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Antiparasitic activity of piplartine (piperlongumine) in a mouse model of schistosomiasis

Ana.C. Mengarda[a], Poliana S. Mendonça[b], Cristiane S. Morais[a], Ramon M. Cogo[a], Susana F. Mazloum[a], Maria C. Salvadori[c], Fernanda S. Teixeira[c], Thiago R. Morais[a], Guilherme M. Antar[d], João Henrique G. Lago[b], Josué Moraes[a,*]

[a] Núcleo de Pesquisa em Doenças Negligenciadas, Universidade Guarulhos, Praça Tereza Cristina, 229, Centro, 07023-070, Guarulhos, SP, Brazil
[b] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, São Paulo, 09210-180, Brazil
[c] Instituto de Física, Universidade de São Paulo, São Paulo, SP, Brazil
[d] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, 05508-090, São Paulo, Brazil

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ABSTRACT

Schistosomiasis is one of the most important parasitic infections in terms of its negative effects on public health and economics. Since praziquantel is currently the only drug available to treat schistosomiasis, there is an urgent need to identify new anthelmintic agents. Piplartine, also known as piperlongumine, is a biologically active alkaloid/amide from peppers that can be detected in high amounts in the roots of *Piper tuberculatum*. Previously, it has been shown to have *in vitro* schistosomicidal effects. However, its anthelmintic activity in an animal host has not been reported. In the present work, *in vivo* antischistosomal properties of isolated piplartine were evaluated in a mouse model of schistosomiasis infected with either adult (patent infection) or juvenile (pre-patent infection) stages of *Schistosoma mansoni*. A single dose of piplartine (100, 200 or 400 mg/kg) or daily doses for five consecutive days (100 mg/kg/day) administered orally to mice infected with schistosomes resulted in a reduction in worm burden and egg production. Treatment with the highest piplartine dose (400 mg/kg) caused a significant reduction in a total worm burden of 60.4% ($P < 0.001$) in mice harbouring adult parasites. *S. mansoni* egg production, a process responsible for pathology in schistosomiasis, was also significantly inhibited by piplartine. Studies using scanning electron microscopy revealed substantial tegumental alterations in parasites recovered from mice. Since piplartine has well-characterized mechanisms of toxicity, is easily available, and is cost-effective, our results indicate that this bioactive molecule derived from medicinal plants could be a potential lead compound for novel antischistosomal agents.

1. Introduction

Schistosomiasis, also known as snail fever and bilharzia, is a major parasitic disease caused by blood-dwelling flatworm trematodes of the genus *Schistosoma*. The disease infects more than 200 million people with approximately 800 million at risk of infection, especially in poor and rural communities without access to safe drinking water and adequate sanitation. Schistosomiasis transmission has been reported in 78 countries, and there are six known human-pathogenic species, of which *Schistosoma mansoni, Schistosoma haematobium*, and *Schistosoma japonicum* are the most prevalent. *S. mansoni* is found where its intermediate host, the freshwater snail *Biomphalaria glabrata*, is available, notably in South America and the Caribbean, Africa, and the Middle East (WHO, 2019). Disease morbidity, due to inflammation and fibrosis associated with the parasite's eggs, is typically chronic, and can be painful and debilitating, hampering both personal productivity and community development (McManus et al., 2018).

As a disease of poverty, also known as a neglected disease, schistosomiasis has attracted little pharmaceutical investment despite exerting a huge influence globally on health and welfare (Lago et al., 2019). In fact, therapy and control of schistosomiasis rely on just one drug, praziquantel. Although effective against all *Schistosoma* species, the drug mainly targets the adult worms, while the immature stages are less susceptible. Additionally, considering that *Schistosoma* isolates with reduced susceptibilities to praziquantel have already been identified, parasite resistance remains a major concern (Vale et al., 2017). As a result, the need to develop alternative antischistosomal drugs has been stressed (Mafud et al., 2016a; Lago et al., 2018).

* Corresponding author.
E-mail address: josuem@usp.br (J. Moraes).

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Natural products, especially from medicinal plants, present a diversity of molecules and have been a reliable source of chemotherapeutic agents, including in anthelmintic drug discovery (de Moraes, 2015). Piplartine, as known as piperlongumine, is an alkaloid/amide found in several species of the genus *Piper* (Piperaceae family), especially in the roots of *Piper tuberculatum* (Cícero Bezerra Felipe et al., 2007). Detected in high amounts, piplartine is well known for its promising anticancer properties. Previous reports indicated that piplartine exhibited a wide range of pharmacological activities, including antiplatelet aggregation, anti-atherosclerotic, antidepressant, anxiolytic, antinociceptive, antidiabetic, antibacterial, antifungal, leishmanicidal, and trypanocidal properties (for review see Bezerra et al., 2013). Moreover, toxicological examinations of piplartine indicate a good safety profile (Bezerra et al., 2013). Our group previously demonstrated that piplartine at submicromolar concentrations possesses *in vitro* schistosomicidal activity against adult (Moraes et al., 2011) and immature stages of *S. mansoni* (de Moraes et al., 2012). We also showed that piplartine caused morphological alterations in the tegument of parasites in a concentration-dependent manner (Moraes et al., 2011; de Moraes et al., 2012). In addition, egg production was reduced when worms were exposed to piplartine (Moraes et al., 2011). However, *in vivo* studies to determine the chemotherapeutic potential of piplartine in the treatment of schistosomiasis have not yet been described.

The aim of the present study was to evaluate the *in vivo* antischistosomal activity of piplartine administered by the oral route in mice infected with either adult (patent infection) or immature (prepatent infection) stages of *S. mansoni*.

2. Material and methods

All experiments were conducted in conformity with Brazilian law following the Guidelines for Care and Use of Laboratory Animals [Law 11790/2008]. The protocol for experimental design was approved by the Comissão de Ética no Uso de Animais (CEUA), Brazil [protocol no. 31/2017]. Animal studies are reported in compliance with the ARRIVE guidelines.

2.1. General experimental procedures

Silica gel (Merck, 230−400 mesh) was used for column chromatography (CC) and silica gel 60 PF_{254} (Merck) was used for analytical TLC separations. NMR spectra were recorded a Bruker Avance II spectrometer at 300 MHz (^{1}H nucleus) and 75 MHz (^{13}C nucleus) using $CDCl_3$ (Aldrich) as solvent and TMS as the internal standard. HRESIMS spectrum was recorded on a Bruker Daltonics MicroTOF QII spectrometer.

2.2. Plant material

Roots of *Piper tuberculatum* were collected in Rio de Janeiro Botanical Garden, Rio de Janeiro State, Brazil, in August 2018 (registration code at SISGEN A4123E4). The plant material was identified by MSc. Guilherme M. Antar and a voucher specimen (A2372) has been deposited in the Herbarium of Institute of Biosciences, University of São Paulo, SP, Brazil.

2.3. Extraction and isolation of piplartine

Dried and powdered roots of *P. tuberculatum* (253 g) were exhaustively extracted using a mixture of *n*-hexane:EtOAc 1:1. After removal of the solvent under reduced pressure, 6.6 g of crude extract were obtained. Part of this material (6.0 g) was subjected to a silica gel column eluted with increasing amounts of EtOAc in *n*-hexane to obtain pure piplartine (2049 mg).

Piplartine. White amorphous solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H 7.68 (d, J = 15.5 Hz, H-3), 7.43 (d, J = 15.5 Hz, H-2), 6.90 (m, H-2′), 6.81 (s, H-5 and H-9), 6.04 (dt, J = 9.7 and 1.7 Hz, H-3′), 4.04 (t, J = 6.5 Hz, H-5′), 3.89 (s, 6-OC$\underline{H}_3$ and 8-OC$\underline{H}_3$), 3.88 (s, 7-OC$\underline{H}_3$) 2.48 (ddt, J = 6.5, 4.4 and 1.7 Hz, H-4′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_C 168.8 (C-1), 165.8 (C-1′), 153.3 (C-6 and C-8), 145.6 (C-3′), 143.7 (C-4), 139.9 (C-7), 130.6 (C-3), 125.7 (C-2′), 121.1 (C-2), 105.4 (C-5 and C-9), 60.9 (7-O$\underline{C}H_3$), 56.1 (6-O$\underline{C}H_3$ and 8-O$\underline{C}H_3$), 41.6 (C-5′), 24.8 (C-4′). HRESIMS *m/z* 318.1349 $[M + H]^+$ (calculated for $C_{17}H_{20}O_5N$, 318.1341).

2.4. Animals and experimental design

Schistosoma mansoni (BH strain) was used in all experiments performed within this research. The parasite's life cycle is maintained locally by routine passage through a rodent *M. musculus* (definitive host) and snail *Biomphalaria glabrata* (intermediate host) (de Moraes, 2012). All procedures performed at this stage are in accordance with pre-established requirements, as previously reported in rather more details (Lago et al., 2019). To investigate the *in vivo* potency of piplartine, three-week-old Swiss female mice (n = 40, ~14 g) were acquired from AniLab (Animal Diagnostic Laboratory - São Paulo, Brazil). Rodents were kept in groups of five, with free access to food and water. Controlled conditions for animal sustenance included temperature (~22 °C), humidity (~50%), and a light/dark cycle (12/12-h). After a habit-forming interval, the mice were infected by subcutaneously injecting with an intermixture containing ~80 cercariae.

For *in vivo* assays, piplartine was dissolved in ethanol (0.3%) and prepared at a dose of 100, 200 or 400 mg/kg. First, four groups of mice harbouring adult *S. mansoni* infection were orally treated with a single dose of piplartine (100, 200 or 400 mg/kg) or once a day for five consecutive days (100 mg/kg/day) at 42 days post-infection (patent infection). Second, considering the *in vivo* activity of piplartine against patent infection, mice harbouring juvenile *S. mansoni* infection were orally treated with a single dose of piplartine (400 mg/kg) or daily doses for five consecutive days (100 mg/kg/day) at 21 days post-infection (pre-patent infection). Untreated mice (n = 5) served as controls in all experiments.

Following standard and international protocol, all mice (treated and control) were euthanized and dissected 56 days post-infection. Systematically, the worms were collected, separated by sex, and counted. The Kato-Katz method was used for quantitative fecal examination. Techniques of quantitative and qualitative oograms using a fragment (10 mm) of the ascending colon were implemented for additional evaluation of the therapeutic efficacy (Silva et al., 2017; Guerra et al., 2019). In the oogram pattern, eggs were scored as immature, mature and dead.

2.5. Scanning electron microscopy analysis

To determine whether piplartine causes morphological alterations in schistosomes living inside the host, two additional mice were orally treated with 400 mg/kg piplartine and were dissected at 24 h post-treatment (Silva et al., 2017). Parasites were harvested from the mesenteric veins and liver, and collected samples were rinsed twice in PBS, and fixed in 1 mL 2.5% glutaraldehyde for 3–24 h at room temperature, as previously described (Guimarães et al., 2015). Afterwards, specimens were air-dried, mounted on stubs and metalized with gold using a Sputter Coater. Samples were then envisioned with the aid of a high-resolution Scanning Electron Microscope with an accelerating voltage of 20 kV (Jeol-JSM-6460LV, Tokyo, Japan).

2.6. Randomization and blinding

The comprised animals were randomly assigned to their experimental groups, and treatment plan. They were also euthanized in a similarly random manner within their corresponding group. All results

obtained were analysed by investigators blinded to the group conditions. Integral research procedures, including worm and egg count, were conducted by two different investigators (Lago et al., 2019; Roquini et al., 2019).

2.7. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0. Results and obtained data were compared to control values using Dunnet's test. A P value of <0.05 was considered significant (de Lima et al., 2018).

3. Results and discussion

3.1. Chemical characterization of piplartine

NMR (^{1}H and ^{13}C) and HRESIMS data of the isolated compound from roots of *P. tuberculatum* were compared with those reported in the literature (Araújo-Vilges et al., 2017), allowing the identification of piplartine at 100% of purity as indicated by HPLC (**Fig. S1-S6**).

3.1. Anthelmintic properties of piplartine in a mice model of schistosomiasis

3.1.1. Oral treatment with piplartine in mice harbouring patent infections significantly reduced worm burden and egg production

Obtained data from infected mice with adult *S. mansoni* (patent infection) treated orally with piplartine, firmly imply that the conducted treatment strategies led to considerable reductions in worm burden, overall. A single dose of 400 mg/kg was able to reduce the worm burden in treated mice by 60.4% ($P < 0.001$), resulting in the highest effect on *S. mansoni* within the implemented treatment options. Moreover, a moderate worm reduction of 50.3% ($P < 0.01$) was observed in mice treated with a single dose of 200 mg/kg. At 100 mg/kg, the minimal administered dose, piplartine discernibly demonstrated a significant effect on worm burden, with a 38.8% ($P < 0.05$) reduction, and complementarily, the group of mice treated with 100 mg/kg of piplartine for five consecutive days, exhibited a moderate worm burden reduction of 58.9% ($P < 0.001$) (**Table 1**). The 50% effective dose (ED_{50}) of 204.2 mg/kg was calculated for piplartine.

Furthermore, oral treatment with piplartine exhibited reduced egg production, as well as an increase in the count of dead eggs (**Table 1**). Eggs of all developmental stages were found in samples from the walls of the intestines; however, the frequency of immature eggs was reduced significantly. More precisely, when compared to the control group, the groups that were administered a single dose of 100 mg/kg, 200 mg/kg and 400 mg/kg, visibly succeeded to reduce the number of immature eggs with 47.2% ($P < 0.05$), 58.1% ($P < 0.01$), and 66.1% ($P < 0.001$), respectively. Immature egg count was further reduced in mice treated with 100 mg/kg once a day for five consecutive days, resulting in an egg burden reduction of 65.9% ($P < 0.001$) was displayed, observably proximate to the percentage obtained from mice treated with a single dose of 400 mg/kg. Over and above that, the Kato-Katz method applied to the fecal samples collected from treated mice, revealed that piplartine consequently reduced the number of eggs within all given doses. Moderate reductions of 41.3% ($P < 0.05$) and 55.8% ($P < 0.01$) were seen in mice treated orally with 100 mg/kg and 200 mg/kg, respectively, and at 400 mg/kg an egg burden reduction of 63.8% ($P < 0.001$) was observed. Remarkably, the highest effect was seen in the 5-day regime with an egg burden reduction of 66.5% ($P < 0.001$). This decrease in egg production was directly correlated with worm burden reduction (Roquini et al., 2019). The reduction of eggs can also be associated with a loss of male/female pairing (Silva et al., 2015; de Castro et al., 2015; Mafud et al., 2016b) or when severe motor activity alterations are recorded (de Brito et al., 2017). The reduction of total immature eggs in the oogram pattern is considered a significant indication of drug efficacy (Guerra et al., 2019) and was specifically evident in this work at the highest doses tested.

3.1.2. Tegumental changes in adult Schistosoma mansoni harboured in mice treated with piplartine

Scanning electron microscopy analysis was employed to investigate and compare tegumental damages induced by piplartine inside the rodent host (**Fig. 1**). The structural features of schistosomes in the control group remained normal and were similar to that in reported earlier (Silva et al., 2017) (**Fig. 1A**). The analysis of adult schistosomes recovered from treated mice demonstrated that piplartine at a single dose of 400 mg/kg revealed marked alteration of structural features of the tegument (**Fig. 1B**). Specifically, male worms exhibited changes in the tubercles, namely protuberance, compression, as well as damage to the spines on their surface. Blisters were additionally observed encircling tubercles. Similar tegumental damages have been documented for various natural products with antischistosomal activity (*e.g.* Godinho et al., 2014; Quelemes et al., 2015).

3.1.3. Oral treatment with piplartine in mice harbouring pre-patent infections significantly reduced worm burden and egg production

As described, piplartine administered at a single dose of 400 mg/kg exerted the highest therapeutic effect on *S. mansoni* adult worms when compared to the different treatment groups and control group. Therefore, the same dose (400 mg/kg) was tested in mice harbouring a pre-patent infection of *S. mansoni*. Additionally, one group of mice was treated with 100 mg/kg once a day for five consecutive days. The treatment of juvenile *S. mansoni*-infected mice with a single 400 mg/kg oral dose of piplartine demonstrated a moderate reduction in worm burden, immature eggs and egg burden ($P < 0.01$) of 54.1%, 51.9% and 50.4%, respectively, when compared to the control group, as well as the adult *S. mansoni* treatment groups. Similar results were observed

Table 1
Effect of piplartine in a mouse model of schistosomiasis infected with either adult (patent infection) or juvenile (pre-patent infection) stages of *Schistosoma mansoni*.

Infection	Dose (mg/kg)	Worm burden reduction (%)[a]	Intestinal egg burden reduction (%)[b]	Faecal egg burden reduction (%)[c]
Patent infection	100	38.8 (± 6.2)*	47.2 (± 3.5)*	41.3 (± 8.6)*
	200	50.3 (± 4.9)**	58.1 (± 4.1)**	55.8 (± 7.2)**
	400	60.4 (± 9.5)***	66.1 (± 6.4)***	63.8 (± 9.3)***
	5 × 100	58.9 (± 6.3)***	65.9 (± 4.9)***	66.5 (± 5.1)***
Pre-patent infection	400	54.1 (± 8.4)**	50.4 (± 9.5)**	51.9 (± 8.7)**
	5 × 100	59.5 (± 5.7)***	53.9 (± 6.3)**	49.2 (± 5.9)**

Values are the mean ± SD ($n = 5$).
[a] Total worm burden (male plus female).
[b] Immature eggs determined by oogram analysis.
[c] Faecal eggs determined by Kato-Katz technique.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ compared with untreated groups.

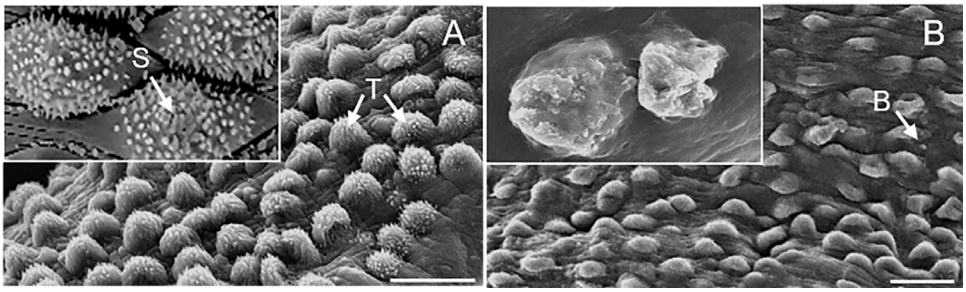

Fig. 1. Microscopy investigation of the tegument of adult *Schistosoma mansoni* recovered from mice. Animals harbouring a 42-day-old adult *S. mansoni* infection were treated with a single oral dose of piplartine 400 mg/kg. Mice were euthanized at 24 h post-treatment and images were obtained by scanning electron microscopy. A: Male worms from infected and non-treated animal (control); dorsal tegumental surface showing tubercles (T) and spines (S) on the surface. B: Male worms from infected animals and treated with piplartine; dorsal tegumental surface showing collapse of the tubercles with loss of the spines on the surface and blisters (B). Scale bars, 25 μm.

in mice treated with 100 mg/kg once a day for five consecutive days. In more detail, worm, immature egg and total egg burden displayed a reduction of 59.5%, 49.2% and 53.9% ($P < 0.01$), respectively (**Table 1**).

Despite the potent *in vitro* activity of piplartine (Moraes et al., 2011), only moderate reductions in worm burdens were observed *in vivo*. Interestingly, similar to praziquantel, which acts primarily against adult schistosomes (worm burden reduction > 90% for adult worms and ~25% for juvenile stages), oral treatment with piplartine was more effective against adult worms (patent infection) than the immature stage (pre-patent infection). Such a difference in anthelmintic susceptibility between the early and the late developmental stages of the parasite was reported previously (de Moraes et al., 2012). A differential expression of piplartine molecular target(s) between the parasites life-stages or differences in the permeability of the tegument (or both) may account for it (de Moraes et al., 2012).

In tandem, the exact mechanism by which piplartine exerts its effect on *S. mansoni* is still not clear. Piplartine's antischistosomal effects may be related to the inhibition of neurotransmission system pathways in *S. mansoni* (Moraes et al., 2011; de Moraes et al., 2012). By binding to proteins known to regulate oxidative stress (ROS), piplartine also may modulate redox and ROS homeostasis (Kim et al., 2014). Further, apoptotic processes induced by piplartine (da Nobrega et al., 2018) may alter main molecular pathways for schistosome development and egg production.

4. Conclusion

Schistosomiasis affects over 200 million people and there are concerns whether the current chemotherapeutic control strategy with praziquantel, the only available drug, is sustainable, necessitating the development of new drugs (Lago et al., 2018). In this study, we describe the efficacy of piplartine in a murine model of schistosomiasis. Interestingly, oral treatment with piplartine in mice harbouring either patent or prepatent infections significantly reduced worm burden and egg production. In addition, scanning electron microcopy analysis demonstrated that piplartine caused morphological alterations in schistosomes recovered from mice. Since piplartine has well-characterized mechanisms of toxicity, is easily available, and is cost-effective, our results indicate that this bioactive molecule derived from medicinal plants could be a potential lead compound for novel antischistosomal agents. Furthermore, the use of synthetic analogues of piplartine on schistosomes may reveal more potent compounds whose studies are in progress.

CRediT authorship contribution statement

Ana.C. Mengarda: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft. **Poliana S. Mendonça:** Methodology, Investigation. **Cristiane S. Morais:** Investigation. **Ramon M. Cogo:** Investigation. **Susana F. Mazloum:** Investigation. **Maria C. Salvadori:** Resources. **Fernanda S. Teixeira:** Resources. **Thiago R. Morais:** Validation. **Guilherme M. Antar:** Resources. **João Henrique G. Lago:** Methodology, Resources, Funding acquisition. **Josué Moraes:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105350.

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