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Cyclohexene-fused 1,3-oxazines with selective antibacterial and antiparasitic action and low cytotoxic effects

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ABSTRACT

Oxazine derivatives, a class of heterocyclic compounds, exhibit a variety of biological properties, such as anticonvulsant and antitumor activities. In this study, we evaluated the effect of two cyclohexene-fused 1,3-oxazines (*cis*-1-benzyl-*N*-phenyl-1,4,4a,5,8,8a-hexahydro-3,1-benzoxazin-2-imine (**1**) and *trans*-*N*-phenyl-1,4,4a,5,8,8a-hexahydro-3,1-benzoxazin-2-imine (**2**)) in cultures of *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Serratia marcescens*, *Shigella flexneri* and *Staphylococcus aureus* by the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Additionally, the *ex vivo* antiparasitic activity of oxazines was assessed against *Schistosoma mansoni*, a helminth that is one of the major agents of the disease schistosomiasis Also, oxazines were evaluated on three tumor cell lines, NCI-H292 (human lung carcinoma), MCF-7 (human breast adenocarcinoma) and HEp-2 (human cervix carcinoma), and two normal cell lines (Vero and red blood cells). Bioassays revealed that oxazine **2** is more effective against bacteria than oxazine **1**, with the lowest MIC and MBC values of 3.91 and 32.5 μg/mL, respectively. Similarly, compound **2** demonstrated higher antiparasitic activity than **1**, and scanning electron microscopy analysis showed several morphological alterations in the tegument of worms in a concentration-dependent manner. In contrast, both oxazines exhibited low cytotoxic effects on cancer and normal cell lines. These results indicated that oxazines exerted direct effects on bacteria and parasite schistosomes. More importantly, since schistosomiasis control programs rely on one drug, praziquantel, oxazines may have the potential to become new antischistosomal agents.

1. Introduction

Chronic diseases such as schistosomiasis and cancer reach a large proportion of the world's population, which may significantly affect the quality of life and even cause death. Cancer affects approximately 15 million people per year and, more than half die. Only in 2013, there were 14.9 million incident cancer cases worldwide and 8.2 million cancer deaths (Global Burden of Disease Cancer Collaboration et al., 2015). On the other hand, schistosomiasis, caused by blood-dwelling flatworms of the genus *Schistosoma,* affects > 250 million people worldwide, with mortality around 280,000 (Inobaya et al., 2014; WHO, 2016). Schistosomiasis is considered the most important helminthic disease of humanity in terms of morbidity and mortality, and is one of the major neglected diseases (Colley et al., 2014). However,

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praziquantel (PZQ) is unfortunately the only drug available for treatment of this disease (Cioli et al., 2014). Thus, the development of new antischistosomal compounds to curb the concern about the development of PZQ resistance should be a high priority (de Moraes, 2015; Mafud et al., 2016a). Regarding PZQ resistance, recent review has been published indicating that mass drug administration, especially in Africa, has led to appearance of reduced efficacy of PZQ, which portends the selection of drug resistant forms of schistosomes (Vale et al., 2017).

Microbial infections are the cause of other serious impacts on human health, especially due to multi-drug resistance. So too are opportunistic mycoses, which have become increasingly common and are identified as a major cause of morbidity and mortality. The rapid emergence and spread of multidrug-resistant bacteria increases the need for identification and control of the pathogens involved (Luepke et al., 2017). Bacterial resistance is very dangerous because of its high prevalence in hospitals as well as its relationship with the failure in treatment with antimicrobials agents. These factors increase the mortality and the costs related to the treatment of patients suffering from those diseases (Sartelli et al., 2016).

In recent years, an increasing number of studies have shown that oxazine derivatives, a class of heterocyclic compounds that contain one nitrogen and one oxygen atom, exhibit a variety of biological properties, such as sedative, anticonvulsant, analgesic, antipyretic, anticoagulant, and antimalarial activities (Sindhu et al., 2013). Due to the biological significance of these compounds, they could be used for the development of new chemical entities to combat various diseases. On the other hand, the antibacterial, antitumor, and mainly anthelmintic properties of oxazine derivatives have been little studied.

The present study investigated the antimicrobial and antischistosomal properties of two partially saturated derivatives of oxazines, named *cis*-1-benzyl-*N*-phenyl-1,4,4a,5,8,8a-hexahydro-3,1-benzoxazin-2-imine (**1**) and *trans*-*N*-phenyl-1,4,4a,5,8,8a-hexahydro-3,1-benzoxazin-2-imine (**2**). Additionally, the cytotoxicity of oxazines was evaluated on three tumor and two normal cell lines.

2. Material and methods

2.1. Drugs and instrumentation

The oxazine derivatives *cis*-1-benzyl-*N*-phenyl-1,4,4a,5,8,8a-hexahydro-3,1-benzoxazin-2-imine (**1**) and *trans*-*N*-phenyl-1,4,4a,5,8,8a-hexahydro-3,1-benzoxazin-2-imine (**2**) were prepared by modification according to previously reported procedures (Peláez et al., 2008; Fülöp et al., 1987). The synthesis of both oxazines started from commercial *cis*-1,2,3,6-tetrahydrophtalic anhydride, which was used as received, to prepare the *cis* derivative (**1**), and turned into its *trans* isomer to start the synthetic path ending in compound (**2**). After some synthetic steps already reported, (**1**) there were new reactions under new conditions, and these included the esterification reaction of *cis*-6-aminocyclohex-3-ene-1-carboxylic acid to afford *cis*-methyl-6-aminocyclohex-3-ene-1-carboxylate, while the *trans*- derivative was prepared from the ester *trans*-methyl-6-aminocyclohex-3-ene-1-carboxylate generated, in turn, from the *trans*-6-aminocyclohex-3-ene-1-carboxylic acid.

Melting points were determined in open glass capillaries with a Scientific Electrothermal Digital apparatus (model IA9100). NMRs were recorded in $CDCl_3$ with a Bruker Avance II 400 MHz spectrometer (BBI probe, z gradient) (^1H at 400 MHz and ^{13}C at 100 MHz). Chemical shifts are reported in parts per million (ppm) downfield from TMS. The spectra were measured at 22 °C. The infrared spectra were recorded at room temperature, with a Bruker IFS-28 spectrometer, in the range 4000–400 cm^{-1}. The IR spectra were recorded from solid samples in KBr pellets (spectral resolution 2 cm^{-1}), at room temperature.

2.2. Bacteria and determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Bacterial strains used in this study were obtained from the National Institute for Quality Control in Health (INCQS/FIOCRUZ, Brazil). The microorganisms used were: *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 10708), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923). The antibacterial effect was evaluated by microdilution methods, as recommended by Clinical and Laboratory Standards Institute (CLSI). Stock solutions of oxazine derivatives (**1** and **2**) were prepared using as a solvent a 20% DMSO aqueous solution. After this procedure, 100 μL of this dilution was transferred to a microplate with 100 μL of Muller-Hinton broth, and eight serial dilutions were performed, which led to concentrations from 500 to 3.91 μg/mL of both oxazines. The inoculum with 5×10^5 CFC/mL was added to each well and Muller-Hinton broth and gentamicin were used, respectively, as a sterility control and an antimicrobial reference control, the latter at concentrations from 0.8 to 0.0125 μg/mL. The microplates were incubated under aerobic conditions for 24 h at 37 °C, and 2,3,5-triphenyltetrazolium chloride (TTC, 10 μL, 2%) was added to each tissue well to detect active bacterial metabolism (the colorless solution changed to red). The MIC was defined as the lowest concentration of the oxazine (**1** and **2**) that inhibited bacterial growth. To determine the MBC, aliquots of these microplates (10 μL) were transferred to Petri dishes with Muller-Hinton agar and were incubated for 24 h at 37 °C. The appearance of colonies of bacteria indicates that some solutions of oxazines were not able to kill 99.9% (or more) of the bacterial inoculum used. The assays were performed in triplicate.

2.3. Parasite and determination of schistosomicidal activity

Schistosoma mansoni (BH strain Belo Horizonte, Brazil) worms are maintained in *Biomphalaria glabrata* snails as intermediate hosts and *Mesocricetus auratus* hamsters as definitive host at the Adolfo Lutz Institute (São Paulo, Brazil), according to standard procedures previously described (Moraes, 2012). All experiments were authorized by the Committee for Ethics in Animal Care of Institute Adolf Lutz, in accordance with nationally and internationally accepted principles for laboratory animal use and care (CEUA, 11.794/08). The study was conducted in adherence to the institution's guidelines for animal husbandry. Female hamsters, weighting 20–22 g, were infected by subcutaneous injection of 150 cercariae. After 7 weeks, the *S. mansoni* adults were recovered from a hamster previously infected with 150 *cercariae*, by infusion in RPMI 1640 medium with heparin. For preparations and culture of *S. mansoni*, the parasites were washed in RPMI 1640 medium maintained at pH 7.5 with 20 mM HEPES buffer (N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)) supplemented with 10% fetal bovine serum, 200 μg/mL of streptomycin and 200 IU/mL of penicillin. After washing, a pair of adult parasites were transferred to each well (24 pairs in total) with the same medium at 37 °C in an atmosphere of CO_2 5%, as previously described (de Moraes et al., 2011; de Moraes et al., 2013). Compounds **1** and **2** were dissolved in 0.5% DMSO and tested at concentrations of 100, 50, 25, and 10 μM. The control groups were performed with incubation of parasites in RPMI 1640 as a negative control group and 5 μM of PZQ in the positive control group. The parasites were maintained for 120 h and monitored every 24 h. The effect of both oxazines and the standard drug (PZQ) was evaluated using microscopes, with emphasis on motor activity, alteration in the tegument, and mortality rate (Veras et al., 2012; de Almeida et al., 2016).

2.4. Scanning electron microscopy studies

To show morphological alterations in adult schistosomes caused by

oxazine **2**, the most active compound, worms were rinsed twice in PBS, and fixed in 1 mL 2.5% glutaraldehyde for 3–24 h at room temperature. Samples were prepared as previously described (Guimarães et al., 2015). Briefly, specimens were air-dried, mounted on stubs and metalized with gold using a Sputter Coater. Samples were then visualized using a high-resolution SEM accelerating voltage of 20 kV (Jeol-JSM-6460LV).

2.5. Cell cultures and determination of the effective inhibition concentration against cell growth

The human lung carcinoma cells (NCI-H292), human breast adenocarcinoma cells (MCF-7), and human cervix carcinoma cells (HEp-2) were obtained from the Cell Bank of Rio de Janeiro (BCRJ, Brazil). Vero, a monkey kidney cell line, was obtained from the American Type Culture Collection (ATCC CCL-81; Manassas, VA). All cell lines were cultured in RPMI 1640 or DMEM media supplemented with 10% fetal bovine serum and 1% antibiotic, and then incubated at 37°°C in an atmosphere containing 5% CO_2 (Silva et al., 2015; de Moraes et al., 2015). Oxazines **1** and **2** were solubilized in sterile dimethylsulfoxide (DMSO) and diluted with culture medium to achieve a final concentration of 1% DMSO. Compounds were serially diluted in RPMI 1640 medium to obtain the final concentration of 100 μM and added to cells that were seeded at 1×10^5 cells/mL in 96-well plates. The plates were incubated for 72 h at 5% of CO_2 and 37 °C. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added (25 μL, 5 mg/mL) and the plates were further incubated for 3 h. After dissolving the precipitate in DMSO, the absorbance was read in a plate spectrophotometer at 595 nm (Berridge et al., 1996).

2.6. Hemolytic activity

The hemolytic assay was performed using sheep red blood cells (Marani et al., 2015; Wei et al., 2016). Erythrocytes were separated from plasma by centrifugation and washed three times (3000 × *g*, 5 min) with 150 mM NaCl. Red blood cells suspension was added to an equal volume of each compound solution at 100 μM. The mixtures were incubated for 1 h at 37 °C and centrifuged at 3000 g for 2 min. The value of absorbance of the supernatant was measured at 550 nm. NaCl 150 mM and Triton-X (0.1% v/v) were used as negative and positive controls, respectively. Experiments were performed in triplicate.

2.7. Statistical analysis

Statistical tests were performed with GRAPHPAD PRISM (version 6.0) software. The results were expressed as mean ± SEM (standard error of mean) and statistical evaluation was performed using analysis of variance (ANOVA) followed by Student-Newman-Keuls test with *post hoc* test. The results of the statistical analysis among the groups were considered significant when $p < 0.05$.

3. Results

3.1. Synthesis of oxazine derivatives

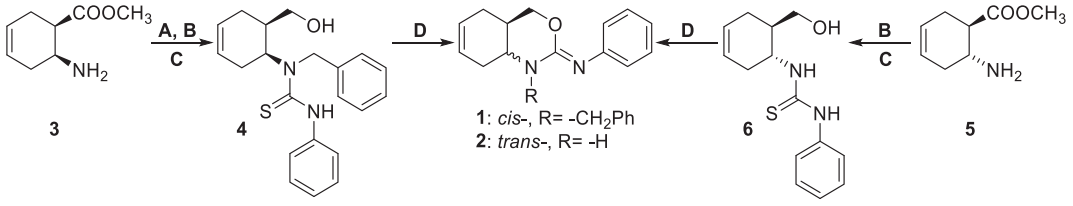
Oxazines **1** and **2** were obtained through the synthetic pathways described in Scheme 1. When *cis*-methyl-6-aminocyclohex-3-ene-1-carboxylate (**3**) was treated with benzoyl chloride (step A), *cis*-methyl-6-benzamidocyclohex-3-ene-1-carboxylate was formed in good yield. Simultaneous LAH reduction of the ester and carboxamide function resulted in *cis*-(6-(benzylamino)cyclohex-3-en-1-yl)methanol (step B), which after treatment with phenyl isothiocyanate afforded *cis*-1-benzyl-1-(-6-(hydroxymethyl)cyclohex-3-en-1-yl)-3-phenylthiourea (**4**) (step C). Starting from *trans*-methyl-6-aminocyclohex-3-ene-1-carboxylate (**5**) and following steps B and C, *trans*-1-(6-(hydroxymethyl)cyclohex-3-en-1-yl)-3-phenylthiourea (**6**) was obtained. Reacting thioureas **4** and **6** with methyl iodide, thiomethyl ethers were formed which were transformed without isolation in alkaline medium to oxazine derivatives **1** and **2** with methyl mercaptol elimination (70–80% yield, step D).

The synthesis of compounds **1** and **2** followed literature procedures (Peláez et al., 2008; Fülöp et al., 1987). All chemical and physical properties of **1** and **2** were similar to those reported by Vainiotalo et al. (1991) for **1** and Fülöp et al. (1987) for **2**. Compound **1** was synthesized in 79% yield and obtained with 97.3% purity, while compound **2** was produced in 80% yield and 99.8% purity. Characterization of synthesized compounds: **Compound 1**: **mp** (°C): 86–88 (Lit.: 89–91), Purity: 97.3% Yield: 79%. **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.86 (1H, d, J = 18.3 Hz); 2.15 (1H, m); 2.15 (1H, m); 2.36 (1H, dm, J = 16.4 Hz); 2.43 (1H, m); 2.47 (1H, m); 3.42 (1H, m); 4.06 (1H, dd, J_1 = 10.3 Hz, J_2 = 5.4 Hz, J_3 = 1.4 Hz); 4.14 (1H, dd, J_1 = 12.4 Hz, J_2 = 10.8 Hz); 4.32 (1H, d, J = 15.4 Hz); 5.30 (1H, d, J = 15.3 Hz); 5.55 (2H, s coalesced signals); 6.91 (1H, tt, J_1 = 7.4 Hz, J_2 = 1.2 Hz); 6.98 (2H, dd, J_1 = 8,5 Hz, J_2 = 1.2 Hz); 7.22 (2H, t, 8.3 Hz); 7.27 (1H, d, J = 7.2 Hz); 7.33 (2H, t, J = 7.3 Hz); 7.4 (2H, d, J = 7.4 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 26.0, 27.2, 30.5, 50.5, 51.5, 67.6, 121.3, 123.2, 123.8, 124.2, 127.3, 128.1, 128.5, 128.6, 138.6, 148.5, 148.8. **IR** (KBr, cm^{-1}): 3057, 3028, 2922, 2908, 2895, 1616, 1575, 1487, 1450, 1440, 1261, 1224, 1114, 1035, 773, 732, 696.

Compound 2: **mp** (°C): 190–191 (Lit.: 192–193). **Purity**: 99.8%. **Yield**: 80%. **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.76 (1H, tm, J = 14.1 Hz), 1.94 (2H, m), 2.17 (1H, dm, J = 16.8 Hz), 2.45 (1H, d, J = 15.3 Hz), 2.28 (1H, td, J_1 = 10.8 Hz, J_2 = 5.4 Hz), 3.90 (1H, dd, J_1 = 10.9 Hz, J_2 = 10.9 Hz), 4.27 (1H, dd, J_1 = 10.3 Hz, J_2 = 4.3 Hz), 5.69 (2H, s colesced signals), 6.94 (1H, t, J = 7.2 Hz), 7.26 (5H, m). **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 27.3, 34.2 (× 2), 52.7, 70.2, 119.5, 121.8, 125.0, 126.7, 128.9. **IR** (KBr, cm^{-1}): 3449, 3225, 3132, 3107, 3020, 2916, 2893, 2860, 2829, 1618, 1585, 1485, 1489, 1468, 1294, 1202, 1184, 1157, 1082, 854, 781, 704, 669.

3.2. Antibacterial activities

In this study, the MIC and the MBC of oxazines **1** and **2** were investigated against the Gram-positive bacteria and Gram-negative bacteria and the results are summarized in Table 1. In general, oxazine **2** was more effective against bacteria than oxazine **1**. Interestingly, oxazine **2** was able to inhibit both Gram-positive and Gram-negative strains, especially for *S. aureus* and *S. flexneri*, which had the same MIC values of 3.91 μg/mL (about 10 μM). Oxazine **2** also showed moderate action against *E. coli* with MIC values of 62.5 μg/mL, and relatively weak activity against *B. cereus*, *E. faecalis*, *S. enterica*, and *S. marcescens,* with the same MIC values of 500 μg/mL. All tested strains presented the predicted susceptibility to the standard antibiotic gentamicin



Scheme 1. Synthesis of oxazines **1** and **2**. A: Benzoyl chloride/TEA. B: $LiAlH_4$ (LAH)/THF. C: Phenyl isothiocyanate/Toluene. D: CH_3I/MeOH then KOH/MeOH.

Table 1
Antimicrobial activities of oxazine **1** and **2.**

Strains	Gentamicin		Oxazine 1		Oxazine 2	
	MIC (μg/mL)	MCB (μg/mL)	MIC (μg/mL)	MCB (μg/mL)	MIC (μg/mL)	MCB (μg/mL)
Gram +						
B. cereus	0.40	0.40	500	500	500	> 500
E. faecalis	0.40	0.40	> 500	> 500	500	500
S. aureus	0.025	0.025	250	500	3.91	31.25
Gram −						
E. coli	0.025	0.40	500	500	62.5	125
K. pneumoniae	0.05	0.05	> 500	> 500	> 500	> 500
S. enterica	0.05	0.05	125	> 500	500	> 500
S. marcescens	0.025	0.025	250	500	500	> 500
S. flexneri	0.025	0.025	500	500	3.91	125

Minimum Inhibitory Concentration (MIC); Minimum Bactericidal Concentration (MCB).

(MIC ≤ 0.5 μg/mL). In order to address the bacteriostatic and bactericidal properties of the oxazines, the MBC values were also evaluated. As shown in Table 1, the MBC values ranged from 31.25–500 μg/mL.

3.3. Antischistosomal properties

The effect of incubation with different concentrations of oxazines **1** and **2** on the motor activity and viability of both male and female *S. mansoni* adult worms for up to 72 h is shown in Table 2. Both **1** and **2** exhibited anthelmintic activity, but **2** had considerably higher activity than **1** against schistosomes, reducing motility and causing death at concentration 25 μM, while **1** was inactive at concentrations < 100 μM. Interestingly, male worms were more susceptible to oxazines than females. For example, when tested at concentrations of 50 μM of oxazine **2**, all *S. mansoni* males showed reduced motor activity and died after 24 h, whereas no mortality was observed in the female worms. Positive control (PZQ, 5 μM) resulted in the death of all parasites within 24 h, whereas no mortality was observed in schistosomes of the negative (RPMI medium) and solvent control (RPMI medium plus 0.5% DMSO) groups.

Table 2
Effect of oxazine **1** and **2** against *S. mansoni* adult worms.

Groups	Period of incubation (h)	Mortality(%)[a]		Reduction in motor activity (%)[a]	
		Male	Female	Male	Female
Control[b]	24	0	0	0	0
	48	0	0	0	0
	72	0	0	0	0
DMSO 0.5%	24	0	0	0	0
	48	0	0	0	0
	72	0	0	0	0
Praziquantel	24	100	100	100	100
5 μM	48	100	100	100	100
	72	100	100	100	100
Oxazine **1**					
100 μM	24	20	0	20	0
	48	50	0	100	0
	72	50	0	100	0
50 μM	24	0	0	0	0
	48	0	0	0	0
	72	0	0	0	0
Oxazine **2**					
100 μM	24	100	50	100	60
	48	100	50	100	70
	72	100	50	100	0
50 μM	24	100	0	100	0
	48	100	0	100	0
	72	100	40	100	40
25 μM	24	0	0	0	0
	48	0	0	20	0
	72	40	10	50	10
10 μM	24	0	0	0	0
	48	0	0	0	0
	72	0	0	0	0

[a] Percentages relative to 20 worms investigated.
[b] RPMI 1640.

In addition to the *in vitro* antiparasitic activity against *S. mansoni* described above (Table 2), we also analyzed the effects of oxazine **2** on the tegument of schistosomes using a scanning electron microscope to examine the surface of male worms exposed to different concentrations of **2**. As shown in Fig. 1, morphological alterations of the tegument on the *S. mansoni* surface were detected after exposure to a medium containing oxazine **2** at concentrations of 25 μM, 50 μM, and 100 μM, whereas no tegumental surface alterations were observed in the negative control group. More specifically, the teguments of schistosome incubated with **2** showed massive sloughing, as well as shrinking and disintegration of the tubercles in a concentration-dependent manner.

3.4. Cytotoxicity and hemolytic activities

The inhibitory activity against three tumor cell lines (NCI-H292, MCF-7 and HEp-2) and normal cell line (Vero) was evaluated by MTS assay. Any compound should present, at least, 50% of cell growth inhibition to be considered as cytotoxic. As shown in Table 3, oxazine **1** and **2** had a low cytotoxic effect on tumor cells lines when tested at concentration of 100 μM. Indeed, both compounds affected < 30% of inhibition of cell growth, whereas positive control (doxorubicin) caused 62 to 88% of inhibition. In addition, oxazines at 100 μM did not exhibit any hemolytic effect on sheep red blood cells.

4. Discussion

Recently, a large number of 1,3-oxazines have been synthesized and subsequently demonstrated to possess biological and pharmacological activities, such as antimicrobial, anticancer, analgesic, and anti-inflammatory properties (Sindhu et al., 2013). In this study, we evaluated, for the first time, the effect of two cyclohexene-fused 1,3-oxazines (**1**) and (**2**) against Gram-positive and Gram-negative bacteria, *S. mansoni* adult worms, and three tumor cell lines. Results showed, in general, that oxazine **2** was more active than oxazine **1** when exposed to bacteria and parasites, and both compounds had a low cytotoxic effect.

In order to perform the tests of antibacterial activity for oxazines, strains with different patterns of pathogenicity and susceptibility to antimicrobial agents were used. The MIC procedure was important to evaluate the activity of compounds. Based on this, our results showed that oxazine **2** was capable of inhibiting the bacterial growth of both Gram-positive and Gram-negative bacteria. The most susceptible bacteria strains were *Staphylococcus aureus* and *Shigella flexneri* with a MIC value of 3.91 μg/mL (about 17 μM). Despite lower than gentamicin, the antibacterial activities of cyclohexene-fused 1,3-oxazine **2** against *S. aureus* are similar to some of the best 1,2- or 1,3-oxazine derivatives previously synthesized (D'Andrea et al., 2005; Verma et al., 2012), which displayed activity with MIC values ranging from 2 to 8 μg/mL. However, it is difficult to compare the antibacterial activities of **2** with other previous synthesized oxazine derivatives, since they are structurally different. Also, it is important to point out that no previous antibacterial studies with cyclohexene-fused 1,3-oxazine derivatives has been reported. No significant difference in susceptibility between Gram-positive bacteria and Gram-negative bacteria was found, similarly to studies with other oxazine derivatives which revealed inhibition of both Gram-positive and Gram-negative strains (Mathew et al., 2010; Mayekar et al., 2011; Didwagh and Piste, 2013; Kamala et al., 2016).

With respect to the anthelmintic tests, both oxazines **1** and **2** exhibited anthelmintic activity, but **2** was more potent than **1** in reducing motility and causing death of the *S. mansoni* adult worms. Also, despite

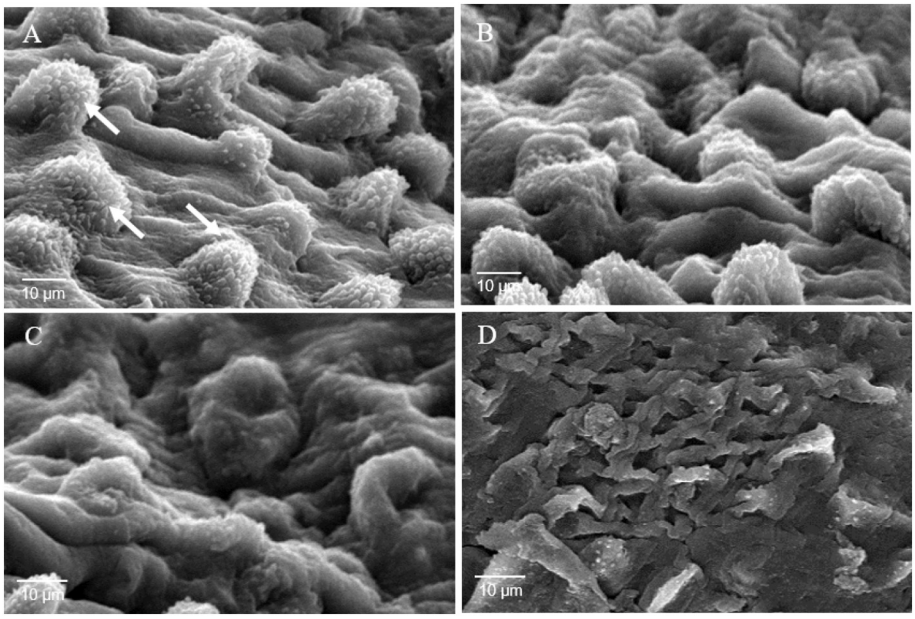


Fig. 1. Scanning electron microscopy observations of *ex vivo S. mansoni* male worms after incubation with oxazine **2**. Adult worms were incubated in 24-well culture plates containing RPMI 1640 medium with 0.5% DMSO and treated with oxazine **2** at different concentrations. Control after 72 h, showing tubercles with numerous spines (white arrow) (A). Worm treated with oxazine **2** at concentrations of 25 µM (B), 50 µM (C), and 100 µM (D).

the schistosomicidal activity of **2** is lower than PZQ at the same comparative concentration, it could be considered a promising lead schistosomicidal compound, since there are little no cytotoxic compounds able to cause death in adult schistosomes. The results also showed that oxazines acted preferentially against male rather than female worms. These differences in the mortality between male and female parasites have been observed with several antischistosomal compounds. For example, studies with the terpene phytol showed that females were more susceptible than males (de Moraes et al., 2014), whereas studies with the terpene nerolidol showed that male worms were more susceptible than females (Silva et al., 2014). Similar results described here, which showed differences between male and female schistosomes, also suggest that the mechanism of action in relation the sexes of schistosomes remains to be investigated.

The schistosome's tegument, which is the interface between host and parasite, is an important target for antischistosomal drugs, and alterations in the surface topography of worms have been described in several *in vitro* and *in vivo* studies (Guimarães et al., 2015; de Moraes et al., 2014; Silva et al., 2014; Mafud et al., 2016b; Quelemes et al., 2015). Because oxazine **2** was active against adult schistosomes, we further analyzed its effects on *S. mansoni* tegument. To better understand the effects of oxazine **2** on the worm's tegument, we used a scanning electron microscope to examine the surface of worms under different concentrations. Oxazine **2** induced severe tegumental damage in male schistosomes and pronounced changes in the aspect of tubercles, which often appeared collapsed and disrupted. These effects were similar to those reported in studies with other compounds, such as piplartine (Moraes et al., 2011), (+)-limonene epoxide (Moraes et al., 2013), cardamonin (de Castro et al., 2015), and licoflavone B (Aleixo de Carvalho et al., 2015). Likewise, it is known that PZQ exhibits potent *in vitro* effects against schistosomes and it causes extensive tegumental alterations in a concentration-dependent manner (Cioli et al., 1995; Pica-Mattoccia and Cioli, 2004).

Also, the tegument of schistosomes is usually considered for its key role in nutrient uptake, secretory functions and parasite protection against the host immune system. Also, in case of severe damages on the tegument, the host's immune response can affect the reparation process (Veras et al., 2012). The tegument destruction induced by oxazine **2** will probably not allow reparation, since the damages lead to extensive peeling of the tegument surface, as well as formation and collapsing of tubercles, indicating straight damage to the cells in a direct dose-dependent effect. Since schistosomiasis control programs rely on one drug, PZQ, these results suggest that oxazine **2** may have the potential to become a new antischistosomal agent.

During recent years, despite lack of interest from the pharmaceutical industry, considerable efforts have been made in the development of novel schistosomicidal agents. As a result, many compounds with promising antischistosomal properties have been identified by researchers. Among these, piplartine (piperlongumine) and curcumin have also been described as promising anticancer agents (Bar-Sela et al., 2010; Bezerra et al., 2013). Additionally, artemisinin and its derivatives, some of the most interesting antischistosomal compounds (Keiser and Utzinger, 2012), have also been shown to exhibit anticancer properties (Hooft van Huijsduijnen et al., 2013). Furthermore, the antitumor activity of 1,3-oxazine derivatives has been described in different tumor cell lines (Ouberai et al., 2006; Narita et al., 2009). In this study, we assessed the toxicity properties of oxazines **1** and **2** on three human tumor cell lines (NCI-H292, MCF-7, and HEp-2) and two normal cell lines (Vero and erythrocytes). Nevertheless, our results show that both compounds show no cytotoxicity toward the tested cancer and normal cell lines when tested at 100 µM. Thus, oxazine **2** exhibited antiparasitic activity without cytotoxicity to mammalian cells.

Table 3
Cell growth inhibition and hemolytic activity of oxazine **1** and **2**.

Compounds	NCI-H292 inhibition (%)	MCF-7 inhibition (%)	HEp-2 inhibition (%)	Vero inhibition (%)	Hemolytic activity (%)
Dox	88.4 ± 11.5	62.2 ± 0.7	80.0 ± 0.4	n.d.	n.d.
1	13.2 ± 1.4	21.8 ± 8.9	28.0 ± 1.4	10.7 ± 3.1	0.9 ± 0.5
2	18.4 ± 0.4	28.4 ± 4.9	19.3 ± 3.1	12.4 ± 3.6	1.1 ± 0.8

Compounds were tested at 100 µM.
Doxorubicin (DOX). Human lung carcinoma (NCI-H292). Human breast carcinoma (MCF-7). Human cervix carcinoma (HEp-2). Animal kidney cells (Vero). n.d: Not determined.

Considering only the chemical structures of oxazines **1** and **2** and their influence on the activity of both compounds, it can be speculated that the -NH- moiety in **2** appears to have a vital role in the activity of this molecule, since in the oxazine **1** N-H bound is not present. Then, we hypothesized about the capability of oxazine **2** acting as a hydrogen bond donor, allowing better interactions than the bulky benzyl substituent (Ph-CH_2-) in **1**. Nevertheless, this observation is based only on two compounds and should be confirmed. Regarding this and due to the complexity of drug mechanisms and their mode of action, future biological experiments are necessary to clarify mechanisms of action, as well as the structure-activity relationship.

In conclusion, hexahydro-benzoxazin imine derivatives have displayed selective antibacterial and schistosomicidal actions, and low cytotoxic effects. Both Gram-positive and Gram-negative bacteria were susceptible to oxazine **2**. Additionally, a remarkable reduction in the motor mobility of the *S. mansoni* parasite, followed by mortality and tegumental alterations, was detected in the presence of **2**. Considering the obtained results, oxazine **2** is a promising compound that could be evaluated in additional chemotherapeutic investigations.

Conflict of interest

We declare no conflict of interest.

Transparency document

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