Chapter from the book *Special Topics in Drug Discovery*

Downloaded from: http://www.intechopen.com/books/special-topics-in-drug-discovery

Interested in publishing with InTechOpen?
Contact us at book.department@intechopen.com
Schistosomiasis: Setting Routes for Drug Discovery

Naiara Clemente Tavares,
Pedro Henrique Nascimento de Aguiar,
Sandra Grossi Gava, Guilherme Oliveira and
Marina Moraes Mourão

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65386

Abstract

Schistosomiasis is the second most prevalent parasitic disease in the world. Currently, the treatment of this disease relies on a single drug, praziquantel, and due to the identification of resistant parasites, the development of new drugs is urged. The demand for the development of robust high-throughput parasite screening techniques is increasing as drug discovery research in schistosomiasis gains significance. Here, we review the most common methods used for compound screening in the parasites life stages and also summarize some of the methods that have been recently developed. In addition, we reviewed the methods most commonly implemented to search for promising targets and how they have been used to validate new targets against the parasite Schistosoma mansoni. We also review some promising targets in this parasite and show the main approaches and the major advances that have been achieved by those studies. Moreover, we share our experiences in schistosomiasis drug discovery attained with our S. mansoni drug screening platform establishment.

Keywords: Schistosoma mansoni, drug screening, histone-modifying enzymes, protein kinases, inhibitors, RNA interference

1. Introduction

Schistosomiasis is a chronic parasitic disease caused by flatworms of the genus Schistosoma. The main species of medical relevance are Schistosoma mansoni, Schistosoma japonicum, and Schistosoma haematobium, which infect around 258 million people worldwide, causing 300,000
deaths yearly, according to WHO statistics. The economic and health effects of schistosomiasis are considerable as this disease can be highly debilitating. To date, there is no licensed vaccine against the disease, and treatment is based on a single drug, praziquantel [1, 2]. Despite its effectiveness, the heavy reliance on a single drug bears a risk of drug resistance development and, indeed, resistant parasites have already been reported [3–7]. Additionally, praziquantel presents poor efficacy against immature schistosome life stage, and there is no pediatric formulation available. Hence, drug discovery in schistosomiasis is still of great importance.

2. Drug screening techniques in schistosomiasis

Several approaches are used to search new drugs for infectious diseases. Among them, we can highlight: selective, empirical, biochemical, and genomic approaches. In the selective approach, compounds targeting molecules important for parasite survival and/or development (a “chokepoint”) are tested. The empirical method consists of a blind random test of a large number of compounds, without any previous knowledge. The biochemical approach verifies whether the compounds are capable to change parasite metabolism. Finally, the genomic approach aims to search for new drug targets based on parasite and/or host genome analysis [8, 9].

Drug screening in Schistosoma has been performed using a myriad of techniques and, most of the time, using a single life stage. In order to optimize drug screening in a parasite presenting such distinct and complex life cycle, the search for inhibitors must be performed in the different human infecting life cycle stages such as the schistosomula and adult worms (males and females) as drug sensitivity can differ between the stages and sexes [7]. The screening should also be conducted in vitro and in vivo since drug activity can be diverse in different systems.

A wide range of in vitro methods for drug screening in S. mansoni is described; however, microscopic observation to identify the presence of intracellular granularity and changes in the shape and movement of the parasite remains the most used technique, and it is considered the “gold standard” for parasite viability evaluation [10, 11]. However, this analysis can be subjective: (1) it relies in one observing person; (2) schistosomula may exhibit characteristics of dead specimens even when viable [12]; and (3) it is time consuming.

Recently, new methods based on high-throughput (HTS) and whole-organism screens in helminths have emerged. The HTS method is based on screening a parasite target against a large number of compounds in parallel (minimum of 10,000) and may be performed manually or automatically using robotic systems [13–15]. In contrast, the whole-organism screen intends to test a small number of compounds against the pathogen and the drug effect is usually individually analyzed [16]. These new methods monitor the parasite by video, impedance, enzymatic activity, colorimetry, and fluorimetry among others [17–23].

An example of fluorimetric method is the quantification of lactate excreted by the parasite in the culture medium. The parasite tegument presents two glucose transporters, SGTP1 and SGTP4, which acquire glucose present in the host bloodstream [24]. Lactate is the final product
after glycolysis, and it is excreted through aquaporin SmAQP, an aquaglyceroporin homologue [25]. The amount of lactate excreted by the parasite can be measured by fluorimetric assays with probes that bind to lactate and emit fluorescence. The measurement of lactate produced by cells has frequently been used for the analysis of cells or whole-organism viability [26, 27]. This method was used by Howe and collaborators [17] and was proved feasible in *S. mansoni*, owing to its sensitivity to measure the viability of adult worms and schistosomula.

Fluorescence viability analysis in schistosomula was also performed by Peak and collaborators [23] combining the use of propidium iodide (PI) (544 nm excitation/620 nm emission) and fluorescein diacetate (FDA) (485 nm excitation/520 nm emission). The PI intercalates into DNA if the membrane of cells is permeable in damaged or dead parasites, but in viable schistosomula, PI is incapable to cross the membrane. Breach of the membrane permeability allows PI to stain nucleic acids. On the other hand, FDA is able to penetrate the membrane of live schistosomula, and due to the parasite esterase activity, it is converted into a highly fluorescent and charged fluorescein, and FDA cannot readily exit living cells. This test requires a fluorescence inverted microscope to evaluate each spectrum. However, even without a fluorescence readout, staining with PI is a quantitative, simple and low-cost method that has been used for a long time for viability evaluation in *S. mansoni* by our group [28].

The blue dye resazurin has been widely used in drug testing in *Trypanosoma* and *Leishmania* species [29, 30]. This method relies on an oxidation-reduction reaction, wherein resazurin suffers a colorimetric change in response to cellular metabolic reduction. The reduced form is pink, and thus, the intensity of fluorescence produced is proportional to the number of living cells. Despite being described as an effective drug test for *S. mansoni* and demonstrating that they were able to detect schistosomula viability, its use is questionable since, when compared to the visual test, this method showed low sensitivity [22]. In our hands, this method has proven very variable among replicates and requires a very specific fluorescence plate reader.

Movement-based assays are widely used for anthelmintic drug screening in adult worms and might be the first phenotype tested in most screenings, whereas movement measures are not explored as much for testing viability of the *S. mansoni* larval stage. Kotze et al. [31] performed visual assays to test drug sensitivity in *Strongyloides* species. This method consisted in scoring the larvae that exhibited movement after 48 h of drug incubation and stimulation with hot water. The approach proved to be an efficient assay for testing new drugs and for detection of resistance; nevertheless, it is quite subjective. Meanwhile, Paveley et al. [19] performed a phenotype analysis in *S. mansoni* schistosomula exposed to drugs. The authors developed an image-based method that collects images in time-lapse every 6 s and analyzes the cumulative change in the area occupied by each schistosomulum. The researchers concluded that this method is suitable for drug screening in schistosomula, although requiring a complex analysis involving machine training.

Recently, Rinaldi et al. [11] have adapted a cell viability method, which is based on impedance, called xCELLigence, to evaluate the movement of cercariae, schistosomula and adult worms, and measure egg hatching of *S. mansoni*. This assay consists in analyzing the changes in electric conductivity due to parasite contact with electrodes; therefore, more parasite movement is
measured as a large electric conductivity change. The possibility of using this method in drug screening using cercariae, adult worms, and eggs was also verified. However, this method is at a preliminary stage of standardization, since only one drug was tested and exhibited large variation across electrical frequencies tested; in addition, the cost of the equipment and its plates is elevated.

A very promising method that provides a viability assay in a high-throughput fashion and with semiquantitative measurements of movement of helminth worms was developed by Marcellino and collaborators [18]. This method has demonstrated to be efficient and sensitive for drug screening using *S. mansoni* adult worms, since worm movement is considered an important parameter for high performance tests. In this assay, a video camera and a free software, called WormAssay, are required and output adult worm movement units. The smaller the value of movement units generated by the software, the lower the parasite viability. Later, this methodology, named it as Worminator, was adapted to be used with microscopic parasites as third-stage larvae of *Cooperia* spp. and *Brugia malayi* and *Dirofilaria immitis* microfilariae. Therefore, Worminator could be an efficient alternative for measuring schistosomula motility [32].

Another promising method based on video capture was developed by Lee and collaborators [14]. This assay is an automated method to analyze images of schistosomula or other parasites in 96, 384, or 1536 well plate format and qualify innumerous phenotypes. A machine-trained algorithm was able to quantitatively describe the following characteristics: size, shape, movement, texture, and color. With this method, it is possible to perform a high-throughput whole-organism drug screening. The web server called quantal dose-response calculator (QDREC), which was described by Asarnow and collaborators [33], is based on this methodology. QDREC compares drug-treated parasites with untreated parasites and automatically determines dose-response. This method is the only automated method to date that provides EC50 (half maximal effective concentration) values based on phenotypic analysis. QDREC was validated using schistosomula and proved to be a high-throughput and reliable method.

For *in vivo* assays, mice are suggested as the animals of choice, owing to their susceptibility to experimental infection [8, 34]. Thus, mice are infected with cercariae and treated with drugs. After infection, one should observe eggs and granuloma numbers in the liver and intestine, oogram alterations, and number of adult worms recovered after perfusion [35]. This method has been employed using single or combination of praziquantel and in association with lovastatin, clonazepam, among others.

3. Case studies

In order to perform *in vivo* assays, mice infected with *S. mansoni* for 45 days were treated with a single dose of imatinib. After 15 days of treatment, the animals were killed and perfused. *In vitro* tests with imatinib impaired movement of adult worms; however, when mice were treated with this drug, no alterations were found in the oogram and adult worm recovery, demonstrating the need of a complete assay during drug screening [36]. In addition, Pereira and
collaborators [37] tested the compound (−)-6,6′-dinitrohinokinin (DNK), a dibenzylbutyrolactone lignin, using in vitro and in vivo assays. This compound seems to affect the development and reproduction of the parasite as it caused reduction in adult worm recovery and significantly decreased egg count, corroborating the results of in vitro tests. These findings reaffirm the need for in vivo testing to confirm drug efficacy, since the biological effects highly rely on host metabolism.

Drug combinations for infectious diseases therapy represent an alternative to retard drug resistance. Based on that premise, Araújo et al. [38] treated infected mice with a single dose of clonazepam associated with praziquantel or oxamniquine. The results showed alterations in the oogram and a higher number of dead worms recovered from liver of mice treated with both clonazepam and praziquantel, in comparison with mice treated only with praziquantel. In another study, the association of praziquantel and oxamniquine with lovastatin was investigated in vivo, and a higher number of dead adult worms, as well as oogram changes, were observed in mice treated with the combination lovastatin and oxamniquine compared to animals treated with praziquantel [38]. These findings suggest that drug combination is a promising alternative for schistosomiasis treatment.

Thus, it is highly relevant to follow systematic procedures during drug screening. Here, we present a schematic workflow of the above-mentioned methods and different life stages that could be included in drug screening experiments (Figure 1).

Figure 1. Schistosoma mansoni compound screening methods. The workflow represents in vivo and in vitro assays which can be employed for drug/compounds screening in S. mansoni.

4. Establishment of an anti-Schistosoma mansoni drug screening platform

The absence of efficient alternative for schistosomiasis treatment demonstrates the need for new research involving the development of new schistosomicidal compounds. Accordingly,
development of a *S. mansoni* drug screening platform, which aims at the study of compound/drug efficacy in different parasite life stages using distinct methodological approaches is required. To date, our group has immersed in this field seeking new methodologies, standardization of existing ones, and validation of results between different laboratories working in the field. It is important to highlight that *Schistosoma* strains vary in response to treatment, and in order to attain relevant leads, a concerted effort is important. Here we present some data of drug screening in schistosomula and adult worm stages and the comparison of four *in vitro* methodologies.

For drug screening in schistosomula, the methods of choice were: resazurin fluorescence assay, lactate quantitative analysis, and visual assay using propidium iodide staining (PI). In order to perform drug screening in adult worms, the movement analysis software WormAssay was employed.

The standardization for schistosomula drug screening was performed using 96-well plate format with 100, 200, 400, 600, and 800 parasites per well. Schistosomula were submitted to three different treatments: parasites exposed to 0.1% dimethyl sulfoxide (DMSO; vehicle control); heat-killed parasites (negative control); and parasites exposed to 20 μM of the sirtuin inhibitor Salermide (half maximal inhibitory concentration—IC50). Lancelot et al. [39] demonstrated that Salermide induces death and apoptosis of schistosomula, separation of adult worm pairs, as well as a reduction in egg laying.

Our results have shown that mortality evaluation by phenotype observation in bright-field light microscope was overestimated when compared to PI staining results. This result reinforces the subjectivity problem of bright-field visual analysis since parasites that presented a dead phenotype (intracellular granularity and absence of movement) not always stain with PI, indicating their viability.

In viability assays using resazurin, we observed large variability between technical and biological replicates and a low value of relative fluorescence units (RFU). Mansour and Bickle [22] described RFU values >1000 in wells containing 100 schistosomula, and such high RFU values were possible only with 500 parasites per well in our assays conditions. Overall, using this fluorescence assay, we were unable to discriminate schistosomula killed by Salermide treatment from the vehicle control (0.1% DMSO). Issues in assay sensitivity measured by RFU values could be due to differences in the fluorescence reader platform, indicating that the assay can present a reproducibility issue. Limitations in this methodology such as low sensitivity and reliability, when compared to visual analysis, were described by the authors who proposed the application of resazurin as *S. mansoni* viability test [22].

Standardization of lactate quantitative analysis demonstrated low variability and significant differences between RFU values of schistosomula exposed to 20 μM Salermide from 0.1% DMSO. RFU values were similar to those described in the work of Howe and collaborators [17] confirming the method’s reproducibility. Mortality of the parasites detected in the lactate quantitative analysis was confirmed by PI staining and observation under the microscope, in
contrast to our previous results with resazurin assay. Considering the fluorescence-based viability assays, lactate quantitative analysis has shown to be more reliable in our hands. However, the test may also be subject to interference, since some compounds crystallize in contact with culture medium and can emit fluorescence at the wavelength utilized (530 nm excitation/590 nm emission).

The use of viability assays that target schistosome metabolism is important for drug screening, since some drugs may reduce parasite viability but do not cause parasite death, and hence these parasites may not stain by PI. Therefore, we believe that the combination of these two approaches to evaluate parasite viability/mortality is a good strategy for drug screening as they are complementary methodologies.

Regarding adult worm drug screening, Howe and collaborators [17] proposed the use of one male adult worm per well exposed for 72-h treatment with praziquantel and mefloquine, and detected a reduction in medium lactate. The use of only males and one parasite per well does not validate this methodology for drug testing in adult worms, as males and females may react differently to treatments and each individual has distinct susceptibility/metabolism. Therefore, the use of this method would require larger number of worms including both sexes.

The method of trematode movement analysis using impedance was described by Smout and collaborators [20] and was recently validated in S. mansoni [11]. In this study, one worm per well was also challenged and wide variation in sensitivity of the experiment was reported due to variation in the electric frequency (kHz). Due to the limitations in those methods, we implemented the WormAssay [18] to analyze adult worm exposure to drugs.

In our platform, eight adult worms were established as the minimum number of specimens to be analyzed. Most importantly, paired females and males should not be analyzed because a drug could be active in only sex, disturbing the movement analysis due to female presence in male gynaecophoric channel. The plates containing the worms are filmed for 1 min and 30 s every day for 10 days. The software is freely available for download, recognizes the wells, and provides the total of movement units for each. Moreover, handling is simple and our tests confirm its sensitivity and accuracy.

For validation of our anti-S. mansoni drug screening platform, we used epigenetic modification factor inhibitors. A total of 137 compounds were tested on S. mansoni schistosomula and adult worms as part of the A-PARADDISE consortium (http://a-paraddise.cebio.org/) using the visual assay with PI, the quantitative analysis of lactate for schistosomula, and the WormAssay for adult worms. Among these compounds, 114 (83%) exhibited some effect on the parasite. From 114 active compounds, 29 were active in all methods used, and in both sexes and stages tested, 12 compounds were active only in schistosomula, 35 were active at least in one sex of adult worm and, among them, 24 were active only in female worms (Figure 2).

These data confirm the need to perform an assay aimed at identifying compounds capable of altering the parasite metabolism and, consequently, their viability, since the compound cannot cause parasite death, hindering the observation of drug effect in a visual assay. Clustering methodologies, life stages, and parasite strains could change the outcomes of large screening studies such as those using a single method and stage; for example, Li et al. [40] performed a
screening of 59,360 thioredoxin glutathione reductase (TGR) inhibitors against *S. mansoni* thioredoxin glutathione reductase (SmTGR) recombinant protein using a fluorometric assay, 74 were active and tested in *S. mansoni*, and among them, 53% were active in schistosomula and only 2.7% were effective against adult worms. Additionally, Yousif et al. [41] carried out a screen of 309 plant extracts on adult worms employing visual analysis of viability and identified 14% as active.

**Figure 2.** Venn diagram of the active compounds tested in propidium iodide, lactate quantification, and WormAssay. The diagram represents the distribution of the active compounds according to stage and used methods. The schistosomula assay using staining with PI is identified by the color green, schistosomula assay using the quantitative analysis of lactate is identified by the color yellow, the test in male adult worms by WormAssay is indicated by the color blue, and the test in female adult worms by WormAssay is represented by the color red.

Our work demonstrates the need to use parallel and complementary methods, since only using PI, for example, a lower number of compounds would be selected as active and consequently, potential compounds would be excluded. In view of the foregoing methods, the contributions from studies aimed at identifying new therapeutic compounds against schistosomiasis could, perhaps, be most effective if they employ more than a single method for screening drugs and different parasite stages. The association of fluorescence with PI staining enables the selection of compounds capable of altering parasite viability and/or inducing mortality. Moreover, male and female adult worms develop and have different metabolisms, and thus, their susceptibility to a specific drug also differs. One clear example is the studies demonstrating that female adult worms are more sensitive to praziquantel than males [4, 42] and approximately 1341 genes are up-regulated in female adult worms when compared to male [43]. Regarding the different life stages, results demonstrate that some drugs, including praziquantel, are active solely in the mature stage of *S. mansoni* [44].
These results indicate the need to employ different methods for drug screening as one can find a larger number of hits than when the tests are performed using only one method or in only one stage of the parasite. In addition, it substantiates the importance of using the selective approach to find active compounds, thus using a rational approach targeting previous validated targets, allowing direct design of specific compounds.

5. Validation of \textit{S. mansoni} drug targets

The most common strategies used in drug discovery are the empirical and the rational approaches. The first is based on testing various compounds randomly looking for biological activities. The second is also called the selective approach as it proposes identifying a biological target and then designing or looking for a specific inhibitor for that target. In this sense, there are many strategies to assess gene function in parasites in order to elucidate their role in development, mechanisms of drug resistance, and speculate its use as a parasite control method. Among them, we can mention comparative “omics,” RNA interference, heterologous complementation using model organisms (i.e., \textit{Caenorhabditis elegans}) and \textit{in silico} approaches. Comparative “omics” has been used to identify potential target proteins that appear to be essential for the parasite, while RNA interference has been used to validate a variety of drug target molecules, such as histone-modifying enzymes (HMEs), protein kinases, and others.

5.1. Histone-modifying enzymes

Among the most studied targets are epigenetic modulators, and among them are regulators of chromatin epigenetic modifications, named histone-modifying enzymes (HMEs), which act on the epigenome resulting in a change in the gene transcription profile. HMEs are involved in a wide range of reactions including methylation, demethylation, acetylation, deacetylation, phosphorylation, ADP-ribosylation, deimination, sumoylation, ubiquitination, etc. Yet, recent findings have described a myriad of lysine modifications, among others: formylation, succinylation, crotonylation, and malonylation [45].

The enzymes involved in the insertion and removal of methyl groups are called histone methyltransferase (HMT) and histone demethylases (HDM) [46]. The histone-modifying enzymes involved in the insertion and removal of acetyl groups are called histone acetyltransferases (HAT) and histone deacetylases (HDAC and sirtuins). Acetylation of lysine residues and methylation of lysine and/or arginine residues in histones H3 and H4 tails are two changes of particular importance [47, 48]. The insertion of acetyl and methyl groups neutralizes the positive charge of histones, destabilizing the structure of nucleosome and allowing the DNA to separate from histones. This results in the facilitation of the access of transcription factors and RNA polymerase to the DNA stimulating gene expression. The removal of these groups has the opposite effect, increasing the positive charge, condensing chromatin, and thereby, repressing transcription [49]. The heterochromatin is transcriptionally inactive when highly methylated at lysine 9 of histone H3 and not methylated at lysine 4, and hypoacetylated in histones H3 and H4 [50]. It is important to highlight that HDACs and sirtuins also deacetylate
other nonhistone substrates such as chaperones, peroxiredoxins, transcription factors, signaling mediators, and structural proteins (e.g., [51–55]).

These modifications allow specific combinations that affect the overall structure of chromatin and the transcription of genes, the so-called histone code, which is, in many cases, conserved among organisms [50]. Aberrant epigenetic states are often associated with human diseases such as cancer, inflammation, metabolic, and neuropsychiatric disorders, and thus HMEs are implicated and intensively studied as therapeutic targets in various diseases [56–59].

One of the most promising approaches for drug discovery among HMEs is the development of HDAC inhibitors, which targets the highly studied lysine deacetylases. These targets are also key for parasites, including schistosomes, which present, similarly to tumors, dependence on lactate fermentation as energy source, host independent growth, high metabolic activity, and host immune evasion through mimetism of molecules [60]. In addition to schistosomes, HMEs have been highly explored as drug targets for parasitic diseases such as Trypanosoma brucei, Plasmodium falciparum, Leishmania spp., Toxoplasma sp., among others [61–63].

Many different types of HDAC inhibitors (HDACi) are under development. The inhibitors targeting class I and II HDACs are classified into four families according to their structure: inhibitors containing short-chain fatty acids (butyrate and the valproic acid—VPA), compounds derived from hydroxamic acid (the Trichostatin A—TSA and the acid-suberoylanilide hydroxamic—SAHA or vorinostat), and the group of cyclic tetrapeptides and benzamides. Among these inhibitors, SAHA was approved by Food and Drug Administration (FDA) for use in adult individuals with cutaneous T-cell lymphoma [64]. In vitro tests with butyrate, VPA and TSA, performed with human cells demonstrated that these inhibitors lead to apoptosis, differentiation, and cell cycle arrest [65].

Some studies have shown that HDACi, such as TSA, triggers histone H4 hyperacetylation in S. mansoni schistosomula at low concentration (2 μM), leading to apoptosis and affecting significantly gene transcription [66, 67]. According to Pierce and collaborators [65], in vitro exposure of schistosomula and adult worms with VPA inhibitors (TSA and SAHA) inhibited 80% of the total HDAC activity and caused the death of parasites.

In addition to pan HDAC inhibitors, one international consortium has been focusing in strategic epigenetic druggable targets for diverse parasites [65]. Recently, SmHDAC8 has risen as a promising target to treat schistosomiasis. First, SmHDAC8 was validated and proved to be essential for parasite infectivity, since parasites knocked down for SmHDAC8 were unable to normally develop in the mammalian host and showed, approximately, 50% reduction in oviposition [68]. Structural analysis has also shown that HDAC8 of S. mansoni presents important structural differences when compared to the human orthologue, despite a single amino acid substitution in the active site [68]. In this work, schistosomula larvae were exposed to hydroxamate derivative, HDAC8-specific inhibitor (J1075), which led to apoptosis and parasite death. These studies are a proof of concept that HMEs are important therapeutic targets, and potential new drugs based on HDACi can be developed against schistosomiasis.

To date, a way to expand the repertoire of specific Schistosoma, HDACi is utilizing a “piggy-back” strategy, which accelerates the exploration for novel antischistosomal compounds. These
strategies are founded in the principle of using structure-based inhibitors, previously validated for other illnesses or other targets, to add a variety of chemical scaffolds and backbones, facilitating the development of selective inhibitors specifically aiming the schistosoma HMEs [69].

Besides deacetylases, HAT inhibitors and some derivatives of medicinal herbs, such as curcumin, also demonstrated their potential as inhibitors, since they induce hypoacetylation and lead tumor cells into apoptosis [70, 71]. Magalhães and collaborators [72] demonstrated the efficacy of curcumin in *S. mansoni*. In this study, the authors reported that in the presence of curcumin (5 and 20 μM), adult worms lose 50% of viability and reduce oviposition, and higher concentrations (50 and 100 μM) cause 100% mortality of worms. These data corroborate the results observed by our group, that 20 μM of this KDM1 and HAT inhibitor, causes a 95.5% motility reduction of female adult worms, confirming the importance of LSD1 and HAT for the development of *S. mansoni* (unpublished data).

Recent studies in *S. mansoni* demonstrated that the HAT inhibitor, PU139, at a high concentration (50 μM), affects adult worm pairing, the reproductive system of female adult worms, and the maturation of viable eggs [73]. In our hands, with a lower dose (20 μM), this compound was able to reduce female motility, confirming that it is active in the worm and highlighting the aforementioned (Section 2) necessity of assaying drug tests in female and male separately in order to reduce false negatives in drug screening in schistosomes.

Studies using HMT and HDM inhibitors are less common, but some results have shown that the chloroacetyl derivative, allantodapsone, a PRMT1 (arginine methyltransferase) inhibitor, showed selective inhibition affecting the growth of tumor cells [74]. Inhibitors of KDM1 (LSD1), a histone demethylase, are considered promising compounds for cancer therapy [75]. Studies performed by our group, knocked down for PRMT3 and KDMs in schistosomula by RNA interference, show that these enzymes are important for *S. mansoni* reproduction (unpublished data).

Due to the wide range of HME functions as “erasers” and “writers” of the epigenome, boosted by the use of histone and many nonhistone protein substrates, and taking into account the cellular localizations of these enzymes, it has been demonstrated, as expected, that they are essential and attractive targets for development of therapy for a number of infectious diseases, including schistosomiasis.

5.2. Protein kinases

The *Schistosoma* complex life cycle involves different life stages and requires sophisticated coordination of its physiological systems to ensure success of infection and survival in both hosts. Therefore, signals of the environment and hosts stimulate physiological, morphological, and biochemical changes [76, 77]. It involves nonlinear signaling networks that switch protein activity by phosphorylation (by protein kinases—PKs) and dephosphorylation (by protein phosphatases) of amino acid residues, or by incorporation of GTP. Protein kinase phosphorylation and the subsequent activation of signaling cascades result in the activation of transcription factors that target specific genes promoting or blocking their transcription. Furthermore,
they can alter enzymatic activity, interaction with other proteins and molecules, cellular localization, and susceptibility to proteases degradation [78].

Eukaryotic protein kinases (ePKs) participate in phosphorylation cascades that regulate diverse cellular processes. PKs are among the largest gene families in eukaryotes and have been extensively studied and considered potential targets for drug development. The development of PK inhibitors has culminated in the approval of some drugs for the treatment of various human diseases such as cancer and diabetes. Furthermore, PKs have gained interest as potential drug targets against many parasites, including S. mansoni [79–82].

The S. mansoni predicted proteome is composed of about 2% of PKs, a total of 252 proteins, of which only about 30 have some functional experimental evidence [83]. The scarcity of data on S. mansoni PKs has motivated studies that will contribute to a better understanding of the roles of this protein family in parasite development and survival.

Four mitogen-activated protein kinases (MAPKs) were studied by our group using double-stranded RNA-mediated interference to elucidate their functional roles. Mice were infected with schistosomula after gene knockdown, and the development of adult worms was observed. Andrade et al. [79] showed that c-Jun N-terminal kinase SmJNK participates in the maturation and survival of the parasite, associated with the presence of undifferentiated oocytes and damage in the adult male tegument. SmERK-1 and SmERK-2 are involved in egg production, since females were recovered with undeveloped ovaries and immature oocytes, and the infected mice harbored significantly fewer eggs. Furthermore, the Smp38 kinase seems to have an important role in the development and survival of parasites and in their protection against reactive oxygen species (unpublished data). Thus, we demonstrated that MAPK proteins are important for parasite survival in vivo and are essential for the development and reproduction of the parasite.

Guidi et al. [84] used RNA interference to investigate the function of 24 proteins in adult worms and schistosomula, and among those, kinases were included. For atypical protein kinase C (SmaPKC), knockdown resulted in decreased viability in both stages. Knockdown of polo-like kinase 1 (SmPLK1) and p38 MAPK (Smp38) increased mortality only in larvae. The SmPLK1 inhibition with BI2536, a specific inhibitor, also increased mortality and interfered with egg production. Knockdown of SmPLK1 and SmaPKC also resulted in lower worm recoveries in vivo.

Ressurreição et al. [85] reported that phosphorylation of PKC, ERK, and p38 MAPK kinases is modulated by light and temperature. Furthermore, in response to linoleic acid, these kinases appear to coordinate the release of components of the cercarial acetabular gland, and PKC and ERK, when activated, are located in putative sensory receptors in the tail, thus demonstrating the importance of PKC, ERK, and p38 MAPK signaling pathways in the mechanisms for host penetration.

As mentioned, PKs are conserved and widely studied in many organisms; therefore, a range of PK inhibitors is already available, which are valuable tools. The function of some kinases was studied by parasite exposure to these inhibitors to explore PK functions in S. mansoni. Matsuyama et al. [86] demonstrated that cAMP-dependent protein kinase is involved in the
osmosis-regulated ciliary movement of miracidia by exposure to the inhibitors PKI(14-22)amide, H89, and H88, verifying the complete inhibition of miracidia swimming in artificial pond water. Inhibition of protein kinase C (SmPKC) by GF109203X accelerates the rate of larval development of *S. mansoni*, with miracidia shedding its ciliary plates significantly faster and developing into mother sporocyst [87]. Another example, the polo-like kinase SmPlk1 has a potent and selective inhibitor (the anticancer drug BI 2536), which induces changes in schistosome gonads, an indication that SmPlk1 participates in parasite gametogenesis [88]. More recently, Long et al. [89] showed that knockdown of SmPlk1 using RNA interference induced abnormal phenotypes in schistosomula. They also tested a panel of 38 benzimidazole thio- phene PLK1 inhibitors and 11 commercially available human PLK1 inhibitors in schistosomula and adult worms using microscopical observation and the QDREC approaches to verify changes in the parasite. Many of these inhibitors caused deleterious changes in the parasite.

Knobloch et al. [90] used the inhibitor Herbimycin A to demonstrate that protein tyrosine kinases (PTKs) regulates gonad development and egg production through changes in gene expression of eggshell proteins, and suggested PTKs as novel anti-*Schistosoma* drug targets. Transcriptome analyses of female worms after treatment with the inhibitors Herbimycin A and TRIKI, or both, revealed a number of genes that were transcriptionally affected. Herbimycin A specifically inhibited the Src kinase SmTK3, and TRIKI (TGF-β receptor type I kinase inhibitor) inhibited the TGFβ receptor, SmTβR. The expression of genes with recognized function in eggshell formation was investigated using quantitative polymerase chain reaction (qPCR) and proved to be regulated by the signaling pathways containing Src and TβRI [91].

Imatinib, an Abl-kinase-specific inhibitor used in human cancer therapy, was tested against adult worms and caused effects on morphology and physiology of *S. mansoni* couples *in vitro* [92]. Transcriptome analyses of adult worms treated with imatinib were performed using microarray and qPCR. Genes related to surface, muscle, gut, and gonad processes were differentially expressed. In addition, a comparative analysis of microarray data with previous data after TRIKI inhibition was performed and provided evidence of an association between TGFβ and Abl kinase signaling pathways [93].

Syk kinase (SmTK4) function was studied in adult worms using RNA interference and the specific inhibitor piceatannol. Prominent morphological changes in testes and ovaries were observed, demonstrating the role of SmTK4 in gametogenesis. In addition, the authors used yeast two-/three-hybrid library screenings and identified a Src kinase (SmTK6) acting upstream and a MAPK-activating protein and a mapmodulin acting downstream of SmTK4 [94].

A set of commercial kinase inhibitors was tested by Morel et al. [95] in schistosomula and adult worms, conforming deleterious effects on parasite physiology, as well as the importance of kinases in parasite biology and reproduction. In that study, five protein kinase B (SmPKB or SmAkt) inhibitors were tested and three affected pairing and oviposition of adult worms, in addition cause mortality in larvae. These data, along with other studies [96], suggest that SmAkt is a key regulator of schistosoma reproduction processes [95].

The roles of protein kinases C (PKCs) and extracellular signal-regulated kinases (ERKs) were studied through modulation of PKC and ERK activity by kinase activators and inhibitors in
adult worms. Results have shown that this modulation induced worm uncoupling, suppressed egg output, male worm detachment, worm paralysis, and provoked sustained coiling. The authors also reported that praziquantel, the drug of choice for schistosomiasis treatment, induced activation of *S. mansoni* PKCs and ERKs. Activated PKC and ERK in adult worms are associated with muscular, tegumental, and reproductive structures [97].

5.3. Other targets

In addition to epigenetic modification, effectors and protein kinases, numerous proteins related to proteolytic, xenobiotic metabolism, redox processes, nucleotide biosynthesis and proteins involved in the nervous system of *Schistosoma* have been tested as therapeutic targets.

5.3.1. Peptidases

Peptidases are enzymes that perform proteolytic reactions and peptide bond hydrolysis. *Schistosoma mansoni* peptidases are attractive drug targets because they act in the host-parasite interaction during parasite invasion, migration, nutrition, and immune evasion [98]. The parasite serine peptidase involved in skin penetration of the human host, called cercarial elastase (SmCE), has been well studied [99, 100]. SmCE could have a role in immune evasion, as a highly purified SmCE was able to cleave IgE and other key molecules involved in immune regulation [101]. The use of serine protease inhibitors prevented IgE cleavage by cercariae and schistosomula extracts in a previous study, indicating that this could be a promising path for therapeutic strategies [102].

The *S. mansoni* cysteine and aspartic peptidases have drawn attention of many researchers as they participate in digestion of the blood meal. A previous study has shown that the use of inhibitors of these peptidases impairs hemoglobin degradation and arrests schistosome development and egg production [103]. The *S. mansoni* cathepsin B1 (SmCB1), which is a highly abundant digestive protease, was the focal point of various studies (reviewed in Ref. [104]). SmCB1 has been validated as a molecular target for therapy against schistosomiasis in a murine model of *S. mansoni* infection [105]. Moreover, SmCB1 crystal structure was determined, and specific inhibitors were designed, which are potential drug leads [106–108]. Another schistosome aspartic peptidase under study as a target is cathepsin D (SmCD). Morales et al. [109] have shown that silencing SmCD transcripts by RNA interference promoted schistosomula growth retardation as well as reduced worm and egg burden in infected mice. SmCD is currently under consideration for vaccine development against *S. mansoni* [110].

Prolyl oligopeptidases of the S9 family of serine peptidases have been investigated in *S. mansoni* by Fajtová et al. [111] who characterized the *S. mansoni* prolyl oligopeptidase (SmPOP) activity and showed that it is localized in the tegument and parenchyma of adult worms and schistosomula. Additionally, the authors designed specific inhibitors of SmPOP that were able to induce schistosoma death, suggesting that SmPOP could be a potential target for antischistosomal drug development.
5.3.2. Xenobiotic metabolism

The biotransformation of xenobiotics involves pathways that can be divided into three phases: (I) oxidation, reduction, or hydrolysis of xenobiotics; (II) conjugation of metabolites with endogenous compounds; and (III) excretion of modified molecules through membrane-bound transport proteins [112]. The xenobiotic metabolism can be a promising area for drug development since it implicates mechanisms that the parasite uses to eliminate drugs or toxic compounds; additionally, it plays vital roles in providing essential molecules for parasite survival. Among the S. mansoni biotransformation proteins, the phase I enzyme CYP450 has been studied by Ziniel et al. [113] who demonstrated that CYP450 RNA interference-mediated knockdown resulted in worm death. Additionally, imidazole antifungal CYP450 inhibitors had schistosomicidal activity against adult and larval worms, and blocked embryonic development in the egg.

The glutathione S-transferase (GST) family, from biotransformation pathway phase II, has been extensively studied in schistosomes. The knockdown of SmGST26 and SmGST28 in sporocysts by dsRNA exposure increased their susceptibility to exogenous oxidative stress and to Biomphalaria glabrata hemocytes-mediated killing [114]. The GST family of proteins is currently important vaccine candidate as it has been shown that these enzymes bind to several commercially available anthelmintics [115, 116].

The phase III membrane-bound transport proteins are currently under study, and the ABC transporters are the most studied among them [117]. The involvement of these ABC transporters such as P-glycoprotein and multidrug resistance-associated protein (SmDR1, SmDR2, SmMRP1, ABCA4, ABCB6, and ABCC10/MRP7) in drug susceptibility and development of drug resistance in schistosomes is clear, and this makes them excellent candidate targets for inhibitors that could potentiate the effect of existing drugs against schistosomes [118] or as new therapeutic targets themselves [119].

5.3.3. Redox mechanisms

Redox balance mechanisms are essential for schistosome worm survival, and differences between schistosome and human host redox networks were shown in previous studies (reviewed in Ref. [120]). The S. mansoni thioredoxin glutathione reductase (SmTGR) has been shown to be an important drug target. The use of oxadiazole-2-oxides as novel TGR inhibitors produced significant activity against various S. mansoni stages ex vivo and in vivo [40, 121, 122]. Another drug target in schistosome redox biology is the peroxiredoxins (SmPrx), which may be responsible to neutralize H$_2$O$_2$ due to the fact that schistosomes lack the catalase enzyme, resulting in a limited capacity to cope with this oxidant [120]. Sayed et al. [123] have shown that SmPrx1 knockdown by dsRNA exposure can potentially lead to schistosome death. Schistosoma mansoni antioxidant system relevance was also demonstrated in sporocysts by Mourão et al. [114]. RNAi-mediated knockdown of glutathione peroxidase (SmGPx), SmPrx1, and SmPrx2 increased larvae susceptibility to H$_2$O$_2$ oxidative stress. Additionally, treatment of parasites with SmPrx1/2 dsRNA increased hemocyte-mediated killing in vitro.
5.3.4. Purine biosynthesis

The purine nucleotide de novo synthesis pathway is absent in *S. mansoni*, which makes the parasite purine salvage pathway an attractive target for antischistosomal therapy development. A key component of this pathway is the purine nucleoside phosphorylase. The *S. mansoni* purine nucleoside phosphorylase (SmPNP) activity and structure have been well characterized [124–126]. Selective inhibitors for SmPNP have been tested in enzymatic assays *in vitro*; however, experiments with parasite larval or adult stages have not been reported yet [127–129]. The hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) was also considered as a potential drug target [130]. Pereira et al. [131] performed with siRNA directed against SmHGPRTase the first successful demonstration of an *in vivo* RNAi-based treatment against schistosomiasis.

5.3.5. Neurotransmitter transporters

*Schistosoma mansoni* nervous system is very well developed with a rich diversity of neurotransmitters. The neurotransmitter serotonin is one of the most abundant neuroactive substances in the *S. mansoni* nervous system [132], stimulating worm movement, muscle contraction, glycogenolysis, and glucose utilization in schistosomes [133, 134]. The *S. mansoni* serotonin transporter (SmSERT) function and localization have been studied [135, 136], and apparently, it acts as a neuronal transporter playing a key role in serotonergic control of parasite motility. Some classical selective serotonin reuptake inhibitors that usually target this type of transporters have shown potent schistosomical effect in drug screening [10, 137]. Some of these inhibitors presented different potency and selectivity for SmSERT when compared to the human hSERT, indicating that this evolutionary distance could be explored for the development of novel anti-*Schistosoma* therapies.

The inhibitory neurotransmitters, norepinephrine (NE) and dopamine (DA), are also present in *S. mansoni*, and they cause muscular relaxation and worm body lengthening [134]. A dopamine/norepinephrine transporter (DAT) from *S. mansoni* (SmDAT) has been characterized [138], and it would be responsible for clearance of NE and DA following their release to terminate the signal. SmDAT pharmacological studies showed that its response to tricyclic antidepressants and to selective serotonin reuptake inhibitors was higher than that shown for human DAT. Once again, the differences in ligand binding activity of schistosome neurotransmitter transporters reinforce them as good candidates for selective drug targeting. Nevertheless, inhibitors of schistosome neurotransmitter transporters have not been tested in an animal infection model yet, so the concerns over psychoactivity and undesirable side effects in the host could not be ruled out.

5.3.6. Neurotransmitter receptors

The *S. mansoni* genome sequence predicts several putative neurotransmitter receptors from the two main classes: ligand-gated ion channels and G protein-coupled receptors (GPCR) [139]. Receptors of neurotransmitters dopamine, histamine, glutamate, serotonin, and acetylcholine have been cloned and characterized [140–147]. Many of these receptors have
shown divergences from host receptors in structural and pharmacological aspects, indicating a possible track for antischistosomal therapy development. MacDonald et al. [148] demonstrated through RNA interference phenotypic assay that the knockdown of *S. mansoni* GPCR for acetylcholine (SmGAR) can disrupt larval motility. The importance of a serotonin receptor (Sm5HTR) for parasite motor activity has been also demonstrated by RNA interference [145]. Furthermore, it has been shown that the use of a heterologous system based on a fluorescent mammalian cell high-throughput functional assay can contribute as a new tool in the search for schistosomicidal drugs in the neurotransmitter receptors field [144].

6. Future directions and new approaches

While schistosomiasis still has a high socioeconomic impact, with the total number of disability-adjusted life years (DALY) lost to schistosomiasis estimated at 4.5 million per year, and treatment relies only on praziquantel since the early 1980s, drug discovery is still of great relevance. Our results with a *S. mansoni* drug screening platform reinforce that the use of parallel and complementary methods to assess parasites viability is essential in future studies. Drug discovery studies that employ different methods for drug screening are more prone to find new lead compounds. Additionally, drug screening should be performed in more than one parasite stages as some compound may present activity in schistosomula larval stage but be inactive in the adult worm stage or vice versa. It is worth to mention that praziquantel has little effect on immature worms; however, this drug acts in <1 h on adult worms damaging the tegument and paralyzing the worms. A compound that acts in all parasite stages and in both sexes is desirable, as such drug could be used to combat the acute and chronic phases of schistosomiasis, as well as to prevent the dissemination of viable eggs.

It is noteworthy that after identifying potential anti-*Schistosoma mansoni* drugs, several strict tests for validation are needed, those include bioavailability, stability, absorption, metabolism, distribution, excretion/pharmacokinetics, and toxicity in *in vivo* assays. The present chapter depicted the most studied drug targets on *S. mansoni*. The extensive publications on *Schistosoma* gene characterization studies and selective inhibitors design may pave the way for new therapeutic approaches against schistosomiasis.

Acknowledgements

This work has been supported by funding from the European Commission’s Seventh Framework Programme for research, under Grant agreement no. 602080 (A-ParaDDisE), CAPES Programa PCDD-Programa CAPES/Nottingham University (3661/2014), FAPEMIG (CBB-APQ-00520-13). GO received funds from CNPq (470673/2014-1 and 309312/2012-4) and is funded by CAPES (003/2014, REDE 21/2015), CNPq (304138/2014-2), and FAPEMIG (PPM-00189-13).
Author details

Naiara Clemente Tavares¹, Pedro Henrique Nascimento de Aguiar¹, Sandra Grossi Gava¹, Guilherme Oliveira² and Marina Moraes Mourão¹

*Address all correspondence to: marinamm@cpqrr.fiocruz.br

1 Rene Rachou Research Center, FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil

2 Vale Institute of Technology – ITV, Belém, Pará, Brazil

References

[1] Hotez PJ, Fenwick A. Schistosomiasis in Africa: an emerging tragedy in our new global health decade. PLoS Negl Trop Dis 2009; 3: e485.

[2] Gryseels B. Schistosomiasis. Infect Dis Clin North Am 2012; 26: 383–97.

[3] Botros S, Bennett J. Praziquantel resistance. Expert Opin Drug Discov 2007; 2: S35–S40.

[4] Coeli R, Baba EH, Araujo N, et al. Praziquantel treatment decreases Schistosoma mansoni genetic diversity in experimental infections. PLoS Negl Trop Dis 2013; 7: e2596.

[5] Liang YS, Dai J-RR, Zhu Y-CC, et al. Genetic analysis of praziquantel resistance in Schistosoma mansoni. Southeast Asian J Trop Med Public Health 2003; 34: 274–80.

[6] Melman SD, Steinauer ML, Cunningham C, et al. Reduced susceptibility to praziquantel among naturally occurring Kenyan isolates of Schistosoma mansoni. PLoS Negl Trop Dis 2009; 3: e504.

[7] Pica-Mattoccia L, Cioli D. Sex- and stage-related sensitivity of Schistosoma mansoni to in vivo and in vitro praziquantel treatment. Int J Parasitol 2004; 34: 527–33.

[8] Carvalho O dos S, Coelho PMZ, Lenzi HL. Schistosoma mansoni e esquistossomose: uma visão multidisciplinar. Rio de Janeiro: Editora Fiocruz, 2008.

[9] Suggitt M, Bibby MC. 50 Years of preclinical anticancer drug screening: Empirical to target-driven approaches. Clin Cancer Res 2005; 11: 971–981.

[10] Abdulla M-H, Ruelas DS, Wolff B, et al. Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening. PLoS Negl Trop Dis 2009; 3: e478.

[11] Rinaldi G, Loukas A, Brindley PJ, et al. Viability of developmental stages of Schistosoma mansoni quantified with xCELLigence worm real-time motility assay (xWORM). Int J Parasitol Drugs Drug Resist 2015; 5: 141–148.
[12] Keiser J. In vitro and in vivo trematode models for chemotherapeutic studies. Parasitology 2010; 137: 589–603.

[13] Inglese J, Johnson RL, Simeonov A, et al. High-throughput screening assays for the identification of chemical probes. Nat Chem Biol 2007; 3: 466–79.

[14] Lee H, Moody-Davis A, Saha U, et al. Quantification and clustering of phenotypic screening data using time-series analysis for chemotherapy of schistosomiasis. BMC Genomics 2012; 13: S4.

[15] Soufan O, Ba-Alawi W, Afeef M, et al. Mining chemical activity status from high-throughput screening assays. PLoS One 2015; 10: 1–16.

[16] Lemieux GA, Liu J, Mayer N, et al. A whole-organism screen identifies new regulators of fat storage. Nat Chem Biol 2011; 7: 206–213.

[17] Howe S, Zöphel D, Subbaraman H, et al. Lactate as a novel quantitative measure of viability in Schistosoma mansoni drug sensitivity assays. Antimicrob Agents Chemother 2015; 59: 1193–1199.

[18] Marcellino C, Gut J, Lim KC, et al. WormAssay: a novel computer application for whole-plate motion-based screening of macroscopic parasites. PLoS Negl Trop Dis 2012; 6: e1494.

[19] Paveley RA, Mansour NR, Hallyburton I, et al. Whole organism high-content screening by label-free, image-based bayesian classification for parasitic diseases. PLoS Negl Trop Dis 2012; 6: 1–11.

[20] Smout MJ, Kotze AC, McCarthy JS, et al. A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. PLoS Negl Trop Dis 2010; 4: e885.

[21] Tritten L, Silbereisen A, Keiser J. Nitazoxanide: In vitro and in vivo drug effects against Trichuris muris and Ancylostoma ceylanicum, alone or in combination. Int J Parasitol Drugs Drug Resist 2012; 2: 98–105.

[22] Mansour NR, Bickle QD. Comparison of microscopy and Alamar blue reduction in a larval based assay for schistosome drug screening. PLoS Negl Trop Dis 2010; 4: e795.

[23] Peak E, Chalmers IW, Hoffmann KF. Development and validation of a quantitative, high-throughput, fluorescent-based bioassay to detect schistosoma viability. PLoS Negl Trop Dis 2010; 4: e759.

[24] Krautz-Peterson G, Simoes M, Faghiri Z, et al. Suppressing glucose transporter gene expression in schistosomes impairs parasite feeding and decreases survival in the mammalian host. PLoS Pathog 2010; 6: e1000932.

[25] Faghiri Z, Camargo SMR, Huggel K, et al. The tegument of the human parasitic worm Schistosoma mansoni as an excretory organ: the surface aquaporin SmAQP is a lactate transporter. PLoS One 2010; 5: e10451.
[26] Massie CE, Lynch A, Ramos-Montoya A, et al. The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. EMBO J 2011; 30: 2719–33.

[27] Matos P, Horn JA, Beards F, et al. A role for the mitochondrial-associated protein p32 in regulation of trophoblast proliferation. Mol Hum Reprod 2014; 20: 745–755.

[28] de Moraes Mourão M, Dinguirard N, Franco GR, et al. Phenotypic screen of early-developing larvae of the blood fluke, schistosoma mansoni, using RNA interference. PLoS Negl Trop Dis 2009; 3: e502.

[29] Bowling T, Mercer L, Don R, et al. Application of a resazurin-based high-throughput screening assay for the identification and progression of new treatments for human african trypanosomiasis. Int J Parasitol Drugs Drug Resist 2012; 2: 262–270.

[30] Shimony O, Jaffe CL. Rapid fluorescent assay for screening drugs on Leishmania amastigotes. J Microbiol Methods 2008; 75: 196–200.

[31] Kotze AC, Clifford S, O’Grady J, et al. An in vitro larval motility assay to determine anthelmintic sensitivity for human hookworm and Strongyloides species. Am J Trop Med Hyg 2004; 71: 608–616.

[32] Storey B, Marcellino C, Miller M, et al. Utilization of computer processed high definition video imaging for measuring motility of microscopic nematode stages on a quantitative scale: ‘The Worminator’. Int J Parasitol Drugs drug Resist 2014; 4: 233–43.

[33] Asarnow D, Rojo-Arreola L, Suzuki BM, et al. The QDREC web server: determining dose-response characteristics of complex macroparasites in phenotypic drug screens. Bioinformatics 2015; 31: 1515–8.

[34] Farah IO, Kariuki TM, King CL, et al. An overview of animal models in experimental schistosomiasis and refinements in the use of non-human primates. Lab Anim 2001; 35: 205–12.

[35] Pellegrino J, Katz N. Experimental chemotherapy of Schistosomiasis mansoni. Adv Parasitol 1968; 6: 233–90.

[36] Katz N, Couto FFB, Araújo N. Imatinib activity on Schistosoma mansoni. Mem Inst Oswaldo Cruz 2013; 108: 850–3.

[37] Pereira AC, Silva MLA e, Souza JM, et al. In vitro and in vivo anthelmintic activity of (-)-6,6’-dinitrohinokinin against schistosomula and juvenile and adult worms of Schistosoma mansoni. Acta Trop 2015; 149: 195–201.

[38] Araujo N, Mattos ACA de, Coelho PMZ, et al. Association of oxamniquine praziquantel and clonazepam in experimental Schistosomiasis mansoni. Mem Inst Oswaldo Cruz 2008; 103: 781–5.

[39] Lancelot J, Caby S, Dubois-Abdesselem F, et al. Schistosoma mansoni sirtuins: characterization and potential as chemotherapeutic targets. PLoS Negl Trop Dis 2013; 7: 1–13.
[40] Li T, Ziniel PD, He P, et al. High-throughput screening against thioredoxin glutathione reductase identifies novel inhibitors with potential therapeutic value for schistosomiasis. Infect Dis Poverty 2015; 4: 40.

[41] Yousif F, Wassel G, Boulos L, et al. Contribution to in vitro screening of Egyptian plants for schistosomicidal activity. Pharm Biol 2012; 50: 732–9.

[42] Mwangi IN, Sanchez MC, Mkoji GM, et al. Praziquantel sensitivity of Kenyan Schistosoma mansoni isolates and the generation of a laboratory strain with reduced susceptibility to the drug. Int J Parasitol Drugs Drug Resist 2014; 4: 296–300.

[43] Anderson L, Amaral MS, Beckedorff F, et al. Schistosoma mansoni egg, adult male and female comparative gene expression analysis and identification of novel genes by RNA-Seq. PLoS Negl Trop Dis 2015; 9: e0004334.

[44] Doenhoff MJ, Cioli D, Utzinger J. Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. Curr Opin Infect Dis 2008; 21: 659–67.

[45] Choudhary C, Weinert BT, Nishida Y, et al. The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol 2014; 15: 536–50.

[46] de la Cruz X, Lois S, Sánchez-Molina S, et al. Do protein motifs read the histone code? Bioessays 2005; 27: 164–75.

[47] Berger SL. Histone modifications in transcriptional regulation. Curr Opin Genet Dev 2002; 12: 142–148.

[48] Kouzarides T. Chromatin modifications and their function. Cell 2007; 128: 693–705.

[49] Cavalcante AGDM, de Bruin PFC. The role of oxidative stress in COPD: current concepts and perspectives. J Bras Pneumol 2009; 35: 1227–37.

[50] Jasencakova Z, Soppe WJJ, Meister A, et al. Histone modifications in Arabidopsis—high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin. Plant J 2003; 33: 471–480.

[51] Dowling DP, Gantt SL, Gattis SG, et al. Structural studies of human histone deacetylase 8 and its site-specific variants complexed with substrate and inhibitors. Biochemistry 2008; 47: 13554–63.

[52] Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet 2009; 10: 32–42.

[53] Beumer JH, Tawbi H. Role of histone deacetylases and their inhibitors in cancer biology and treatment. Curr Clin Pharmacol 2010; 5: 196–208.

[54] Delcuve GP, Khan DH, Davie JR. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin Epigenet 2012; 4: 5.
Van Dyke MW. Lysine deacetylase (KDAC) regulatory pathways: an alternative approach to selective modulation. ChemMedChem 2014; 9: 511–22.

Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat Rev Drug Discov 2014; 13: 673–91.

Mottamal M, Zheng S, Huang T, et al. Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. Molecules 2015; 20: 3898–3941.

Kumar R, Li D-Q, Müller S, et al. Epigenomic regulation of oncogenesis by chromatin remodeling. Oncogene 2016; 35: 4423–36.

Wang Z, Xue X, Sun J, et al. An ‘in-depth’ description of the small non-coding RNA population of Schistosoma japonicum schistosomulum. PLoS Negl Trop Dis 2010; 4: e596.

Oliveira G. Cancer and parasitic infections: similarities and opportunities for the development of new control tools. Rev Soc Bras Med Trop 2014; 47: 1–2.

Ouaissi M, Ouaissi A. Histone deacetylase enzymes as potential drug targets in cancer and parasitic diseases. J Biomed Biotechnol 2006; 2006: 134–74.

New M, Olzscha H, La Thangue NB. HDAC inhibitor-based therapies: Can we interpret the code? Mol Oncol 2012; 6: 637–656.

Andrews KT, Haque A, Jones MK. HDAC inhibitors in parasitic diseases. Immunol Cell Biol 2012; 90: 66–77.

Duvic M, Talpur R, Ni X, et al. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). Blood 2007; 109: 31–39.

Pierce RJ, Dubois-Abdesselem F, Lancelot J, et al. Targeting schistosome histone modifying enzymes for drug development. Curr Pharm Des 2012; 18: 3567–78.

Azzi A, Cosseau C, Grunau C. Schistosoma mansoni: developmental arrest of miracidia treated with histone deacetylase inhibitors. Exp Parasitol 2009; 121: 288–91.

Dubois F, Caby S, Oger F, et al. Histone deacetylase inhibitors induce apoptosis, histone hyperacetylation and up-regulation of gene transcription in Schistosoma mansoni. Mol Biochem Parasitol 2009; 168: 7–15.

Marek M, Kannan S, Hauser A-T, et al. Structural basis for the inhibition of histone deacetylase 8 (HDAC8), a key epigenetic player in the blood fluke Schistosoma mansoni. PLoS Pathog 2013; 9: e1003645.

Marek M, Oliveira G, Pierce RJ, et al. Drugging the schistosome zinc-dependent HDACs: current progress and future perspectives. Future Med Chem 2015; 7: 783–800.

Balasubramanyam K, Varier RA, Altaf M, et al. Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/
nonhistone proteins and histone acetyltransferase-dependent chromatin transcription. J Biol Chem 2004; 279: 51163–71.

[71] Stimson L, Rowlands MG, Newbatt YM, et al. Isothiazolones as inhibitors of PCAF and p300 histone acetyltransferase activity. Mol Cancer Ther 2005; 4: 1521–1532.

[72] Magalhães LG, Machado CB, Morais ER, et al. In vitro schistosomicidal activity of curcumin against Schistosoma mansoni adult worms. Parasitol Res 2009; 104: 1197–1201.

[73] Carneiro VC, de Abreu da Silva IC, Torres EJL, et al. Epigenetic changes modulate schistosome egg formation and are a novel target for reducing transmission of schistosomiasis. PLoS Pathog 2014; 10: e1004116.

[74] Bissinger E-M, Heinke R, Spannhoff A, et al. Acyl derivatives of p-aminosulfonamides and dapsone as new inhibitors of the arginine methyltransferase hPRMT1. Bioorg Med Chem 2011; 19: 3717–31.

[75] Wang J, Lu F, Ren Q, et al. Novel histone demethylase LSD1 inhibitors selectively target cancer cells with pluripotent stem cell properties. Cancer Res 2011; 71: 7238–7249.

[76] Amiri P, Locksley RM, Parslow TG, et al. Tumour necrosis factor alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. Nature 1992; 356: 604–7.

[77] LoVerde PT, Hirai H, Merrick JM, et al. Schistosoma mansoni genome project: an update. Parasitol Int 2004; 53: 183–192.

[78] Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298: 1911–2.

[79] Andrade LF De, Mourão MDM, Geraldo JA, et al. Regulation of Schistosoma mansoni development and reproduction by the mitogen-activated protein kinase signaling pathway. PLoS Negl Trop Dis 2014; 8: e2949.

[80] Dissous C, Ahier A, Khayath N. Protein tyrosine kinases as new potential targets against human schistosomiasis. BioEssays 2007; 29: 1281–1288.

[81] Naula C, Parsons M, Mottram JC. Protein kinases as drug targets in trypanosomes and Leishmania. Biochim Biophys Acta 2005; 1754: 151–159.

[82] Ward P, Equinet L, Packer J, et al. Protein kinases of the human malaria parasite Plasmodium falciparum: the kinase of a divergent eukaryote. BMC Genomics 2004; 5: 79.

[83] Andrade LF, Nahum LA, Avelar LGA, et al. Eukaryotic protein kinases (ePKs) of the helminth parasite Schistosoma mansoni. BMC Genomics 2011; 12: 215.

[84] Guidi A, Mansour NR, Paveley R a, et al. Application of RNAi to genomic drug target validation in schistosomes. PLoS Negl Trop Dis 2015; 9: e0003801.
[85] Ressurreição M, Kirk RS, Rollinson D, et al. Sensory protein kinase signaling in Schistosoma mansoni cercariae: host location and invasion. J Infect Dis 2015; 212: 1787–97.

[86] Matsuyama H, Takahashi H, Watanabe K, et al. The involvement of cyclic adenosine monophosphate in the control of schistosome miracidium cilia. J Parasitol 2004; 90: 8–14.

[87] Ludtmann MHR, Rollinson D, Emery AM, et al. Protein kinase C signalling during miracidium to mother sporocyst development in the helminth parasite, Schistosoma mansoni. Int J Parasitol 2009; 39: 1223–1233.

[88] Dissous C, Grevelding CG, Long T. Schistosoma mansoni polo-like kinases and their function in control of mitosis and parasite reproduction. An Acad Bras Cienc 2011; 83: 627–635.

[89] Long T, Neitz RJ, Beasley R, et al. Structure-bioactivity relationship for benzimidazole thiophene inhibitors of polo-like kinase 1 (PLK1), a potential drug target in Schistosoma mansoni. PLoS Negl Trop Dis 2016; 10: e0004356.

[90] Knobloch J, Kunz W, Grevelding CG. Herbimycin A suppresses mitotic activity and egg production of female Schistosoma mansoni. Int J Parasitol 2006; 36: 1261–72.

[91] Buro C, Oliveira KC, Lu Z, et al. Transcriptome analyses of inhibitor-treated schistosome females provide evidence for cooperating Src-kinase and TGFβ receptor pathways controlling mitosis and eggshell formation. PLoS Pathog 2013; 9: e1003448.

[92] Beckmann S, Grevelding CG. Imatinib has a fatal impact on morphology, pairing stability and survival of adult Schistosoma mansoni in vitro. Int J Parasitol 2010; 40: 521–526.

[93] Buro C, Beckmann S, Oliveira KC, et al. Imatinib treatment causes substantial transcriptional changes in adult Schistosoma mansoni in vitro exhibiting pleiotropic effects. PLoS Negl Trop Dis 2014; 8: e2923.

[94] Beckmann S, Buro C, Dissous C, et al. The Syk kinase SmTK4 of Schistosoma mansoni is involved in the regulation of spermatogenesis and oogenesis. PLoS Pathog 2010; 6: e1000769.

[95] Morel M, Vanderstraete M, Cailliau K, et al. Compound library screening identified Akt/PKB kinase pathway inhibitors as potential key molecules for the development of new chemotherapeutics against schistosomiasis. Int J Parasitol Drugs Drug Resist 2014; 4: 256–266.

[96] Vanderstraete M, Gouignard N, Cailliau K, et al. Venus kinase receptors control reproduction in the platyhelminth parasite Schistosoma mansoni. PLoS Pathog 2014; 10: e1004138.
[97] Ressurreição M, De Saram P, Kirk RS, et al. Protein kinase C and extracellular signal-regulated kinase regulate movement, attachment, pairing and egg release in Schistosoma mansoni. PLoS Negl Trop Dis 2014; 8: e2924.

[98] McKerrow JH, Caffrey C, Kelly B, et al. Proteases in parasitic diseases. Annu Rev Pathol Mech Dis 2006; 1: 497–536.

[99] Ingram JR, Rafi SB, Eroy-Reveles AA, et al. Investigation of the proteolytic functions of an expanded cercarial elastase gene family in Schistosoma mansoni. PLoS Negl Trop Dis 2012; 6: e1589.

[100] Yang Y, Wen YJ, Cai YN, et al. Serine proteases of parasitic helminths. Korean J Parasitol 2015; 53: 1–11.

[101] Aslam A, Quinn P, McIntosh RS, et al. Proteases from Schistosoma mansoni cercariae cleave IgE at solvent exposed interdomain regions. Mol Immunol 2008; 45: 567–74.

[102] Pleass RJ, Kusel JR, Woof JM. Cleavage of human IgE mediated by Schistosoma mansoni. Int Arch Allergy Immunol 2000; 121: 194–204.

[103] Wasilewski MM, Lim KC, Phillips J, et al. Cysteine protease inhibitors block schistosome hemoglobin degradation in vitro and decrease worm burden and egg production in vivo. Mol Biochem Parasitol 1996; 81: 179–89.

[104] Jílková A, Horn M, Řezáč P, et al. Activation route of the Schistosoma mansoni cathepsin B1 drug target: structural map with a glycosaminoglycan switch. Structure 2014; 22: 1786–98.

[105] Abdulla M-H, Lim K-C, Sajid M, et al. Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. PLoS Med 2007; 4: e14.

[106] Fanfrlík J, Brahmkshatriya PS, Řezáč J, et al. Quantum mechanics-based scoring rationalizes the irreversible inactivation of parasitic Schistosoma mansoni cysteine peptidase by vinyl sulfone inhibitors. J Phys Chem B 2013; 117: 14973–82.

[107] Horn M, Jílková A, Vondrásek J, et al. Mapping the pro-peptide of the Schistosoma mansoni cathepsin B1 drug target: modulation of inhibition by heparin and design of mimetic inhibitors. ACS Chem Biol 2011; 6: 609–17.

[108] Jílková A, Rezácová P, Lepsík M, et al. Structural basis for inhibition of cathepsin B drug target from the human blood fluke, Schistosoma mansoni. J Biol Chem 2011; 286: 35770–181.

[109] Morales ME, Rinaldi G, Gobert GN, et al. RNA interference of Schistosoma mansoni cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade. Mol Biochem Parasitol 2008; 157: 160–168.

[110] Ahmad Fuaad AAH, Roubille R, Pearson MS, et al. The use of a conformational cathepsin D-derived epitope for vaccine development against Schistosoma mansoni. Bioorg Med Chem 2015; 23: 1307–12.
[111] Fajtová P, Štefanic S, Hradilek M, et al. Prolyl oligopeptidase from the blood fluke *Schistosoma mansoni*: from functional analysis to anti-schistosomal inhibitors. *PLoS Negl Trop Dis* 2015; 9: e0003827.

[112] Cvilink V, Lamka J, Skálová L. Xenobiotic metabolizing enzymes and metabolism of anthelmintics in helminths. *Drug Metab Rev* 2009; 41: 8–26.

[113] Ziniel PD, Karumudi B, Barnard AH, et al. The *Schistosoma mansoni* cytochrome P450 (CYP3050A1) is essential for worm survival and egg development. *PLoS Negl Trop Dis* 2015; 9: e0004279.

[114] Mourão MDM, Dinguirard N, Franco GR, et al. Role of the endogenous antioxidant system in the protection of *Schistosoma mansoni* primary sporocysts against exogenous oxidative stress. *PLoS Negl Trop Dis* 2009; 3: e550.

[115] Brophy PM, Barrett J. Glutathione transferase in helminths. *Parasitology* 1990; 100 Pt 2: 345–9.

[116] Mo AX, Agosti JM, Walson JL, et al. Schistosomiasis elimination strategies and potential role of a vaccine in achieving global health goals. *Am J Trop Med Hyg* 2014; 90: 54–60.

[117] Greenberg RM. Schistosome ABC multidrug transporters: from pharmacology to physiology. *Int J Parasitol Drugs Drug Resist* 2014; 4: 301–309.

[118] Kasinathan RS, Sharma LK, Cunningham C, et al. Inhibition or knockdown of ABC transporters enhances susceptibility of adult and juvenile schistosomes to praziquantel. *PLoS Negl Trop Dis* 2014; 8: 1–11.

[119] Kasinathan RS, Morgan WM, Greenberg RM. Genetic knockdown and pharmacological inhibition of parasite multidrug resistance transporters disrupts egg production in *Schistosoma mansoni*. *PLoS Negl Trop Dis* 2011; 5: e1425.

[120] Huang H-H, Rigouin C, Williams DL. The redox biology of schistosome parasites and applications for drug development. *Curr Pharm Des* 2012; 18: 3595–611.

[121] Sayed A a, Simeonov A, Thomas CJ, et al. Identification of oxadiazoles as new drug leads for the control of schistosomiasis. *Nat Med* 2008; 14: 407–412.

[122] Rai G, Sayed AA, Lea WA, et al. Structure mechanism insights and the role of nitric oxide donation guide the development of oxadiazole-2-oxides as therapeutic agents against schistosomiasis. *J Med Chem* 2009; 52: 6474–83.

[123] Sayed AA, Cook SK, Williams DL. Redox balance mechanisms in *Schistosoma mansoni* rely on peroxiredoxins and albumin and implicate peroxiredoxins as novel drug targets. *J Biol Chem* 2006; 281: 17001–17010.

[124] da Silveira NJF, Uchôa HB, Canduri F, et al. Structural bioinformatics study of PNP from *Schistosoma mansoni*. *Biochem Biophys Res Commun* 2004; 322: 100–4.
[125] Pereira HD, Franco GR, Cleasby A, et al. Structures for the potential drug target purine nucleoside phosphorylase from Schistosoma mansoni causal agent of schistosomiasis. J Mol Biol 2005; 353: 584–99.

[126] Pereira HM, Cleasby A, Pena S SDJ, et al. Cloning, expression and preliminary crystallographic studies of the potential drug target purine nucleoside phosphorylase from Schistosoma mansoni. Acta Crystallogr D Biol Crystallogr 2003; 59: 1096–9.

[127] Castilho MS, Postigo MP, Pereira HM, et al. Structural basis for selective inhibition of purine nucleoside phosphorylase from Schistosoma mansoni: kinetic and structural studies. Bioorg Med Chem 2010; 18: 1421–1427.

[128] De Moraes MC, Cardoso CL, Cass QB. Immobilized purine nucleoside phosphorylase from Schistosoma mansoni for specific inhibition studies. Anal Bioanal Chem 2013; 405: 4871–4878.

[129] Postigo MP, Guido RVC, Oliva G, et al. Discovery of new inhibitors of schistosoma mansoni PNP by pharmacophore-based virtual screening. J Chem Inf Model 2010; 50: 1693–1705.

[130] Craig SP, McKerrow JH, Newport GR, et al. Analysis of cDNA encoding the hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) of Schistosoma mansoni; a putative target for chemotherapy. Nucleic Acids Res 1988; 16: 7087–101.

[131] Pereira TC, Pascoal VDB, Marchesini RB, et al. Schistosoma mansoni: evaluation of an RNAi-based treatment targeting HGPRTase gene. Exp Parasitol 2008; 118: 619–623.

[132] Bennett J, Bueding E, Timms AR, et al. Occurrence and levels of 5-hydroxytryptamine in Schistosoma mansoni. Mol Pharmacol 1969; 5: 542–5.

[133] Day TA, Bennett JL, Pax RA. Serotonin and its requirement for maintenance of contractility in muscle fibres isolated from Schistosoma mansoni. Parasitology 1994; 108 ( Pt 4: 425–32.

[134] Pax RA, Siefker C, Bennett JL. Schistosoma mansoni: differences in acetylcholine, dopamine, and serotonin control of circular and longitudinal parasite muscles. Exp Parasitol 1984; 58: 314–24.

[135] Patocka N, Ribeiro P. Characterization of a serotonin transporter in the parasitic flatworm, Schistosoma mansoni: Cloning, expression and functional analysis. Mol Biochem Parasitol 2007; 154: 125–133.

[136] Patocka N, Ribeiro P. The functional role of a serotonin transporter in Schistosoma mansoni elucidated through immunolocalization and RNA interference (RNAi). Mol Biochem Parasitol 2013; 187: 32–42.

[137] Ribeiro P, Patocka N. Neurotransmitter transporters in schistosomes: structure, function and prospects for drug discovery. Parasitol Int 2013; 62: 629–638.
[138] Larsen MB, Fontana ACK, Magalhães LG, et al. A catecholamine transporter from the human parasite Schistosoma mansoni with low affinity for psychostimulants. Mol Biochem Parasitol 2011; 177: 35–41.

[139] Berriman M, Haas BJ, LoVerde PT, et al. The genome of the blood fluke Schistosoma mansoni. Nature 2009; 460: 352–8.

[140] El-Shehabi F, Ribeiro P. Histamine signalling in Schistosoma mansoni: immunolocalisation and characterisation of a new histamine-responsive receptor (SmGPR-2). Int J Parasitol 2010; 40: 1395–1406.

[141] El-Shehabi F, Vermeire JJ, Yoshino TP, et al. Developmental expression analysis and immunolocalization of a biogenic amine receptor in Schistosoma mansoni. Exp Parasitol 2009; 122: 17–27.

[142] El-Shehabi F, Taman A, Moali LS, et al. A novel G protein-coupled receptor of Schistosoma mansoni (SmGPR-3) is activated by dopamine and is widely expressed in the nervous system. PLoS Negl Trop Dis 2012; 6: e1523.

[143] Hamdan FF, Abramovitz M, Mousa A, et al. A novel Schistosoma mansoni G protein-coupled receptor is responsive to histamine. Mol Biochem Parasitol 2002; 119: 75–86.

[144] MacDonald K, Buxton S, Kimber MJ, et al. Functional characterization of a novel family of acetylcholine-gated chloride channels in Schistosoma mansoni. PLoS Pathog 2014; 10: e1004181.

[145] Patocka N, Sharma N, Rashid M, et al. Serotonin signaling in Schistosoma mansoni: a serotonin-activated G protein-coupled receptor controls parasite movement. PLoS Pathog 2014; 10: e1003878.

[146] Taman A, Ribeiro P. Investigation of a dopamine receptor in Schistosoma mansoni: functional studies and immunolocalization. Mol Biochem Parasitol 2009; 168: 24–33.

[147] Taman A, Ribeiro P. Glutamate-mediated signaling in Schistosoma mansoni: a novel glutamate receptor is expressed in neurons and the female reproductive tract. Mol Biochem Parasitol 2011; 176: 42–50.

[148] MacDonald K, Kimber MJ, Day TA, et al. A constitutively active G protein-coupled acetylcholine receptor regulates motility of larval Schistosoma mansoni. Mol Biochem Parasitol 2015; 202: 29–37.