**Schistosoma mansoni**: *In vitro* schistosomicidal activity and tegumental alterations induced by pipartine on schistosomula

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**HIGHLIGHTS**

- Pipartine is able to kill *Schistosoma mansoni* schistosomula of different ages.
- The lethal effect occurred in a dose-dependent manner and was also dependent on the age of the parasite.
- Confocal microscopy observation revealed that pipartine induced morphological changes on the tegument of the worms.
- Pipartine is a promising compound that could be used for the development of a new schistosomicidal agent.

**ABSTRACT**

Schistosomiasis is one of the most important parasitic infections in humans that occur in many tropical and subtropical countries. Currently, the control of schistosomiasis rests with a single drug, praziquantel, which is effective against adult worms but not the larval stages. Recent studies have shown that pipartine, an amide isolated from plants of the genus *Piper* (Piperaceae), reveals interesting antischistosomal properties against *Schistosoma mansoni* adult worms. Here, we report the *in vitro* antischistosomal activity of pipartine on *S. mansoni* schistosomula of different ages (3 h old and 1, 3, 5, and 7 days old), and examine alterations on the tegumental surface of worms by means of confocal laser scanning microscopy. Pipartine at a concentration of 7.5 μM caused the death of all schistosomula within 120 h. The lethal effect occurred in a dose-dependent manner and was also dependent on the age of the parasite. Microscopy observation revealed extensive tegumental destruction, including blebbing, granularity, and a shorter body length. This report provides the first evidence that pipartine is able to kill schistosomula of different ages and reinforce that pipartine is a promising compound that could be used for the development of a new schistosomicidal agent.

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1. Introduction

Schistosomiasis or bilharziasis is a neglected tropical disease caused by worms of the genus *Schistosoma*. The disease continues to threaten millions of people, particularly in poor regions (King, 2010). It has been estimated that there are more than 207 million people infected worldwide, with 779 million people at risk of infection (Steinmann et al., 2006). At least 280,000 people die each year of schistosomiasis and the subtle morbidities associated with chronic infection have a more serious impact than hitherto credited (van der Werf et al., 2003; King et al., 2005). The bloodfluke *Schistosoma mansoni* is the major causative agent of human schistosomiasis, affecting people in Africa, the Middle East, South
America, and the Caribbean. Infection is acquired when skin in contact with infested fresh water. Following the penetration of the host skin, cercariae lose their tails and transform into schistosomula, which reside in the skin for up to 72 h before entering a blood vessel. After a lung passage, the parasites move to the portal venous system, where they mature into male and female worms and mate, producing hundreds of eggs daily. Schistosomes can live for years or even decades in human hosts and, hence, the disease runs a chronic and debilitating course (Gryseels et al., 2006).

Despite the public health importance of schistosomiasis, the treatment and control of this disease relies on a single drug, praziquantel, an isoquinoline–parazine derivative. However, drug resistance is an imminent threat, which underscores the need for alternative therapies (Caffrey, 2007; Doenhoff et al., 2008). In addition, there is an important deficiency in the therapeutic profile of praziquantel, as it lacks activity against larval developing stages of the parasite; hence, retreatment is necessary to kill those parasites that have since matured (Utzinger et al., 2003). Accordingly, it is imperative to develop new effective drugs for treatment and prevention of the infection. Currently, there is also an urgent need to search for drugs as prophylactic agents, being hence able to kill immature forms. Ideally, a single drug should have broad effectiveness against schistosomula and adult worms (Moraes, 2012; Veras et al., 2012).

Piplartine, 5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxy-phenyl)-2-propenyl]-2(1H)-pyridinone, is an amide found in several plants of the genus Piper (Piperaceae family). It is one of the major amides isolated from Piper tuberculatum, whose plant has been largely used in the folk medicine by several communities. As an advantage, Piper species are widely distributed in tropical and subtropical countries and piplartine is found in various parts of the plants, especially in the root (Jaramillo and Manos, 2001; Navickiene et al., 2003). Several biological activities of piplartine have been explored such as antitumor, antinociceptive, antidepressant and anxiolytic properties, as well as antiplatelet aggregation, insecticidal, antifungal, and antiparasitic activities (Navickiene et al., 2000, 2003; Vasques da Silva et al., 2002; Tsai et al., 2005; Bezerra et al., 2007; Bodiwala et al., 2007; Cícero Bezerra Felipe et al., 2007; Cotinguiba et al., 2009; Rodrigues et al., 2009). Additionally, recent study has shown that piplartine reveals interesting antischistosomal properties (Moraes et al., 2011). This natural compound is highly effective against S. mansoni adult male and female worms, and it is able to reduce egg production when worms are treated with sub-lethal concentrations. In this study we present, to our knowledge, the first description of the effect of piplartine on schistosomula and adult worms (Moraes, 2012; Veras et al., 2012).

For in vitro drug assays, schistosomula of different ages in vitro (3 h old and 1, 3, 5, and 7 days old) were used. After the culturing period for each age, these schistosomula were transferred to 96-well culture microplates (approximately 150 parasites per well) and cultured as already described (37 °C, 5% CO₂) in 169 medium in the presence of piplartine at various concentrations. The final volume in each well was 200 μl. Schistosomula incubated in medium containing 0.5% DMSO, the highest concentration of drug solvent used, served as a negative control group. In some experiments, praziquantel at 20 μM (Merck, Rio de Janeiro, RJ, Brazil) was used as positive control group. All the experiments were performed in triplicate. The culture plates were monitored on a daily basis for 5 days using an inverted microscope. Parasite death was defined as no movement for at least 1 min of examination (Moraes, 2012). Schistosomula death was also confirmed by a trypan blue dye exclusion test (Harrop and Wilson, 1993). The activity was measured by calculating the percentage of the number of dead worms compared to the total number of worms.

2. Material and methods

2.1. Plant material

The plant material was collected in September of 2005 from a specimen of P. tuberculatum growing under greenhouse conditions at the Institute of Chemistry (Universidade de São Paulo, SP, Brazil), and it was identified by Dr. Guillermo E. D. Paredes (Universidad Pedro Ruiz Gallo, Lambayeque, Peru). The voucher specimen (Kato-169) was deposited at the Herbarium of the Institute of Biosciences (Universidade de São Paulo, SP, Brazil) as previously described (Moraes et al., 2011).

2.2. Extraction and isolation of piplartine

Piplartine was isolated from P. tuberculatum Jacq as previously described (Navickiene et al., 2000). Briefly, a dry powder of inflorescences from P. tuberculatum (500 g) was extracted with MeOH (2 x 2 l) at room temperature during 3 days. The solutions were filtered and concentrated under vacuum conditions, yielding brown syrup (47 g). Part of this extract (2 g) was submitted to a silica chromatography column using a gradient of hexane–EtOAc at increasing polarities, which yielded 35 fractions. Fraction 20 (350 mg) was re-crystallized with hot MeOH; a white crystalline compound was obtained (150 mg) and was identified as piplartine (Fig. 1) by ¹H NMR analysis.

2.3. Parasite

S. mansoni (BH strain Belo Horizonte, Brazil) worms have been maintained in Biomphalaria glabrata snails as intermediate hosts and Mesocricetus auratus hamsters as definitive hosts at the Parasitology Laboratory (Butantan Institute, São Paulo, Brazil).

2.4. Preparation of schistosomula and assessment of drug effect

Cercariae were shed from infected snails by exposure to light at 37 °C for 2 h and subsequently converted to schistosomula by mechanical transformation, using a Vortex mixer as described by Ramalho-Pinto et al. (1974). Schistosomula were cultivated in 169 medium containing antibiotics and supplemented with 10% fetal bovine serum at 37 °C in a 5% CO₂ atmosphere (Basch, 1981). The schistosomula were cultured until day 7, which corresponds to the skin- and lung-stage worm (Moraes, 2012; Veras et al., 2012).

In order to observe morphological changes on tegument, schistosomula were monitored using a confocal laser scanning microscope in vitro.
of death, schistosomula were fixed in a formalin-acetic acid-alcohol solution (FAA) and analyzed under a confocal microscope (Confocal Laser Scanning Microscopy, LSM 510 META, Zeiss) at 488 nm excitation and 505 nm emission (Moraes et al., 2009).

2.6. Ethics statement

This study was approved by the Institutional Review Board of the Instituto Butantan (approval number: 814/11). All the animals were handled in strict accordance with good animal practice as defined by the Animals Use Ethics Committee of the Instituto Butantan (São Paulo, Brazil). The study was conducted in adherence to the institution’s guidelines for animal husbandry.

2.7. Statistical analysis

Statistical tests were performed with GRAPHPAD PRISM (version 5.0) software. Significant differences were determined by a one-way analysis of variance (ANOVA) and by applying Tukey’s test for multiple comparisons with the level of significance set at $P < 0.05$.

3. Results and Discussion

The control of schistosomiasis relies on a single drug, praziquantel, which is effective against adult worms but not the larval stages. Recently, many scientific research centers around the world are screening substances originating from plants for schistosomicidal activity (Magalhães et al., 2009, 2010; Caixeta et al., 2011; Pereira et al., 2011; Moraes, 2012). In this sense, in vitro promising antischistosomal properties of piplartine, an amide found in *Piper* species, against adult schistosomes has been reported (Moraes et al., 2011). However, no study with piplartine against schistosomula has been performed. To further deepen our understanding of the activity of this natural compound against schistosomes, we investigated the in vitro effect of piplartine on *S. mansoni* of different ages (3 h old and 1, 3, 5, and 7 days old schistosomula) as well as tegumental alterations of piplartine on parasites.

3.1. Antischistosomal activity of piplartine on *S. mansoni* schistosomula of different ages

*S. mansoni* schistosomula of different ages (3 h old and 1, 3, 5, and 7 days old) were cultured in vitro in the presence of different concentrations of amide piplartine and mobility and death were monitored. In the presence of piplartine, all schistosomula died at concentrations of 7.5 μM after 120 h. In contrast, the worms in the control group remained viable until the end of the incubation period (Fig. 2 and data not shown). Interestingly, the lethal effect occurred in a dose-dependent manner and was also dependent on the age of the parasite. As shown in Fig. 2, piplartine at a concentration of 15 μM caused the death of 100% of 5 and 7 day old schistosomula, after 72 and 48 h, respectively. Furthermore, at this same concentration (15 μM), the compound was able to kill all 3 h old and 1 and 3 day old schistosomula only after 96 h. Therefore, older (lung-stage) schistosomula appeared highly more sensitive to piplartine schistosomicidal activity than younger schistosomula (newly transformed- and skin-stage).

As previously mentioned, schistosomula are not susceptible to praziquantel (Utzinger et al., 2003). In this study, we also evaluated the effect of praziquantel on schistosomula of different in vitro age, but no death was detected after treatment at concentration as high as 20 μM (results not shown). In contrast, our in vitro data demonstrated that piplartine exhibits a strong schistosomicidal action against schistosomula at concentrations as low as 7.5 μM. Additionally, piplartine has been as effective as praziquantel to kill adult stage of *S. mansoni* and their schistosomicidal activity was demonstrated at a concentration of 9.5 μM (Moraes et al., 2011). Thus, piplartine is a promising compound that could be used for the development of new schistosomicidal agent.

3.2. Confocal laser scanning microscopy investigations

In addition to mortality rate, morphological changes on tegument were monitored microscopically. In the absence of the drug, all schistosomula, regardless of age, showed normal viability without any morphological changes for up to 120 h. In contrast, tegumental changes were observed when schistosomes have been exposed to the piplartine at lethal concentrations above 7.5 μM.
Interestingly, before the death of the 3 h old and 1, 3, 5, and 7 day old schistosomula, all parasites showed minimal activity, including convulsions and contractions. However, after occurrence of death, several morphological changes on the body region such as several scattered blebs, granularity, and a shorter body length were observed due to exposure to piplartine at lethal concentrations (Fig. 3 and data not shown).

The major interface between the schistosome and its external environment is through the tegument (Skelly and Alan Wilson, 2006). Thus, the tegument has been identified as a key target of some schistosomicidal compounds. The surface topography of S. mansoni schistosomula as studied by microscopy has been reported in great details in previous study (Crabtree and Wilson, 1980; Basch and Basch, 1982). The structure and appearance of S. mansoni schistosomula incubated in absence of piplartine were similar to those described in the literature (Manneck et al., 2010; Veras et al., 2012). Likewise, morphological effects and tegumental alterations induced by piplartine on schistosomula were similar to those observed for parasites incubated with other antischistosomal compound such as mefloquine (Manneck et al., 2010).

Previous studies have shown that piplartine exhibits broad range of biological activities in vitro and in vivo (Bezerra et al., 2005, 2006, 2008a, 2009). For example, Tsai et al. (2005) showed that piplartine has potent anti-platelet aggregation activity induced by collagen in vitro (Tsai et al., 2005). More recently, Fontenele et al. (2009) demonstrated that antiplatelet effects of piplartine are due to the possible involvement of cyclooxygenase blockade and antioxidant activity; moreover, the authors also reported that piplartine shows anti-inflammatory effects similar to that occurring with aspirin, a well-known inhibitor of cyclooxygenase.

Furthermore, piplartine isolated from different Piper species possesses antitumor activity both in vitro and in vivo, which is due to induced toxicity, and this amide may be a promising candidate to combat cancer (Bezerra et al., 2005, 2006, 2008a). In this context, toxicological aspects of the treatment with piplartine have been studied. For example, piplartine had no effect on the spleen of treated animals, and apparently has the kidney as the main toxicological target; however, the kidney damage observed in piplartine-treated animals could also be considered reversible (Bezerra et al., 2006, 2008a). Hematological analysis showed that piplartine also normalize the leukocytes proportions and this amide did not induced hemolysis of mouse erythrocytes (Bezerra et al., 2005, 2008a). Conversely, piplartine possesses DNA damaging potential in mammalian cells as well as mutagenic and recombinogenic effects on yeast (Bezerra et al., 2008b, 2009). Regarding the antischistosomal properties, it was previously shown that piplartine has selective antiparasitic activity and exhibited no cytotoxicity when incubated monkey kidney fibroblasts (Moraes et al., 2011).

In vitro sensitivity testing has been important in the screening of new anthelmintic agents, but the assessment of therapeutic activity using in vivo models should be performed (Moraes, 2012). Importantly, in vivo studies with piplartine have previously demonstrated in animal models. For example, Bodiwala et al. (2007) showed significant antileishmanial activity of piplartine at 30 mg/kg, administered intraperitoneally, in Leishmania donovani-infected hamsters. Additionally, it has been demonstrated that piplartine (0.0001–30 mg/kg, administered intraperitoneally) exhibited significant and dose-related antinociceptive effects against acetic acid-induced visceral pain in mice, supporting the ethnomedical use of Piper species as analgesic and sedative (Rodrigues et al., 2009).

The mechanism by which piplartine exert their in vitro schistosomicidal effects is not clear. However, it has been reported that the in vitro effects of piplartine on S. mansoni adults better correlate with the muscular function (motor activity) than with the tegumental destruction (Moraes et al., 2011). Recently, Cicero Bezerra et al. (2012)Importantly, when incubated monkey kidney fibroblasts (Moraes et al., 2011), piplartine has selective antiparasitic activity and exhibited no cytotoxicity chistosomal properties, it was previously shown that piplartine.

Fig. 3. Effect of piplartine on the tegument of 3 h old and 1, 3, 5, and 7 days old S. mansoni schistosomula. Parasites of different ages were incubated in 24-well culture plates containing 169 medium and treated with piplartine. After occurrence of death, schistosomula were fixed in FAA solution and fluorescent images were obtained using confocal laser scanning microscopy (Carl Zeiss LSM 510 META). The images were analyzed with the Zeiss LSM Image Browser software; (A) 3 h old schistosomula; negative control, (B) 3 h old schistosomula; piplartine 7.5 μM, (C) 1 day old schistosomula; negative control, (D) 1 day old schistosomula; piplartine 7.5 μM, (E) 3 day old schistosomula; negative control, (F) 3 day old schistosomula; piplartine 7.5 μM, (G) 5 day old schistosomula; negative control, (H) 5 day old schistosomula; piplartine 7.5 μM, (I) 7 day old schistosomula; negative control, and (J) 7 day old schistosomula; piplartine 7.5 μM. Bars = 25 μm.
Felipe et al. (2007) showed that piplartine present anxiolytic and antidepressant effects in mice; the authors suggest that this amide interacts with the neurotransmission system, which make this drug potentially useful in anxiety and depression. Therefore, the in vitro antischistosomal effects of piplartine may be related to the inhibition of neurotransmission system pathway in S. mansoni. Importantly, the nervous systems of schistosomes and other helminths have been considered as important targets for drugs (Geary et al., 1992; Cioli et al., 1995; Thompson et al., 1996; Sangster et al., 2005). However, further studies are required to correlate these neural effects with the schistosomical action of piplartine.

Taken together, those previous findings mentioned and the results presented here indicate the potential of piplartine towards schistosomiasis treatment. Nevertheless, considering that piplartine induces in vivo and in vitro mutagenicity in eukaryotic models (Bezerra et al., 2008b, 2009) further studies are needed to identify the mechanisms involved in the biological activity of this compound. Since piplartine showed selective antischistosomical activity and exhibited no cytotoxicity to mammalian cells (Moraes et al., 2011), experiments to evaluate this amide in vivo in mice infected with S. mansoni and in vitro assays with structural analogs are also important to characterize the antischistosomal effect in greater detail.

3.3. Conclusions

The results of this study indicated that piplartine is an efficient compound against S. mansoni schistosomiasis in vitro. In addition, results obtained from direct observation of the schistosomula viability and confocal microscopy strongly suggest that piplartine is more effective than praziquantel. Therefore, considering the schistosomal effects against larval- and adult-stage of S. mansoni, piplartine may represent a step forward in the search for novel antischistosomal agents, at a time when there is an urgent need for novel drugs. Experiments to evaluate this natural amide in vivo onto mice infected with S. mansoni are under progress, besides biological studies to elucidate their mechanism(s) of schistosomical action.

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