely increase of mature and dead eggs and decrease of immature eggs), along with the oviposition reduction (from the in vitro assay), it is possible that the decrease in eggs was not only a consequence of the decrease in worm burden, but also due to a disruption in female parasite reproductive system. This possibility is further supported since arthemeter and DQHS have been described to cause alterations in the female parasite ovaries and vitelline glands (Li et al. 2013; Xiao et al. 2004) thus, it is conceivable that AcART and AcDQHS would cause similar effects. The results, regarding egg reduction and changes in oogram pattern, are of great significance, since the pathology is associated with the inflammatory response to the large number of eggs stuck in the host tissues and organs (de Oliveira et al. 2017a; Gryseels et al. 2001). In addition, disrupting the parasite biological cycle would interfere with the transmission of schistosomiasis in endemic areas (de Oliveira et al. 2017a).

Conclusion

In this work, we tested several ARTs against *S. mansoni*. Through optical microscopy, scanning electron microscopy and using Hoescht 33258 probe, we observed severe morphological damage caused by these derivatives. Our results also showed that AcART and AcDQHS caused a significant parasite burden reduction compared to PZQ, as well as a significant reduction in fecal egg count and changes in the oogram pattern when applied 21 dpi. Also, treatment with these derivatives 45 or 60 dpi needed lower dosages than PZQ to have similar results. The efficiency of both AcART and AcDQHS against both juvenile and adult parasites, shows great potential has alternative drugs against *S. mansoni.*

Acknowledgments The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their support, Finance Code 001. The authors also thank Dr. Paul Ormond for revising the manuscript.

Compliance with ethical standards

Conflict of interest The authors confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

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