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Antischistosomal Natural Compounds: Present Challenges for New Drug Screens

Josué de Moraes
Supervisão de Vigilância em Saúde, SUVIS Casa Verde,
Secretaria de Saúde da Cidade de São Paulo,
Rua Ferreira de Almeida, São Paulo, SP
Brazil

1. Introduction

Schistosomiasis, or bilharzias, is a neglected disease that remains a considerable public health problem in tropical and subtropical regions. This parasitic disease is the most important human helminth infection in terms of morbidity and mortality and is a growing concern worldwide. It is estimated that more than 200 million people have been infected and that 779 million are at risk of infection, resulting in 280,000 deaths annually (van der Werf et al., 2003; Steinmann et al., 2006). Schistosomiasis is caused by blood-dwelling fluke worms of the genus *Schistosoma* and is endemic in African, Asian and South American countries. The main disease-causing species are *S. mansoni*, *S. haematobium*, and *S. japonicum. S. mansoni* is the most widely distributed, affecting people in Africa, the Middle East, South America, and the Caribbean, while *S. japonicum* is confined to China, Indonesia, and the Philippines. *S. haematobium* is found in Africa and the Middle East. The adult worms colonise the veins of either the portal system (*S. mansoni* and *S. japonicum*) or the urinary bladder plexus (*S. haematobium*) and can live for years or even decades in human hosts; thus, the disease runs a chronic and debilitating course. Egg production is responsible for both the transmission of the parasite and the aetiology of the disease. Schistosomal species are distinguished by differences in their morphology, both in their parasite stages and in their eggs; further species distinction is made by the species of intermediate host snails that support transmission of the parasite (Gryseels et al., 2006).

The global strategy for the control of schistosomiasis is by chemotherapy. Systematic searching for chemotherapeutic drugs began almost a century ago, and the development of praziquantel (PZQ) in 1970 was essential for a reduction in morbidity and mortality due to schistosomiasis. Currently, treatment is still based on the use of PZQ, but the long-term application of PZQ results in decreased efficiency and serious concerns regarding the onset of resistance. In addition, PZQ has no prophylactic properties and is ineffective against larval stages of parasites (schistosomula), meaning that for effective treatment and sustainable control, PZQ must be given on a regular basis. Thus, it is prudent to search for novel therapeutics, and recent discussions have focused on reawakening the need to search for alternatives to PZQ (Caffrey, 2007; Doenhoff et al., 2008; Fenwick et al., 2003; Hagan et al., 2004; Keiser & Utzinger, 2007).

Natural products, mainly plants, have been the source of medicines for thousands of years. The discovery of pure compounds as active principles in plants was first described at the beginning of the 19th century, and the art of exploiting natural products has become part of the molecular sciences (Kayser et al., 2003). Several extracts or bioactive constituents from living organisms have been used in many communities worldwide against parasitic diseases, including schistosomiasis, and in the past decades, natural products have attracted renewed interest (Kayser et al., 2003; Mølgaard et al., 2001; Ndamba et al., 1994; Sanderson et al., 2002; Tagboto & Townson, 2001).

In vitro screening systems are useful and affordable ways to discover potential anthelmintic candidates for *in vivo* tests (Keiser, 2010; Ramirez et al., 2007; Yousif et al., 2007). Because a molecular-target approach is still rarely employed in schistosomicidal drug discovery, a more common strategy has been the complementary approach of whole-organism phenotypic screening *in vitro* to measure compound efficacy (Keiser, 2010; Ramirez et al., 2007; Yousif et al., 2007). In this context, screening for natural products that are active against schistosome is important in the establishment of future strategies for new antischistosomal drug discovery to control schistosomiasis (Yousif et al., 2007).

Considerable efforts are ongoing to develop novel schistosomicidal agents. As a result, many natural compounds with promising antischistosomal properties have been identified (Braguine et al., 2009; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011; Mølgaard et al., 2001; Parreira et al., 2010; Sanderson et al., 2002). The efficacy of these new compounds against schistosome is defined using three strategies: a) curative, by killing the adult worm; b) prophylactic, by killing schistosomula; and c) suppressive, by inhibiting worm egg-laying. Thus, several parameters, such as motor activity, tegumental changes, and oviposition, are often evaluated as indicators of biological activity and toxicity in studies with schistosome species.

This chapter reviews the present state of *in vitro* drug screening strategies used to discover new compounds active against *S. mansoni*, the most important species infecting humans, with an emphasis on natural products. Also highlighted are the best practices and challenges for drug screenings. Furthermore, information is provided about toxicity, susceptible *Schistosoma* stages, and other interesting laboratory studies on potential antischistosomal compounds, both natural products and natural product-derived compounds.

2. Antischistosomal drugs

For the control of schistosomiasis, which at present is dependent on chemotherapy, it is not satisfactory to have only one single effective treatment (Caffrey, 2007; Doenhoff et al., 2008; Fenwick et al., 2003). Ideally, other antischistosomal drugs would be available so that the classical strategy of alternating treatments to avoid the development of resistance could be used. Unfortunately, the other drugs used before the advent of PZQ, oxamniquine and metrifonate, are restricted in their use. Metrifonate, a drug that exhibits activity against *S. haematobium*, has recently been withdrawn from the market because of medical, operational, and economic criteria (Reich & Fenwick, 2001; Utzinger et al., 2003). Oxamniquine is the only alternative antischistosomal drug, but it is effective only against *S. mansoni*. In the 1970s, oxamniquine was used for individual and mass treatment of schistosomiasis, with satisfactory results regarding efficacy and tolerance. However, its use is currently declining and is being replaced by praziquantel (Cioli, 2000; Reich & Fenwick, 2001; Utzinger et al.,

2001, 2003). As there is currently no available vaccine for this disease in people (Bergquist et al., 2008), chemotherapy may now be at a crucial point.

Chemotherapy against schistosomiasis was reviewed extensively by Cioli et al. (1995), with an emphasis on compounds that were used in the past. Additionally, Cioli (1998) summarised some interesting laboratory studies on potential antischistosomal compounds and the possible emergence of praziquantel-resistant schistosomes. More recently, Ribeiro-dos-Santos et al. (2006) reviewed results from a comprehensive search of the scientific literature for substances and compounds tested for schistosomiasis therapy over the past century. The authors gathered information on the therapeutic action in humans or animal models and the mechanisms of action of over 40 drugs.

Briefly, antimonial compounds were introduced in 1918, and this group of drugs has been the major point of schistosome chemotherapy for approximately 50 years. However, they cause numerous side effects, such as nausea, vomiting, diarrhoea, anorexia, and cardiovascular, hepatic, and dermatological disturbances. Lethality from cardiac syncope and anaphylactic shock was also reported. Emetine, a drug used to treat amoebiasis, was employed in the second decade of the past century, but the doses required against schistosomiasis were at the very limit of toxicity. The introduction of 2,3-dehydroemetine reduced the toxicity of the parent compound, but patients had to be hospitalised over a month for treatment. Thus, the use of 2,3-dehydroemetine as an antischistosomal agent was abandoned (Cioli et al., 1995). Only in the 1960s was there a breakthrough in the treatment of schistosomiasis, with the rise of metrifonate, nitrofurans, lucanthone, niridazole, hycanthone, and, finally, oxamniquine. In the 1970s, several schistosomicidal drugs emerged, such as tubercidin, amoscanate, PZQ and its benzodiazepine derivative Ro11-3128, and oltipraz. Nevertheless, the therapeutic doses of most of these drugs were found to cause major side effects. PZQ, an isoquinoline-pyrazine derivative, immediately proved to be superior to any other schistosomicidal drug and quickly became the drug of choice in most endemic areas (Cioli et al., 1995; Fenwick & Webster, 2006). Because of the reliance on a single drug for the treatment and control of schistosomiasis and the considerable concern regarding the development of PZQ resistance, it is timely to review potential alternatives, with an emphasis on natural products.

2.1 Antischistosomals: Natural product and natural product-derived compounds

The use of natural products for curative and therapeutic purposes has a long history, and compounds derived from natural products have made a big impact on the pharmaceutical industry (Newman, 2003; Newman & Cragg, 2007). In addition to microbes and plants, there has been growing interest in other living organisms, such as arthropods and amphibians, as important sources of biologically active compounds (Kayser et al., 2003). However, the potential for using living beings as sources of new antischistosomal drugs is still poorly explored. In recent decades, there has been a growing interest in the scientific community to search for extracts and pure compounds, especially those derived from plants, that exhibit potential schistosomicidal properties, as one alternative method to the conventional chemical control.

Plants have been traditionally used in the treatment of different diseases, including schistosomiasis, especially in Africa and Asia (Ndamba et al., 1994). In general, medicinal plants are prepared by traditional healers, who have empirical knowledge and

cultural communities throughout the world. For example, in Zimbabwe, Ndamba et al. (1994) investigated the herbal remedies used in the treatment of schistosomiasis. Based on interviews with 286 traditional healers, they composed a list of 47 plant species most widely used to treat urinary schistosomiasis. Based on this survey, the seven most commonly used plants, *Abrus precatorius* (Leguminosae) *Ozoroa insignis* (Anacardiaceae), *Dicoma anomala* (Cornpositae), *Ximenia caffra* (Oleaceae), *Lannea edulis* (Anacardiaceae), *Elephantorrhiza goetzei* (Leguminosae) and *Pterocarpus angolensis* (Leguminosae), were collected, prepared as described by the traditional healers, their efficacy was evaluated using laboratory animals previously exposed to *S. haematobium* cercariae, and the activity from the extract of *P. angolensis* bark was almost comparable to that of praziquantel. Later, Mølgaard et al. (2001) screened extracts of 23 plant species, popularly used against schistosomiasis in Zimbabwe, for their anthelmintic effect against schistosomula of *S. mansoni*, and the best results against larval forms were obtained with stem and root extracts from *Abrus precatorius* (Fabaceae) and stem bark from *Elephantorrhiza goetzei* (Mimosaceae). All families and names of the plants that are used by traditional healers to treat urinary schistosomiasis in Zimbabwe are described by Ndamba et al. (1994).

Some of the most interesting antischistosomal compounds are derivatives of artemisinin, such as artemether and artesunate (Utzinger et al., 2001; Xiao et al., 2002). They are highly effective in the treatment of malaria and have also been shown to exhibit antischistosomal properties. Artemisinin is a sesquiterpene lactone with an endoperoxide group, which was isolated from the leaves of *Artemisia annua* L. This plant has been used for centuries in Chinese traditional medicine as antidote to many different ailments (Lee, 2007; Utzinger et al., 2001). Artemisinin has been used as an antimalarial since the early 1970s, and its antischistosomal activity was discovered in 1980 by a group of Chinese scientists. In 1982, antischistosomal properties were confirmed for artemether, the methyl ether derivative of artemisinin. Interestingly, artemether has been shown to be active against immature schistosome in experimentally infected animals, but it is less effective against adult worms (Utzinger et al., 2001). Significant progress has been made with artemether and its potential for the control of schistosomiasis, which has been reviewed by Utzinger et al. (2001). The mechanism of action of artemisinin and its derivatives appears to involve an interaction with heme, which cleaves the endoperoxide bridge of the drug to produce carbon-centred free radicals that then alkylate parasite proteins (Golenser et al., 2006). In addition, scanning electron microscopy showed that artemether caused extensive and severe damage to the tegument in 21-day-old *S. mansoni* harboured in mice (Xiao et al., 2000). Considering that artemether and praziquantel exhibit the highest activity against schistosomula and adult worms, respectively, combined treatment has been proposed to enhance the reduction in worm burden (Utzinger et al., 2003). Currently, new trials to use artemisinin and its synthetic derivatives as lead molecules for drug discovery against schistosomiasis and various other diseases are rapidly growing, and the studies are ongoing (Lee, 2007; Utzinger et al., 2003, 2007; Xiao et al., 2002). Likewise, research on other natural products and natural product-derived compounds against schistosome has been performed by many groups. Accordingly, several plants with antischistosomal properties have been described in the literature (Braguine et al., 2009; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011; Mohamed et al., 2005; Mølgaard et al., 2001; Parreira et al., 2010; Sanderson et al., 2002).

Here, data on natural products and related natural product-derived compounds are reviewed, especially those from recent years or that have received considerable attention due to their antischistosomal properties (Table 1).

Date *	Extract/ Compound and Biological Source	Relevant notes
1980	Artemisinin, active principle from the plant *Artemisia annua* L. (Asteraceae)	Artemisinin derivatives: artemether (1982) and artenusate (1983); effective against immature schistosome in experimentally infected animals; morphological alteration on the tegument (Utzinger et al., 2001)
1989	Extracts from the plant *Pavetta owariensis* P. Beauv (Rubiaceae) contain proanthocyanins	Effective in mice infected with *S. mansoni* (Baldé et al., 1989)
1997	Goyazensolide isolated from the plant *Eremanthus goyazensis* (Gardner) Sch. Bip. (Compositae)	*In vitro* activity on *Schistosoma* adult worms; inhibitory effect on egg-laying; female more susceptible than male; not tested on schistosomula (Barth et al., 1997)
2000	Extract of leaf from the plant *Vernonia amygdalina* Del (Compositae)	Active against *S. mansoni* in mice (Ogboli, 2000)
2001	Mirazid myrrh, an oleo-gumresin from the stem of the plant *Commiphora molmol* (Burseraceae)	There is a great debate about the efficacy and effectiveness of myrrh in the treatment of schistosome infections, both in laboratory and clinical settings (Abdul-Ghani et al., 2009; Badria et al., 2001; Botros et al., 2004, 2005; Sheir et al., 2001)
2002	Oil from the plant *Nigella sativa* L. (Ranunculaceae)	Active on *S. mansoni*-infected mice; crushed seed also has *in vitro* effects against *S. mansoni* miracidia, cercariae, and adult worms, and an inhibitory effect on egg-laying; not tested on schistosomula (Mahmoud et al., 2002; Mohamed et al., 2005)
2002	Extract of the seeds and isoflavonoids from the plant *Millettia thonningii* (Schum. et Thonn.) Baker (Leguminosae)	*In vitro* against *S. mansoni* adult worms; no egg production was observed for experimental worms; not tested on schistosomula. Schistosomicidal activity against *S. mansoni* cercariae and miracidia has been previously described (Lyddiard et al., 2002)

* Dates of introduction or publication are only approximate.

Table 1. *In vitro* and *in vivo* antischistosomal characteristics of natural products.

Date *	Extract/ Compound and Biological Source	Relevant notes
2005	Extract of rhizomes from the plant *Zingiber officinale* Roscoe (Zingiberaceae)	*In vitro* male worms seemed more susceptible than female; reduction in egg output; activity against *S. mansoni* in mice was conflicting between Mostafa et al. (2011) and Sanderson et al. (2005); morphological alteration on the tegument; not tested on schistosomula
2007	Extract from plant *Curcuma longa* L. (Zingiberaceae)	Effective on *S. mansoni*-infected mice (El-Ansary et al., 2007; El-Banhawey et al., 2007); the *in vitro* schistosomicidal activity of curcumin, the major constituent in the rhizome, and reduction in egg production has been reported (Magalhães et al., 2009)
2007	Extract from garlic *Allium sativum* L. (Liliaceae)	Active against *S. mansoni* in mice (50 mg/kg) and not effective in high dose (100 mg/kg); affects the development and maturity of *S. mansoni* eggs in mice and seems to be an agent in protecting hepatic tissue against oxidative damage due to *S. mansoni* infection. *In vitro* allicin (2011), the main constituent of garlic, causes alterations on the tegument of male worm in high doses (10 to 20 µg/ml), but toxicity not assessed; not tested on schistosomula (El Shenawy et al., 2008; Lima et al., 2011; Riad et al., 2007)
2009	Extract from the plant *Clerodendrum umbellatum* Poir (Verbenaceae)	Effective in *S. mansoni* mice model (Jatsa et al., 2009)
2009	Extract from the plant *Zanthoxylum naranjillo* Griseb (Rutaceae) and its isolated compounds	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cells (Braguine et al., 2009)
2010	Phloroglucinol compounds from plants of the *Dryopteris* genus (Dryopteridaceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cells (Magalhães et al., 2010)
2010	Essential oil from the plant of *Baccharis dracunculifolia* DC. (Asteraceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cell (Parreira et al., 2010)
2011	Essential oil from plant *Ageratum conyzoides* L. (Asteraceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cells (de Melo et al., 2011)

Table 1. Continued

Date *	Extract/ Compound and Biological Source	Relevant notes
2011	Sulfated polysaccharide α-D-glucan extracted from lichen *Ramalina celastri* (Spreng.) Krog. & Swinsc	Effective in *S. mansoni*-infected mice (Araújo et al., 2011)
2011	Piplartine, an amide isolated from plant *Piper tuberculatum* Jacq. (Piperaceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; causes alterations on the tegument of worms; not tested on schistosomula; not toxic in mammalian cells (Moraes et al., 2011)
2011	Dermaseptin 01, an antimicrobial peptide found in the skin of frog of the genus *Phyllomedusa* (Hylidae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; causes alterations on the tegument of worms; not tested on schistosomula; not toxic in mammalian cells (de Moraes et al., 2011)
2011	Epiisopiloturin, an alkaloid isolated from plant *Pilocarpus microphyllus* Stapf ex Holm (Rutaceae)	*In vitro* against *S. mansoni* adult worms; causes alterations on the tegument of worm; not tested on schistosomula; not toxic in mammalian cells (Leite et al., 2011)
2011	Extract from plants of the *Artemisia* genus (Asteraceae)	*In vitro* against *S. mansoni* adult worms; not tested on schistosomula (Ferreira et al., 2011)

Table 1. Continued

As shown in Table 1, several *in vitro* studies have been conducted to search for new natural substances with schistosomicidal activity. These natural products and natural product-derived compounds mostly come from plants. Extensive phytochemical investigations of many species have revealed the presence of a large number of novel compounds belonging to different classes (Kayser et al., 2003; Kato & Furlan, 2007; Parmar et al., 1997; Prassad et al., 2005). For example, various secondary metabolites have been isolated from the family Piperaceae, and these plants have generated great interest as a result of their biologically active metabolites, such as pyrones, terpenes, lactones, chromenes, chalcones, lignoids, amides, and alkaloids (Kato & Furlan, 2007; Parmar et al., 1997). Regarding the variety of biological properties in particular, Moraes et al. (2011) demonstrated the *in vitro* schistosomicidal activity of piplartine, an amide found in several *Piper* species. The authors showed that at low concentrations (9.5 μM) this amide can kill *S. mansoni* adult worms (male and female coupled) and that the sub-lethal concentration of piplartine (6.3 μM) caused a 75% reduction in egg production. Additionally, piplartine was not cytotoxic against mammalian cells when given at concentrations up to three times higher than what is needed for a schistosomicidal effect (31.5 μM). Furthermore, *Piper* species are widely distributed in tropical and subtropical regions of the world, and they are among the most important medicinal plants used in various systems of medicine (Jaramillo & Manos, 2001; Parmar et

al., 1997). In addition to the wide geographical distribution and their use in folk medicine, the interest in these compounds and plant extracts is based on the fact that it is easy to isolate secondary metabolites and to propagate the plant, which has a short reproductive cycle. Thus, considering the *in vitro* schistosomicidal activity of the amide piplartine, the importance of more research on the biological activity of the natural compounds isolated from the family Piperaceae and other plants is apparent.

3. *Schistosoma mansoni* life cycle and maintenance in the laboratory

Schistosome species are dioecious (having male and female reproductive organs in separate individuals) platyhelminthes and have complex life cycles comprising multiple morphologically distinct phenotypes in definitive mammalian and intermediate snail hosts. *S. mansoni* is one of the most common etiological agents of human schistosomiasis and is the most widely used schistosome model for chemotherapeutic studies.Schistosome infection of humans (or another definitive host) occurs by direct contact with freshwater containing free-swimming larval forms of the parasite, known as cercariae. Cercariae penetrate the intact human skin and transform into schistosomula, which reside in the skin for up to 72 hours before entering a blood vessel. Within the vascular system, schistosomula migrate via complex routes to their final venous destination, where they mature into male and female adults. The mature flukes dwell in the human portal vasculature, depositing eggs in the intestinal wall that either pass to the gut lumen and are expelled in the faeces or travel to the liver and trigger immune-mediated granuloma formation and peri-portal fibrosis. Egg production commences 5 to 6 weeks after infection and continues for the life of the worm. The life cycle is completed when the eggs passed in the faeces hatch in the water, releasing the larval form miracidia, which then infect freshwater snails of the *Biomphalaria* spp. The infected snails, bearing schistosomal sporocysts, release cercariae into the water, which in turn penetrate the skin of their definitive host (Gryseels et al., 2006).

To complete a life cycle in the laboratory, *S. mansoni* is commonly maintained using rodents, ranging from hamsters to mice, as the definitive hosts and *Biomphalaria glabrata* as the intermediate host snail species (Figure 1). Infections of rodents of the same gender, 3 to 4 weeks of age and weighing 18 to 25 g, with *S. mansoni* are commonly initiated by subcutaneous injection of 100 to 150 cercariae (infective larvae). At 42, 49 or 56 days postinfection, animals are sacrificed with CO_2, dissected, and miracidia are hatched from *S. mansoni* eggs taken from animal livers. Each intermediate host snail is exposed to approximately 10 miracidia. All animals should be handled in strict accordance with good animal practice adhering to the institutional guidelines for animal husbandry. Thus, all studies should have a statement from their ethics committee or institutional review board indicating the approval of the research.

The use of *in vivo* animal models in drug discovery and the techniques used for these studies in the laboratory have recently been described in detail elsewhere by Keiser (2010) and Ramirez et al. (2007). This chapter will focus on the techniques used for *in vitro* studies. Many methods have been described that aim to determine the antischistosomal activity of drugs *in vitro*. The assessment of the viability of different stages of schistosome, tegumental changes, oviposition, toxicity in mammalian cells and other parameters are important in the search for antischistosomal substances. The techniques detailed here will be the key to better assess the methodology employed during screening tests.

4. Parasite culture system

In vitro studies with schistosomula, juvenile and adult worms of *S. mansoni* are frequently used in screening strategies for the discovery of new antischistosomal drugs (Abdulla et al., 2009; Keiser, 2010; Mølgaard et al., 2001; Peak et al., 2010; Ramirez et al., 2007; Smout et al., 2010; Yousif et al., 2007). Parasites at different stages might show differences with regard to drug sensitivity. The *in vitro* methods currently utilised have recently been reviewed, and following the establishment of the *S. mansoni* life cycle in the laboratory, *in vitro* parasite culture techniques were developed (Keiser, 2010; Ramirez et al., 2007). For *in vitro* trials, parasites of different ages are used, such as 3-h-old and 1-, 3-, 5- and 7-day-old schistosomula, 21 day-old juveniles, and 42- to 56-day-old adults. Figure 1 shows the life cycle of *S. mansoni* in the laboratory, illustrating the collection points for *in vitro* chemotherapeutic studies.

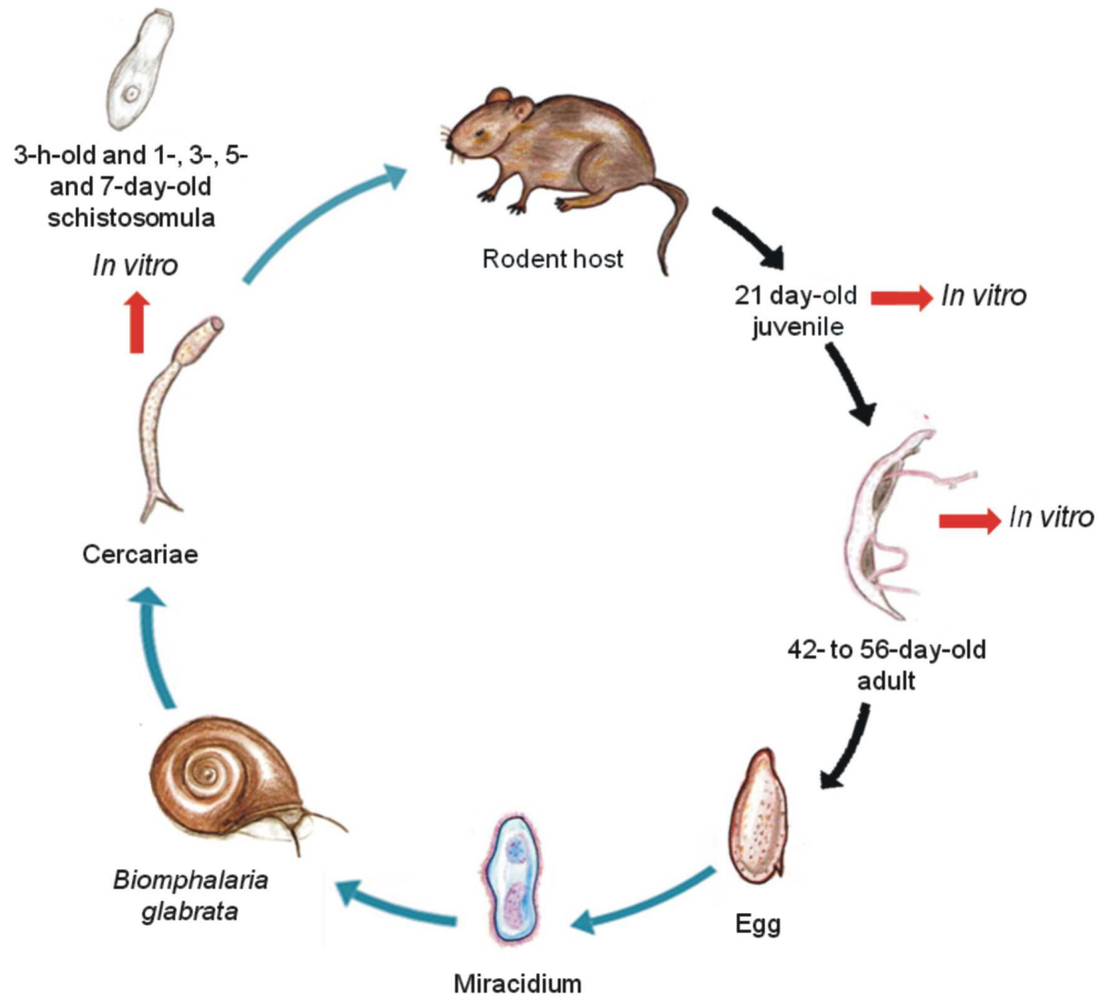

Fig. 1. Life cycle of *S. mansoni*, illustrating the collection points for *in vitro* chemotherapeutic studies. Black arrow: maturation of parasite within final host. Blue arrow: aquatic phase

4.1 Juvenile and adult schistosomes

Adult worms have been more commonly used for antischistosome drug discovery. Today, *in vitro* chemotherapeutic studies using juvenile worms are also highly recommended. For these assays, each rodent is commonly infected with either 100-150 or 400-500 cercariae. Rodents exposed to 400-500 cercariae are sacrificed 21 days after infection for juvenile recovery, while mice exposed to 100-150 cercariae are sacrificed 42 to 56 days after infection for adult recovery. Juveniles or adults are collected using a perfusion technique with Hanks' balanced salt solution (HBSS), Dulbecco's Modified Eagle's Medium (DMEM), or Roswell Memorial Park Institute (RPMI) 1640 medium, containing an anticoagulant such as heparin at a concentration range of 5-20 U/ml (Smithers & Terry, 1965). The worms are washed in RPMI 1640 medium, kept at pH 7.5 with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) containing 200 U/ml penicillin, 200 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. After washing, ten to fifteen juveniles or one pair of adult worms (male and female coupled) are transferred to each well of a 24-well culture plate containing 2 ml of the same medium supplemented with 10% bovine foetal serum and incubated at 37 ^{o}C in a humid atmosphere containing 5% CO_2. Only viable, contractile worms showing total tegument integrity as assessed by light microscopy should be included in the different investigations (Figure 2).

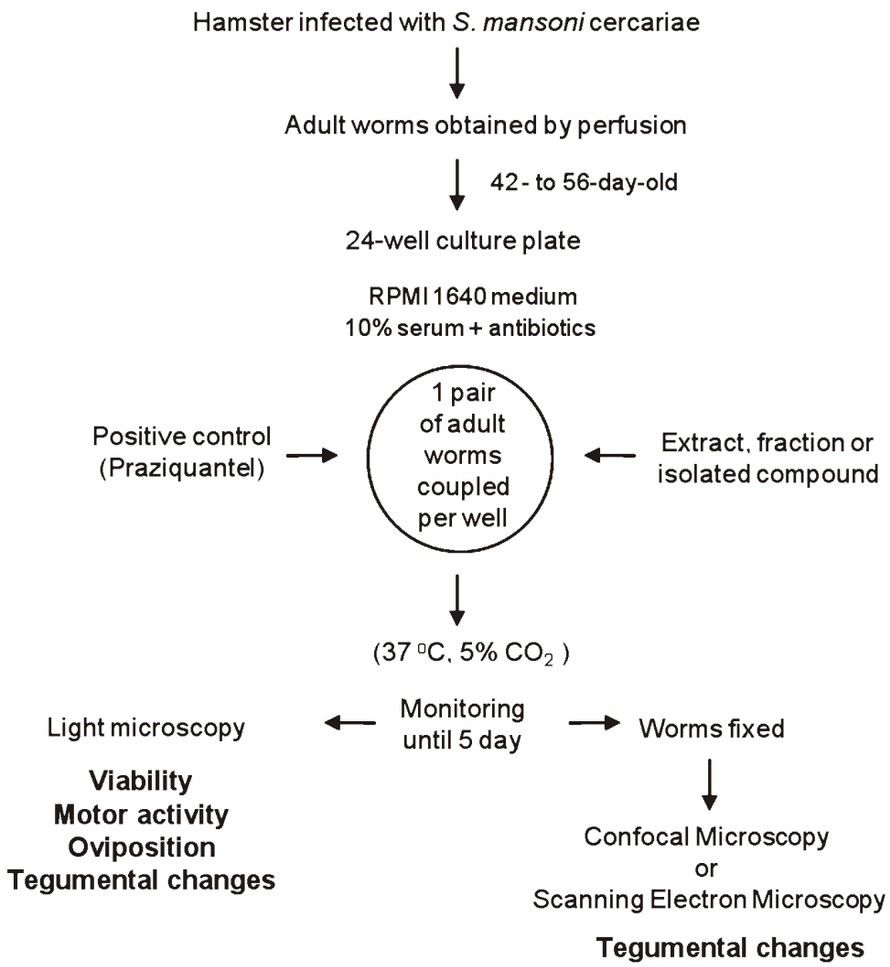

Fig. 2. *In vitro* assay models with *Schistosoma mansoni* adult worms.

4.2 Schistosomula

Obtaining sufficient quantities of schistosomula directly from skin or lung tissue for most research purposes is time consuming and involves working with mammalian hosts. These difficulties led to the development of techniques for transforming cercariae and maintaining schistosomula. In fact, schistosomula can be obtained by transforming cercariae using simple techniques, such as centrifugation, vortexing, repeated aspiration through a syringe needle, or chemical stimulation, and these schistosomula are easily maintained *in vitro* for several days (Basch, 1981). These *in vitro* strategies are advantageous because they confer uniformity in parasite maturation, which cannot be achieved *in vivo* due to the variation in the time required for individual parasites to penetrate the host skin and enter the vasculature. In addition, the use of mechanically obtained schistosomula is an alternative method that reduces, refines and replaces the use of animals in laboratory research in accordance with animal protection principles (Broadhead & Bottrill, 1997).

Among the techniques available for the production of schistosomula, the current methods most commonly used for the removal of tails from cercariae are the repeated aspiration through a syringe needle, based on Colley and Wikel (1974), and the use of a Vortex mixer, based on Ramalho-Pinto et al. (1974). In our laboratory, for example, schistosomula are mechanically transformed according to Ramalho-Pinto et al. (1974) and cultured *in vitro* in 169 medium, as described by Bash (1981). This method is recommended over the syringe-passage technique, which severely stresses and damages the parasites. In addition, the mechanical transformation procedure in a Vortex mixer is simpler than the passage of the parasites under pressure many times through a needle. Briefly, to obtain cercariae, *B. glabrata* snails infected with miracidia are exposed to incandescent light for 2 h, and then cercariae are collected, concentrated in glass conical centrifuge tubes and cooled in a ice bath for 10 minutes to reduce the motility of the worms. The ice-cold cercarial suspension is centrifuged, resuspended in RPMI 1640 medium with 25 mM HEPES, 200 UI/ml penicillin, 200 µg/ml streptomycin, and 0.25 µg/ml amphotericin B, and vortexed for 2 minutes to trigger tail loss. The resulting cercarial bodies are isolated from free tails by centrifugation through a 60% Percoll gradient (Lazdins et el., 1982) or decantation (Ramalho-Pinto et al., 1974). Microscope examination is used to assess the quantity and quality of purified schistosomula. Finally, schistosomula are cultivated in 169 medium containing antibiotics and supplemented with 10% bovine foetal serum at 37 ^{o}C in a 5% CO_2 atmosphere. Schistosomula are cultured until day 7, which corresponds to the lung-stage worm. For *in vitro* drug screening assays, schistosomula are transferred into 96-well culture microplates, with approximately 50 parasites per well, and maintained in 200 µl 169 medium under the conditions described above.

After penetrating the definitive host, significant morphological, physiological, and biochemical changes occur in the developing schistosomula (Gobert et al., 2007; Skelly & Alan Wilson, 2006). Although mechanically transformed schistosomula are different from the schistosomula that have penetrated the skin, these larval cultures have been maintained in *vitro* for different amounts of time to produce all the mammalian host stages, including skin- and lung-stage schistosomula and paired, mature adult males and females (Basch, 1981). Mechanically transformed schistosomula are now commonly used in studies of behaviour, development, metabolic activity, biochemistry, molecular biology, immunology,

and in vaccine development and drug screening protocols (Abdulla et al., 2009; Gobert et al., 2007; Harrop & Wilson, 1993; Peak et al., 2010). There is evidence that mechanically transformed schistosomula are structurally similar to their lung schistosomula counterparts (Chai et al., 2006), and after 7 days in culture, the larvae have the morphological features of lung worms and are capable of maturation when introduced into the portal vein of mice (Harrop & Wilson, 1993). Furthermore, mechanically transformed schistosomula are able to develop steadily until adult worm pairing (Basch, 1981). Because of these reasons, drug-screening assays in our laboratory are based on mechanically transformed schistosomula of different ages *in vitro* (3-h-, 1-, 3-, 5- and 7-day-olds). It takes roughly 3 h for the cercariae to secrete the contents of their acetabular glands; the 1- to 7-day-olds correspond to the skin- and lung-stage schistosomula.

5. Operating procedures for antischistosomal drug screening and the techniques employed

In recent years, the search for new anthelmintics has intensified, but little significant progress has been made in developing new techniques. The *in vitro* drug screening approaches must take into account some specific concerns, particularly to be simple and inexpensive. Important methodologies can objectively and rapidly distinguish helminth viability or phenotype. *In vitro* screening could identify novel anthelmintics and could eventually translate into practical applications. Herein, a general overview is given of the most common methodologies used for screening antischistosomal compounds and their effects on the whole organism.

5.1 Compound storage and handling

To test new compounds, including synthetic or natural products and crude extracts or fractions of a natural source, it is necessary to consider factors such as solubility and stability. The compounds are usually stored in hermetically sealed glass containers, covered with aluminium foil to protect the contents from light, and kept refrigerated at 8 ^{o}C or at ambient temperature until used. The compounds are commonly dissolved in dimethyl sulfoxide (DMSO), which should not exceed a final concentration of 2% in the culture medium containing parasites. Control schistosomes are incubated in the presence of the highest concentration of solvent used.

For *in vitro* screening assays, there is not a set maximum concentration to evaluate the activity of compounds on schistosomes, as long as no toxicity occurs on mammalian cells. However, in our laboratory, *in vitro* screenings are performed at final maximum concentrations of up to 500 μg/ml for crude extracts or fractions and 1,000 μM for isolated compounds. The reference drug praziquantel is used as a positive control at final concentrations ranging from 5 to 10 μM (Figure 2).

5.2 Assessment of parasite viability

The effects of compounds on *S. mansoni* are commonly assessed by phenotypic changes. The parasites are kept for 5 days as described above, and monitored at different time points (e.g., 24, 48, 72, 96, and 120 h) to evaluate their general condition, using parameters such as motor activity, morphological changes, and mortality rate (Figure 2).

Current methods utilised to assess schistosomal viability have recently been reviewed, and most of these methods involve microscopic techniques (Keiser, 2010; Ramirez et al., 2007). The phenotypic changes are scored by using a viability scale. For example, a scale of 0 – 4, where 4= normally active, 3= slowed activity, 2= minimal activity, 1= absence of motility apart from gut movements, and 0= total absence of mobility, is based on standard procedures for compound screening at the Special Programme for Research and Training in Tropical Diseases, World Health Organization, WHO-TDR (Ramirez et al., 2007). Alternatively, as described by Manneck et al. (2010, 2011), drug activity is defined as 3= totally vital, normally active, and no morphological changes; 2= slowed activity, primary morphological changes and visible granularity; 1= minimal activity, severe morphological changes and granularity; 0= all worms dead, severe morphological changes and granularity; the granularity is characterised only for schistosomula. The regular movement of both larval and adult schistosomes has proven to be a valuable trait in assessing schistosome viability *in vitro* because lack of movement is a good indicator of death. Worm death is usually defined as no movement observed for at least 2 min of examination (Manneck et al., 2010). In this context, the viability of worm during the culture period is also assessed by motor activity reduction, and it is defined as "slight " or "significant". This subjective criterion is commonly used by several research groups (Braguine et al., 2009; de Melo et al., 2011; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011, Parreira et al., 2010; Pereira et al., 2011; Xiao et al., 2007). Size measurements of parasites are also employed to study phenotypic changes.

In addition to the phenotypic approaches, another *in vitro* drug-screening assay method is based on microcalorimetry. Manneck et al. (2011) analysed the effects of drugs on the metabolic activity of schistosomula and adult *S. mansoni* by comparing their heat flow. In this study, a multi-channel isothermal microcalorimeter equipped with 48 measuring channels was used to monitor the heat production by schistosomes as a result of their metabolism over time. The results show that microcalorimetry can be a valuable tool to study antischistosomal drugs, and the microcalorimetric measurements confirmed, in part, the results of the phenotypic evaluation. However, the level of agreement between microscopy and microcalorimetry data requires further investigation (Manneck et al., 2011). In the following section, other methods are described that are used to determine the effect of drugs on schistosomula and adult *S. mansoni.*

Phenotypic changes are determined as mentioned above. However, because of the lack of standardisation between laboratories, the replication of results obtained by microscopic means is not always possible. In an effort to avoid the subjective nature of quantifying schistosome viability from the microscopic examination of phenotype alone, further adaptations have been developed and are based on the differentiating potential of some colorimetric vital dyes. Diamidinophenylindole (DAPI) has been used as a differential stain of dead schistosomula during microscopy; in addition, the low DAPI concentration (1 μg/ml) in the medium proved not to be toxic to the schistosomula, nor did it cause any background fluorescence (Van Der Linden & Deelder, 1984). Trypan blue has also been shown to be a reliable dye for differentially staining dead schistosomula (Harrop & Wilson, 1993) and by means of a methylene blue dye exclusion test (Gold, 1997). The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is also a vital dye that has been successfully used to assess the viability of worms. The use of this assay on helminths was pioneered by Comley et al. (1989), and several nematode

species have been assessed. Nare et al. (1991) used the MTT marker to evaluate the viability of adult schistosomes; currently, some *in vitro* bioassays used to study the effects of drugs have demonstrated the ability of MTT to assess the viability of adult worms (Braguine et al., 2009; de Melo et al., 2011; Magalhães et al., 2009, 2010; Parreira et al., 2010; Pereira et al., 2011).

Recently, Peak et al. (2010) validated a high-throughput system for detecting the viability of schistosomula using a microtiter plate-based method. In this study, the authors combined the use of propidium iodide with fluorescein diacetate to allow the easy assessment of the percent of viable schistosomula present in a sample. This helminth fluorescent bioassay was developed into a method of wide-scale application because it is sensitive, relevant to industrial high-throughput (384-well microtiter plate compatibility, 200 schistosomula/well) and academic (96-well microtiter plate compatibility, 1000 schistosomula/well) settings, translatable to drug screening assays, does not require a priori knowledge of schistosome biology or extensive training in parasite morphology, and is objective and quantitative.

The development of high-content screening systems is an important step for the assessment of parasite viability in a high-throughput format. A novel assay for anthelmintic drug screening by real-time monitoring of parasite motility was developed by Smout et al. (2010). This technological advance is based on the detection of changing electrical currents running through mini gold electrodes on the bottom of tissue culture plates. In this assay, the authors assessed the motility of *S. mansoni* using an xCELLigence system (Real Time Cell Assay, RTCA SP instrument), which monitors cellular events in real time without the incorporation of labels by measuring the electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture plates. This technology was applied to adult schistosome using one pair (one coupled male and female worm) in 200 µl per well of an E-plate, which is a 96-well plate for cell-based assays on the RTCA instruments. Because the real-time system measures changes in worm motility with the high level of precision necessary for high-throughput studies, it is widely applicable to a range of helminth species and developmental stages (Smout et al., 2010). This motility assay may provide a superior methodology to microscopy by removing the subjectivity from helminth phenotype characterisation and making available a technology that could allow the direct comparison of results from different laboratories. However, the initial cost of this RTCA system and E-plates may restrict its use, especially in an academic laboratory.

5.3 Assessment of changes in the tegument of parasites

The tegument is the major interface between the schistosome and its external environment. In addition to providing protection, the tegument is an important site of the uptake of nutrients and other molecules. Moreover, the tegument is extremely important for infection success and survival in the host, and it has been a major target for the development of antischistosomal drugs (Skelly & Wilson, 2006; Van Hellemond et al., 2006). Therefore, most of the drugs currently used against schistosome act by damaging the worm tegument (Doenhoff et al., 2008; Fenwick et al., 2003; El Ridi et al., 2010; Keiser, 2010; Manneck et al., 2010, 2011; Mostafa et al., 2011; Xiao et al., 2000).

The schistosome tegument is often approached as a drug target in schistosomiasis and is associated with the subjective assessment of parasite viability described here. The

morphological alterations of the tegument of *S. mansoni* are also assessed by methods that involve microscopic analysis. Indeed, during the assay, the parasite is manipulated *in vitro*, and the effect of such manipulation is assessed by bright-field examination of the morphology of the parasite (Figure 2). The criteria used to assess morphological changes induced by a drug require visual scoring by skilled operators and is assessed subjectively. Morphological changes are usually defined qualitatively as "partial" or "extensive" (Braguine et al., 2009; de Melo et al., 2011; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011; Parreira et al., 2010; Pereira et al., 2011; Xiao et al., 2007). Therefore, the effect of anthelmintic drugs on the tegument of schistosome cannot be evaluated in a dose-dependent manner. In addition, a question remains: is the death of the parasite is associated with damage to the coat?

The inherent subjectivity of this qualitative analysis led Moraes et al. (2011) and de Moraes et al. (2011) to develop a quantitative method for evaluating the effect of drugs on the tegument of *S. mansoni* using confocal microscopy. In this quantitative analysis, areas of the tegument of male worms are assessed, and the numbers of tubercles are counted. Briefly, the parasites are fixed in Formalin-Acetic-Alcohol solution (FAA) and analysed under a confocal microscope at 488 nm (excitation) and 505 nm (emission), as described by Moraes et al. (2011) and de Moraes et al. (2011). During the microscopic analysis of the three-dimensional images captured using LSM Image Browser software (Zeiss), areas of the tegument of parasite are assessed, and the numbers of tubercles on the dorsal surface of male helminths are counted in a 20,000 μm^2 area, which is calculated using the same software image capture. Importantly, the area chosen is in the dorsal region of the male adult worm, close to the ventral sucker (acetabulum) region, because there is no significant variation in the number of tubercles in this region. Furthermore, schistosome and others trematodes are self-fluorescent (Moraes et al., 2009), and this fluorescence is increased when the parasites are in the FAA solution. This is advantageous because it allows images to be captured without using a fluorescent fluorophore. The FAA solution consists of a 2:9:30:59 mixture of acetic acid, formaldehyde, ethanol (95%) and distilled water. This methodology is summarised in Figure 3.

As previously mentioned, drug effects are currently assessed by observing morphological changes in parasites using light microscopy methods. However, these techniques do not allow the microscopic analysis of the tegument in detail. The quantitative analysis described by Moraes et al. (2011) and de Moraes (2011) must be performed with high-resolution microscopy, such as confocal or scanning electron microscopy. Therefore, the drug effects assessed by phenotypic changes, such as tegumental alterations, cannot be trusted. For example, Moraes et al. (2011) used confocal microscopy to evaluate the *in vitro* schistosomicidal activity of piplartine, an amide isolated from *Piper tuberculatum*, and demonstrated that the tegumental damage occurs after incubation with doses higher than the lethal concentrations, suggesting that worm death is caused by different mechanisms. Thus, tegumental damage may not always result in death, and a quantitative assessment technique is needed to understand the mechanisms of action of newly discovered antischistosomal drugs. In addition, the advent of screening methods that allow high-throughput, scalable, automated, and objective assays for helminth viability, combined with knowledge of the molecular biology of the schistosome to identify possible new drug targets, will make drug development for schistosomiasis easier.

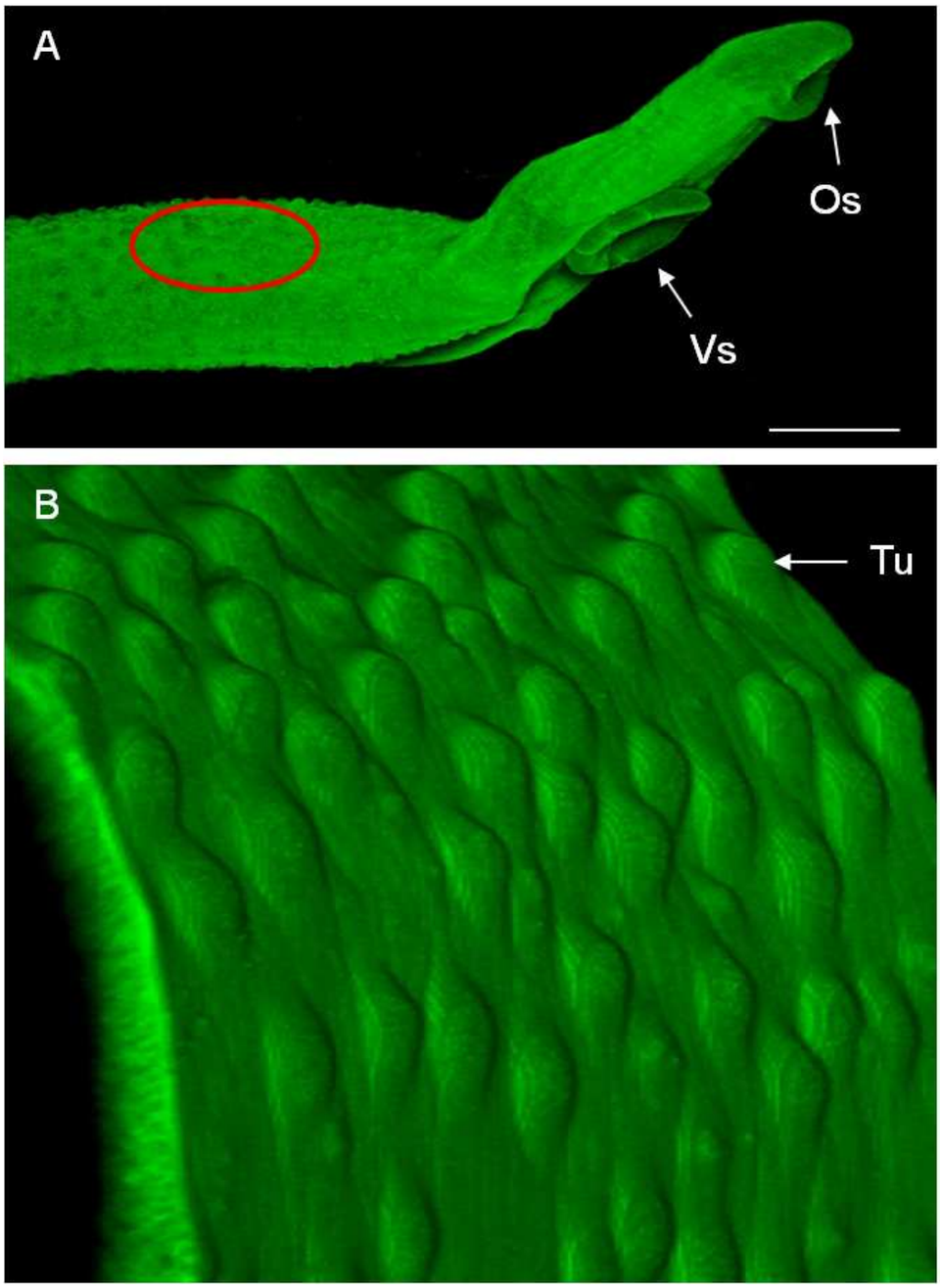

Fig. 3. Dorsal region of a *Schistosoma mansoni* adult male worm, on which the effect of antischistosomal compounds on the tegument is evaluated quantitatively. The parasite was fixed in FAA solution, and fluorescent images were obtained using a confocal microscope. A: General view of the anterior helminth region showing, in red, the location where tubercles were counted. Bar = 500 µm. B: View of an area of 20,000 µm², calculated with the Zeiss LSM Image Browser software, showing the tubercles. This image is a higher magnification of the dorsal region of the *S. mansoni* adult worm marked in red in panel A. X and Y: three-dimensional images obtained from laser scanning confocal microscopy. Os: oral sucker; Vs: ventral sucker; Tu: tubercles

5.4 In vitro assessment of the reproductive fitness of adult worms

The effects of natural or synthetic products on the reproductive fitness of *S. mansoni* have been previously reported in several studies (Braguine et al., 2009; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Mohamed et al., 2005; Moraes et al., 2011; Sanderson et al., 2002). To evaluate drug effects on schistosome during *in vitro* screening drug assays, cultures are continually monitored to assess the sexual fitness of worms treated with sub-lethal concentrations of drug. In this case, the following parameters are assessed: (1) changes in the pairing, an indicator of the mating process; (2) egg production, an indicator of egg output per worm; and (3) egg development.

In the experiments, adult worm pairs (male and female coupled) are incubated in a 24-well culture plate, as previously described here, and parasites are monitored on daily basis for 5 days using an inverted microscope and a stereomicroscope (Figure 2). Therefore, it is important that, after collection by the perfusion technique, the parasites are carefully washed to prevent the separation of the worm pairs.

Schistosome egg output *in vitro* is usually determined by counting the number of eggs. Egg development can be analysed quantitatively and scored as developed or undeveloped on the basis of the presence or absence of the miracidium (de Melo et al., 2011; Magalhães et al., 2009, 2010). This is a simple and recommended method because conventional light microscopy is able to distinguish morphologic differences in eggs. However, the characterisation of the viability of immature eggs is very difficult. Alternatively, the analysis of egg viability, distinguishing live immature eggs from dead immature ones, can be performed using a fluorescent label, as described by Sarvel et al. (2006). In this assay, the eggs obtained in culture are stained with the Hoescht 33258 probe and observed with fluorescent microscopy. The authors evaluated fluorescent labels and vital dyes, aiming at differentiating live and dead eggs, and showed the only the fluorescent Hoechst 33258 can be considered a useful tool to differentiate between dead and live eggs.

5.5 Cytotoxicity assays

Finding a new compound capable of killing a parasite is not difficult. However, it is difficult to find a substance that can kill the parasite without affecting the host. Therefore, early *in vitro* studies of new compounds must include comparative cytotoxicity data from human or animal cells in tissue culture to establish that the compound has selective antischistosomal activity and may be a realistic prospect for future clinical use in humans. In our operating procedures for antischistosomal drug screening, mammalian cells are exposed to concentrations of at least two times higher than what is needed to elicit a schistosomicidal effect. Thus, the *in vitro* schistosomicidal activity of compounds cannot be associated with cytotoxic effects.

General toxicity tests can be conducted in many cell types (e.g., fibroblasts and epithelial and hepatoma cells). Peripheral blood mononuclear cells and erythrocytes are widely used in *in vitro* studies to detect cytotoxicity or cell viability following exposure to antischistosomal compounds. Vero mammalian cells (African green monkey kidney fibroblasts) are also commonly used to examine whether natural or synthetic antiparasitic compounds are tolerated by mammalian cells (da Silva Filho et al., 2009; Moraes et al., 2011; Parreira et al., 2010).

The crystal violet staining method and the neutral red and MTT assays are the most common methodologies used to detect cytotoxicity or cell viability following exposure to toxic substances. In our *in vitro* cytotoxicity assays with cultured cells, the crystal violet

staining method is routinely used because it is rapid and inexpensive. This method measures the effects of compounds on cell growth through the colorimetric evaluation of fixed cells stained with crystal violet. Briefly, cells maintained in culture medium are seeded into 96-well culture microplates in the presence of different concentrations of extracts, fractions or isolated compounds. After different timepoints of incubation (e.g., 2, 24, 48, 72, and 96 h), the supernatants are removed and the remaining live cells are assessed by fixing and staining them with crystal violet (0.2% in 20% methanol). Viable cells attach to the bottom of the well plate, and the absorbance is measured by reading each well at 595 nm in a microplate reader (Moraes et al., 2011).

Neutral red is another cell viability assay often used to determine cytotoxicity following exposure to toxic substances (Borenfreund & Puerner, 1985). It has been used as an indicator of cytotoxicity in cultures of primary hepatocytes and other cell lines (Fautz et al., 1991; (Fotakis & Timbrell, 2006; Morgan et al., 1991). Living cells take up the neutral red, which is concentrated within the lysosomes of cells.

The MTT assay is also used to measure cell viability. MTT is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan upon the cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes, and therefore, it accumulates in healthy cells. The MTT assay has been tested for its validity in various cell lines (Fotakis & Timbrell, 2006; Mossmann, 1983).

Alternatively, the lactate dehydrogenase (LDH) leakage assay and protein assay are also used to detect cytotoxicity, despite the fact that they have low sensitivity when compared to the methods already described. The LDH leakage assay is based on the measurement of LDH activity in the extracellular medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage (Decker & Lohmann-Matthes, 1988; Fotakis & Timbrell, 2006). The protein assay is an indirect measurement of cell viability because it measures the protein content of viable cells. Despite the existence of several protocols to establish total protein concentration (e.g., biuret, bicinchoninic acid, Lowry and Bradford protocols), the two most commonly used methods for protein quantification are the Lowry and Bradford assays (Bradford et al., 1976; Lowry et al., 1951).

Finally, tritiated thymidine-based methods, which act through the incorporation of tritium into the DNA of cells, have also been used currently to detect cytotoxicity, especially in immune cells (Pechhold et al., 2002).

6. Conclusions

Schistosomiasis is a neglected disease that is one of the most common chronic infections among the poorest people in the world. Most chemotherapeutic-based programs attempting to eradicate schistosomiasis in the developing world rely on the effectiveness of a single drug, praziquantel; therefore, there is an urgent need to identify new parasite targets and effective antischistosomal compounds. Secondary plant metabolites have attracted the attention of many researchers over the years as a result of the variety of their chemical structures and their broad range of biological activities that may provide lead structures for the development of new drugs. Recently, marine organisms have also been recognised as an attractive source of antiparasitic compounds, and it can be expected that other living organisms, such as insects and amphibians, will emerge as additional sources in the future.

Discovering untapped natural sources of new anthelmintic compounds remains a major challenge and a source of novelty in the era of combinatorial chemistry and genomics. To find new anthelmintics, all sources of natural, synthetic and semi-synthetic lead compounds must be investigated. *In vitro* bioassays using parasitic worms have played a central role in the early pre-clinical stages of most research on potential natural anthelmintics. The identification of the antiplasmodial and antischistosomal activity of the sesquiterpene lactone artemisinin has stimulated interest in natural products, and soon, promising leads will be identified with new chemical types and active agents against schistosomiasis. Therefore, bioprospecting programmes related to the isolation of bioactive compounds must be rewarded, and the screening *in vitro* of chemical constituents belonging to different classes must be evaluated on the blood fluke *S. mansoni.*

The literature regarding antischistosomal compounds contains a large number of natural products screened for their schistosomicidal properties. However, only a few of these may be promising drug leads in the development of a therapeutic reserve for schistosomiasis. Therefore, it is important to continue to identify new drugs and to explore alternative strategies to improve screening efficacy. Most of the extracts or natural compounds were only evaluated with *in vitro* studies; it is expected that they will be evaluated using *in vivo* experimental models. Further, it must be mentioned that the results of *in vitro* assays with many drugs do not correspond to what is observed *in vivo*; however, *in vitro* screening could identify novel anthelmintics that could eventually translate into practical applications. Thus, while *in vitro* tests are recommended initially, the assessment of therapeutic activity using *in vivo* models should be performed.

The analysis of the *S. mansoni* genome and transcriptome offers great possibilities for identifying possible new drug targets and will facilitate further exploration of differences between host and parasite metabolic pathways. In addition to the isolation and structural determination of new drugs from natural products and information from the originating plant, the integration of the pharmacological properties of natural products with the functional genomic and proteomic studies in schistosome and *in vitro* screening methods with improved automatic high-content screening will be important tools to identify possible new drugs in the future and shed light on the approaches of helminth chemotherapy. Attempting new combinations of natural or synthetic drugs will be also important in discovering alternative drugs to replace the use of praziquantel.

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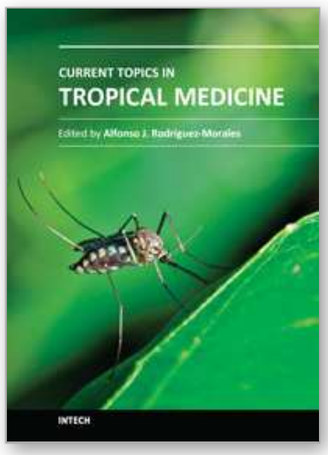

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Tropical Medicine has emerged and remained as an important discipline for the study of diseases endemic in the tropic, particularly those of infectious etiology. Emergence and reemergence of many tropical pathologies have recently aroused the interest of many fields of the study of tropical medicine, even including new infectious agents. Then evidence-based information in the field and regular updates are necessary. Current Topics in Tropical Medicine presents an updated information on multiple diseases and conditions of interest in the field. It Includes pathologies caused by bacteria, viruses and parasites, protozoans and helminths, as well as tropical non-infectious conditions. Many of them are considering not only epidemiological aspects, but also diagnostic, therapeutical, preventive, social, genetic, bioinformatic and molecular ones. With participation of authors from various countries, many from proper endemic areas, this book has a wide geographical perspective. Finally, all of these characteristics, make an excellent update on many aspects of tropical medicine in the world.

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