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Evaluation of the Anti-*Schistosoma mansoni* Activity of Thiosemicarbazones and Thiazoles

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Schistosomiasis is a chronic and debilitating disease caused by a trematode of the genus *Schistosoma* and affects over 207 million people. Chemotherapy is the only immediate recourse for minimizing the prevalence of this disease and involves predominately the administration of a single drug, praziquantel (PZQ). Although PZQ has proven efficacy, there is a recognized need to develop new drugs as schistosomicides since studies have shown that repeated use of this drug in areas of endemicity may cause a temporary reduction in susceptibility in isolates of *Schistosoma mansoni*. Hydrazones, thiosemicarbazones, phthalimides, and thiazoles are thus regarded as privileged structures used for a broad spectrum of activities and are potential candidates for sources of new drug prototypes. The present study determined the *in vitro* schistosomicidal activity of 10 molecules containing these structures. During the assays, parameters such motility and mortality, oviposition, morphological changes in the tegument, cytotoxicity, and immunomodulatory activity caused by these compounds were evaluated. The results showed that compounds formed of thiazole and phthalimide led to higher mortality of worms, with a significant decline in motility, inhibition of pairing and oviposition, and a mortality rate of 100% starting from 144 h of exposure. These compounds also stimulated the production of nitric oxide and tumor necrosis factor alpha (TNF-α), thereby demonstrating the presence of immunomodulatory activity. The phthalyl thiazole LpQM-45 caused significant ultrastructural alterations, with destruction of the tegument in both male and female worms. According to the present study, phthalyl thiazole compounds possess antischistosomal activities and should form the basis for future experimental and clinical trials.

Schistosomiasis is a chronic and debilitating disease caused by a trematode of the genus *Schistosoma* and is one of the most prevalent and neglected diseases of tropical and subtropical regions. This parasitic disease ranks second after malaria in terms of its public health importance and has a significant economic and social impact. It is estimated that more than 207 million people have been infected worldwide, while 779 million people remain at risk of infection (1–6).

According to the World Health Organization, schistosomiasis is the cause of more than 200,000 deaths per year in sub-Saharan Africa, and this may still be an underestimate (5). *Schistosoma mansoni* is one of the most common etiological agents of human schistosomiasis, and the disease is triggered by the inflammatory granulomatous reaction that occurs during deposition of parasite eggs in the liver and other host tissues (7).

The eggs released during the progression of schistosomiasis produce antigens that induce a stronger Th2 response, leading to the formation of granulomas, whereas the parasite antigen induces a Th1 response, with a predominantly Th1 response being observed in the acute phase that is replaced by a Th2 immune response upon egg antigen production (8–10).

Current schistosomiasis treatment is based on the use of praziquantel (PZQ), a pirazylisoquinoline, which is effective against all *Schistosoma* species infecting humans (11, 12) and has been successfully used over the last 20 years as the drug of choice in most areas where the disease is endemic (3, 13, 14). Even though PZQ the antihelminthic drug of choice and despite its advantages, which include tolerability, safety, efficacy, and low cost, PZQ does not protect individuals from reinfection and is not active against the immature stages of the worm, such as the schistosomula and preadult and juvenile adult stages (13, 15).

Furthermore, the appearance of drug-resistant strains of *Schistosoma* is a constant concern for public health authorities (16–18). Hence, the massive use of PZQ in zones of endemicity with the possibility of the emergence of drug-resistant *Schistosoma*, combined with the lack of any other effective antischistosomal drug, requires new effective schistosomicidal compounds to be developed and further studies to be carried out with a view to developing alternative therapies that could either replace or complement the use of PZQ for treatment of *S. mansoni* infection (4, 13).

It is well known that hydrazones, thiosemicarbazones, and phthalimides as well as thiazoles are considered privileged structures as leads in medicinal chemistry (19–21). These core structures have figured prominently in a vast number of structural subunits used for a broad spectrum of activities, and the mode of action of the nuclei of these pharmacophores is usually attributed to the inhibition of multiple targets. For example, hydrazones and thiosemicarbazones have been shown to possess antimicrobial,

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anticonvulsant, analgesic, anti-inflammatory, antiplatelet, antitubercular, and antitumor properties, among others (22).

Likewise, hydrazones, thiazolidinone, and their bioisoster thiazole derivatives are known for their potential pharmaceutical applications and possess antimicrobial (23), antischistosomal (24), antifungal (25), antimalarial (26), herbicidal (27), antiviral (28), antidiabetic (29), and antioxidant (30) properties. Thiazole derivatives have been used to prepare various drugs that are important for antimicrobial (31), antibacterial (32, 33), antifungal (32), anti-inflammatory (34), and antitubercular (35) treatment, and some of the thiazole derivatives are used as antiprotozoals (36).

On the other hand, phthalimide derivatives, such as thalidomide, are known for their immunomodulatory activities, inhibiting the cytokine tumor necrosis factor alpha (TNF-α), interleukins-1β (IL-1β), IL-6, and IL-12, and granulocyte-macrophage colony-stimulating factor. They also activate the Th1 response, by increasing gamma interferon (IFN-γ) and IL-2, have antiangiogenic and antiproliferative properties, activate apoptosis, T cells, and NK cells, and inhibit cell adhesion (37, 38).

With this in mind, we performed a synthesis of a set of molecules whose structures have a hydrazine and/or thiazole nucleus as a common group. With a view to ascertaining whether the new thiosemicarbazone, phthalyl thiosemicarbazone, phthalyl thiazole, and phthalyl thiazolidinone pharmacophores are an essential requirement for antischistosomal activity, 10 compounds were synthesized, and their antischistosomal potentials were determined.

The efficacy of the compounds was examined in terms of (i) schistosome survival, (ii) egg output (oviposition), (iii) motor activity, (iv) ultrastructural alterations in the tegument of *S. mansoni* as determined by scanning electron microscopy (SEM), and (v) cytotoxicity and immunomodulatory activity induced by these new compounds on splenocytes and macrophages, respectively.

MATERIALS AND METHODS

Compounds. The compounds 2-(1-phenoxypropan-2-ylideno)thiosemicarbazide (LpQM-01), 2-(1-phenoxypropan-2-ylideno)-4-phenylthiosemicarbazide (LpQM-02), and 2-(1-phenoxypropan-2-ylideno)-4-methylthiosemicarbazide (LpQM-03) were prepared as described by Moreira et al. (39). The compounds 2-(2-(1,3-dioxoisoindolin-2-yl)ethylidene)-1-methylthiosemicarbazide (LpQM-38), (3-methyl-4-oxothiazolidin-2-ylidene)hydrazono)ethyl) isoindoline-1,3-dione (LpQM-40), 2-(2-(2-(4-(4-fluorophenyl)thiazol-2-yl) hydrazono)ethyl)isoindoline-1,3-dione(LpQM-43), 2-(2-(2-(4-(4-methoxyphenyl)thiazol-2-yl)hydrazono)ethyl)isoindoline-1,3-dione (LpQM-45), and 2-(2-(2-(4-(4-chlorophenyl)thiazol-2-yl)hydrazono)ethyl)isoindoline-1,3-dione (LpQM-47) were prepared as described by Pessoa et al. (40); compounds 2-(2-(1-(3-bromophenyl)propylidene)hydrazinyl)-4-methoxyphenyl)thiazole (LpQM-14) and 2-(2-(1-(3-bromophenyl)propylidene)hydrazinyl)-4-(4-nitrophenyl)thiazole (LpQM-17) were also used (C. L. Leite and P. A. T. Gomes, unpublished data). All compounds were chemically characterized by nuclear magnetic resonance (NMR), infrared, and mass spectra and by elemental analysis and presented purity of >95%. PZQ (catalog no., 4668; bench, BCBD3257V) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Parasites and intermediary and definitive hosts. The LE strains (Belo Horizonte, Minas Gerais, Brazil) of *S. mansoni* were used throughout this study. These strains were maintained in *Biomphalaria glabrata* snails and Swiss mice, in the laboratory at the Aggeu Magalhães Research Center (CPqAM) of the Oswaldo Cruz Foundation (FIOCRUZ, PE, Brazil).

Female Swiss mice weighing 20 ± 2 g were used as the definitive host and were infected transcutaneously with about 120 cercariae (LE strain). The animals were kept in a controlled temperature and light environment and had access to food and water *ad libitum*. After 55 days of infection, adult *S. mansoni* specimens were recovered from the mice by perfusion, using the technique developed by Duvall and De Witt (41).The experiments were approved by the Ethics Committee on Animal Use (CEUA), FIOCRUZ (process number 22/2011).

***In vitro* assay.** *S. mansoni* worms harvested from Swiss mice were kept in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) buffered to pH 7.5, supplemented with HEPES (20 mM), 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 $\mu g \cdot ml^{-1}$). Incubation was carried out at 37°C in a humid atmosphere containing 5% CO_2 gas. LpQM-43, LpQM-45, LpQM-47, and LpQM-14 compounds were dissolved in 1.6% dimethyl sulfoxide (DMSO) and used in concentrations varying from 40 to 100 $\mu g \cdot ml^{-1}$; compounds were added to the medium containing the worms after a 2-h period of adaptation to the culture medium. One pair of adult worms/well was used in this study. The control worms were assayed in RPMI 1640 medium with 1.6% DMSO as a negative-control group. All experiments were carried out in five replicates and were repeated at least three times. The motor activity, egg output (oviposition), tegumental alterations, and survival of the parasites were monitored every 24 h for 192 h using an inverted microscope (SMZ 1000; Nikon).

SEM. Male and female worms after *in vitro* exposure to LpQM-45 compound over a period of 24 and 48 h were fixed overnight at room temperature with 2.5% glutaraldehyde, 4% formaldehyde, and 0.1 M cacodylate buffer at pH 6.8. They were then postfixed in 2% osmium tetroxide (OsO_4) in a 0.1 M cacodylate buffer at pH 6.8 for 60 min in the absence of light at room temperature. The next steps included washing and dehydration in a graded ethanol series for 15 min each. The worms were critical-point dried using liquid CO_2, directly sputter coated with colloidal gold for 1 min, and examined under a JEOL-5600LV microscope.

Animals used for cytotoxicity and immunological assays. Male BALB/c mice (6 to 8 weeks old) were raised at the animal facility of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) and maintained at the animal facility of the Aggeu Magalhães Research Center, Oswaldo Cruz Foundation, in Recife, Brazil. All mice were euthanized, and their spleens were removed in accordance with the guidelines of the Oswaldo Cruz Foundation Commission for Experiments with Laboratory Animals (Ministry of Health, Brazil, 0266/05).

Spleen cell harvesting. Spleen cells were harvested according to a previous protocol (42). After the BALB/c mice were euthanized with CO_2 gas, the spleen of each mouse was removed aseptically and placed in a Falcon tube containing incomplete RPMI 1640 medium with fetal calf serum (complete medium). In a vertical flow, each spleen was transferred to a petri dish where it was soaked. The cell suspensions obtained were transferred to Falcon tubes containing approximately 10 ml of incomplete medium per spleen and centrifuged at 4°C and 200 × *g* for 5 min. After the supernatant was discarded, distilled water was added to the sediment to trigger red blood cell lysis. The supernatant (containing no cell debris) was collected and centrifuged at 4°C and 200 × *g* for 5 min. The resulting sediment (containing cells) was resuspended in complete RPMI 1640 medium. An aliquot of each cell suspension was separated and diluted in trypan blue for quantification in a Neubauer chamber, and the viability of cells was determined.

***In vitro* cytotoxicity assay.** Spleen cells (6×10^5 cells/well), obtained as described in the previous paragraph, were cultured in 96-well plates containing RPMI 1640 medium. These cells were incubated with the compounds at six concentrations (1, 5, 10, 25, 50, and 100 $\mu g \cdot ml^{-1}$) in the presence of [^{3}H]thymidine (Amersham Biosciences, USA) (1 $\mu Ci \cdot well^{-1}$) for 24 h at 37°C and 5% CO_2. Cells treated with saponin (0.05%) were used as a positive control, and cells treated with DMSO (1%) were used as a negative control. Each drug was tested in triplicate.

The contents of the plate were then harvested to determine [^{3}H]thymidine incorporation using a beta-radiation counter (Wallac 1209; Rackbeta Pharmacia, Stockholm, Sweden). Compound toxicity was determined by comparing the percentage of [^{3}H]thymidine incorporation (as an indicator of cell viability) in treated cells with that in untreated cells. Noncytotoxic concentrations were defined as those where [^{3}H]thymidine incorporation was

30% lower than the level in untreated controls. Six concentrations were also used for PZQ (1, 5, 10, 25, 50, and 100 $\mu g \cdot ml^{-1}$).

Measurement of cytokine levels in macrophage supernatants. Cytokines were quantified in supernatants of macrophage cultures treated *in vitro* after 48 h and 72 h with LpQM-43, LpQM-45, LpQM-47, LpQM-14, and PZQ at 56, 58, 55, 50, and 51 $\mu g \cdot ml^{-1}$, respectively (50% cytotoxic concentration [CC_{50}] in macrophages). As a positive control, cells were stimulated with the mitogens lipopolysaccharide (LPS at 50 $\mu g \cdot ml^{-1}$) and concanavalin A (ConA at 2.5 $\mu g \cdot ml^{-1}$), while for the negative controls, cells did not receive either mitogen or drugs. The levels of the IL-6, IL-10, IL-12, and TNF-α cytokines were measured using sandwich enzyme-linked immunosorbent assays (ELISAs), according to the manufacturer's suggested protocols. The monoclonal antibodies used were the OptEIA (BD Biosciences) kit, and these were used after titration. Plates with 96 wells (NalgeNunc International Corp.) were sensitized with specific anticytokine antibodies (according to the manufacturer's instructions) and incubated overnight at 4°C. Cytokine standards were added after serial dilutions from their initial concentrations (16,000 $pg \cdot ml^{-1}$). After a washing step, 50 μl of all samples and standards was added in duplicate, and the plate was incubated for 2 h at room temperature. The specific antibodies were then combined with biotin (according to the manufacturer's instructions) and incubated for 1 h 30 min at room temperature. Revealer solution containing 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was added. The reaction was blocked with 1 M sulfuric acid, and the reading was carried out on a spectrophotometer (3550; Bio-Rad, Hercules, CA) at 415 nm. Sample concentrations were calculated in the linear region of the titration curve of cytokine standards, and final concentrations were expressed in $pg \cdot ml^{-1}$, using Microplate Manager, version 4.0, software (Bio-Rad Laboratories).

***In vitro* nitrite analysis.** Nitric oxide (NO) production was measured as nitrite (a stable breakdown product of NO) accumulated in the supernatant of the macrophage culture stimulated with LpQM-43, LpQM-45, LpQM-47, LpQM-14, and PZQ at 56, 58, 55, 50, and 51 $\mu g \cdot ml^{-1}$, respectively (the 50% cytotoxic concentration [CC_{50}] in the macrophages) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and LPS (50 $ng \cdot ml^{-1}$) were used as positive and negative controls, respectively.

The Griess indirect method (43) was used, and nitrite levels were quantified using 50 μl of each supernatant and an equal volume of Griess reagent [1% sulfanilamide, 0.1% dihydrochloride of *N*-(1-naphthyl)-ethylenediamine, 2.5% H_3PO_4], and samples were incubated at room temperature for 10 min. Absorbance was measured on a 540-nm reader (Multiskan FC; Thermo Scientific) at 540 nm, and the nitrite levels in each sample after 24 h, 48 h, and 72 h were determined by extrapolation from a previously determined standard curve.

Statistical analysis. The differences between groups were analyzed using Mann-Whitney *U* and Dunnett's nonparametric tests. All results are expressed as mean values of groups ± standard deviations, and a *P* value of <0.05 was taken to be statistically significant.

RESULTS

Effect of new compounds on adult *S. mansoni* survival. Ten derivatives containing the pharmacophore phthalimide, thiazole, thiazolidinone, and thiosemicarbazone nuclei were tested for schistosomicidal properties (Table 1). Compounds were evaluated at a concentration of 40 to 100 $\mu g \cdot ml^{-1}$ every 24 h for a period of 192 h, and mortality, motility, and alterations in the integument of the worms were observed. The mortality after 144 h of exposure at a concentration of 100 $\mu g \cdot ml^{-1}$ was initially used to perform a screening of compounds. Of the tested compounds, those formed of thiazole and phthalimide led to higher mortality among worms (Fig. 1). One exception was LpQM-17, a thiazole derivative that did not kill worms under the conditions cited. We investigated the action of four compounds, LpQM-43, LpQM-45, LpQM-47, and LpQM-14, in greater detail, examining the effect of these compounds on the mortality rate with respect to concentration and incubation time. Other factors evaluated included changes in motility and integument and the 50% inhibitory concentration (IC_{50}; 50% mortality).

All compounds were tested in concentrations of 5, 10, and 20 $\mu g \cdot ml^{-1}$, but they did not cause worm mortality in these concentrations (data not shown). Because of this, only concentrations above 40 $\mu g \cdot ml^{-1}$ are described in Table 2. The phthalyl thiazoles LpQM-45 and LpQM-14 caused 100% worm mortality at concentrations of 100 and 80 $\mu g \cdot ml^{-1}$ within 144 and 168 h, respectively. Similarly, the phthalyl thiazoles LpQM-43 and LpQM-47 caused 67% and 95% worm mortality, respectively, in 192 h at a concentration of 100 $\mu g \cdot ml^{-1}$ (Table 2). Interestingly, oviposition by adult worms was not seen with any of the four compounds examined. All of the worms in the control group remained viable until the end of the experiment.

As can be seen in Table 3, there was a significant reduction in motility under the treatment with the LpQM-45 compound at all concentrations. The LpQM-47 compound brought about a reduction in motility at all concentrations, and LpQM-14 reduced motility at 60 to 100 $\mu g \cdot ml^{-1}$. LpQM-43 caused only a partial reduction in worm motility. Many physiological alterations were observed in adult worms exposed to the new compounds (Table 2). The IC_{50}, the concentration of compound required to cause 50% mortality of worms, was another parameter used to evaluate schistosomicidal activity, and the results are shown in Table 2.

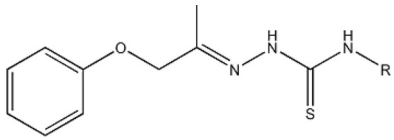
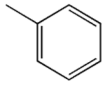
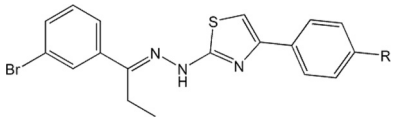
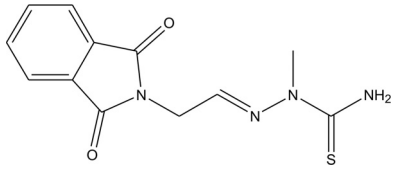
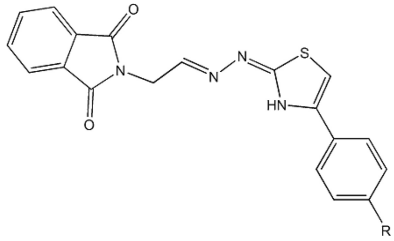
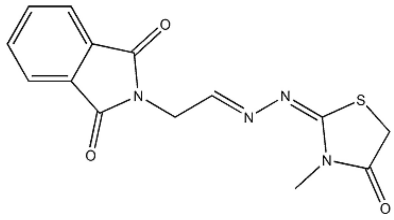
The activity of compounds was evaluated after 120 h of exposure in terms of changes in motility and the tegument of the worms and according to the criteria established by Ramirez et al. (44). According to these criteria, LpQM-43 was considered partially active at a concentration range of 40 to 100 $\mu g \cdot ml^{-1}$, while LpQM-45 and LpQM-47 were considered active at a concentration of 40 to 100 $\mu g \cdot ml^{-1}$, and LpQM-14 was active at a concentration of 60 to 100 $\mu g \cdot ml^{-1}$ and partially active at a concentration of 40 $\mu g \cdot ml^{-1}$.

Scanning electron microscope examination. The compound LpQM-45 caused 100% worm mortality, and it was the most potent of the compounds; therefore, we studied its effects on the worm morphology.

The tegument surface of male and female *S. mansoni* worms after *in vitro* exposure to LpQM-45 for periods of 24 and 48 h was examined using scanning electron microscopy (SEM). The worms exposed to DMSO and to medium alone (controls) were also examined using SEM. The control male and female worms that were not exposed to any drugs (negative controls) presented normal surface membrane topography. The male worms exhibited a large number of tubercles with typical spines, sensory papillae, oral and ventral suckers, and no abnormality, and, in the anterior part of the body, the gynecophoral canal showed no abnormality after 24 and 48 h (Fig. 2A to D). In the female worms, parallel fissures, tegument spines, and sensory papillae with no abnormality were observed at 24 and 48 h (Fig. 2E to H).

Exposure of the worms to compound LpQM-45 resulted in ultrastructural alterations, which were already apparent during the first period of exposure (24 h), revealing a variety of changes in the tegument surface. In the male worm, complete destruction of some tubercles was found, with extensive sloughing and exposure of the subtegument layer of muscle tissue (Fig. 3A to D). The

TABLE 1 Structure of new thiosemicarbazone analogs used in this study

Analog	Structure	Compound	Identification of R
Phenoxy-thiosemicarbazones		LpQM-01	—H
		LpQM-02	
		LpQM-03	—CH_3
Phenyl-thiazoles		LpQM-14	—O—CH_3
		LpQM-17	—NO_2
Phthalyl-thiosemicarbazone		LpQM-38	
Phthalyl-thiazoles		LpQM-47	—Cl
		LpQM-43	—F
		LpQM-45	—O—CH^3
Phthalyl-thiazolidinone		LpQM-40	

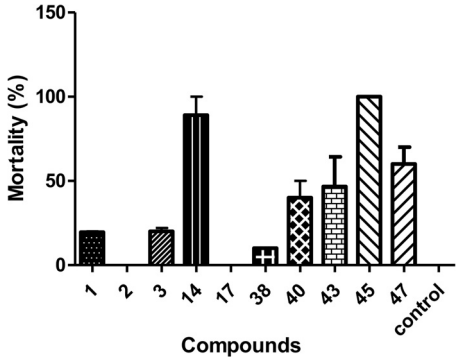


FIG 1 Effects of LpQM compounds on the mortality rate of *S. mansoni* at a concentration of 100 $\mu g \cdot ml^{-1}$ after 144 h. Compounds are indicated by the numerical suffix following LpQM.

severity of tegument damage was higher in the males after 48 h of exposure, with an increasing number of tubercles completely destructed or eroded, a roughened surface in the same areas, and disintegration of the tegument in this area, as revealed by higher magnification (Fig. 3E to H).

The female worms exposed to LpQM-45 showed serious damage, with extensive sloughing and disintegration of the tegument and exposure and injury of the layer of muscle tissue after 24 and 48 h (Fig. 4).

Cytotoxic activity in splenocytes. After determining the antiparasitic activity against *S. mansoni* worms, we determined the cytotoxicity in splenocytes of BALB/c mice for the most potent antiparasitic compounds. The evaluation of cytotoxic compounds showed that LpQM-43, LpQM-45, and LpQM-47 presented nontoxic effects at concentrations up to 100 $\mu g \cdot ml^{-1}$, while LpQM-14 presented nontoxic effects at concentrations up to 25 $\mu g \cdot ml^{-1}$ (Table 2). On the other hand, PZQ showed higher toxicity in splenocytes (<1 $\mu g \cdot ml^{-1}$) at all concentrations tested.

TABLE 2 *In vitro* effects of LpQM-43, LpQM-45, LpQM-47, and LpQM-14 against adult worms of *S. mansoni*

Drug	Time (h)	Mortality (%) at the indicated concn (μg/ml)				IC_{50} (μg/ml)	Cytotoxicity (μg/ml)[a]	Worm characteristic(s) observed
		100	80	60	40			
LpQM-43	24	13	0	7	0	82.24	>100	Not paired
	48	23	3	10	0	84.13		No sucker adherence
	72	30	7	10	0	83.53		Absence of eggs
	96	37	13	16	0	73.65		Nontransparent blackish tegument
	120	40	20	24	0	64.13		
	144	47	37	29	0	58.01		
	168	60	40	45	0	55.83		
	192	67	53	56	53	32.93		
LpQM-45	24	5	36	9	32	46.20	>100	Not paired
	48	40	41	28	41	19.97		No sucker adherence
	72	60	60	57	51	26.36		Absence of eggs
	96	70	80	62	56	31.53		Integument morphology altered (nontransparent blackish tegument, appearance of bubbles)
	120	90	95	76	61	33.78		
	144	100	100	90	70	32.09		
	168	100	100	90	85	25.88		
	192	100	100	90	95	24.69		
LpQM-47	24	5	9	10	5	40.08	>100	Not paired
	48	5	9	19	15	51.38		No sucker adherence
	72	15	25	39	21	41.90		Absence of eggs
	96	20	29	53	44	37.48		Integument morphology altered (nontransparent, blackish tegument, appearance of bubbles)
	120	40	46	66	66	23.78		
	144	60	54	74	73	24.92		
	168	80	58	88	83	26.97		
	192	95	58	91	88	30.95		
LpQM-14	24	5	0	5	0	65.70	25	Not paired
	48	25	0	20	0	72.77		No sucker adherence
	72	50	10	25	0	76.17		Absence of eggs
	96	50	40	25	5	60.15		Integument morphology altered (nontransparent, blackish tegument, appearance of bubbles)
	120	78	75	39	22	53.95		
	144	89	95	52	37	48.06		
	168	100	100	57	47	43.41		
	192	100	100	67	61	33.52		

[a] The highest nontoxic concentration on spleen cells of BALB/c mice. Saponin (<1.0 μg/ml) was used as a positive control.

Immunomodulatory activity in macrophages treated with the compounds. The ability of the compounds to stimulate the secretion of IL-6, IL-10, IL-12, and TNF-α was investigated through the measurement of these cytokines in the supernatants of macrophage cultures. Results are shown in Fig. 5. In comparison to the untreated cells (negative control), a significant ($P < 0.001$) production of TNF-α was observed after 48 h of treatment with the compound LpQM-47. A similar finding was observed for PZQ-treated cells. After 72 h of treatment, compounds LpQM-43, LpQM-45, and LpQM-47 caused significant TNF-α secretion. In contrast, compound LpQM-14 caused significant TNF-α secretion only after 72 h of treat-

TABLE 3 Motility score of control and worms treated with PZQ and LpQM-43, LpQM-45, LpQM-47, and LpQM-14 for 120 h

Group	Percentage of worms by motility score after drug treatment at:[a]															
	100 μg/ml				80 μg/ml				60 μg/ml				40 μg/ml			
	3	2	1	0	3	2	1	0	3	2	1	0	3	2	1	0
Control	100	0	0	0	100	0	0	0	100	0	0	0	100	0	0	0
PZQ	0	0	0	100	0	0	0	100	0	0	0	100	0	0	0	100
SC-43	17	29	17	38	3	50	27	20	13	46	18	23	4	48	10	38
SC-45	0	0	10	90	0	5	0	95	0	5	19	76	0	20	19	61
SC-47	5	10	45	40	8	13	34	45	0	4	30	66	0	13	21	66
PT-1.4	5	11	6	78	0	20	5	75	0	23	39	39	16	42	20	22

[a] The measurement of mean worm motility is scored on a scale of 0 to 3 as follows: 3, normally active; 2, slowed activity; 1, minimal activity with occasional movement of head and tail and absence of motility apart from gut movements; 0, total absence of mobility.

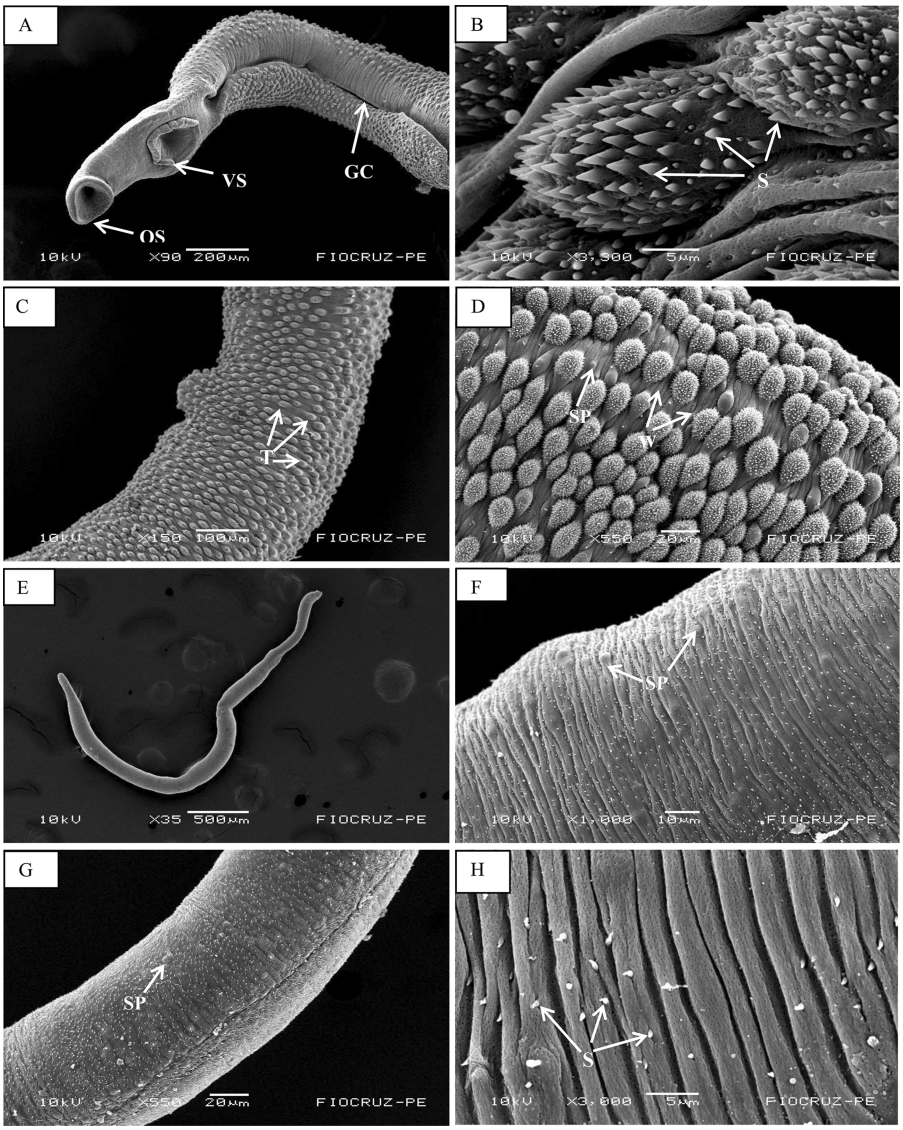


FIG 2 Scanning electron micrographs of adult male and female *S. mansoni* worms from the control group. (A) Male worms kept in medium and DMSO after 24 h, showing the anterior portion of the body with oral (OS) and ventral suckers (VS) and the gynecophoral canal (GC) with no abnormalities. (B) Male worms kept in medium and DMSO after 48 h, showing in detail numerous spines (S) covering the tubercules (T). (C) The dorsal region of male worms kept in medium alone after 48 h, showing numerous tubercles distributed along the body. (D) Male worms kept in medium only after 24 h, showing in detail the dorsal region with sensory papillae (SP) and parallel wrinkles (W) visible. (E) Female worms kept in medium and DMSO after 24 h, showing the whole extent of the body. (F) Female worms kept in medium and DMSO after 48 h, showing the sensory papillae (SP). (G) Female worms kept in medium alone showing the integrity of the tegument. (H) Female worms kept in medium alone, showing spines (S) in detail.

ment. In addition, we observed that the TNF-α content under treatment with compound LpQM-14 was quite similar to that observed for LPS-stimulated cells (positive control), indicating that this compound substantially modulates the immune response. After the TNF-α content was measured, the secretion of IL-6, IL-10, and IL-12 was evaluated under compound treatment. However, in comparison to the LPS-stimulated cells (positive control), none the compounds induced the secretion of IL-6, IL-10, and IL-12 in macrophages until 72 h after drug incubation (data not shown).

NO content in macrophages. In another set of experiments, the nitrite content was determined in the supernatant of macrophages treated with the drugs LpQM-43, LpQM-45, LpQM-47, and LpQM-14. LPS-treated cells (positive control) and untreated cells (negative control) were included in the experiment (Fig. 6). In comparison to untreated cells, the production of nitrite was significantly higher under compound LpQM-14 treatment, and this was observed during the three data points (24, 48, and 72 h after incubation). In contrast, compound LpQM-45 produced a statistically significant amount of nitrite only at a time point under 48 h after incubation, while PZQ had no effect on the nitrite production.

DISCUSSION

The 10 tested compounds all had a phthalimide nucleus, a hydrazone moiety, and/or a thiazole ring system. In general, compounds that possessed a phthalimide and thiazole ring system showed significant

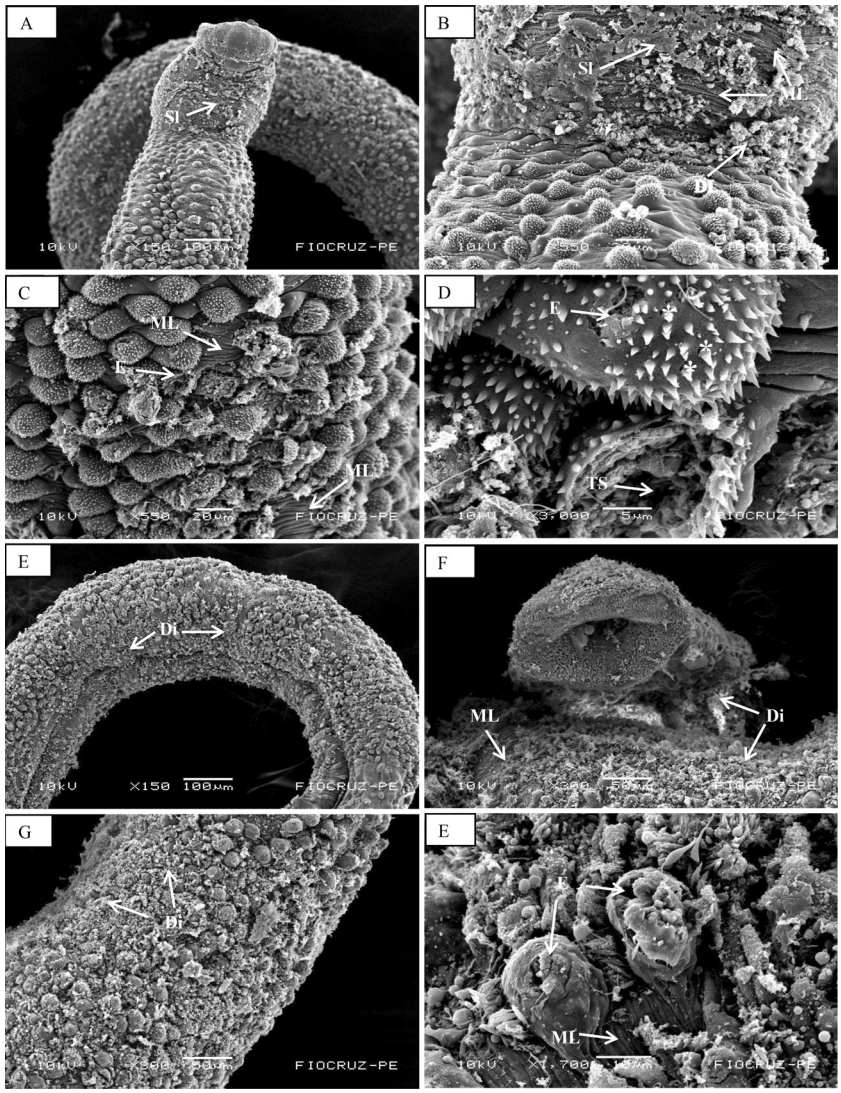


FIG 3 Scanning electron micrographs of adult male *S. mansoni* worms exposed to 80 μg · ml^{-1} LpQM-45. (A to D) Worms after 24 h of incubation showing extensive sloughing (Sl), erosion (E), loss of spines (*) and disintegration (Di) of tegument with exposure of subtegumental tissue (TS) and muscle layer (ML). (E to H) Worms after 48 h of incubation showing a greater area of erosion (E) and disintegration (Di) of the tegument and exposure of muscle layer (ML).

antiparasitic activity against *S. mansoni* worms. No antiparasitic activity was observed in the case of LpQM-17, a thiazole derivative that also has a nitro group in the 4-position of the phenyl ring. The cytotoxicity in mouse cells of PZQ was greater than that of any of the tested compounds that showed anti-*S. mansoni* activity; therefore, the compounds described here were more selective. In terms of the oviposition profile of adult worms (absence), the tested compounds exhibited activity similar to that observed for PZQ.

The thiazole nucleus, which is present in an important class of heterocyclic compounds present in many biologically potent active molecules (45), is also present in the most active compounds of the whole series. Some thiazoles have, in fact, been shown in the literature as schistosomicidal agents (19, 46). The phthalyl thiazoles LpQM-43, LpQM-45, and LpQM-47 and the thiazole LpQM-14 have been shown to have antischistosomal properties, with the IC_{50}s ranging from 82.24 to 32.93 μg · ml^{-1}, 46.20 to 24.69 μg · ml^{-1}, 40.08 to 30.95 μg · ml^{-1}, and 65.70 to 33.52 μg · ml^{-1}, respectively. These results suggest that the efficacy varies according to the substituent in the 4-position of the phenyl group. LpQM-45 exhibited the best schistosomicidal properties in relation to other compounds, with 100% mortality within 144 h at concentrations of 100 and 80 μg · ml^{-1}. LpQM-14 also showed significant schistosomicidal activity, with 100% mortality within 168 h at concentrations of 100 and 80 μg · ml^{-1}.

The compounds LpQM-14 and LpQM-45 have in common a methoxyl group attached in the 4-position of the phenyl ring.

In comparison to the literature findings, it was also observed that heterocyclic compounds containing a methoxyl group exhibited higher schistosomicidal activity than compounds without this group (47). Another interesting structure-activity relationship is observed for the compound LpQM-47, which produced a mortality rate of 95% at 100 μg · ml^{-1} and 91% at 60 μg · ml^{-1} after 192 h of incubation. This compound has a chloro atom attached to the 4-position of the phenyl ring. In agreement with this, it is described in the literature that the attachment of a chloro atom im-

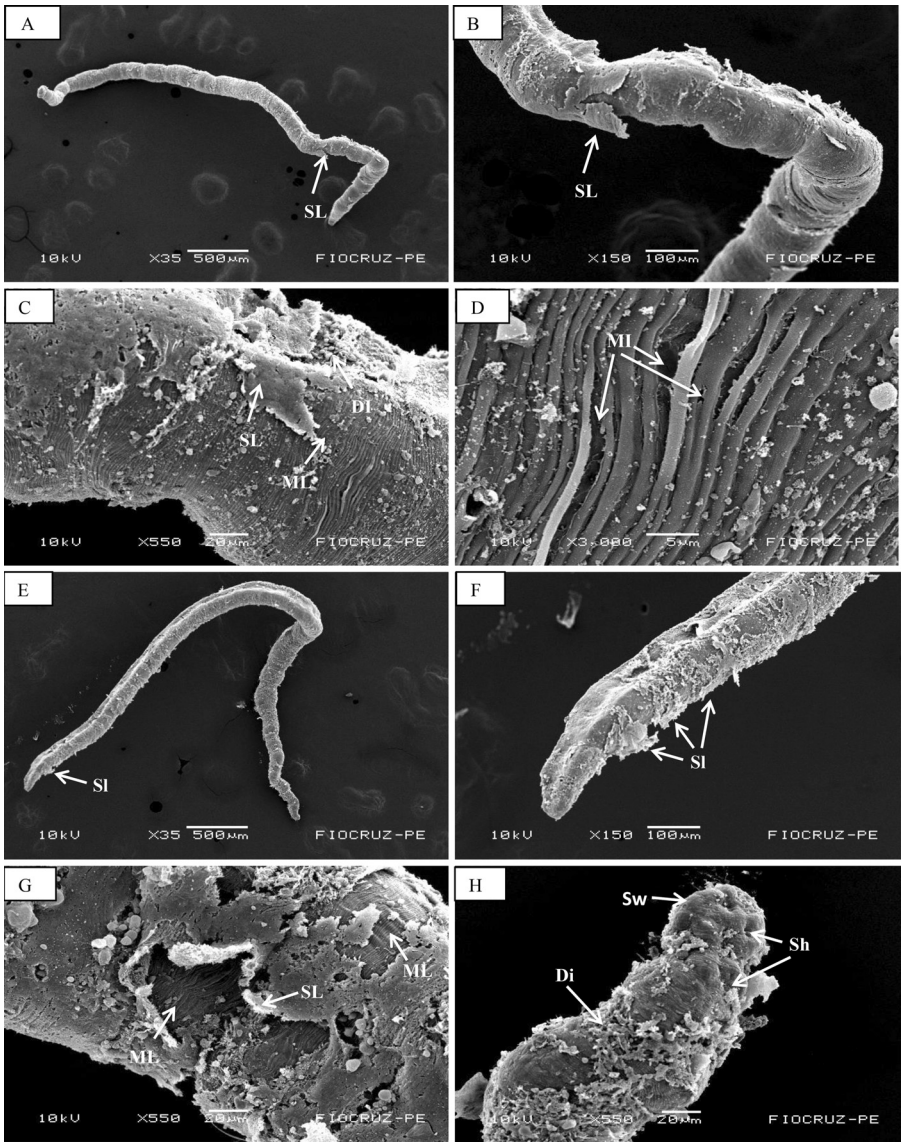


FIG 4 Scanning electron micrograph of adult female *S. mansoni* worms exposed to 80 μg · ml^{-1} LpQM-45. (A to D) Worms after 24 h of incubation showing sloughing (Sl), disintegration (Di) of tegument, and exposure of subtegumental muscle layer (ML) with muscle damage (MI). (E to H) Worms after 48 h of incubation showing sloughing (Sl), disintegration (Di) of the tegument with exposure of muscle layer (ML), swelling (Sw), and shrinking (Sh).

proves the schistosomicidal activity for 2-thioxoimidazolidin-4-one compounds (48).

Another common feature of all active compounds described here is the presence of a hydrazone moiety. Some 9-acridanone hydrazones were found to be effective against *S. mansoni* in mice, killing almost all of the skin schistosomules, when administered at a dose of 100 mg/kg (49). In another study, 9-acridanones derived from thiazoles were effective against *S. mansoni* in the skin phase, killing almost all of the parasites in mice at a dose of 100 mg/kg, 24 h after penetration by cercariae. This same study showed that when the compound is administered to monkeys at a dose of 25 mg/kg, worms and eggs are absent from liver tissue and rectal mucosa 7 days after infection, which constitutes cure (20).

Detailed microscopic observation showed that LpQM-43, LpQM-45, LpQM-47, and LpQM-14 molecules caused alterations in the teguments of the worms compared with the untreated control group. These compounds also appear to influence the oviposition of parasites since no eggs were found in the culture medium.

The thick tegument that covers the entire surface of schistosomes is an important target for drugs because the functioning of the surface membrane and the integrity of the tegument are critical for the survival and proliferation of the *Schistosoma* parasite (13). These structures play vital roles in the immune evasion, nutrient absorption, and cholesterol metabolism of the host (13, 50). Alterations in the surface ultrastructure of schistosome worms have been investigated by a number of authors in order to evaluate antischistosomal drugs (13, 24, 50, 51, 52, 53). The present study thus examined the surface topography of male and female worms to determine the schistosomicidal effect of LpQM-45. We found extensive damage to the tegument in both male and female worms after 24 and 48 h of exposure.

SEM analysis revealed progressive damage to the tegument

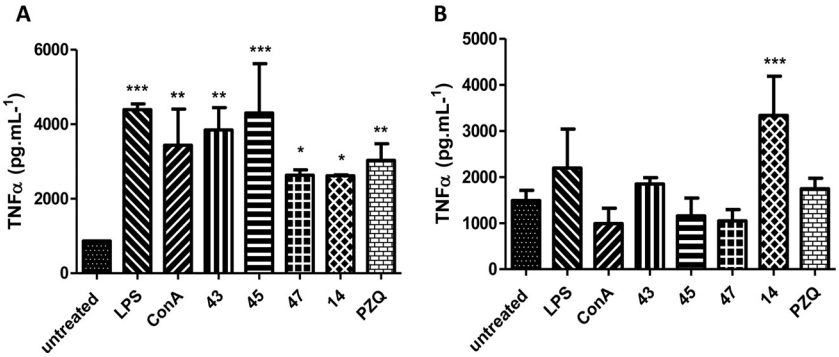


FIG 5 TNF-α production in supernatants of the macrophage culture in the presence of LpQM-43,-45,-47, and -14 and PZQ at 56, 58, 55, 50, and 51 $\mu g \cdot ml^{-1}$, respectively. Assays were performed at 48 h (A) and 72 h (B). The horizontal bars represent median values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

surface, causing destruction of tubercles in the male, extensive sloughing with disintegration of the tegument in the same areas, and exposure of subtegumental tissue and the layer of muscle tissue in both male and female worms. Similar changes occurred in response to different drugs (13, 50). For instance, Manneck et al. (50) have presented a detailed study of tegument surface alterations caused by 100 $\mu g \cdot ml^{-1}$ of mefloquine in *S. mansoni* worms, finding in females extensive sloughing, with the base membrane exposed along with roughened teguments which have already started to disintegrate. The males showed a roughened surface with disintegration of the tegument, resulting in a fibrous appearance, loss of tubercles, spines, and parallel wrinkles. Bertão et al. (13) also found severe damage to the surface of adult male schistosomes caused by exposure to miltefosine, which was characterized by peeling of the tegument, reduction in the size of the spine, erosion, the formation and rupturing of blisters, and the emergence of holes with exposure of layers of muscle tissue.

After 3 h of exposure, PZQ cause severe muscle contraction; the worms became curved, resulting in a decrease in body size (13, 54). In contrast, LpQM-45 caused severe damage to the surface of the worm but no muscle contraction. The lesions were more numerous in parasites exposed to LpQM-45 than in those treated with PZQ. Similarly, miltefosine (13) and thioxoimidazolidine (54) have also been shown to be more effective than PZQ in causing tegument damage in *S. mansoni*. In terms of differences between the changes in males and females, it was noted that the tegument of female worms was slightly more affected and that mefloquine (13) and artemether (55) likewise tend to have a greater effect on females.

The outer membrane and lipid bilayers of *S. mansoni* worms proved to be extremely sensitive to LpQM-45. The morphological

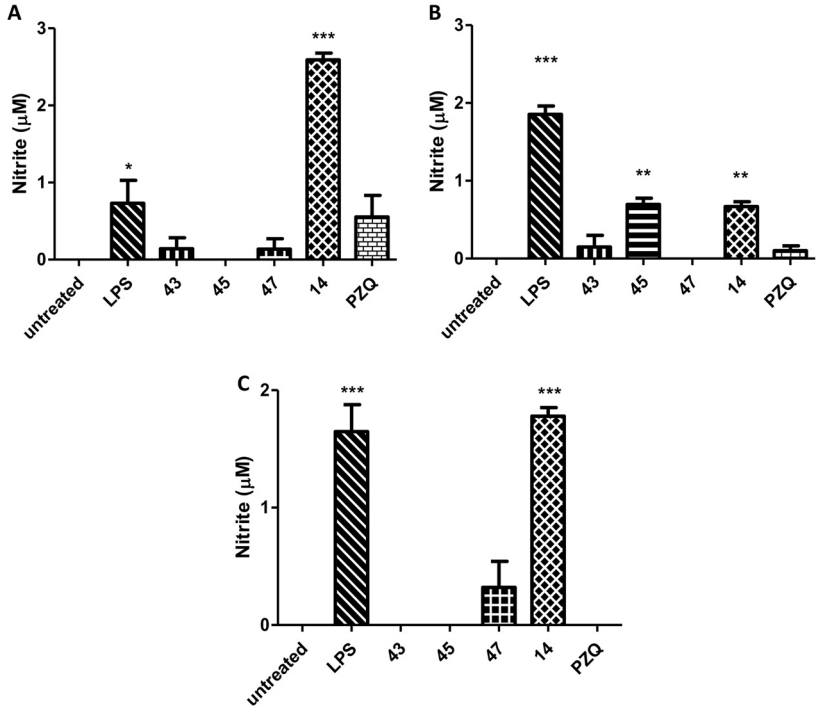


FIG 6 Nitrite production in supernatants of the macrophage culture in the presence of LpQM-43, -45, -47, and -14 and PZQ at 56, 58, 55, 50, and 51 $\mu g \cdot ml^{-1}$, respectively. Assays were performed at 24 h (A), 48 h (B), and 72 h (C). The horizontal bars represent median values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

changes brought on by LpQM-45 may therefore have exerted a profound effect upon the metabolic activity of the parasite and may be the mechanism that causes these compounds to kill the worms. The damage to the tegument along the worm's body may impair the functioning of the tegument and destroy the worm's defense system so that it is easy prey to the host's immune system (24).

The morbidity caused by human schistosomiasis is attributed to the granulomatous inflammation caused by an immune response to the egg antigen. Many studies have shown that in schistosomotic patients, there is a balance between the Th1 and Th2 responses (12). Furthermore, these same studies strongly suggest that resistance to infection is multifactorial and that it cannot be clearly correlated with a single immune mechanism. The Th1 immune response, generated by tumor necrosis factor alpha (TNF-α), IL-1, and IL-6, seems to predominate in the acute phase, but it is replaced by a Th2 immune response upon egg antigen production. The main Th2 cytokine responsible for fibrosis is IL-13 (56). Some mediators such as IL-12, TNF-α, NO, and gamma interferon (IFN-γ) prevent production of excess IL-13 during *S. mansoni* infection (9). For immunological assays, the present study investigated IL-6, IL-10, IL-12, and TNF-α cytokines and NO production in the supernatants of macrophage cultures stimulated *in vitro* with LpQM-43, LpQM-45, LpQM-47, and LpQM-14 compounds.

In terms of cytokine production, statistically significant levels of TNF-α were observed, compared to those of the negative control, for all four compounds analyzed. In the case of LpQM-47, peak production of TNF-α was observed after 24 h, decreased production was noted after 48 h, and there was no significant production after 72 h. LpQM-43 and LpQM-45 affected peak production after 48 h, and LpQM-14 had an effect after 72 h. These results demonstrate that the compounds analyzed stimulate a response with a Th1 cytokine profile. Some compounds that stimulate production of TNF-α have shown antiparasitic activity, including meglumine antimoniate, which has been shown to have antileishmania properties involving increased production of TNF-α (57).

NO has been shown to be an important cytotoxic and cytostatic effector for a number of pathogens, including viruses, bacteria, fungi, and parasites (58). It is implicated as an integral component of the host armament against invading parasites, and evidence has been put forward for the beneficial role of NO during helminthic infections. In the case of *Schistosoma mansoni*, for example, NO plays a role in regulation of egg-induced inflammation, acting as an antifibrogenic substance and preventing hepatocyte death and widespread tissue damage, in addition to being toxic to the schistosomula (9, 59, 60). It is significant that schistosomes are known to be more susceptible to oxidative stress than the hosts (61). The production of NO may also contribute to worm mortality by way of *S*-nitrosylation of cysteine proteases, given that schistosomes express cysteine proteases that play a role in digestion, reproduction, and protein turnover and that this appears to be a common and widespread mechanism (58, 62). Studies have also shown that compounds that induce the NO release possess potential immunomodulatory properties (63–65). The present study found that LpQM-14 stimulated NO production for the three incubation times analyzed, while LpQM-45 stimulated production only after 48 h of exposure. This behavior may indicate the immuno-stimulant properties of these compounds, especially when it is noted that the same compounds caused production of TNF-α.

Conclusions. To sum up, our results indicate that the thiazoles, especially those containing a methoxyl or chloro group, are antiparasitic agents and that they were more potent than their analogs, the thiosemicarbazones. These compounds showed substantial schistosomicidal properties against adult *S. mansoni* worms, with a significant reduction in motility, severe alterations in the integument and mortality of worms, lower toxicity than the reference drug (PZQ), and production of nitric oxide, inhibiting oviposition. The present study revealed LpQM-45 to possess the most effective schistosomicidal properties, suggesting its use as a prototype for the development of new schistosomicidal compounds. The present findings provide a sound basis for further in-depth studies of the antischistosomal properties of phthalyl thiazoles, particularly LpQM-45. It is also important that, in view of the results obtained, further biological studies are needed to shed light on the mechanism(s) of schistosomicidal action.

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