Screening open access compound libraries and repurposing drugs to identify and characterize new molecules active against schistosomiasis and other helminthiases

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Summary

Far from the baneful impact of the so-called “big three” (HIV/AIDS, tuberculosis and malaria), schistosomiasis and other worm infections, such as the soil-transmitted helminths (STH)[1] for example, have a backstairs influence on poverty in the tropical belt, mainly in sub-Saharan Africa and South-East Asia. The lack of sanitary infrastructure in endemic areas is contributing to the transmission of the parasites and the maintenance of their life-cycles in the environment. People, especially children, get infected while carrying out their daily activities or simply by walking barefoot.

Preventive chemotherapy is the most widely used control strategy for schistosomiasis and STH. It relies on praziquantel for schistosomiasis and on the benzimidazoles for STH. A corollary of this repeated and large-scale use of the same drugs is that the risk of resistance emergence is rising and neither alternative treatment nor vaccine are available against these diseases. Also, the current anthelminthic formulations are not optimized for all patient categories like pregnant women or pre-school-age children. Unfortunately, because of the lack of return on investment, the industry had turned its back on anthelminthic research and development and the academic research in this area is scarce and generally underfunded. Hence, there is a dramatic lack of molecules in the development pipeline, especially in pre-clinical or clinical phases.

It is a crucial moment to intensify research on antischistosomal drugs. However, it remains the appanage of only a few academic institutions throughout the world that use different screening methods and that often have a long-standing know-how on life-cycle maintenance. In order to promote early antischistosomal drug discovery and help researchers starting to work in this area, we developed a protocol unifying cultivation methods with *in vitro* and *in vivo* drug testing.

[1] STH include infections with the roundworm *Ascaris lumbricoides*, the hookworms *Ancylostoma duodenale*, *Necator americanus*, the whipworm *Trichuris trichiura* and *Strongyloides stercoralis*.

In this context, the main objective of this thesis was to expand the pool of antischistosomal candidates by screening two libraries of 400 compounds each, both compiled and provided by the non-profit product development partnership Medicines for Malaria Venture (MMV). One library, the Pathogen Box, was composed of drug-like molecules with already-known activity against one or more infectious agents. The other library, the Stasis Box, was a set of drugs that were abandoned at advanced stages of their clinical development. The activity of the compounds from the two libraries was first screened *in vitro* on the larval stage of *Schistosoma mansoni*. The hits were then tested on adult worms. In both cases, the viability of the parasite was assessed phenotypically. This straightforward approach enabled the identification of 22 antischistosomal leads with satisfying *in vitro* activity (IC_{50} < 10 µM) and selectivity. However, the good efficacy *in vitro* did not translate *in vivo*, as the 16 molecules that were tested in mice harboring an infection with *S. mansoni* failed to significantly reduce the burden of infection. Whereas this lack of efficacy *in vivo* might be imputable to a strong albumin-binding effect for the Stasis Box drugs, this was not the case for the Pathogen Box compounds. In addition to test the activity of both compound libraries, a series of more than a hundred analogues from three of the leads identified in the Pathogen Box screening was also tested *in vitro*. Screening this set of analogues enabled to launch a preliminary structure-action relationship analysis that paves the bases for future compound synthesis programs and selected, new leads. Hundreds of Pathogen Boxes were distributed to research teams across the globe, including some that tested its compounds on schistosomes. The variability of results between laboratories screening the same set of compounds might be important, especially since there is no consensus on drug screening methods for schistosomes. Also, the reliability of activity-based phenotypic screening approach can be limited. For these reasons, our results from the Pathogen Box larval stage screening were compared to the ones obtained at the University of California in San Diego (UCSD) and the ones from metabolic assays performed by the Fiocruz Foundation in Brazil. This resulted in a 74%

overall agreement between the three laboratories and confirmed that activity-based phenotypic assays on the larval stage are a reliable and cost-effective method to screen large compound libraries. In order to potentially improve the quality and increase the speed of the larval assays read-outs, we tested an image-based motility assessment method in collaboration with the team of Prof. Britta Lundström-Stadelmann at the University of Bern. Good correlations with the phenotypic assessment were found but this system must be validated with more drugs and under different conditions.

Additionally, in the framework of the Master thesis of Tanja Karpstein, the screening work on schistosomes was extended to other parasites. The veterinary anthelminthic emodepside was tested not only on schistosomes but also on five different species of nematodes, including hookworms and the whipworm. While its efficacy on *S. mansoni* and *S. haematobium* remained moderate, emodepside revealed very promising *in vitro* and *in vivo* activity that should encourage its repositioning as treatment for human soil-transmitted helminthiases.

In conclusion, this work describes, applies and explores different methods in anthelminthic early drug development with a strong emphasis on schistosomiasis. Activity-based screening of open access libraries has identified new potent antischistosomal molecules. Investigating the activity of some lead analogues has also enabled to single out different patterns that could be used for further compound development. Finally, this work highlights the importance of open access libraries, drug repurposing and non-profit product development partnerships to stimulate the development of new anthelminthic drugs.

List of abbreviations

AIDS	Acquired immunodeficiency syndrome
DALYs	Disability adjusted life years
DNDi	Drugs for Neglected Diseases initiative
ED_{50}	Effective dose 50%
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
IC_{50}	Inhibitory concentration 50%
MDA	Mass drug administration
MMV	Medicines for Malaria Venture
NTD	Neglected tropical diseases
NTS	Newly-transformed schistosomula
PC	Preventive chemotherapy
PZQ	Praziquantel
R.& D.	Research and development
STH	Soil-transmitted helminth(iase)s
TDR	Special Programme for Research and Training in Tropical Diseases
WASH	Water, sanitation and hygiene
WHO	World Health Organization

Chapter 1: General introduction

1.1. The global burden of helminthiases, preventive chemotherapy and fear of resistance

Schistosomiasis and the diseases grouped under the appellation of soil-transmitted helminthiases (STH) are responsible for millions of disability-adjusted life years (DALYS). Despite a possible underestimation of its real burden, schistosomiasis was recently evaluated to be causing between 1.4 and 2.5 million DALYS because of its associated chronic morbidities (Colley et al., 2017; Kassebaum et al., 2016; King and Galvani, 2018; Kyu et al., 2018).

With the exception of a few imported cases in Western countries, helminthiases affect mostly people living in low-resource settings of tropical and subtropical regions (Grobusch et al., 2003; Gryseels et al., 2006; Pullan et al., 2014). Without appropriate treatment, the immunopathological events, the blood losses or the obstructions induced by the parasites can severely impair the function of internal organs and result in chronic anemia, that can have dramatic effects on growth and cognitive development of children (Hotez et al., 2008).

In addition to these deleterious consequences on health, helminthiases have an important socio-economic impact. While they keep patients away from their studies or professional activity, which catalyze impoverishment, their transmission is favored by the cruel lack of sanitary infrastructure worldwide, that put millions of people at risk of infection (Conteh et al., 2010; Kassebaum et al., 2016). Schistosomiasis and STH therefore play the dual role of being both determinant and result of poverty (King, 2010). Moreover, these infections are often concomitant with other pathologies such as malaria, tuberculosis or HIV/AIDS (Bustinduy et al., 2014; McManus et al., 2018). Polyparasitism – the infection of a patient by different species of parasites at the same time – is also very common and increases the severity of the symptoms (Ezeamama et al., 2008).

The transmission cycle of these parasites can be interrupted thanks to different strategies, namely preventive chemotherapy (PC), water and sanitation programs (WASH), health education and

snail/vector control in the case of schistosomiasis (Bergquist et al., 2017b; Chitsulo et al., 2000; Knopp et al., 2013). Preventive chemotherapy is a public health measure that aims at limiting the morbidities associated with helminthiases thanks to the regular administration of a drug to populations at risk. Preventive chemotherapy is carried out in large-scale drug distribution programs called mass drug administration (MDA) (WHO, 2017, 2006). Although its efficacy has been debated, this strategy remains the most widely used because of its cost-effectiveness and safety (Andrews et al., 2017; Gabrielli et al., 2011; Hotez et al., 2009; Montresor et al., 2012).

Building on the World Health Organization (WHO) roadmap against neglected tropical diseases (NTDs) (WHO, 2012), different stakeholders from philanthropy, the public sector and the pharma industry ratified in 2012 the London Declaration – an international commitment to control and eliminate selected NTDs including schistosomiasis and STH (Anderson et al., 2012). This agreement has been translated into larger donations of medicines and intensified MDA programs (WHO, 2017).

With PC as their cornerstone, integrated control strategies significantly reduced the burden of schistosomiasis and STH. However, the potential emergence of resistance would jeopardize any control or elimination programs (Montresor et al., 2012; Schulz et al., 2018). Additionally, current anthelminthic drugs neither protect from re-infection nor are adapted to all categories of patients (e.g. pre-school aged children) (Schmidlin et al., 2013; Schulz et al., 2018). Hence, the development of new anthelmintic medicines is urgent and requires expanding the array of active molecules by encouraging innovation and drug discovery (Geary et al., 2015).

1.2. Schistosomiasis

1.2.1. Epidemiology

Human schistosomiasis, also known as bilharzia, accounts for more than 200 million cases around the world, mainly in sub-Saharan Africa and South-East Asia (Colley et al., 2014; Gryseels et al., 2006). People get infected when in contact with contaminated water in their daily-life activities, often when laundering or washing cars. The disease is more prevalent in children because of their more frequent contacts with potential contaminated water streams while playing or bathing (Colley et al., 2014; Hotez and Kamath, 2009; Kabatereine et al., 2004; King et al., 1988; Osakunor et al., 2018).

The parasites – the schistosomes – actively penetrate the skin, reproduce and lay eggs that will be excreted in urine or feces. Many different structural, behavioral or environmental reasons can explain the ongoing transmission in different regions of the world (figure1). The lack of access to proper sanitation infrastructure and hence frequent open urination or defecation enhances water contamination (Grimes et al., 2015; Schmidlin et al., 2013). Moreover, climate change and modifications of the local ecosystem, i.e. dam or irrigation channels, create new living sites for the intermediate host and are likely to increase the area of repartition of the host in certain regions (McCreesh et al., 2015; Steinmann et al., 2006)

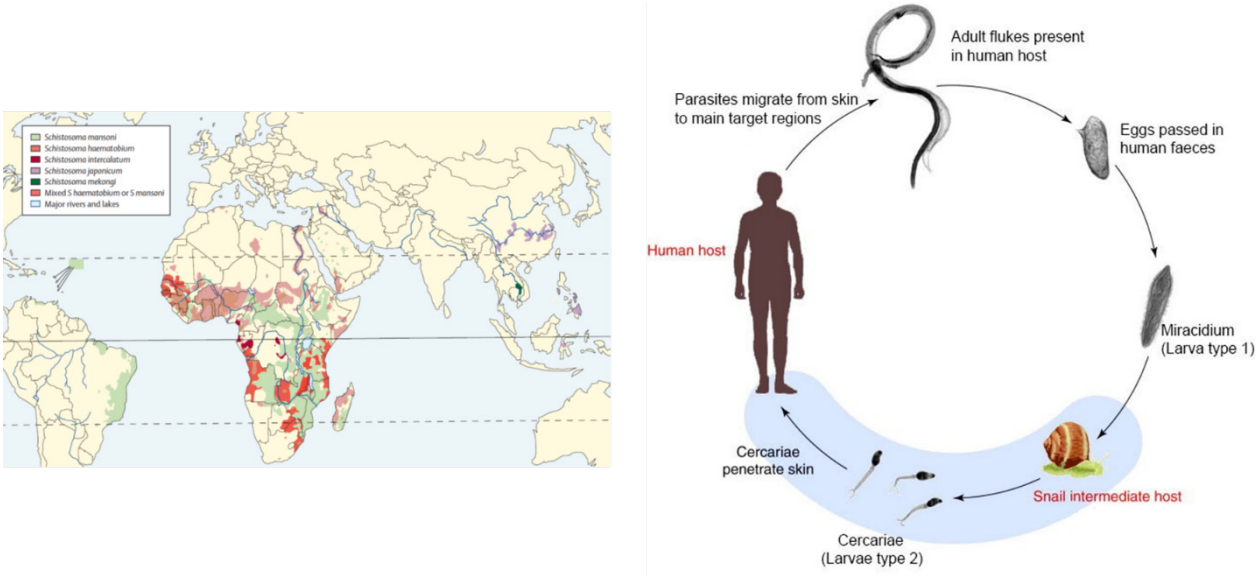

Figure 1: Worldwide distribution and the life cycle of schistosomiasis. Adapted from Colley (2014), Gryseels and colleagues (2006) and www.schistosomiasisaware.wordpress.com.

1.2.2. Biology of the schistosomes

Six different species of trematodes from the *Schistosoma* genus are responsible for the human disease. Schistosomiasis has two forms, either digestive or urogenital, depending on the species of infection. *Schistosoma mansoni*, *S. japonicum*, *S. guineensis*, *S. intercalatum* and *S. mekongi* are causing intestinal schistosomiasis while *S. haematobium* is responsible for the urogenital form (Colley et al., 2014; McManus et al., 2018).

Once released in the environment through feces or urine (*S. haematobium*), the eggs eventually hatch and release the free-swimming ciliated stage called miracidium. The miracidia can survive for hours in the water before infecting an aquatic snail, i.e. *Biomphalaria* spp (Anderson et al., 1982; Walker, 2011). Asexual reproduction (schizogony) takes place within the snail. After 4 to 5 weeks post-infection, the other free-swimming stage and human infective stage, the cercariae, are released in the water. Cercariae are characterized by a motile tail. They seek and actively

invade their definitive host by the skin (Lee et al., 2013; Walker, 2011). The cercariae can remain infectious for up to three days (Colley et al., 2014; Gryseels et al., 2006; Weerakoon et al., 2015). Once in contact with the skin, the cercariae secrete proteases and collagenase and the tail is lost once in the dermis. The heads, now referred as schistosomula reach the blood circulation thanks to which they will reach different tissues. They migrate to the lungs and end up in the liver, where the parasites mature and reproduce. After mating, the worms go to the mesenteric veins or the vesical plexus (*S. haematobium*) to lay their eggs. Finally, the eggs migrate through the mucosa to be released in the intestinal or vesical lumen and contaminate stools or urine (Colley and Secor, 2014; Pearce and MacDonald, 2002; Schwartz and Fallon, 2018).

1.2.3. Clinical manifestations and morbidities

1.2.3.1. Early schistosomiasis

Acute schistosomiasis is referred as “Katayama syndrome” and develops during a period of a few weeks to several months (2-12 weeks) after the initial infection. It is caused by the hypersensitivity immune reaction towards the egg antigens (Colley et al., 2014; McManus et al., 2018; Ross et al., 2007). This syndrome is characterized by eosinophilia and high fever. Other symptoms include malaise, abdominal pain, gastro-intestinal disorders and non-productive cough. It is sometimes preceded by a dermatitis caused by the penetration of the cercariae into the skin a few hours after infection. A spontaneous recovery generally occurs after 2 to 10 weeks (Colley et al., 2014; Da Silva et al., 2005; Gryseels et al., 2006; Puylaert and van Thiel, 2016).

1.2.3.2. Chronic infection

Schistosoma worms can subsist for decades in their host, laying eggs on a regular basis (Fulford et al., 1995). The immunopathologic events triggered by the deposition of the eggs in the tissues are responsible for the chronic morbidities associated with schistosomiasis (Colley and Secor,

2014; Schwartz and Fallon, 2018). With the exception of *S. haematobium* that live in the vesical plexus, other schistosomes reside in the mesenteric veins (Colley et al., 2014). After attachment, their eggs penetrate the tissue (e.g. intestinal) and elicit a TH2-response. In presence of endothelial cells and immune cells (macrophages, eosinophils, etc.), granulomas develop. While some eggs are released in the intestinal or vesical lumen by different processes that have not been fully understood yet, some eggs remain trapped in the tissue. The granulomas lead to the development of fibrosis and calcification that can, in severe cases, result in organ malfunction (Colley et al., 2014; Schwartz and Fallon, 2018). Chronic digestive schistosomiasis generally causes abdominal pain, diarrhea and induce the development of polyps. The urogenital form of the disease includes hematuria as a classical symptom but also leads to a wide range of possible complications, from painful and difficult urination to renal failure. *S. haematobium* infection has been associated with neoplastic events such as bladder carcinomas (King et al., 1988). Often neglected, granulomas can also affect the genitals and expose patients to acute risks of viral infections such as HIV or HPV (Downs et al., 2011).

The eggs that did not attach to the endothelial cells generally end up in the hepatic portal system, where the subsequent tissue fibrosis can lead to portal hypertension, known as “Symmers pipestem fibrosis” (Da Silva et al., 2005). The eggs can sometimes also be found in ectopic locations (Gryseels et al., 2006). Neuroschistosomiasis is one of the most common and impairing ectopic forms. It results from the deposition and dissemination of the eggs in the brain or in the cerebrospinal fluid (CSF) (Colley et al., 2014). The most frequent manifestation of neuroschistosomiasis is generally an encephalitis that is associated with *S. japonicum* or a myelopathy that can also be caused by other species (Carod Artal, 2012; Colley et al., 2014; Ferrari and Moreira, 2011; Ross et al., 2012).

1.3. Drug(s)

1.3.1. Praziquantel

Different drugs like antimony derivatives were used to treat schistosomiasis before the introduction of praziquantel in the late 1970s. Praziquantel is a derivative of pyrazinoisoquinoline and has been commercialized under different names (e.g. Biltricide®). It is sometimes used in combination therapies like Profender® in the veterinary field. Praziquantel was developed initially as a tranquilizer then repositioned as a veterinary and human broad-spectrum anthelmintic by a partnership between the German firms Merck and Bayer (Cioli et al., 2014; Doenhoff et al., 2008; Mäder et al., 2018). It combines a broad activity against platyhelminths with a good safety profile and mild side-effects. Additionally, its tablets are inexpensive to produce (Cioli et al., 2014). These properties made it the ideal candidate for preventive chemotherapy and propelled it as the reference drug, not only against schistosomiasis but against many other trematode infections (Keiser and Utzinger, 2009). Part of the WHO essential medicines list[2], praziquantel has been recommended for MDA since the mid-80s (Colley et al., 2014).

Despite its undeniable qualities, praziquantel has several limitations that urge the development of more potent alternatives and combinatory drugs. Praziquantel is notably unable to block the transmission of the parasite (Bergquist and Elmorshedy, 2018). In addition, it is inactive against juvenile worms (Pica-Mattoccia and Cioli, 2004). Hence, the intake of multiple doses is generally required to completely clear the infection (Cioli et al., 2014).

While schistosomiasis is more prevalent in children, most of the drawbacks of praziquantel concerns its pediatric use. It is broadly acknowledged that “children are not small adults” and neither the dosage nor the current formulation of praziquantel are optimized for children (Gillis and Loughlan, 2007; Marsot, 2018). The tablets are rather big and difficult to swallow. Together

[2] https://www.who.int/medicines/publications/essentialmedicines/en/

with the fact that they taste extremely bitter due to the presence of the S-enantiomer of the molecule, the tablets themselves are critical factors limiting the compliance to the treatment (Lovis et al., 2012; Meyer et al., 2009; Stothard et al., 2013). To overcome this lack of adapted treatment, the Pediatric Praziquantel Consortium[3] – a non-profit public-private partnership – was established in 2012. Thanks to this initiative, a new praziquantel formulation recently entered in clinical phase III (N'Goran et al., 2019).

Despite its broad use, the mechanism of action of praziquantel remains unresolved (McManus et al., 2018). However, it is known to act on calcium homeostasis and is likely to result in spastic contractions and damages to the tegument of the worms, as it has been observed both *in vitro* and *in vivo* (Greenberg, 2005; Xiao et al., 2018).

1.3.2. Antischistosomal drug candidates

There is no advanced clinical candidate against schistosomiasis and the development pipeline is dreadfully empty (Panic and Keiser, 2018). The latter is limited to a handful of pre-clinical leads that were classified by Panic and Keiser (2018) as follows: the ozonides ("OZs"), the old antischistosomal leads developed by Roche, the oxamniquine derivatives and the organometallic derivatives (figure 2).

[3] https://www.pediatricpraziquantelconsortium.org/

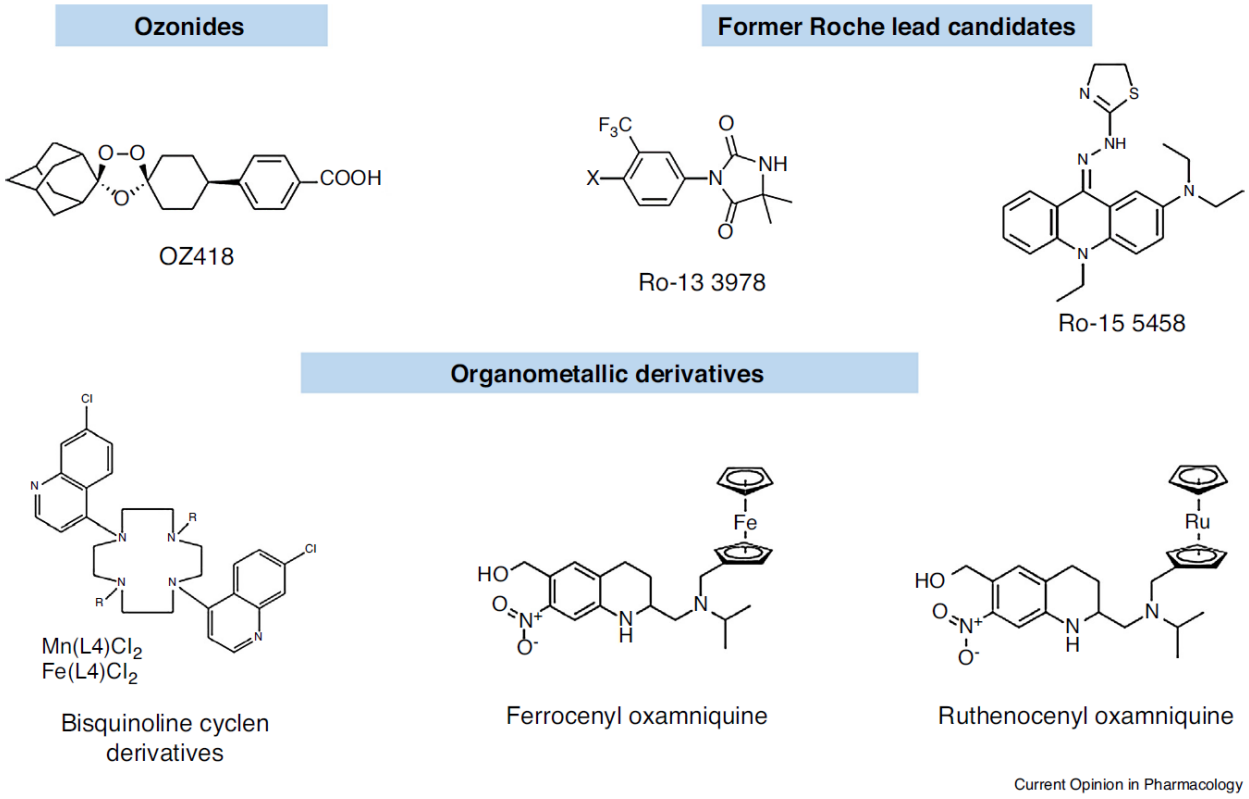

Figure 2 : Structure of the pre-clinical antischistosomal leads highlighted by Panic and Keiser (2018).

1.4. Anthelmintic drug discovery

1.4.1. Limitations of existing therapies

Thanks notably to scaled-up preventive chemotherapy, the prevalence, the morbidity and the mortality associated with helminth diseases spectacularly dropped in less than 20 years (Kassebaum et al., 2016; Kyu et al., 2018; Lo et al., 2017; Pullan et al., 2014; Roth et al., 2018; Schulz et al., 2018). However, millions of people are still in need of anthelmintic treatment. Despite

increasing donations, the preventive chemotherapy coverage for schistosomiasis remains below 50% and is still far from the goals set by the London Declaration (WHO, 2017).

In a context of frequent, repeated and large-scale distribution of the same treatments to the same population, the efficacy of praziquantel and some anti-STH drugs like albendazole is decreasing worryingly (Crellen et al., 2016; Melman et al., 2009; Moser et al., 2017). Whereas anthelmintic resistance is already widespread in the veterinary field, resistance in humans has not yet been confirmed (Geerts and Gryseels, 2001; Kaplan and Vidyashankar, 2012; Rose et al., 2015). However, different indicators point towards an alarming risk of resistance emergence, as revealed in both laboratory and field observations (Caffrey, 2007; Doenhoff et al., 2008; Ismail et al., 1999). For example, praziquantel-resistant strains of *S. mansoni* could easily be developed *in vitro* (Fallon and Doenhoff, 1994; Wang et al., 2012). Pushed forward by larger tablet donations, deworming programs are likely to expand in the next few years. Yet, if the pool of sensitive worms is likely to decrease, it would also favor the diminution of anthelmintic efficacy or, even worse, the development of resistance (Melman et al., 2009).

Most of the current anthelmintic were developed between the 1950s and the 1980s (Campbell, 2016). With the exception of tribendimidine that was approved by the Chinese Food and Drug Administration in 2004[4] for STH infection and moxidectin for onchocerciasis, no new anthelmintic has been marketed in the last decades (Bergquist, 2016; Pedrique et al., 2013; Xiao et al., 2013). In addition, no new molecular entity (NME) active against schistosomes has entered advanced stages of drug development. This is particularly worrisome since praziquantel remains the only therapeutic option against the disease (Bergquist et al., 2017a).

A vaccine would be particularly interesting to overcome the re-infection occurring with preventive chemotherapy against schistosomiasis and STH. Despite ongoing different vaccine research

[4] this medicine has only been approved in China and FDA or EMA approval is still ongoing

programs, none is yet available. Three antischistosomal vaccine candidates recently entered in clinical phases but, so far, conclusions are still hard to draw and implementation of a vaccine in control programs, in the short-term, is rather unlikely (Hotez et al., 2018; Merrifield et al., 2016; Molehin et al., 2016; Tebeje et al., 2016).

1.4.2. Specificity and challenges of anthelmintic drug research

Anthelmintic research is challenging for two main reasons. First, it has been underfunded for decades and second, it relies on very basic tools. With extremely potent broad-spectrum anthelmintic treatments available on the market since the late 1970s, research on new drugs has not been encouraged, especially for human use (Geary et al., 2015, 1999). While the pharma industry invested in more profitable areas, the lack of public funding for parasitologists in academia limited the understanding of the biology of the worms. This resulted in a lack of innovation in this area for decades, especially in regards to the development of mechanism based-assays (Geary, 2012). Hence, the gold standard to evaluate the activity of drugs for most of the helminths is still whole-organism screenings, where both the survival rate and the phenotype of the parasites are assessed visually.

Helminths have very complex life cycles and no reliable “egg to egg” culture methods have been developed yet (Geary et al., 2015, 1999; Keiser, 2010). This drastically limits the availability of raw material to test drugs on and represents an important bottleneck for potential middle and high throughput systems. As phenotypic screenings are very time consuming and can be subjective, alternatives have been investigated (Geary et al., 2015; Ramirez et al., 2007). For antischistosomal drug research, metabolic or fluorescence-based assays (Aguiar et al., 2017; Manneck et al., 2011; Panic et al., 2015), organisms “on a chip” (Chawla et al., 2018) and

computer-assisted systems (Asarnow et al., 2015) represent promising methods, but are not yet as reliable as phenotypic screenings and still need improvements.

1.4.3. Product development partnerships (PDP)

Since the World Health Assembly resolution WHA54.19 and the Millennium Development Goals (MDGs) in the early 2000s, research and development (R. & D.) in the NTD world has been boosted thanks to the creation of global initiatives and large donations from philanthropic foundations (Lustigman et al., 2012; Moran, 2011, 2005; Utzinger and Keiser, 2013). This led to the creation of different product development partnerships (PDP) for developing medicines against diseases with no obvious return on investment. In this model, the initial development steps are often outsourced to public institutions, while later stages, such as production, are delegated to the private sector. Such partnerships presents a significant advantage by limiting R. & D. costs. Non-profit organizations and PDPs often take over the coordination of the drug development effort. Together with different financing schemes set up to delink the development costs from the drug price, this approach tends to enable a fair access to medicines (Balasegaram et al., 2017; Mahoney, 2011). So far, PDPs have been successful at securing new antimalarial drugs and more recently fexinidazole against trypanosomiasis (Ashley and Phyo, 2018; Mesu et al., 2018).

1.4.4. Screening open access libraries and drug repurposing

The Medicines for Malaria Venture (MMV) is a Geneva-based PDP dedicated to malaria research. In 2012, MMV launched the “Malaria Box” as a strategy to expand the number of lead antimalarial molecules. This library was also tested against schistosomes and other helminths (Voorhis et al.,

2016). For example, N,N9-diarylurea and a 2,3-dianilinoquinoxaline were identified as antischistosomal leads (Ingram-Sieber et al., 2014).

Given the success of this approach, MMV decided to launch the "Pathogen Box"[5]. The library gathers drug-like molecules carefully selected by medicinal chemists for their previous efficacy on one or more infectious diseases. At the same time, MMV compiled the "Stasis Box" – a set of 400 drugs that were stopped in their clinical development and for which potential new applications had to be found. This library was distributed to selected partners of MMV including our laboratory. In this thesis, we aimed to identify new active molecules against schistosomes by screening both the Pathogen and the Stasis boxes. In addition to seeking to expend the pool of antischistosomal drugs by screening libraries of compounds, we applied a second common R. & D. strategy, drug repurposing (chapters 4a and 4b). Drug repurposing or repositioning means finding new applications for already developed drugs or advanced candidates (Panic et al., 2014; Ramamoorthi et al., 2015). By short-cutting some of the pre-clinical steps, i.e. toxicity assessment, repurposing is another strategy to reduce the R. & D. costs. In the present work, drug repurposing was used when testing both the Stasis Box on *S. mansoni* (chapter 4a) and emodepside, a veterinary anthelmintic, on STH laboratory models (chapter 4b).

[5] https://www.mmv.org/mmv-open/pathogen-box

1.5 Aim and objectives

The treatment and control of schistosomiasis and STH rely essentially on the large-scale administration of a handful of old drugs. Neither alternative treatments nor vaccines are yet available, and the efficacy of the current drugs is at stake as a result of their overuse in affected regions. In this PhD thesis, we intended to expand the pool of antischistosomal molecules and feed the dry anthelmintic development pipeline. Two main strategies were applied to identify new compounds of interest: first, the screening of open access compound libraries and second, drug repurposing. This was completed by an interlaboratory comparison of the results of one of these libraries and the test of a new screening device for *S. mansoni* schistosomula.

In more detail, the objectives of this work were defined as follows:

1) Elaborate an integrated protocol detailing the cultivation and early antischistosomal drug discovery (Chapter 2);
2) Identify new antischistosomal leads following *in vitro* and *in vivo* testing on schistosomes and conduct structure activity relationship studies of two compound libraries, the MMV Pathogen and Stasis boxes (Chapters 3a and 4a);
3) Validate the drug testing results by comparing the findings of the Pathogen Box screening on schistosomes with those of two other institutions that screened the same library (Chapter 3b);
4) Contribute to an investigation on the activity of emodepside on schistosomes, hookworms and whipworms (Chapter 4b);
5) Test a movement-based system, initially developed for anti-cestodal compound screening, on the larval stage of the schistosomes (Chapter 5).

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Chapter 2

Life cycle maintenance and drug-sensitivity assays for early drug discovery on *Schistosoma mansoni*

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Life cycle maintenance and drug-sensitivity assays for early drug discovery in *Schistosoma mansoni*

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Drug discovery for schistosomiasis is still limited to a handful of academic laboratories worldwide, with only a few novel antischistosomal lead compounds being actively researched. Despite recent international mobilization against the disease to stimulate and promote antischistosomal drug discovery, setting up a drug-screening flow with schistosome parasites remains challenging. Whereas numerous different protocols to obtain and cultivate schistosomes have been published, those describing the drug-screening process are scarce, and none gather together parasite cultivation and early drug discovery procedures. To help overcome this hurdle, we provide here a set of integrated methods either adapted from already-published protocols or based on our long-term experience in schistosomiasis research. Specifically, we detail the establishment and maintenance of the complex and several-week-long *Schistosoma mansoni* life cycle in a laboratory setting, as well as the means of retrieving and culturing the parasites at their relevant life stages. The in vitro and in vivo assays that are performed along the drug-screening cascade are also described. In these assays, which can be performed within 5 d, the effect of a drug is determined by phenotypic assessment of the parasites' viability and morphology, for which stage-specific scoring scales are proposed. Finally, the modalities for testing and evaluating a compound in vivo, constituting a procedure lasting up to 10 weeks, are presented in order to go from in vitro hit identification to the selection of early lead candidates.

Introduction

In May 2012, the World Health Organization (WHO) put forth an ambitious goal to eliminate schistosomiasis, a debilitating parasitic disease caused by trematodes of the *Schistosoma* genus, as a public health problem by 2020 (WHO, https://www.who.int/neglected_diseases/9789241564540/en/). To fulfill this mandate, treatment coverage using the only drug available, praziquantel (PZQ), would need to expand from 35 to 75% of school-aged children in at-risk areas (WHO, https://www.who.int/neglected_diseases/news/WHO_urges_increased_access_to_praziquantel/en/). Yet, because of concerns about drug resistance, along with the drug's other drawbacks, the scientific community has recognized an urgent need for the development of new treatments[1,2]. Despite the pressing need, however, no new drug candidates are currently close to reaching market. As one of the neglected tropical diseases, the drug discovery pipeline for schistosomiasis is, by extension, underfunded[3]. Nonetheless, drug discovery efforts have been extensive in the academic community[4–7]. Moreover, recent product-development partnerships, such as the Drugs for Neglected Diseases *initiative* (DND*i*) and the Medicines for Malaria Venture (MMV), have been productive in securing compound libraries to be tested against a broad range of infectious diseases, including schistosomiasis, which has resulted in an array of hit compounds for the disease[8–10]. Hence, this is a key moment to expand antischistosomal drug discovery efforts.

This protocol aims to support these efforts by providing the necessary basics for establishing a screening cascade. Specifically, it details the procedures used at the Swiss Tropical and Public Health Institute (Swiss TPH) to establish the complex *S. mansoni* life cycle in the laboratory, obtain the relevant life-stage parasites, integrate them into an in vitro and in vivo drug-screening cascade, and lay out best-practice phenotypic assay evaluation procedures. As fewer than 30 institutions worldwide host an in-house *S. mansoni* life cycle[11], different culturing conditions and various screening techniques are being successfully used. The fact that we focus here on the protocols currently used at the Swiss TPH should therefore neither overshadow nor discredit alternative methods, which we discuss when applicable.

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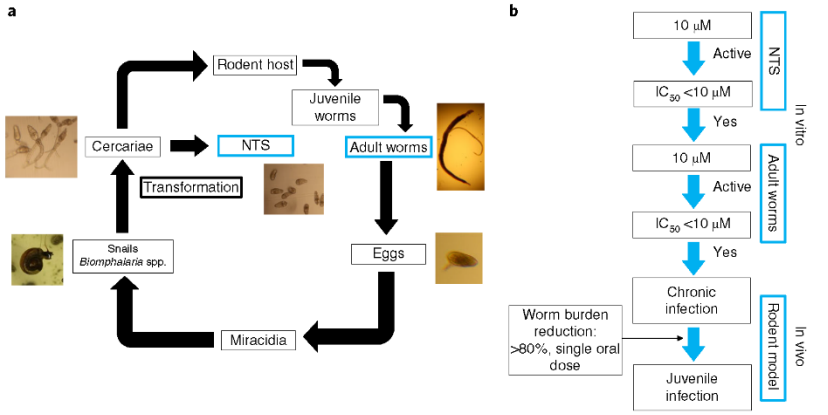

Fig. 1 | Overview of the protocol. a, The *S. mansoni* life cycle (Steps 1–38) requires that eggs obtained from an infected rodent be exposed to water to hatch as miracidia (Steps 11–28), which infect *Biomphalaria glabrata* snails. After a few weeks of asexual reproduction, *S. mansoni* cercariae emerge after snails are exposed to light (Steps 29–34). A cercarial suspension can then be collected to infect a mouse or hamster (Steps 35–38). The life stages used in the drug-screening cascade are indicated in blue boxes in the figure. For other *Schistosoma* species, please refer to Supplementary Table 1. **b**, The antischistosomal screening cascade starts with a pre-screen of compounds on NTS at 10 μM (can be adjusted) (Steps 51–55). Active compounds are tested on adult-stage worms (Steps 72–78) in vitro, and the most active from this screen are further tested in mice harboring *S. mansoni* chronic infection (Steps 79–82). The activity of the compounds that show worm burden reduction >80% can be further characterized on mice bearing juvenile worms.

Development of the protocol

Methods for breeding and maintaining *Biomphalaria glabrata* snails and schistosomes in laboratories have been established since the 1950s[12,13]. The procedure described here to maintain an *S. mansoni* life cycle is based on these original methods, with several modifications over time[11–16], as this life cycle has been running for more than 50 years at the Swiss TPH.

Since the 1970s, different techniques for mechanical cercarial transformation have been described[17–19]. In this protocol, we propose a transformation method adapted from Milligan and Jolly[20] (see video therein) and Colley and Wikel[17], which consists of a series of washing steps and repeated passage between two connected syringes.

Despite recent advances in automated or quantitative methods for drug screening on the different *S. mansoni* stages, there is no broadly accepted consensus on automated assays. Therefore, drug sensitivity assays based on phenotypic evaluation of adult worms have formed the basis of standard operating procedures recommended by the UNICEF/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR) since 2007[11,21]. However, when screening large libraries of chemical compounds, huge quantities of parasites are required. A medium-throughput phenotypic screening procedure based on schistosomula assessment was proposed by Abdulla et al.[22]. This approach was later applied by Mansour and Bickle[23], and modified to be suitable for screening large libraries of compounds. Assessing the viability of the parasite after drug exposure is essential to determining the effect of a drug candidate[11]. For this reason, we adapted the phenotypic scoring scale described by Ramirez et al.[21] and Abdulla et al.[22] for the assessment of newly transformed schistosomula (NTS) in vitro[11,24].

Overview of the procedure

The first part of the procedure (Steps 1–38) details how to set up and maintain the *S. mansoni* life cycle (Fig. 1a), which comprises setting up a *B. glabrata* snail life cycle (Steps 1–10), obtaining *S. mansoni* eggs from the infected hamsters (Steps 11–16), hatching the miracidia from the eggs and infecting the snails (Steps 17–28), inducing cercarial shedding from the snails (Steps 29–34), and infecting mice or hamsters with *S. mansoni* cercariae (Steps 35–38). Once the life cycle is established, it is possible to conduct the in vitro and in vivo screening.

The drug-screening process (depicted in Fig. 1b) often starts with a pre-screen of the compound library on the larval-stage worms, which are more abundant and easier to obtain. This stage of the

protocol therefore begins with the production of larval-stage parasites, hereafter referred to as NTS (Steps 39–50). The drug assay setup, as well as the drug assay evaluation procedure, is then detailed (Steps 51–55). Only the compounds that are found to be active on NTS are tested on the primary target of interest, the adult-stage worms (Steps 72–78). In the last part of the protocol, the methods for testing and evaluating the activity of lead candidates in a mouse model are presented (Steps 79–86).

Alternative methods

Establishment and maintenance of a schistosome life cycle

The methods for establishing a schistosome life cycle vary slightly, depending on the *Schistosoma* species and lab conventions. At Swiss TPH, we generally use a Liberian *S. mansoni* strain, *B. glabrata* (Egyptian) for snail infection, coupled with an *S. mansoni*—hamster or *S. mansoni*—mouse infection model, as described in the Procedure. Variations to the protocols required for *S. haematobium* and *S. japonicum* are described in Supplementary Table 1, in addition to various mouse strain options. We also cite the optimal snail size for infection with miracidia (4–6 mm), development period until cercarial shedding (5–6 weeks) and length of exposure to light to induce cercarial shedding (3–4 h) that we have found to be effective in our drug discovery work[7,9,25,26]. However, this could vary from lab to lab for a number of reasons, such as the *Schistosoma* or snail strains chosen. We therefore recommend that readers experiment and determine their own optimal conditions.

In our experience, the RJHan:AURA hamster (Janvier Labs) is optimal for life cycle maintenance because of its ability to harbor many parasites for a sufficient period of time. However, the alternative rodent hosts listed in Supplementary Table 1 are also suitable.

Research groups that do not want to undertake the task of establishing a life cycle can make use of the NIH National Institute of Allergy and Infectious Diseases (NIAID) Schistosomiasis Resource Center, which maintains a variety of schistosome–snail and schistosome–rodent infections, available freely to any research team that requests them (http://www.afbr-bri.com/schistosomiasis/).

Cercarial transformation

The time requirement for the cercarial transformation using two connected syringes followed by a saline rinse is, in our experience, much shorter than those for other procedures based on pipetting or vortexing[16,18] to trigger separation of the cercarial head from the tail, or using a Percoll gradient[16,27] to rinse the tails.

In vitro drug testing

This procedure allows a certain flexibility and some modifications to it may be required to meet the reader's needs. For example, Basch medium can be used instead of M199 medium for larval-stage assays[11]. Also, the drug concentration range (10–100 μM) and the duration of each assay will depend on the type of drug being tested. Whereas a screen at 10 μM might be suitable for synthetic compounds, a test concentration of ≥100 μM might be more suitable to test sublethal effects of natural product derivatives[28].

Although phenotypic screenings based on microscopy as a readout are beneficial for researchers working in low-resource facilities, they are laborious and undeniably subjective[11]. Intensive research has been conducted to overcome these limitations, and various automated readout alternatives have been proposed. These include notably *S. mansoni* NTS drug assays using luminescent[29] or fluorescent markers[30,31], label-free microcalorimetry[32] or other methods based on electrical recordings[33,34]. In parallel, the development of computer-assisted image analysis is expected to improve the readout of phenotypic assays[22,35,36].

In vivo studies

We commonly use NMRI outbred mice for in vivo experiments, as they are more amenable to testing in larger numbers, easy to handle and can maintain an *S. mansoni* infection for a long period. As stated, we have listed alternative mouse models in Supplementary Table 1. It has been noted that drug efficacy can vary depending on the mouse strain used, and the reason for this variation is not clear[37,38]. It is also debatable whether inbred versus outbred mice are preferred[38]. Inbred mice would reduce experimental variability, but the results may be less reproducible across other breeds and species.

For infection of mice, the concentration of cercariae per injection we use (100 cercariae per mouse), as well as the 7-week infection period, has been optimized for our NMRI mouse strain.

If researchers desire to use other strains, they might need to adjust these parameters. Instead of subcutaneous injection with the cercarial suspension, mice can alternatively be infected by dipping the tail or the shaved abdomen directly into a Petri dish with a cercarial suspension or by percutaneous exposure[12,16,39]. Whereas these techniques allow the development of a natural infection, the parasite load cannot be fully controlled. In addition, the fact that these infection methods require animals to be anesthetized or restrained might be an issue in regard to ethical requirements. On that last concern, the paddling method proposed by Dettmann et al.[40] might provide an alternative, as it allows mouse infection without prior shaving.

In studies with schistosomes, portal perfusion is often used to flush out the adult worms. This technique has been well described[15,16,41]. However, in this protocol, we collect the worms from the mesenteric veins manually. It has the advantage that it is a fairly straightforward method to adopt for labs that are completely new to the process. It also ensures that all worms have been collected. In addition, one can visualize the localization of the worms after drug treatment. For example, the worms can lose their adherence from the mesenteric veins and be shifted to the liver (liver shunt). This could indicate partial activity of a drug[25]. On the other hand, the method we describe has the main disadvantage of being more time consuming as compared to the portal perfusion methods[41].

Applications

Several parts of this protocol are not restricted to antischistosomal drug discovery and can be useful to research teams working in other fields and with other parasites. The methods for establishing and maintaining a *Biomphalaria* snail cycle in a controlled environment can be used by malacologists, for example, in behavioral or ecological studies[42,43]. Schistosomes and their intermediate hosts are model organisms often used in the field of evolutionary biology and host–parasite interactions[44,45]. Similarly, NTS can be useful not only for the analysis of schistosome development but also for a range of biological studies, including genomic and transcriptomic analysis[46–48]. Nonetheless, the reader should note that NTS are not completely identical to the schistosomula present in the host after natural infection. However, only minor differences in morphology, antigenic profile and gene expression have been identified between NTS and 'natural' schistosomula[46,49,50]. Also, at the host–parasite interface, studies investigating hosts' immunity toward helminths—notably characterized by a shift from Th1 to Th2 immune responses—can use *S. mansoni*–mouse infection models[51]. Such immunological studies are also very relevant for developing a potential vaccine, which could prevent continuous reinfections with the parasite[52].

Advantages and limitations

The drug-screening process for *Schistosoma* spp. comes with a few challenges that may hinder research groups (especially in low-resource settings) from establishing their own screening flow. First, as target-based screening for schistosomiasis is still under development[53], antischistosomal drug discovery relies essentially on whole-organism drug sensitivity assays. Although more efficient at producing successful first-in-class compounds[54], whole-organism screening necessitates regular access to parasites that can neither reproduce nor be maintained in vitro for very long[11]. Therefore, a life cycle with both the intermediate and the definitive hosts—snails (e.g., *Biomphalaria spp.* for *S. mansoni*) and rodents (hamsters or mice), respectively—must be established and maintained. Moreover, only a limited number of adult-stage schistosomes (the most relevant parasite life stage for drug screening) can be collected from rodents, and only several weeks after infection. This is not an issue when only a handful of compounds are to be tested, but it poses an ethical and resource bottleneck when screening large compound libraries. This limitation is partly addressed by first screening compounds on NTS. The cercarial transformation method used to produce them and which is presented hereafter is reliable, fast and simple to execute.

Second, as there is no consensus on an acceptable automated reading system, the gold standard method of determining the antischistosomal activity of a drug is still direct observation using an optical microscope, which is simple and low-tech but subjective and laborious[23]. The post-drug exposure assessment of the parasites in vitro is therefore particularly important for the selection of lead candidates. Nonetheless, drug-screening results can vary greatly from lab to lab; a recent study showed that when two independent laboratories tested the same set of compounds, <30% of their hits overlapped[25]. This may be in great part due to differences in evaluation outcomes. In this protocol, we aim to minimize this by providing a graphic guide to parasite viability scoring.

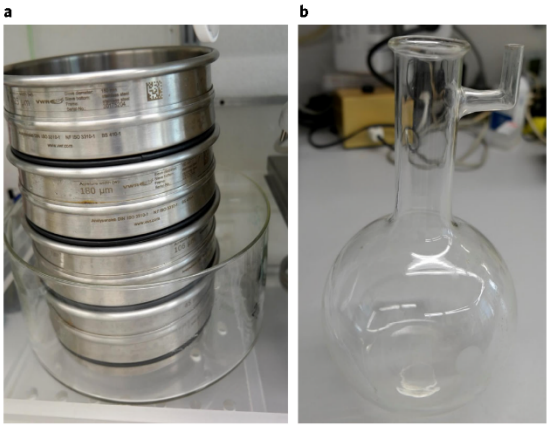

Fig. 2 | Equipment required for collecting eggs and hatching the miracidia. a, Four stainless-steel sieves (45–425 µm) (Step 15). **b**, 0.5-L side-arm flask (Step 18).

Third, the lead selection process described here is rather simple, and it is based on activity thresholds. However, additional tests can be performed to increase the chances of successful in vitro–in vivo translation, such as metabolic or enzymatic assays[23].

Experimental design

Note on animal experimentation

Many parts of this procedure include work on laboratory rodents. Compulsory certifications in animal experimentation are required in many countries, and we also recommend prior practical training. Untrained personnel may improperly inject the material, causing harm to the animal or themselves. We also encourage the reader to comply with the 3Rs principles for in vivo experimental design and statistical analysis, as well as to adhere to the ARRIVE guidelines for reporting animal research[55]. National laws and institutional regulatory board guidelines must be followed.

Establishment and maintenance of a schistosome life cycle

This part of the protocol comprises four essential steps, namely establishing a *B. glabrata* snail cycle (Steps 1–10), hatching miracidia for infecting snails (Steps 11–28), shedding and collecting of the cercariae (Steps 29–34), and infecting the rodent host with the collected cercariae (Steps 35–38). For the snail cycle, simple aquarium systems are used, with ecological water, ambient temperature and light cycle. Snails are kept until they start to produce eggs. Half of them are moved to a new tank containing flat, square polystyrene pieces on which they lay eggs. The eggs are then collected and placed in breeding tanks (Steps 1–10). The other half of the snails are collected for infection with *S. mansoni* (Steps 21–28). To infect the snails, the schistosome eggs are recovered from a saline solution of blended liver of an infected hamster that has been previously filtered (Steps 11–17) (Fig. 2a). The eggs are then placed in warm water in a covered side-arm flask (Fig. 2b) where the side arm is exposed to lamp light (Steps 18–23). After the hatched miracidia swim to the side arm, the suspension is collected and placed in well plates together with snails of appropriate size (4–6 mm) to infect them (Steps 24–28). After 5–6 weeks, the infected snails normally start to shed cercariae. The cercariae are collected by placing the snails under a neon lamp for a couple of hours (Steps 29 and 30) (Fig. 3 (**i**)). The cercariae are shed optimally for 2–4 weeks. Snail shedding of cercariae is highest in the first 2 weeks after initial shedding. After that, the persistence of the infection is highly variable, ranging anywhere from 4 weeks to 3 months. The collected cercarial suspension is then used to infect hamsters by subcutaneous injection (Steps 35–38). After the 7 weeks required for establishing a patent infection, the infected livers can be used to continue the cycle (Steps 11–28).

Cercarial transformation and adult worm collection

Mechanical stress is used to transform cercariae into NTS. The cercarial suspension (Fig. 3 (**ii–iv**)) is pushed between two interconnected syringes, followed by a cold rinse to separate heads from tails

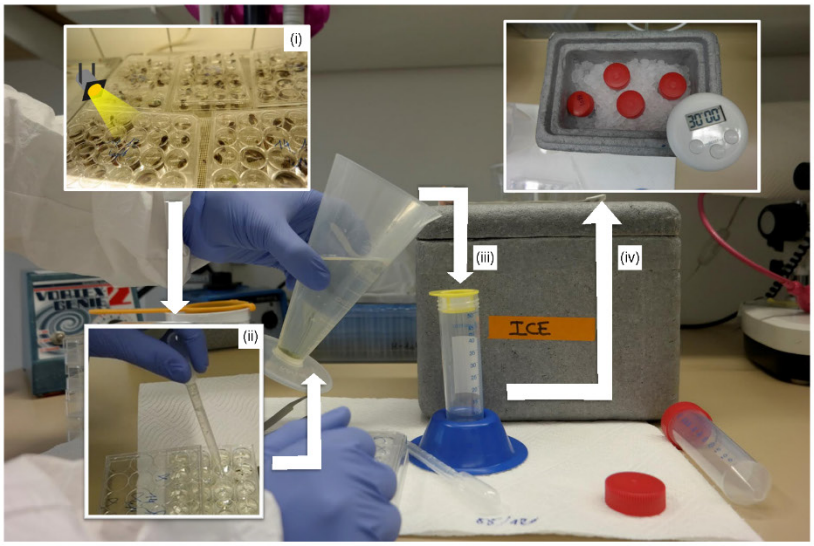

Fig. 3 | Collection of *S. mansoni* cercariae for transformation to NTS. (**i**) Snails are first exposed to light (Step 30). (**ii**) The snails are removed (Step 32), and the pond water containing the cercaria shed by the snails is collected (Step 33). (**iii**) The cercarial suspension is allowed to sediment at RT and is then poured through a 100-µm filter (yellow) into a 50-mL tube (Step 3). (**iv**) The suspension is cooled on ice for 30 min (Step 39) to immobilize the cercariae.

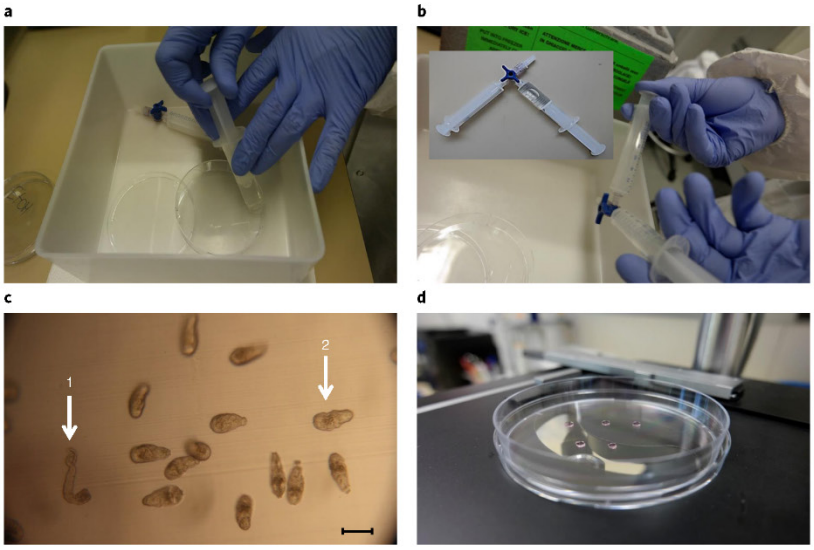

Fig. 4 | Transformation of cercariae to NTS. a, One syringe is filled with cercarial suspension (Step 43). **b**, The syringes are connected (inset) and the contents are pushed back and forth vigorously, three to four times (Step 43). **c**, Most (>90%) of the heads should be separated from the tails. This can be directly visualized under the microscope (Step 43). After several washing steps, the NTS are counted and the suspension is adjusted to the desired concentration (Step 49); tails (1) can be distinguished from the NTS (2). Scale bar, ~100 µm. **d**, Counting the NTS (Step 50).

(Steps 39–50) (Fig. 4). An overnight incubation in culture medium is required to complete the transformation. To obtain adult worms for drug screening, laboratory rodents (hamsters or mice) are injected subcutaneously with the collected cercarial suspension (Steps 35–38). The animals are then

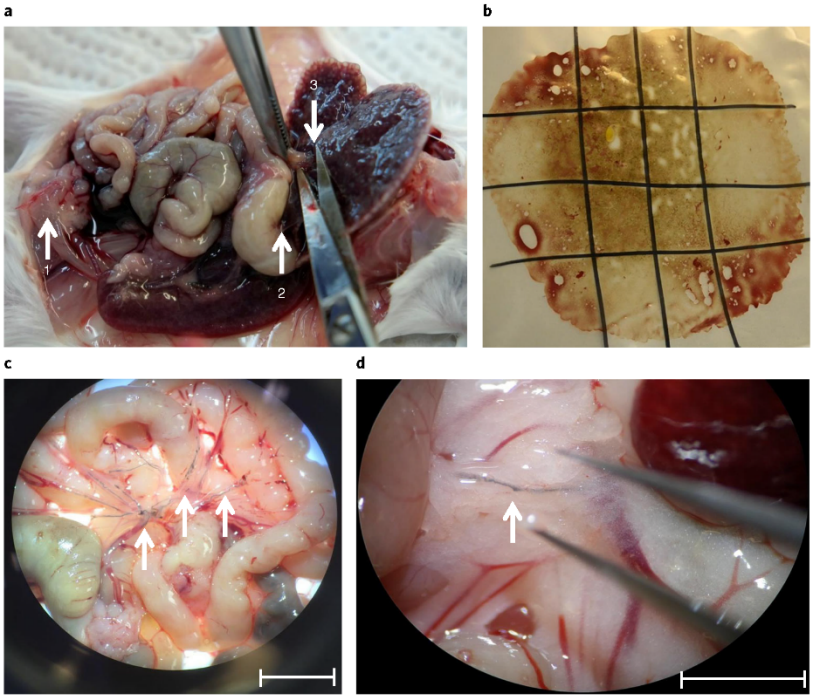

Fig. 5 | *S. mansoni* adult worm recovery. a, The intestines are excised by cutting at the distal part of the rectum (1) and the esophagus (2). The operator must hold the portal vein firmly closed, while excising the liver, to avoid blood spill resulting in the loss of adult worms (3). **b**, The liver is excised and placed between two transparent plastic layers and pressed vigorously. A grid is drawn on the plastic foil to facilitate worm localization (Step 69). **c**, The intestines are excised and placed under a dissection microscope. **d**, Zoomed-in view at ×1.8 magnification. The adult worms present in the mesenteric veins and indicated by the white arrows in **c** and **d** are then collected (Step 70). Scale bars, ~1 cm. For all our experiments involving laboratory animals, we were granted ethical and experimental approval from both the Canton Basel-Stadt and the Swiss federal veterinary authorities (license no. 2070). A certification for animal experimentation was required for all the operators involved.

euthanized, the mesenteries are excised and the adult worms are directly collected from the rodent mesenteric veins using flat-tip tweezers and a dissecting microscope (Steps 67–71) (Fig. 5). The collected worms are incubated with culture medium and can be used for ~2 weeks after the dissection.

In vitro drug testing

When screening large compound libraries, initial efficacy tests on NTS are helpful in selecting candidate compounds of interest. We first describe a pre-screen assay at a mono-concentration to assess the antischistosomal activity of a compound (Steps 51–55). The parasite viability is evaluated over 3 d post compound exposure and compared to the negative control wells containing the highest concentration of the solvent, dimethyl sulfoxide (DMSO) (Steps 51–55). Hits are further tested to determine their 50% inhibitory concentration (IC_{50}). We use a benchmark of $IC_{50} < 10$ μM to select compounds to test on adult worms, as drug concentrations higher than this are rarely found in vivo with realistic dose levels (Steps 56–62). Hits are then tested on adult worms (Steps 72–78) to select compounds with which to pursue in vivo testing (Steps 79–86).

In vivo studies

Drug candidates are tested in laboratory mice harboring an *S. mansoni* infection. Different rodent strains can be used (Supplementary Table 1). We infect 3- to 4-week-old female NMRI mice with *S. mansoni* cercariae (Steps 35–38). To evaluate the activity of a compound, drug candidates are

administered 7 to a maximum of 8 weeks post infection, the time it takes for adults to develop in the mouse model (Steps 79–82).

To test the activity of a drug candidate on juvenile worms, the mice should instead be treated 3 weeks post infection. In addition, drugs can be tested for their protective efficacy by administering the drug before the infection (i.e., day −2 up to day 0) or for efficacy against migrating schistosomula[56]. In both cases, the value used to assess the activity of a drug is the worm burden reduction (WBR). It is determined by comparing the number of live worms found in the treated mice to the numbers of live worms in the control group (Steps 83–86).

Controls

The negative controls for the in vitro procedure are parasites incubated in appropriate medium with the drug solvent only, in general 0.1% (vol/vol) DMSO. For positive-control wells, the experimenter can use auranofin or mefloquine for the NTS assays (Step 54) or praziquantel for the adult worm assays (Step 77). The control mice for in vivo experiments are either untreated or administered with the drug vehicle only (Step 82).

Materials

Biological materials

! CAUTION National and institutional guidelines on animal experimentation must be followed. Refer to the 'Experimental design' section. For all our experiments involving laboratory animals, we were granted ethical and experimental approval from both the Canton of Basel-Stadt and the Swiss federal veterinary authorities (license no. 2070). A certification in animal experimentation was required for all the operators involved.

- *B. glabrata* snails (these can be initially obtained from, e.g., the NIAID Schistosomiasis Resource Center, https://www.niaid.nih.gov/research/schistosomiasis-resource-center)
- Hamster or mouse infected with *S. mansoni* worms (these can be initially ordered from the NIAID Schistosomiasis Resource Center, https://www.niaid.nih.gov/research/schistosomiasis-resource-center)
- RJHan:AURA male hamsters (3 weeks old, e.g., Janvier Labs, cat. no. HAMST-M)
- NMRI female mice (3 weeks old, e.g., Charles River, cat. no. 605NMRI)

Reagents

- 'Pond water' (filtered dechlorinated tap water)
- Fresh lettuce and spinach leaves
- 70% (vol/vol) Ethanol (EtOH; Merck, Switzerland, cat. no. 1.00983.6025) in water
- Milli-Q water (Merck, e.g., Milli-Q Advantage A10 Water Purification System, cat. no. Z00Q0V0WW)
- NaCl (Merck, cat. no. 1.06404.1000)
- Hank's Balanced Salt Solution (1× HBSS; Gibco, cat. no. 24020117)
- Penicillin–streptomycin (pen–strep; 10,000 U/mL; Sigma-Aldrich, cat. no. P4333-100ML)
- Medium 199 (Gibco, cat. no. 22340-020)
- Fetal calf serum (FCS; Bioconcept, cat. no. 2-01F30-1)
- Kanamycin (Fluka, cat. no. 60615)
- Penicillin G (Sigma, cat. no. P3032-10MU)
- 5-Fluorocytosine (Sigma, cat. no. F7129)
- Chloramphenicol (Fluka, cat. no. 23275)
- dH_2O
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. 276855-2L)
- RPMI 1640 (Gibco, cat. no. 51800-043)
- Tween 80 (Fluka, cat. no. 23908273)
- Absolute ethanol (Merck, cat. no. 1.00983.6025)

Equipment

- Personal protective equipment: nitrile gloves, lab coat, sleeve protection, long gloves, facial shield and laboratory goggles
- 5-gallon standard fish tank or a simple, transparent rectangular plastic bin
- Simple fish tank mesh lid (should completely cover the fish tank opening)
- Simple fish tank air pump
- Flat polystyrene (in square pieces of 8–9 cm)

- CO_2 chamber for euthanizing rodents
- Dissection board
- Pins for dissection
- Scissors
- Forceps
- Hamster polycarbonate type 4 cages (Tecniplast, cat. no. 1354G)
- Mouse polycarbonate type 3 cages (Tecniplast, cat no. 1290D)
- Four stainless-steel sieves with pore sizes of 425, 180, 106 and 45 μm (Fig. 2a; VWR, cat. nos. VWRI510-0586, VWRI510-0576, VWRI510-0570 and VWRI510-0560)
- Laboratory immersion blender with variable speed (variable-speed laboratory blender; Waring, cat. no. Z272183)
- Pressure garden sprayer with brass lance and brass nozzle pump (5 L)
- 1-L side-arm flask (e.g., Sigma-Aldrich, cat. no. Z290459-1EA; alternatively, fit an Erlenmeyer filter flask with a custom-made L-shaped adapter (Fig. 2b))
- Water bath (Julabo VC)
- A fitted cardboard or wooden box to cover the side-arm flask (or aluminum foil)
- Transparent plastic foil (Semadeni, cat. no. 2457)
- Incandescent bulb (40 W; Osram)
- Dissecting microscope (Optika, model no. Lab 20)
- Eppendorf pipettes (P20, P200 and P1000; Eppendorf)
- Pyrex glass beakers (10, 30 and 1,000 mL; Corning, cat. nos. CLS100010, CLS100030 and CLS10001L)
- 24-, 48- and 96-well plates (Sarstedt, cat. nos. 83.3922, 83.3923 and 83.3924)
- Light source for cercaria shedding: neon lamp (36 W, 4,000 K, 3,350 lumens, G13; Osram, cat. no. 4050300517872)
- Tweezers (flat tip, no teeth; Fisherbrand, cat. no. 16100127)
- Hypodermic 25-gauge syringe needle (0.5 × 16 mm, sterile; Braun Medical, cat. no. 4657853)
- Filter for sterilization (0.2 μm, Filtropur S 0.2; Sarstedt, cat. no. 83.1826.001)
- Conical graduated cylinder (Dynalon, cat. no. 1211Y42)
- Stericup (0.22 μm, 500 mL; Corning, cat. no. 431097-COR)
- Nylon filters (100 μm, nylon, VWR, cat. no. 732-2759)
- 15- and 50-mL Test tubes (Sarstedt, Germany, cat. nos. 62.547.004 and 62.554.002)
- Serological pipettes (5, 10 and 25 mL; Sarstedt, Germany, cat. nos. 86.1253.001, 86.1254.025 and 86.1685.001)
- Syringes with Luer-Lok tips (10 mL; BD, cat. no. BD 309695)
- Luer-Lok connector (Discofix; B. Braun, cat. no. 40951111)
- Petri dish, 94 × 16 mm (Greiner, cat. no. 633102)
- Upright bright-field microscope with 4, 10 and 20× lenses (Primovert)
- Pyrex borosilicate glass Petri dishes (55-mm diameter; Fisher Scientific, cat. no. 12013333)
- Oral gavage needle (straight; Carl Roth, cat. no. HPY8.1)
- Pasteur pipette (Pastette; Alpha Laboratories, cat. no. LW4111)
- 1-mL Sterile plastic syringes (B. Braun, cat. no. 9161406V)
- Waterproof markers (Stabilo, cat. no. 842/41)
- Mouse identification marker: picric acid solution (1.3% (vol/vol) in H_2O; Sigma-Aldrich, cat. no. P6744-1GA)

Reagent setup

S. mansoni egg rinsing solution

S. mansoni egg rinsing solution is 4 °C physiological saline solution (0.9 or 1.2% NaCl in Milli-Q H_2O at 4 °C). Prepare fresh solution before each experiment.

Hank's Balanced Salt Solution

Hank's Balanced Salt Solution (HBSS 1×) should be supplemented with 1% (vol/vol) pen–strep. It can be stored at 4 °C for up to 4 weeks.

NTS culture medium

M199 medium should be supplemented with 5% (vol/vol) FCS, 1% (vol/vol) pen–strep and 1% (vol/vol) antibacterial/antifungal mix (see preparation below) with 45% (vol/vol) kanamycin, 27% (vol/vol) penicillin G, 23% (vol/vol) 5-fluorocytosine and 5% (vol/vol) chloramphenicol. It can be stored at 4 °C for 2–4 weeks.

Antibacterial/antifungal mix for 10-mL solution

Antibacterial/antifungal mix should be prepared as shown below. It can be stored at −20 °C for up to 3–6 months[57].

- Weigh 10 mg of chloramphenicol and dissolve it in 143 µL of 70% (vol/vol) EtOH.
- Weigh 50 mg of 5-fluorocytosine and dissolve it in 9,857 µL of dH_2O in a 15-mL tube.
- Weigh 100 mg of kanamycin.
- Weigh 60 mg of penicillin G.
- Mix all ingredients together in a 15-mL tube.
- Sterilize by 0.2-µm filtration.
- Prepare aliquots of the solution for convenience.

Adult worm culture medium

RPMI 1640 medium supplemented with 5% (vol/vol) FCS and 1% (vol/vol) pen–strep. It can be stored at 4 °C for 2–4 weeks.

Drug solution for oral administration

Drug solution for oral administration is 70:30 Tween 80/absolute ethanol solution dissolved in Milli-Q H_2O (10% (vol/vol)). Prepare fresh solution before each experiment.

Procedure

Establishing and maintaining a snail cycle ● Timing 30 min

1 Fill the aquarium tank three-quarters full with pond water warmed to 26–28 °C.
! CAUTION Water quality is important: charcoal-filtered water must be used in the case that the water quality is untested. Water must always be dechlorinated. As it will be frequently used, we recommend setting up a large reservoir of pond water: simply fill a large tub with tap water and add a simple air pump.

2 Insert the air pump and turn it on such that bubbles form and float to the top to prevent water fouling.
▲ CRITICAL STEP Never allow the water to become stagnant. Check the air flow in the tanks periodically.

3 Place between 200 and 300 snails of different sizes in the tank and add a few leaves of lettuce and/or spinach.

4 Cover the tank with a weighted mesh lid and place the aquarium under neon lamps.

5 Set and maintain the room temperature (RT) at 26–28 °C.

6 Maintain a 12-h day and night light cycle by, for example, automatically setting lights to turn on at 6 AM and turn off at 6 PM.

7 Feed the snails once daily with fresh lettuce and spinach, and clean the tanks weekly.
▲ CRITICAL STEP The fresh vegetables should not remain longer than 1 d in the tank, as they will start to rot. Feed only as much as the snails consume. Store the food in a wine cooler refrigerator, as this works best for lettuce and spinach.

8 At the time of snail infection, select snails that have reached the adult stage (size ≥4–6 mm) and transfer these to a new tank, filled with 26 –28 °C pond water. Those snails will be infected with *S. mansoni* miracidia (Steps 11–28). This new tank is referred to as the 'infection tank'. Take the remaining snails and place them into a second new tank filled with 26–28 °C pond water. This tank, known as the 'breeding tank', will be used to produce offspring (Steps 1–10).
! CAUTION This step should be performed once for each batch of snails. Therefore, in the breeding tank(s), snails of different sizes will be present. Each tank should have no more than 200–300 snails.

9 Put an 8- to 9-cm^2 flat piece of polystyrene into each breeding tank(s) with the smooth side upside down, so the snails can lay their eggs on the surface. Move the polystyrene pieces with the eggs to a new tank filled with pond water, for breeding the snails.
! CAUTION Snails might lay eggs on the walls of the tank. Collect these eggs and add them to the breeding tank(s). Alternatively, siliconized surface materials can be used to prevent the eggs from attaching.
? TROUBLESHOOTING

10 Set up the new tanks as described in Steps 1 and 2, and maintain them as described in Steps 4–7.
! CAUTION Infecting a new batch of snails every 2–3 weeks is recommended for continuous availability of cercariae.
? TROUBLESHOOTING

Hatching miracidia and infecting snails ● Timing 30 min to collect eggs plus overnight release of eggs; 2.5 h to hatch and infect; maintain tank for 5–6 weeks

! CAUTION National and institutional guidelines on animal experimentation must be followed. Refer to the 'Experimental design' section. For all our experiments involving laboratory animals, we were granted ethical and experimental approval from both the Canton Basel-Stadt and Swiss federal veterinary authorities (license no. 2070). A certification in animal experimentation was required for all the operators involved.

11 Euthanize two infected hamsters by CO_2 inhalation (see the 'Materials' section, or infected mice can be used, from Step 38).
! CAUTION The number of hamsters for *S. mansoni* egg isolation depends on the investigator's needs. The hamsters can be kept between 2 and 12 months post infection at maximum.

12 After checking for the absence of vital signs and reflexes, dissect each hamster upward from the lower abdomen to expose the liver (Fig. 5a).

13 Excise the liver and place it into cold (4 °C) physiological saline overnight (see 'Materials' for saline preperation). This will create small tissue cracks in the liver, which will increase the release of *S. mansoni* eggs.

14 The next day, blend the cracked livers with cold (4 °C) saline solution, using a variable-speed blender progressively from 0 to ~9,000*g* for 10 s. Repeat this step three times.
▲ CRITICAL STEP The blending must ensure that the liver is very well homogenized, so that the maximum number of *S. mansoni* eggs are released. However, it must not be too rough, or the eggs will be destroyed in the blending process.
? TROUBLESHOOTING

15 Pour the liver homogenate sequentially through four sieves (Fig. 2a) in the following order of pore size: 425, 180, 106 and 45 µm. Rinse all the sieves with the cold (4 °C) saline solution using the pump sprayer, until the smallest filter is reached.
▲ CRITICAL STEP If the saline solution becomes warm (25–28 °C), the miracidia could hatch; therefore, it is important to keep the solution cold (at 4 °C) during the filtering process.
? TROUBLESHOOTING

16 Collect the unfiltered fractions from the 425- and 180-µm sieves into a 1,000-mL beaker by rinsing them with the 4 °C saline solution using the pump sprayer; then blend and sieve the unfiltered fractions by repeating Steps 14 and 15 three times.

17 The eggs will have been collected on the 45-µm sieve, as they do not pass through this sieve. Rinse the eggs from the 45-µm sieves into a 1,000-mL beaker by using 50–100 mL of cold (4 °C) saline solution, using the pump sprayer.

18 For hatching, pour the egg suspension into the side-arm flask (Fig. 2b). Add dechlorinated tap water (28 °C) until it reaches and fills the side arm of the flask.

19 Place the side-arm flask into a 30 °C water bath and place an incandescent lamp above. Wait until you see by eye the first hatched miracidia (10–20 min).
? TROUBLESHOOTING

20 Cover the entire side-arm flask with a carton box (or aluminum foil), except for the side arm.

21 Expose the side arm of the flask to the light. The miracidia will concentrate in the side arm, attracted by the light, and will swim up to the surface.

22 Collect the entire volume of miracidial suspension from the side arm with a P1000 pipette and place it in a 30-mL beaker. Check for the presence of miracidia under a bright-field microscope.

23 Determine the concentration of miracidia by sampling 5 × 10-µL drops of the miracidial suspension, placing them in a Petri dish and counting the number of miracidia in each drop.
▲ CRITICAL STEP Miracidia tend to sediment, so shake the solution before each sampling to ensure a homogeneous distribution.

24 Fill each well of a 48-well plate halfway with pond water.

25 Transfer each snail from the infection tank (saved for infection in Step 8) and place one snail per well of the plate prepared in Step 24.
! CAUTION Use flat-tip tweezers carefully to avoid damaging the snails.
▲ CRITICAL STEP Selecting snails that are smaller or larger in diameter than 4–6 mm will result in an unsuccessful or mild infection, and, on average, early mortality of small snails.

26 Add a volume of the miracidial suspension that would correspond to six to eight miracidia per well.

27 Place the 48-well plate under a 40-W incandescent bulb and leave it for 2–3 h at RT. In our experience, this ensures a proper infection of the snails by the miracidia.

28 Place the infected snails into a new tank, referred as the 'working tank', set up as described in Steps 1 and 2, and maintain as described in Steps 4–7.

Shedding and collecting of the cercariae ● Timing 30–60 min

▲ CRITICAL A record of each infection should be kept, with infection rate (number of snails infected per batch) and the intensity of infection (average number of cercariae shedding per snail).

29 After 5–6 weeks post infection, the snails from the working tank (Step 28) should be ready to shed cercariae. Fill each well of a 24-well plate with 1 mL of pond water.

! CAUTION Cercariae can infect humans via direct skin contact. Wear protective clothing that includes a lab coat, nitrile gloves and laboratory goggles. Note: simple hospital latex gloves are potentially insufficient for protection.

30 Place one snail into each well (carefully using flat-tip tweezers). Put the lid on the plate and leave it under a neon lamp for 3–4 h (Fig. 3 (i)).

! CAUTION As the procedure is a source of stress for the snails, we recommend that it not be repeated more than twice a week. Alternatively, if several snail batches are available, we recommend using them in rotation.

▲ CRITICAL STEP Cercarial shedding from the snail follows a circadian rhythm, so it is essential that the snails be placed under light in the morning and retrieved 3–4 h later in the afternoon. In our laboratory, the snails are placed under the light before 9 AM, but each lab should track the cercarial shedding pattern once the cycle is established.

31 Retrieve the plate and examine for cercariae, using a bright-field microscope with a ×10 or ×20 magnification.

? TROUBLESHOOTING

32 Place all the infected snails back into the working tank (Step 28) until the next cercarial shedding.

! CAUTION Steps 29–34 can be repeated six to eight times with the same snails. Snails that are not shedding cercariae after 8 weeks should be killed by placing them into soapy water. Do not use a batch of infected snails more than twice a week (better only once). The snails should have time to recover; otherwise the parasite yield could decrease.

▲ CRITICAL STEP Only uninfected snails lay eggs, but do not keep the snails that are not shedding cercariae for breeding, as this would decrease the overall genetic susceptibility of the breeding pool to *S. mansoni* infection.

33 Use a Pasteur pipette to collect the cercariae from the 24-well plate into a 150-mL conical-bottom beaker at RT before pouring the cercarial suspension through a 100-µm filter into 50-mL tube(s). At this point, the cercarial solution is either immediately adjusted for animal infection and injected (Steps 35–38) or is transformed into NTS (Steps 39–50).

34 Count the cercarial concentration by sampling 5 × 10 µL drops of the suspension on a Petri dish. Use a bright-field microscope to count and average the number of cercariae for 10 µL.

Infecting rodents ● Timing 30 min–2 h, depending on the number of rodents

! CAUTION National and institutional guidelines on animal experimentation must be followed. Refer to the 'Experimental design' section. For all our experiments involving laboratory animals, we were granted ethical and experimental approval from both the Canton Basel-Stadt and Swiss federal veterinary authorities (license no. 2070). A certification in animal experimentation was required for all the operators involved.

35 Adjust the cercarial concentration to 100 cercariae/100 µL by either adding the appropriate volume of pond water to dilute the suspension from Step 33, or by centrifuging it (211*g*, 3 min, RT) and removing the appropriate volume of supernatant to concentrate the suspension.

36 Aspirate 100 µL of the cercarial suspension with the 1-mL syringe, ensuring there are no air bubbles in the aspirate. Make sure to stir the suspension before aspirating.

37 Inject the suspension subcutaneously into the mouse/hamster neck. The number of mice/hamsters to infect depends on the cercarial yield and/or the investigator's needs.

! CAUTION In the case that a higher number of *S. mansoni* adult worms is required, we recommend infecting some of the mice with 200 cercariae instead of 100, or infecting hamsters with a dose of up to 800 cercariae. Although this will increase the yield of adult worms, we do not recommend using the highly infected mice for drug testing, as they will probably not be able to withstand the testing period.

38 After infection, house the animals at 25 °C with a 12-h day/night cycle with free access to rodent diet and water in enriched cages. At 7 weeks post infection, the adult parasite infection is established and the egg production is initiated. For perpetuating the life cycle, return to Step 11. For adult worm collection, refer to Steps 67–71.
? TROUBLESHOOTING

Transformation of cercariae to NTS ● Timing 3–4 h

39 Place the suspension from Step 33 on ice for 30 min to immobilize the cercariae (Fig. 3 (**iv**)).
40 Centrifuge the tube for 3 min at 211*g* at RT.
41 Use a Pasteur pipette to remove the supernatant and discard it.
42 Resuspend the pellet in 7 mL of ice-cold HBSS supplemented with 1% (vol/vol) pen–strep. Alternatively, supplemented M199 medium at 37 °C can be used.
43 Fill a 10-mL syringe with the 7-mL cercarial suspension obtained in Step 42 and connect it to the Luer-Lok (Fig. 4a,b (inset). Connect another empty syringe to the opposite side of the Luer-Lok. Push the liquid back and forth three to four times vigorously (Fig. 4b). The transformation yield can immediately be viewed under the bright-field microscope. The tails must have been separated from the heads for at least 90% of the parasites (Fig. 4c). If this is not the case, repeat this step three to four times more and check for any improvement under the bright-field microscope.
! CAUTION Test the syringe-Luer-Lok connection by passing some water or HBSS before you proceed. An incorrect connection can result in a splattering of infectious cercarial suspension. Personal protection, including a face shield, must be worn in case of spills. Wearing two pairs of gloves, with a long-sleeved pair over the top is advisable.
44 Pour the suspension into 15-mL tubes. Place them on ice and in the dark for 7 min to allow the sedimentation of the heads.
45 Remove the supernatant (and therefore the tails), by slowly pipetting, and discard. Avoid disturbing the sedimented heads (hereafter referred to as NTS).
? TROUBLESHOOTING
46 Resuspend the NTS in 4 °C HBSS supplemented with 1% (vol/vol) pen–strep to wash them.
47 Repeat Steps 44–46 three times to eliminate the remaining tails. Observe the successful separation of the tails from the heads under a bright-field microscope (Fig. 4c).
▲ CRITICAL STEP It is imperative to reduce the number of tails and untransformed cercariae as much as possible, as they can harm the NTS with their rapid whipping movements.
48 Remove the wash solution by pipetting and resuspend the sediment in 5–10 mL of supplemented M199 by inverting the tube or gently pipetting up and down.
! CAUTION Do not vortex, as additional mechanical stress could damage the parasites and negatively influence their overall viability.
49 Count the NTS by sampling 5 × 10-µL drops from the NTS suspension. Pipette each drop into a Petri dish (Fig. 4d) and observe under a bright-field microscope.
▲ CRITICAL STEP To obtain a homogeneous NTS suspension, mix it gently by inverting the tube before each sampling.
50 Adjust the suspension to the appropriate NTS concentration. To increase the concentration of NTS, place the tubes in a vertical position for 5 min to allow them to sediment. The proper volume of medium can then be removed by gently pipetting away the supernatant. For decreasing the concentration, simply top up with an appropriate volume of supplemented M199 medium.
▲ CRITICAL STEP We recommend that the NTS be suspended in an appropriate volume of medium for a maximum concentration of 2,000 NTS/mL.
■ PAUSE POINT Before being used in drug assays, the NTS should be left overnight in the incubator (37 °C, 5% CO_2) for 12 to a maximum of 24 h. This allows the parasites to complete the necessary biochemical and metabolic changes to emulate the larval stage of the parasite in the definitive host[46].

Drug-screening assay with NTS ● Timing 2–3 h

▲ CRITICAL Because not 100% of the cercariae will be transformed, it is important to wear laboratory goggles and use proper biosafety level 2 (BSL 2) equipment.
51 Dilute the initial 10 mM drug stock solution (in DMSO) to a 100 µM solution in supplemented M199 medium.

52 Add 175 µL of supplemented M199 medium per well of a 96-well plate.

53 Add 25 µL of the 100 µM drug solution to each well, then 50 µL of the 2,000 NTS/mL suspension (from Step 50) for a final drug concentration of 10 µM and a final count of ~100 NTS per well. Each drug should be tested in duplicates or triplicates.

54 Repeat Steps 52 and 53 for the control wells, using the solvent (DMSO) instead of the drug. One can also include a positive control such as auranofin or mefloquine, but this is not required for assays based on visual scoring as assessment.

! CAUTION Praziquantel is not a good positive control for NTS in vitro assays because it is not very active on the larval stages of the parasite.

▲ CRITICAL STEP If the drugs are dissolved in DMSO, the final assay concentrations of DMSO should never exceed >1.5% (vol/vol), as this can decrease the parasites' viability.

55 Place the plate in the incubator (37 °C, 5% CO_2). Score the NTS viability every 24 h for up to 72 h to evaluate the drug effect compared to the controls. The evaluation procedure is described below (Steps 56–66).

? TROUBLESHOOTING

Determination of compound IC_{50} values ● Timing 2–3 h

! CAUTION The desired drug concentration range varies from assay to assay. Here, we provide a sample protocol that is used in our lab as a guiding example. The procedure below is for a starting concentration of 10 µM and a dilution factor of 1:2.

56 To determine the IC_{50} values of any drug, first prepare in a 96-well plate a drug dilution series that is ten times higher than the desired final assay concentration. Thereafter, the whole assay is diluted 10× in another 96-well plate, where the NTS are added. To prepare this pre-dilution plate, fill row A of a 96-well plate with 180 µL of supplemented M199 medium and add 20 µL of a 1 mM drug working solution to reach a concentration of 100 µM (one drug per well).

57 To rows B to G, add 100 µL of supplemented M199 medium. Pipette 100 µL from row A to row B for a 1:2 dilution and mix well. Continue by pipetting from row B to row C, and so on. Reject the last 100 µL from row G. This results in concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM.

58 In row H, prepare DMSO controls by adding 20 µL of 10% (vol/vol) DMSO in 180 µL of supplemented M199 medium for a final concentration of 1% (vol/vol) DMSO in the well.

59 In another 96-well plate (test plate), add 175 µL of supplemented M199 medium to all wells. Assign three columns of the plate to each drug dilution (in order to have three replicates for each drug).

60 Transfer 25 µL of column 1 from the pre-dilution plate to columns 1, 2 and 3 of the new plate. Repeat for each drug (e.g., column 2 of the pre-dilution plate to columns 4, 5 and 6 of the new plate, and so on).

61 Add 50 µL of NTS suspension (Step 50) to each well of the new plate. The result is a drug dilution assay with concentrations of 10, 5, 2.5, 1.25, 0.63, 0.31 and 0.16 µM and control wells with 0.1% (vol/vol) DMSO.

62 Incubate at 37 °C and 5% CO_2 and evaluate at 24, 48 and 72 h post drug incubation (Steps 63–66).

? TROUBLESHOOTING

Assessment of the parasites in vitro ● Timing 2 h

63 Evaluate each well, using a bright-field microscope (×4 or ×10 magnification), and assign a score to each well that reflects the phenotype of the majority of the parasites. Score the control wells first. In Figs. 6 and 7, we present the viability score scale and sample phenotypes for NTS and adult worms, respectively. The motility is the most important parameter to take into account when assigning a score. Living parasites move smoothly. An abnormally decreased or enhanced motility, a loss of plasticity, saccades or wobbles would lead to a lower score. The second important aspect to consider is the morphology and the integrity of the tegument. Impaired parasites may lose their original shape by shrinkage or swelling. Blebs on the tegument or a darkened pigmentation can also appear (Figs. 6 and 7, and Supplementary Videos 1–12).

! CAUTION Do not keep the plates outside the incubator for longer than 15 min. The NTS movement decreases when the medium cools; this might bias the scores.

▲ CRITICAL STEP Control wells should have a minimum score of 2 to ensure that the NTS were sufficiently viable for the assay. If not, the assay should be rejected and repeated.

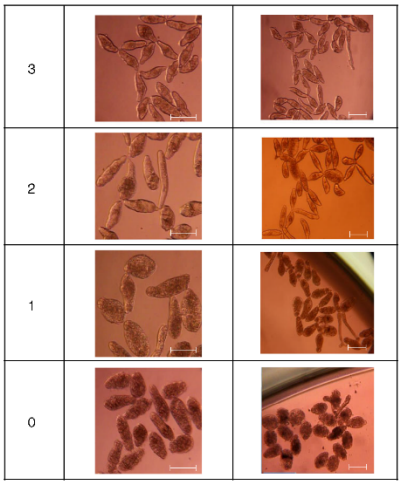

Fig. 6 | NTS phenotypes corresponding to each viability score. 3 = motile, no changes to morphology or transparency; 2 = reduced motility and/or some damage to tegument noted, as well as reduced transparency and granularity; 1 = severe reduction of motility and/or damage to tegument observed, with high opacity and high granularity; 0 = dead. The images of the NTS in the left column were taken with a ×20 magnification. The images in the right column were obtained using a ×10 magnification (Step 63). Scale bars, ~100 µm. See also Supplementary Videos 9–12.

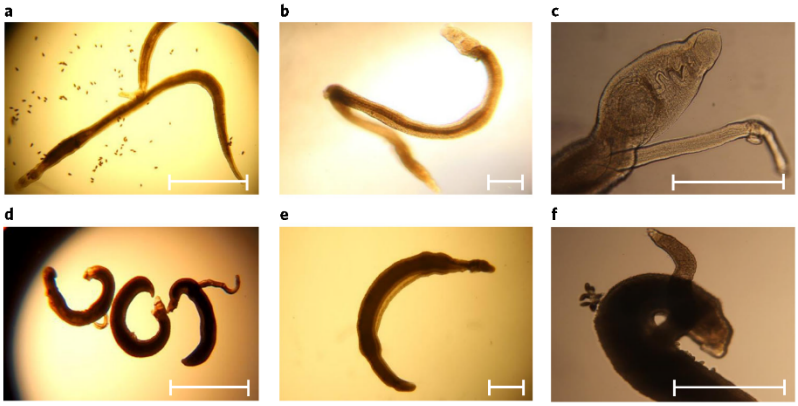

Fig. 7 | Adult worm phenotypes. a–c, The viability scores of 3 = motile, no changes to morphology, transparency and intact tegument, active ventral and oral sucker (see also Supplementary Videos 7 and 8) and (**d–f**) 0 = dead, the worms appear darkened and motility of the ventral and oral sucker is absent (see also Supplementary Videos 1 and 2). Additional characteristics can also help to assign a score (see also Supplementary Videos 3–6 for examples of scores 1 and 2). For instance, the ventral sucker of impaired worms (score <2) is often detached from the bottom of the well, whereas the presence of eggs can be used as a sign of good viability of the worm (score >2). The images were taken with ×4 (**a**,**d**), ×10 (**b**,**e**) or ×20 (**c**,**f**) lenses (Step 63). Scale bars, 1 mm (**b**,**c**,**e**,**f**); 2.5 mm (**a**,**d**).

64 Report all the data in a spreadsheet and calculate the effect score of each drug by normalizing the drug scores to the control well scores, using the following formula: average (Test) is the average score of all test wells containing the drug and Average (Control) is the average score of the control wells.

$$\%\text{Effect} = 100 - \frac{\text{Average(Test)} \times 100}{\text{Average(Control)}}.$$

65 With a single concentration assay (Steps 51–55), determine the threshold effect for what you will consider to be a hit. Usually, this threshold reflects either total mortality of the parasite (100% effect) or substantially reduced parasite viability (e.g., 75% effect).

66 For dose–response assays (Steps 56–62), plot the effect scores against the drug concentrations to generate a dose–response curve in order to determine the IC_{50} value. There are many tools for this; an easy and freely available tool is CompuSyn (http://www.combosyn.com/).

S. mansoni adult worm recovery ● Timing 15–30 min per mouse

! CAUTION National and institutional guidelines on animal experimentation must be followed. Refer to the 'Experimental design' section. For all our experiments involving laboratory animals, we were granted ethical and experimental approval from both the Canton Basel-Stadt and Swiss federal veterinary authorities (license no. 2070). A certification in animal experimentation was required for all the operators involved.

67 Euthanize the infected mice from Steps 34 to 38 with CO_2 for 5 min (see 'Materials' section).

68 After checking for the absence of vital signs and reflexes, dissect the mice by cutting from the lower abdomen up to the sternum to expose the intestines and liver (Fig. 5a).

69 Resect the liver, then excise the intestines and place them into a Petri dish. At this point, the liver can also be removed. Manually, press the liver between two pieces of transparent plastic foil in order to obtain a thin layer of tissue so that the worms can be visualized, counted and sexed under a dissecting microscope. A pair is counted as two worms. Draw a grid on the plastic foil with a marker to facilitate the localization of the worms (Fig. 5b). The live worms can be collected with flat-tip tweezers and placed in 37 °C supplemented RPMI 1640 medium. For in vitro assays, refer to Steps 72–78.

▲ CRITICAL STEP The portal vein must be located and pinched firmly with tweezers in order to prevent the worms from spilling out, because *S. mansoni* are mostly located in the portal vein (Fig. 5a).

? TROUBLESHOOTING

70 Place the intestines under the dissection microscope and, using flat-tip tweezers, gently open the veins and pick out the worms (Fig. 5c,d). Place the recovered worms in supplemented RPMI 1640 medium. Count and sex the worms as in Step 69. Transfer them to a new Petri dish filled with supplemented RPMI 1640 medium and incubate them at 37 °C, 5% CO_2.

▲ CRITICAL STEP It is important to avoid damaging the worms with the flat-tip tweezers during collection. Do not tug the worms.

■ PAUSE POINT Worms can be kept in an incubator for up to 10 d if the medium is replaced regularly (every 3 d). To allow sufficient nutrient supply, a maximum of 15 worms should be placed in a single Petri dish.

? TROUBLESHOOTING

71 Use the worms recovered from Steps 69 to 70 for in vitro assays (Steps 72–78). The worm counts can be used to determine the WBR (Steps 83–86).

In vitro assay of *S. mansoni* adult worms ● Timing 40–60 min

72 From a 10 mM drug stock solution in 100% (vol/vol) DMSO, prepare a 1 mM working solution in supplemented RPMI 1640 medium. We recommend the use of a 10 µM drug concentration as a starting concentration (unless natural crude extracts are being screened). Higher concentrations are unlikely to be achieved in vivo.

73 In each well of a 24-well plate, pipette 15 µL of the 1 mM drug solution, and add 1,485 µL of supplemented RPMI 1640 medium. Each compound should be tested in duplicate or triplicate when possible.

▲ CRITICAL STEP To ensure that the worms remain clearly viable throughout the assay, the volume to add to the well should not be <1.5 mL.

74 Prepare a 10% (vol/vol) DMSO (or any other solvent) solution in supplemented RPMI 1640 medium and transfer 15 µL of it to the control wells to have a final concentration of 0.1% (vol/vol) of the solvent. Praziquantel can be used for positive control wells in this assay; follow Steps 72 and 73.

75 Carefully, place two pairs or three random worms of both sexes per well, using flat-tip tweezers.
▲ CRITICAL STEP To avoid contaminations, clean the flat-tip tweezers by dipping them first into a solution of 70% (vol/vol) ethanol–water and then into supplemented RPMI 1640 as frequently as possible.

76 Check the assay to ensure that each well has the correct number of worms. Place the assay in the incubator (37 °C, 5% CO_2).

77 Score each of the worms individually. Record both the sex and the phenotypic score every 24 h for up to 72 h (Steps 63–66 and Fig. 7).

78 Compounds that are active can then be further tested in a drug dose–response assay to determine their IC_{50} value. The same assay setup is used (Steps 72–78) with a 1:2 or 1:3 serial drug dilution, and at least three drug concentrations.

In vivo drug administration ● Timing >4 h

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79 Prepare the drug solution for oral administration in Tween 80/EtOH solution (or any other vehicle). Intraperitoneal or intravenous administration can also be considered. The drug dosage depends on the researcher's needs, and it should be adapted accordingly.
▲ CRITICAL STEP Make sure that the drug is well dissolved; otherwise, you risk administering different concentrations to each mouse.
? TROUBLESHOOTING

80 Weigh each infected mouse from Step 38 individually.
! CAUTION The mice must be individually identifiable and marked before drug administration using any standard identification (ID) method. We recommend noninvasive and painless methods such as skin or fur marking in order to comply the 3Rs principles[58]. We use picric acid solution (a yellow synthetic marker) on the mouse's back for labeling.

81 Calculate the drug volume to administer to each mouse using the following formula:

$$\text{Volume administration} = (\text{target dose (mg/kg)} \times \text{mouse mass (kg)})/\text{drug concentration (mg/mL)}.$$

82 Prepare the administration volume in a 1-mL syringe. Administer the drug to the infected mouse. The control mice for the experiment can be administered with the vehicle only or left untreated.
▲ CRITICAL STEP Do not administer >100 µL for every 10 g of weight, keeping in mind that it is better to work with small volumes. It is important to administer each drug to several mice. The number of mice used depends on the experimental design[59].
? TROUBLESHOOTING

Data recovery for in vivo results ● Timing >2–3 h

83 After a maximum of 21 d after drug administration, euthanize the mice as in Steps 67–71.

84 Dissect the mice and recover the liver and the intestines as described previously (Steps 67–70). The granuloma distribution and the hepatosplenomegaly provide important information on the magnitude of the infection of each mouse.

85 Evaluate the presence of live adult worms in the pressed liver and in the mesenteric veins of test and control mice (Steps 69–70). Sex, count and include them in the WBR evaluation formula (Step 86).

86 Calculate the WBR using the following equation:

$$\text{WBR}(\%) = 100\% - (100\%/\text{WB Control} \times \text{WB treatments}),$$

which compares the average number of live worms in the treated mice to the live worms found in the mouse control group.
? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
9	No/poor snail egg deposition	• Poor water quality • Poor water tank airflow • Poor-quality snail food • Stagnant water	• Use filtered water • Check air flow • Use fresh leaves (we suggest spinach) • Check water quality
1–10	Snails die	• Poor water quality • Not enough food • Too many snails in the same tank	• Use filtered water • Avoid allowing food to rot in the tanks and change it daily • Subdivide the snail population into separate tanks, keeping the population between 200 and 300 snails
14, 15	Damaged *S. mansoni* eggs	• Too-aggressive blending of the liver(s)	• Reduce the time and/or speed of each blending step
19	No miracidia hatching	• Too-aggressive blending of the liver(s) • Water temperature not correct	• Reduce the time and/or speed of each blending step • Ensure water temperature is 30 °C • Increase the exposure time by 10–15 min
31	No/poor cercarial shedding	• Snails are not permissive • Snails are too old • Circadian cycle is not adequate • Too many cercariae collections	• Make a freshly infected batch of snails • After 6–8 weeks, infected snails should be replaced • Consider starting the light cercarial induction earlier in the morning • Increase the collection time span to ensure one cercarial shedding per week
38	Mice die after infection with *S. mansoni*	• Too many cercariae were injected	• Adjust the cercariae concentration to 100 cercariae/100 µL and inject smaller volumes
45	The NTS pellet is taken away or disturbed	• Pipetting error	• Repeat the washing step (Steps 46 and 47)
55	Dead NTS in the assay control wells	• Overly aggressive transformation • Too many NTS in medium kept overnight • Incorrect medium used	• Reduce the number of syringe passages (three or four instead of four to six) • Control the medium formulation and concentration
62	No changes in NTS viability after testing a known active drug (positive control)	• Suboptimal concentration used, low solubility of the compound and/or low activity on NTS (e.g., praziquantel)	• Increase the drug concentration and/or improve solubility (e.g., by changing the solvent)
69, 70	Dead adult worms recovered from untreated mice	• Worms were recovered too late • Worms were damaged during collection	• Collect the worms at an earlier time point • Avoid pressure with tweezers during worm collection
79, 86	Erratic worm burden in vivo	• Multiple operators perform the mouse infection • Biological variation	• Consider using a single operator for infections • Include a randomization step
82	Mice die a few days after drug treatment	• Too many cercariae • The drug dose was too high • The compound is toxic	• Adjust cercariae concentration for mouse infection • Lower the administered dose

Timing

Steps 1–10, establishing and maintaining a snail cycle—initial setup: 30 min
Steps 11–28, hatching miracidia and infecting snails: 30 min to collect eggs plus overnight release of eggs; 2.5 h to hatch and infect; maintain tank for 5–6 weeks
Steps 29–34, shedding and collection of cercariae: 30–60 min
Steps 35–38, infecting rodents: 30 min–2 h, depending on the number of rodents
Steps 39–50, transformation of cercariae to NTS: 3–4 h
Steps 51–55, drug-screening assay with NTS: 2–3 h
Steps 56–62, determination of IC_{50}: 2–3 h

Steps 63–66, assessment of parasites in vitro: 2 h
Steps 67–71, *S. mansoni* adult worm recovery: 15–30 min per mouse
Steps 72–78, in vitro assay of *S. mansoni* adult worms: 40–60 min
Steps 79–82, in vivo drug administration: >4 h
Steps 83–86, data recovery for in vivo results: >2–3 h

Anticipated results

This protocol describes the establishment of an *S. mansoni* life cycle, which is the basis for conducting in vitro and in vivo drug sensitivity assays. Once a schistosome life cycle has been established in the laboratory, cercariae should be shed by the snails into the water on a regular basis for 5–8 weeks. There is, however, no typical volume of cercarial yield, as this depends on the number of snails, their infection level and the age of the snail infection. In our experience, a cercarial suspension with a minimum range of 250–1,000 cercariae per milliliter is required for performing an effective transformation. Practically, a satisfactory cercarial yield can be visualized under a bright-field microscope, directly in the wells containing the shedding snails, before starting the transformation. Although some cercariae can be very motile, not all are active. The transformation should therefore be attempted even in the absence of fully active cercariae.

The described phenotypic drug assays can be used for testing compounds and libraries in a low-throughput manner, as described in previous studies[7,9,10,25]. These assays allow determination of dose–response relationship or IC_{50} values essential to identifying hits and selecting early lead drug candidates to be tested in vivo. The worm burden obtained from infected mice or hamsters is variable, but in our experience, 10–50 adult worms can be recovered 7 weeks post infection with 100 cercariae (as described in Steps 76 and 77). Some examples of in vivo results for antischistosomal drug discovery can be found in our previous studies[7,9,10,25]. Although anemia and loss of weight are often observed in infected mice, they can survive for a maximum of 5 weeks post treatment without presenting major symptoms or behavioral changes. As a consequence of egg deposition, infected animals usually present an enlarged granulomatous liver and spleen (hepatosplenomegaly, Fig. 5a).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary.

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Author contributions

J.K., F.C.L., V.P. and G.P. developed the concept of the protocol. F.C.L., V.P., G.P. and Y.E. drafted the first version of this manuscript, and J.K. revised the manuscript. Videos and images were taken by F.C.L. and V.P.

Competing interests

The authors declare no competing interests.

Additional information

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Key references using this protocol

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Chapter 3: Pathogen Box

Chapter 3a

Early antischistosomal leads identified from *in vitro* and *in vivo* screening of the Medicines for Malaria Venture Pathogen Box

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Early Antischistosomal Leads Identified from *in Vitro* and *in Vivo* Screening of the Medicines for Malaria Venture Pathogen Box

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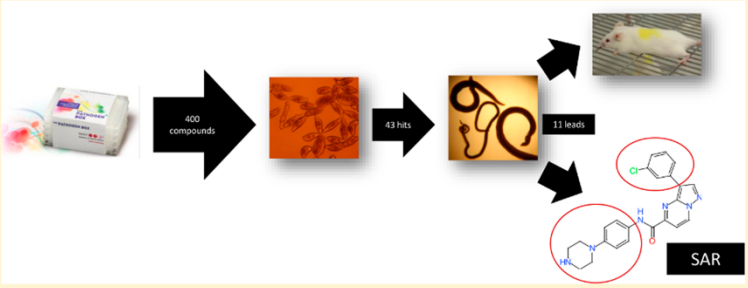
Supporting Information



ABSTRACT: As part of the control and elimination strategy of human schistosomiasis, preventive chemotherapy relies on a single drug, praziquantel. Facing an almost dry drug development pipeline, screening the Pathogen Box from the Medicines for Malaria Venture (MMV), provides a unique opportunity to possibly expand the pool of potent molecules against schistosomiasis. The activity of 400 compounds from this open-access library was first screened *in vitro* on the larval stage of *Schistosoma mansoni*. The hits were then tested on adult worms. Eleven leads were identified and tested for albumin-binding and activity on adult *S. haematobium*. In parallel, a rudimental structure−activity relationship analysis was performed on the 112 available analogues of three leads, yielding another 30 molecules active against both larval and adult stages of *S. mansoni*. Seven leads, selected on druglikeness, pharmacokinetic properties, and availability, plus auranofin were tested in mice harboring a chronic *S. mansoni* infection. MMV022029 and MMV022478 revealed the highest worm burden reductions of 67.8 and 70.7%, respectively. This study provided a series of new potent scaffolds and pharmacophores that could be used to design and develop suitable alternative(s) to praziquantel.

KEYWORDS: *Pathogen Box, schistosomiasis, Schistosoma mansoni, Schistosoma haematobium, drug discovery, anthelminthics*

Human schistosomiasis is a highly prevalent tropical disease associated with various debilitating hepatic, intestinal, and urogenital morbidities. It is caused by three main species of blood flukes: *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*.[1,2] Despite a strong mobilization of public and private stakeholders to eliminate this disease as a public health problem during the past decade, schistosomiasis is still affecting millions of people across the globe.[2−4] Four main interventions are available to control schistosomiasis, namely snail control, behavioral change, WASH programs, and mass drug administration with praziquantel ("preventive chemotherapy"), with the latter the most commonly used.[5−7] Therefore, the emergence of drug resistance toward praziquantel, the only drug currently available, would be a significant drawback in the control of schistosomiasis.[8−10]

The discovery of new anthelminthic drugs is scarce and underfunded, notably because helminthiases lack commercial interest by pharmaceutical companies as the drugs are mainly donated and because of "magic bullets" including praziquantel for schistosomiasis or ivermectin for onchocerciasis.[11,12] Indeed, during the last four decades, no new drug against schistosomiasis progressed into advanced stages of clinical development. Hence, there is a strong need to discover new antischistosomal lead candidates.[6,13]

With the goal to catalyze drug discovery against diseases of poverty, the public−private partnership Medicines for Malaria Ventures (MMV) assembled three available libraries of chemical compounds in the past 6 years. The Malaria Box, launched in 2012, led to the identification of lead compounds on a variety of pathogenic agents.[14] We recently screened the Stasis Box (2016), a library including advanced drugs to repurpose, on *S. mansoni*, yielding eleven lead molecules with

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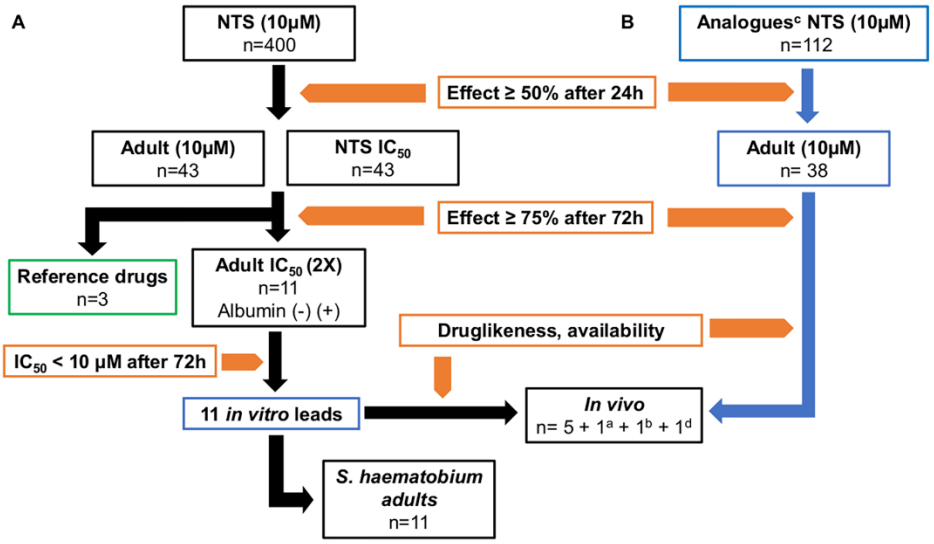

Figure 1. Detailed workflow and hit to lead selection criteria used in the Pathogen Box screening against *S. mansoni* and *S. haematobium*. (A) The 400 compounds were initially screened on NTS at 10 μM. The compounds to proceed to further testing were selected according to the different criteria shown in the orange boxes. The compounds that showed an activity of at least 50% after 24 h on NTS were tested on adult worms at 10 μM. Their IC_{50} values on NTS were also determined. An activity of 75% after 72 h on adult worms was used as a cutoff, and 14 compounds, including 3 drugs with known antischistosomal activity (auranofin, clofazimine, and mefloquine), were identified as hits. The mean IC_{50} of the 11 compounds (excluding the 3 already-known drugs) was determined both in absence and presence of albumin. All of them had an IC_{50} below 10 μM, which was generally not substantially increased in the presence of albumin. These 11 compounds were considered as leads and selected for *in vivo* testing (n = 6) if available, if never investigated before as antischistosomal candidate, and if stable in plasma and/or presenting drug-like physicochemical properties[18] (cLogP < 5, molecular weight <500 g/mol). The 11 leads were also tested on *S. haematobium* adult worms. MMV1578296[a] was tested instead of MMV687812 that was unavailable in sufficient quantity. Auranofin was also tested *in vivo*[b]. (B) Analogues[c] (n = 112) of 3 leads tested in vivo (MMV022029, MMV022478, and MMV687812) were screened as in (A). The screening of the analogues identified MMV1578493[d] that was also tested *in vivo*.

high *in vitro* activity but only moderate *in vivo* activity.[15] Finally, MMV constituted recently the Pathogen Box (2016), including 400 drug-like molecules, which was tested by many researchers around the world.[16,17]

Here, we present the results of the Pathogen Box screening to identify novel drug candidates against schistosomiasis. The compounds were first tested *in vitro* on *S. mansoni* newly transformed schistosomula (NTS). The most active compounds were then studied *in vitro* on adult *S. mansoni* and *S. haematobium*. In addition, more than a hundred analogues of three lead candidates were investigated to explore the structure–activity relationship (SAR) on both parasite stages. Finally, selected compounds were studied in mice harboring a chronic *S. mansoni* infection (Figure 1).

RESULTS

***In Vitro* Studies against *S. mansoni*.** The initial screening of the Pathogen Box at 10 μM identified 43 compounds active on NTS after 24 h (effect ≥50%, IC_{50} range: 0.5–45 μM, Supplementary Table 1). Nineteen of them were lethal (effect = 100%). Considering a 72 h incubation time, the number of active compounds increased to 114, with 63 drugs displaying an effect over 75%.

All 43 fast-acting compounds (effect ≥50% after 24 h) were then tested at 10 μM on adult worms. After 3 days of incubation, 14 compounds showed an activity over 75%, with half of them being lethal (Table 1). These hits included auranofin, clofazimine, and mefloquine. Ten out of the eleven remaining NTS hits had IC_{50} values below 3 μM (Table 2) 3 days postincubation.

Table 1. Number of Hits Found after Screening the Pathogen Box Compounds on *S. mansoni* at 10 μM[a]

	24 h	48 h	72 h
NTS[b] (active)	43	80	144
NTS[b] (high activity)	28	44	63
NTS[b] (lethal)	19	25	34
adult[c] (high activity)	9	12	14
adult[c] (lethal)	5	6	7

[a]The hits correspond to compounds that showed an activity (≥50%) against the parasites. The number of highly active (≥75%) or lethal (100%) compounds over 3 days postincubation are presented. [b]The total number of compounds tested was 400. [c]The total number of compounds tested on adult worms was 43.

The IC_{50} of these 11 hits (MMV687812, MMV022478, MMV022029, MMV272144, MMV687251, MMV687807, MMV688761, MMV68876, MMV688178, MMV687273, and MMV690102 referred as compounds **1**–**11** in this manuscript) were determined in both absence and presence of albumin. Already 1 h after incubation, 7 out of the 11 *in vitro* leads had IC_{50}values below 50 μM in the absence of albumin. After 16 h, all the compounds except compound **11** showed IC_{50}values

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Table 2. IC_{50} of the 11 Lead Compounds Identified after Screening the Pathogen Box *in Vitro* on *S. mansoni* NTS and Adult Worms and Effect at 10 μM on *S. haematobium* Adults[a]

		NTS IC_{50} [μM]			adults IC_{50} [μM]					adults + albumin IC_{50} [μM]			effect (%) 10 μM *S. haematobium* adults		
manuscript ID	MMV ID	24 h	48 h	72 h	1 h	16 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	MMV687812	1.9	1.2	0.8	26.7	3.8	3.2	3.2	3.0	12.9	4.8	6.4	64.5	84.6	100.0
2	MMV022478	1.0	1.1	0.6	42.3	7.0	3.6	2.1	2.0	7.2	5.8	5.9	61.4	93.0	100.0
3	MMV022029	4.2	3.5	2.6	>300	5.4	5.2	2.7	3.0	9.7	11.3	9.1	53.6	70.0	75.4
4	MMV272144	1.9	2.2	0.9	12.2	4.0	6.6	6.7	6.8	71.8	143.5	>300	78.2	89.1	87.7
5	MMV687251	0.9	0.8	0.8	40.4	4.3	3.4	5.3	4.7	36.5	37.9	143.5	89.5	82.5	75.4
6	MMV687807	0.3	0.3	0.2	9.7	2.6	2.2	2.3	2.1	5.7	5.5	5.6	100.0	100.0	100.0
7	MMV688761	0.6	0.5	0.4	NA	3.4	2.9	3.0	3.7	8.1	6.5	6.8	87.7	90.8	90.8
8	MMV688763	0.3	0.3	0.4	NA	5.6	2.4	2.8	2.5	13.0	10.1	5.6	95.9	100.0	100.0
9	MMV688178	3.0	3.7	2.2	38.5	8.9	4.7	2.6	3.4	38.3	49.5	43.6	57.9	54.4	75.4
10	MMV687273	18.6	11.3	2.9	53.5	5.7	4.0	5.7	4.9	15.7	5.3	10.1	50.9	57.9	82.5
11	MMV690102	14.6	29.6	16.7	45.1	22.3	22.6	11.8	7.9	NA	23.3	242.2	67.3	46.8	50.9

[a]NA: not applicable. The chemical structure of the lead compounds is displayed in Figure 2.

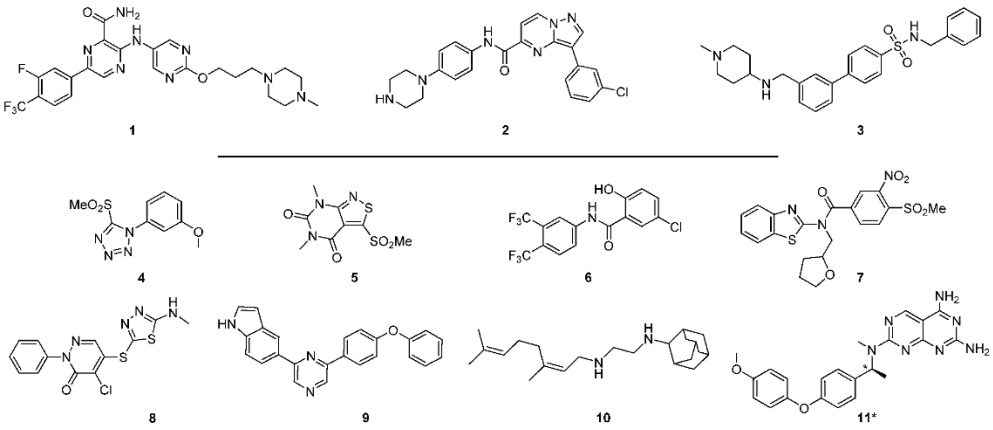

Figure 2. Chemical structure of the compounds that had analogues available for the SAR (upper part) and other *in vitro* lead compounds reported in Table 2 (bottom part) *Single enantiomer but absolute configuration not determined.

Table 3. *In Vitro* and *in Vivo* Activity of the Reference Compounds Included in the Pathogen Box

		in vitro activity (%)			*in vivo* activity	
MMV ID	drug name	24 h NTS | adult	48h NTS | adult	72 h NTS | adult	WBR[c] (%) (dose, route, bolus)	reference
MMV687800	clofazimine	100 | 75	100 | 86	100 | 86	82.7[a] (400 mg/kg, PO, single)	Panic et al., 2015[19]
MMV688978	auranofin	100 | 100	100 | 100	100 | 100	20.25 (100 mg/kg, PO, single)	
MMV000016	mefloquine	95 | 50	100 | 54	100 | 77	77.3%[a] (400 mg/kg, PO, single)	Keiser et al., 2009[20]
MMV002529	praziquantel	30[b]	35	56	99.3[a] (250 mg/kg, PO, single)	Kovač et al., 2017[21]

[a]These results were obtained in previous studies. [b]In this study, the activity of Praziquantel was tested on NTS only. [c]WBR: worm burden reduction.

below 10 μM. It reached this threshold between 48 and 72 h postincubation. For the other compounds, the IC_{50} values remained constant over the three-day examination period (Table 2). In presence of albumin at human plasma concentration, the IC_{50} values were increased more than 10-fold for compounds **4**, **5**, **9**, and **11**. The remaining *in vitro* leads showed moderately increased IC_{50} values in the presence of albumin (between 1.5- to 3-folds; Table 2).

***In Vitro* Studies against Adult *S. haematobium*.** The 11 *in vitro* leads (Figure 2) were all active against adult *S. haematobium* when tested at 10 μM. At the 24 h examination time point, compounds **4**, **5**, **7**, and **8** were highly active (effect ≥75%), and compound **6** was lethal (effect = 100%). After 72 h, with the exception of compound **11** that showed a moderate activity against the worms (51%), all the other compounds were highly active or lethal (n = 5) (Table 2).

Structure–Activity Relationship Studies with Three Lead Analogues. MMV made available different analogues of compounds **1**, **2**, and **3** (n = 112). We tested all these analogues *in vitro* as an opportunity approach to generate rudimentary SAR. They were first tested on NTS, and only the fast-acting ones proceeded to testing on adult worms (Figure 1

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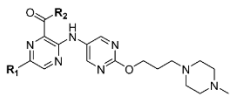
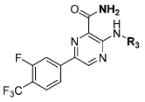
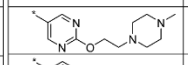
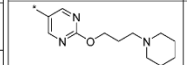
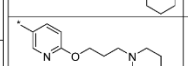
Table 4. SAR around Compound 1 (MMV687812)



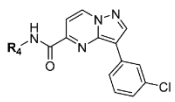


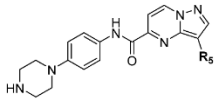
R_1	R_2	ID[a]	NTS effect[b] 24h	NTS effect[b] 72h	Adult effect[b] 24h	Adult effect[b] 72h	ALogP[c]
		1	1	1	0.70	1	3.03
		1a	0.46	0.94			1.87
		1b	0.46	0.60			1.88
		1c	0.95	1	0.93	0.89	4.53

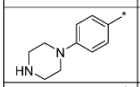
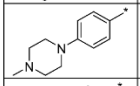
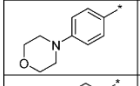
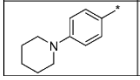
R_3	ID[a]	NTS effect[b] 24h	NTS effect[b] 72h	Adult effect[b] 24h	Adult effect[b] 72h	ALogP[c]
	1d	0.90	1	0.83	1	2.97
	1e	1	1	1	1	3.99
	1f	1	1	0.87	1	3.68

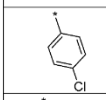
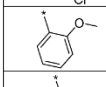
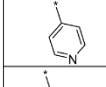
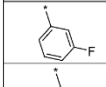
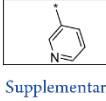
[a]The complete data for all the analogues of compound **1** are available in Supplementary Table 3. [b]The activity values (effect) are given as decimals and not percentage. [c]ALogP calculated using BioVia's ScienceCloud (https://www.sciencecloud.com/).

Table 5. SAR around Compound 2 (MMV022478)





R_4	ID[a]	NTS effect 24h	NTS effect 72h	Adult effect 24h	Adult effect 72h	ALogP[b]
	2	0.73	0.94	0.5	0.91	3.09
	2a	0.81	1	0.61	0.83	3.63
	2b	0.05	0.06			3.35
	2c	0.05	0.13			4.59

R_5	ID[a]	NTS effect 24h	NTS effect 72h	Adult effect 24h	Adult effect 72h	ALogP[b]
	2d	1	1	1	1	3.09
	2e	1	1	1	1	2.40
	2f	1	1	1	1	1.27
	2g	1	1	0.78	1	2.63
	2h	0.52	0.56	0.23	0.08	1.27

[a]The complete data for all the analogues of compound **2** are available in Supplementary Table 3. [b]ALogP calculated using BioVia's ScienceCloud (https://www.sciencecloud.com/).

and Supplementary Table 3). This was the case for 8 analogues of compound **1**, 9 of compound **2**, and 16 of compound **3**, respectively. After 3 days incubation on adult worms, all the analogues of **1** (n = 8, Supplementary Figure 1), 11 of **2** (Supplementary Figure 2), and **3** (Supplementary Figure 3) had an effect above the 75% cutoff.

Table 4 summarizes activity data on NTS and adult worms collected on **1** and structural analogues. The very preliminary SAR obtained from this limited set of compounds suggests that the lipophilicity might play a significant role with reduction of antischistosomal activity when decreasing ALogP (from 3.03 for compound **1** to 1.87 for **1a**). 3-Fluoro-4-(trifluoromethyl)-phenyl was identified as the best R_1 substituent among the ones tested. Replacing the carboxamide at position R_2 by an acid moiety was not tolerated. A methyl ester like in **1c**, **1g**, or **1h** (Supplementary Table 3) showed interesting activity only for the best R_1 substituents. In position R_3, both aminopyridine and aminopyrimidine functionalities appear to be tolerated with ethoxy or propoxylinker in position 2 of the pyrimidine or pyridine ring connected either to *N*-methylpiperazine or a piperidine ring.

The influence of the substitution pattern of the pyrazolopyrimidine-5-carboxamide core of compound **2** was investigated. Table 5 shows the data for a representative set of compounds presenting structural modifications at the R_4 and R_5 positions. The presence of a phenylpiperazine group as R_4 substituent and its basicity appeared to be essential for activity >50% on NTS. Indeed, analogues harboring a piperidine or morpholine and *tert*-butyloxycarbonyl (*N*-Boc) derivatives showed very weak activity (**2b**, **2c**, **2f**, **2i**). Investigation of the R_5 substitution revealed that a range of aryl substituents was tolerated. The highest activity against NTS and adult worms

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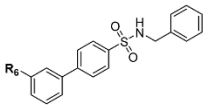
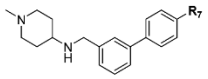
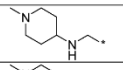
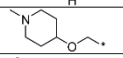
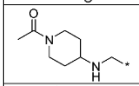
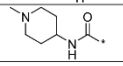
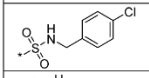
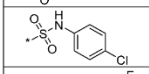
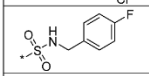
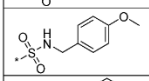
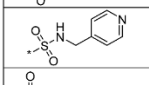
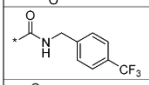
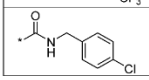
Table 6. SAR around Compound 3 (MMV022029)





R_6	ID[a]	NTS effect 24h	NTS effect 72h	Adult effect 24h	Adult effect 72h	ALogP[b]
	3	0.60	0.94	0.76	0.91	3.5
	3a	0.33	0.31			3.76
	3b	0.14	0.19			2.76
	3c	0.10	0.13			3.17

R_7	MMV ID	NTS effect 24h	NTS effect 72h	Adult effect 24h	Adult effect 72h	ALogP[b]
	3d	0.81	0.81	0.63	1	4.17
	3e	1	1	0.78	0.94	4.15
	3f	0.33	0.94			3.71
	3g	0.33	0.94			3.48
	3h	0.10	0.25			2.34
	3i	0.95	1	0.76	0.92	4.74
	3j	0.57	0.94	0.56	0.76	4.46

[a]The complete data for all the analogues of compound **3** are available in Supplementary Table 3. [b]ALogP calculated using BioVia's ScienceCloud (https://www.sciencecloud.com/).

was obtained with *para*-chloro, *para*-pyridine, *meta*-fluoro, *meta*-trifluoromethyl, and *ortho*-methoxy phenyl substituents, respectively. In contrast, switching the pyridine nitrogen atom in *meta* position in **2h** abolished activity against adult worms compared to lethal activity achieved with **2f**. The presence of an unsubstituted phenyl group in **2j** or incorporation of an isoxazole moiety in **2k** slightly dropped the activity <50% on adult worms.

Table 6 shows a representative set of compounds to highlight SAR at positions R_6 and R_7 of the biphenylsulfonamide core moiety of compound **3**. The basicity of the two nitrogen atoms from the methylpiperidin-4-amino group at position R_6 appeared to be paramount for activity. Modulating the basicity of the piperidine nitrogen atom either with acetyl or sulfonyl substituents like in **3b** and **3k** resulted in a loss of activity on NTS. Replacement of the amino group by an ether in the spacer or incorporation of an amide functionality was also detrimental (**3a** and **3c**, respectively). Regarding R_7 substitution pattern, incorporation of a chlorine into the benzyl substituent seemed to bring beneficial effects. In contrast, fluoro- or methoxy-benzyl as well as picolyl substituents resulted in loss of potency when compared to compound **3**. Compound **3e** showed good activity against both NTS and adult worms. The associated removal of a benzylic position should be beneficial for metabolic stability. The replacement of the sulfonamide moiety by an amide appeared to potentiate the antischistosomal activity of the analogues bearing a *para*-trifluoromethyl benzyl substituent, while it was reduced when a chlorine atom was attached. Generally speaking, the modifications on the biphenylsulfonamide core moiety of compound **3** seem to have nonadditive effects (Supplementary Table 2).

***In Vivo* Studies.** Compounds **2**, **3**, and **7** achieved the highest worm burden reduction ranging from 55.2 to 70.7%. Lower worm burden reduction values from 20.25 to 37.6 were obtained for **10**, **11**, auranofin, and **1e**, the analogue of compound **1**. The analogue of compound **2**, MMV1578493, had no effect on the worm burden (Table 7).

Table 7. *In Vivo* Activity of 7 Lead Molecules from the Pathogen Box Tested at 200 mg/kg in Mice Harboring *S. mansoni* Infection

manuscript ID	no. of mice tested	mean no. worms alive in the mesenteric veins	(SD)	WBR (%)
2	4	4.3	(4)	70.7
3	3[a]	4.7	(5.7)	67.8
7	4	6.5	(5.9)	55.2
10	4	11.3	(14.9)	22.4
11	4	9.8	(6.6)	32.8
1e[b,c]	3	18.3	(8.4)	37.6
2g[d]	3	26.67	(15.63)	0
control 1	8	14.5	(5.8)	
control 2[c]	8	29.4	(6.19)	
control 3[d]	8	24.5	(6.19)	

[a]One mouse was administered with only half the dose; therefore, it was excluded from the statistical analysis. [b]Compound **1** was not available in quantities required for *in vivo* studies. An analogue, **1e**, was tested instead. [c]Worm burden reduction (WBR) for **1e** was calculated using control 2. [d]WBR for **2g** was calculated using control 3.

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A Kruskall–Wallis test found no statistical significance between the different treatment groups and the controls ($p > 0.05$).

DISCUSSION

The emergence of resistance against praziquantel would cast a shadow on the global effort to control schistosomiasis as both treatment and control rely significantly on this drug. Yet, no new chemical entity has reached clinical development. It is therefore essential to discover new molecules active against schistosomes.[2,6,10] Aiming at expanding the pool of prospective antischistosomal drugs, we tested the 400 molecules of the Pathogen Box on *S. mansoni* and *S. haematobium*.

Applying a prescreen on *S. mansoni* NTS, we found that 10% of the Pathogen Box compounds were fast-acting. Testing next the activity of these compounds on adult worms revealed that 11 of them were highly active on both *S. mansoni* and *S. haematobium*. Screening the analogues of 3 of the 11 leads identified, compounds **1**, **2**, and **3**, led to the identification of 30 additional molecules active *in vitro* on both stages of the parasite. The SAR study conducted on these three scaffolds identified different moieties and modifications that are likely to potentiate the antischistosomal activity. For the pyrazolopyrimidine **2** and the aminopyrazinamide **1** several modifications indeed maximized this activity. Suspected mechanisms of action were reported for both of them. While a near neighbor of compound **2** was reported to prevent the assembly of NADPH oxidase complexes by inhibition of protein kinase C,[22,23] Shirude and colleagues showed that compound **1** targeted the ATPase domain of the mycobacterial enzyme GyrB.[24,25] On the contrary, specific conclusions are more difficult to draw from the SAR of **3** for which there is no information on its mechanism of action. The preliminary SAR around compound **3** highlighted key features such as a suitable range of LogP (between 3 and 5), modifications which could be beneficial in terms of physicochemical, drug metabolism, and pharmacokinetics properties (DMPK) or either detrimental or beneficial interactions which could help in the selection of compounds deserving further DMPK evaluation to design a next round of analogues.

In addition to their good *in vitro* activity, compounds **1**, **2**, and **3** showed satisfactory worm burden reduction values *in vivo*. Compound **2** also displayed good pharmacokinetic (PK) properties in preliminary studies in two rats (Supplementary Table 2). While no information on PK data was available for compound **7**, poor exposure was observed for compound **1** (Supplementary Table 2), which could be imputable to high lipophilicity and the basicity induced by the amine moieties. As a matter of fact, lack of permeability and a poor microsomal stability were reported for this compound.[17] The remaining four compounds tested *in vivo* showed a low activity. However, with the exception of compound **11**, which had *in vitro* IC_{50} value considerably increased in the presence of albumin, the IC_{50} values of the other compounds tested *in vivo* increased only moderately. Their lack of efficacy *in vivo* is therefore less likely resulting from a strong plasma protein-binding, as observed in our previous study,[15] but from their rather poor exposure levels (Supplementary Table 2). However, further studies are required to better understand the PK/PD relationship of these compounds.

Three of our *in vitro* leads [compound **7** (LSHTM-2045), compound **8** (LSHTM-00002069), and compound **9** (LSHTM-00002013)] were already identified by Mansour and colleagues (2016) in a high throughput assay.[26] Moreover, compound **2** presents structure similarities with LSHTM-1507 and LSHTM-1945, also identified as promising compounds in this same study. Some of our *in vitro* lead candidates were also identified as hits or leads by other research teams that screened the Pathogen Box on other pathogens. For example, the ethylenediamine compound **10** (SQ-109), currently tested in clinical phase II against tuberculosis,[27] was found active against *Candida albicans*[28] as well as against *Plasmodium* and kinetoplastids.[16] Similarly, compound **6**, a niclosamide analogue, was active on *Toxoplasma gondii* and *C. albicans*. Compounds **2** and **3** that were active against *Plasmodium* also emerged as leads against *Trypanosoma brucei brucei*.[16,24] Preston and colleagues (2016)[29] tested the Pathogen Box on the barber's pole worm, *Haemonchus contortus*, and identified Tolfenpyrad (MMV688934) as a lead candidate. This compound was not identified as a hit during our initial screening on NTS (data not shown).

A set of marketed drugs (the so-called reference drugs) active against different pathogenic agents were included in the Pathogen Box. For schistosomiasis, these included praziquantel that displayed an effect below the 50% cutoff during the prescreen on NTS. Praziquantel was therefore not identified as a hit. Its low activity on NTS was reported in previous studies,[26,30] especially when an early time-point (24 h) was chosen for drug evaluation. This finding highlights the disadvantage of applying a larval based prescreen instead of directly testing on adult schistosome stages. Molecules found inactive on NTS, as it is somewhat the case for praziquantel, might actually be active against adult schistosomes and hence missed. However, performing a prescreen on NTS before testing molecules on adult worms presents major advantages when screening large sets of compounds, notably regarding animal welfare, costs and time.[31,32] Unlike praziquantel, other reference compounds were highly active or lethal on both stages of the parasite. This was the case for the antileprotic drug clofazimine that was already identified in the screening of a FDA compound library[19,31] and mefloquine that is known to be a very potent antischistosomal drug *in vitro* and *in vivo*.[20,33] Auranofin was another reference compound included in the Pathogen Box. Targeting the ubiquitous enzyme thioredoxin reductase involved in the protection of nucleic acid against oxidative stress, auranofin *in vitro* activity has been previously characterized in *S. mansoni*[34,35] and *S. japonicum*.[36] Despite its promising fast-acting and lethal activity *in vitro* observed in this study, auranofin failed to have an effect on the infection *in vivo*. Our *in vivo* results contrast the ones reported by Kuntz and colleagues (2007)[34] that found worm burden reductions of 60%. This might be explained by the choice of administration route and dose regimen. While Kuntz and colleagues administered the mice twice daily with 6 mg/kg IP for 9 days, a single dose 100 mg/kg p.o. was administered 7 weeks postinfection in our study.

CONCLUSION

In conclusion, the present study identified fast-acting molecules able to efficiently kill *S. mansoni* and *S. haematobium* *in vitro*. These leads represent good starting points for the discovery of new antischistosomal drugs. Three of the selected candidates tested *in vivo* revealed moderate worm burden reductions. The preliminary SAR analysis on three lead compounds provided useful information on how to optimize

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new derivatives to potentiate both their druglikeness and their activity on schistosomes.

EXPERIMENTAL SECTION

Media, Compound Dilutions, and Assays Plates. Hank Balanced Salt Solution 1× (HBSS), M199 medium, RPMI 1640, and AlbuMax II were purchased from Gibco (Waltham MA, United States). penicillin/streptomycin 10 000 U/mL and inactivated fetal calf serum (iFCS) were purchased from Bioconcept AG (Allschwil, Switzerland). The 400 Pathogen Box compounds were distributed in five 96-well plates and dissolved in 10 μL of pure DMSO at a concentration of 10 mM. The stock solutions were diluted (1:10) in supplemented M199 medium on the day of the first drug assay and stored at −20 °C upon further use. For IC_{50} determination and additional *in vitro* tests, ∼1 mg of compounds **1**−**11** and analogues were provided by Medicines for Malaria Venture. Stock solutions (10 mM) in DMSO were prepared of these compounds for drug assays and stored at −20 °C upon further usage. Eight compounds were tested *in vivo*: **2**, **2g**, **3**, **7**, **10**, **11**, and auranofin. While compounds **1e**, **2**, **2g**, **3**, **7**, **10**, and **11** were obtained from Medicines for Malaria Venture, auranofin was purchased from Sigma-Aldrich (Buchs, Switzerland). The *in vivo* compounds were dissolved in tap water with Tween80/ethanol (10%, 70:30) before oral application.

Parasites for *in Vitro* Assays. *Biomphalaria glabrata* snails infected with *S. mansoni* (Liberian strain) were kept in water tanks filled with pond water. The snails were maintained at constant temperature and humidity level under UV light that followed the normal day/night cycle. On the day of transformation into NTS, they were placed in individual wells of a 24-well plate filled with fresh pond water and left for 4 h under the same light to enable cercarial shedding. The cercariae were then collected directly from the snail water.

NTS were obtained by syringe transformation followed by different washing, centrifugation, and filtration steps. *S. mansoni* adult worms were collected by dissecting the mesenteric veins of infected mice. *S. hematobium* adult worms developed in Golden Syrian LVG Hamsters (Charles River, United States) that were infected and ordered at the NIH Biomedical Research Institute (BRI). The worms were incubated for maximum 1 week in RPMI medium supplemented with FCS and penicillin−streptomycin.

***In Vitro* Assays.** As depicted in Figure 1A, the 400 compounds from the Pathogen Box were initially tested at 10 μM in duplicate on NTS. The compounds that produced an effect superior to 50% after 24 h postexposure were considered as hits and underwent determination of their IC_{50} using 1:2 serial dilutions from 0.16 to 10 μM. They were also tested in duplicate at 10 μM on adult *S. mansoni*. The IC_{50} of the hits on adult worms (defined as activity over 75%) were then determined using 1:3 serial dilutions ranging from 1.23 to 33.33 μM. Each concentration was tested in duplicate, and the entire assay was repeated once. For all *in vitro* assays, negative controls (using the highest concentration of DMSO) were included. A set of 26 reference drugs was already included in the Pathogen Box itself. Four of them, praziquantel, auranofin, clofazimine, and mefloquine, had established *in vitro* antischistosomal activity and served as positive controls (Table 3). The adult worms were evaluated at fixed time points over the 3 days following incubation with the compounds as described in previous studies.[31,37] The compounds with an IC_{50} < 10 μM were considered as leads. These leads were tested for protein binding *in vitro* by determining an IC_{50} on adult *S. mansoni* in the presence of bovin serum albumin (BSA, Albumax II, Gibco) at the human plasma concentration of 45 g/L. They were also tested at 10 μM on *S. haematobium* adult worms.

Analogues were available for some of the Pathogen Box compounds. To get a first insight into SAR information, we selected and tested *in vitro* the analogues of our leads that presented either an interesting pharmacokinetic profile or a sufficient variety of chemical structures. Similarly to the screening of the Pathogen Box, the 24 h active hits (effect ≥50%) on NTS were then tested on *S. mansoni* adults (Figure 1B). Thereby, 112 analogues of compound **1** (n = 17), compound **2** (n = 39), and compound **3** (n = 56) were screened for antischistosomal activity on NTS.

***In Vivo* Studies.** Female mice (aged 3 weeks) were ordered from Charles River (Germany). After 1 week acclimatization in our animal facility, the mice were injected (SC in the neck) with 100 cercariae. The mice were then randomly assigned to enriched cages with continuous access to rodent food and fresh water. Seven weeks postinfection, 200 mg/kg of compounds **1e**, **2**, **2g**, **3**, **7**, **10**, and **11** were administered orally to groups of 3 or 4 mice using a metal cannula. Auranofin was administered at a single, oral dose of 100 mg/kg. All the treated mice were then kept for 14−21 days more until euthanasia and dissection.

Ethical Statement. *In vivo* work was performed with respect to the 3R recommendations, followed both the Kanton Basel-Stadt and the Swiss federal law on animal experimentation, and was approved by the veterinary cantonal authorities (authorization no. 2070).

Data Analysis. Means and standard deviations for *in vitro* and *in vivo* results were calculated in Microsoft Excel. CompuSyn software (version 1.0; ComboSyn Inc., 2007) was used to calculate IC_{50} values. For *in vivo* studies, the worm burden reduction (%) was determined as described in our previous studies,[19] and a Kruskal−Wallis test was employed for statistical significance in R (version 3.2.2). When required, a Mann−Whitney−Wilcoxon test was applied instead.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00220.

Supplementary Table 1: IC_{50} of the NTS hits 24 h postincubation; Supplementary Table 2: physicochemical properties and PK parameters of the lead compounds from the Pathogen Box lead; Supplementary Table 3: structure and *in vitro* activity of MMV687812, MMV022478, and MMV022029 analogues; Supplementary Table 4: class and potential biological target of the *in vitro* leads identified in the Pathogen Box screening on *S. mansoni*; Supplementary Figure 1: SAR of the lead analogues of MMV687812; Supplementary Figure 2: SAR of the lead analogues of MMV022478; Supplementary Figure 3: SAR of the lead analogues of MMV022029 (PDF)

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Author Contributions

J.K. and V.P. designed the study, analyzed the data, and wrote the first version of the manuscript. V.P. performed the *in vitro* and *in vivo* experiments. B.L. performed the SAR analysis and revised the manuscript. All authors read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Chapter 3b

Multi-center screening of the Pathogen Box collection for schistosomiasis drug discovery

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Multi-center screening of the Pathogen Box collection for schistosomiasis drug discovery

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Abstract

Background: Over the past five years, as a public service to encourage and accelerate drug discovery for diseases of poverty, the Medicines for Malaria Venture (MMV) has released box sets of 400 compounds named the Malaria, Pathogen and Stasis Boxes. Here, we screened the Pathogen Box against the post-infective larvae (schistosomula) of *Schistosoma mansoni* using assays particular to the three contributing institutions, namely, the University of California San Diego (UCSD) in the USA, the Swiss Tropical and Public Health Institute (Swiss TPH) in Switzerland, and the Fundação Oswaldo Cruz (FIOCRUZ) in Brazil. With the same set of compounds, the goal was to determine the degree of inter-assay variability and identify a core set of active compounds common to all three assays. New drugs for schistosomiasis would be welcome given that current treatment and control strategies rely on chemotherapy with just one drug, praziquantel.

Methods: Both the UCSD and Swiss TPH assays utilize daily observational scoring methodologies over 72 h, whereas the FIOCRUZ assay employs XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) at 72 h to measure viability as a function of NAD^+/NADH redox state. Raw and transformed data arising from each assay were assembled for comparative analysis.

Results: For the UCSD and Swiss TPH assays, there was strong concordance of at least 87% in identifying active and inactive compounds on one or more of the three days. When all three assays were compared at 72 h, concordance remained a robust 74%. Further, robust Pearson's correlations (0.48–0.68) were measured between the assays. Of those actives at 72 h, the UCSD, Swiss TPH and FIOCRUZ assays identified 86, 103 and 66 compounds, respectively, of which 35 were common. Assay idiosyncrasies included the identification of unique compounds, the differential ability to identify known antischistosomal compounds and the concept that compounds of interest might include those that *increase* metabolic activity above baseline.

Conclusions: The inter-assay data generated were in good agreement, including with previously reported data. A common set of antischistosomal molecules for further exploration has been identified.

Keywords: *Schistosoma*, Schistosomiasis, Drug discovery, Phenotypic screen, Pathogen Box, MMV

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Background

Schistosomiasis is a parasitic disease caused by trematodes of the genus *Schistosoma*. The disease is endemic in 78 countries and preventive chemotherapy of at least 206 million people was necessary in 2016 [1]. Of concern for such a prevalent disease, treatment and control relies on mass chemotherapy with just one drug, praziquantel (PZQ) [2, 3]. PZQ is safe, active against all species of *Schistosoma* and is affordable, or distributed free

of charge. However, the increasing distribution of what is very often a sub-curative drug may encourage the emergence of PZQ-tolerant, or worse, PZQ-resistant parasites [3–5], an alarming prospect in the absence of any back-up drug.

Unlike the case for other global infectious diseases of poverty, schistosomiasis has lacked the prioritization necessary to establish trans-national, public-private drug development programmes that support the synthesis of small molecule chemical libraries, high throughput screening systems and the discovery expertise to identify and advance compounds to the clinic. Allied to this is the greater difficulty, logistics and costs needed to maintain and handle the schistosome parasite compared to the self-replicative single-celled organisms like malaria or the kinetoplastid parasites that are relatively easy to culture and amenable to automated liquid handling robotics. Accordingly, schistosome drug discovery remains fragmented across the academic sector with its more limited resources.

In the last decade, the realization that the ever-increasing distribution of just one drug to sustain the management of schistosomiasis is untenable has motivated the development of a number of phenotypic screening paradigms to identify new small molecules of interest. Many of these have coalesced around the application of *Schistosoma mansoni* schistosomula (post-infective larvae) [6–8]. These can be harvested in their tens of thousands, are reasonably amenable to liquid handling and have been used as the entry-point to screening and triaging the larger 'industrial-scale' of small molecule collections for subsequent tests against the more limiting adult parasite which, although directly responsible for disease morbidity *via* their eggs, can only be obtained in small numbers and from small vertebrate animals. A plethora of schistosomula assays with different read-outs has been developed [6, 8–11].

Over the same period, the Medicines for Malaria Venture (MMV) has made available to the drug discovery community a number of box sets each containing 400 well-annotated small molecules that have been validated in various disease contexts. These include the Malaria Box [12], Pathogen Box [13], Stasis Box and, most recently, the Pandemic Box. All, bar the last box, have been screened against schistosomes in culture/*in vivo* and the data made publicly available [12, 14–16]. Because the general interpretability of data arising from the phenotypic screening of schistosomula with the various MMV-supplied boxes (and other small molecules) may have been influenced by the particular assay methodology employed, we took the opportunity to perform an inter-institutional phenotypic evaluation of the Pathogen Box. The study involved teams from the University of California San Diego (UCSD), the Swiss Tropical and Public Health Institute (Swiss TPH), and the Fundação Oswaldo Cruz (FIOCRUZ). With the same chemical matter in hand, the goals were to identify a core set of active anti-schistosomal compounds of interest for future preclinical pursuit and highlight some distinct features of each particular assay.

Methods

Compounds

The Pathogen Box collection contains 400 compounds distributed in five 96-well plates and dissolved in 10 µl of pure dimethyl sulfoxide (DMSO) at a concentration of 10 mM. Plates were shipped on dry ice to each of the three institutions by the MMV. Supporting information for the compound collection can be found at https://www.mmv.org/mmv-open.

Life-cycle of *S. mansoni* at UCSD and preparation of schistosomula

The NMRI isolate of *S. mansoni* is maintained by passage through *Biomphalaria glabrata* snails (NMRI line) and 3–5 week-old, female Golden Syrian hamsters (Envigo, Placentia, CA, USA) as intermediate and definite hosts, respectively [6, 17]. The acquisition, preparation and *in vitro* maintenance of post-infective larvae (schistosomula) are described as follows. Briefly, infected patent snails were induced to shed infective larvae (cercariae) under a lamp for up to 2 h. Cercariae were concentrated over ice for up to 1 h and then mechanically transformed to schistosomula *via* tail-shearing through a 22 gauge micro-emulsifying needle attached to two 10 ml syringes (passaged back and forth 12 times) [18]. Schistosomula were washed in ice-chilled Basch Medium 169 [19] (containing 100 U/ml penicillin and 100 mg/ml streptomycin). Schistosomula were collected and washed three times in the same medium and kept on ice for a maximum of 1 h before distribution into assay plates containing compounds.

In vitro phenotypic assay of schistosomula at UCSD

The assay was performed as described [6, 20, 21]. Essentially, stock Pathogen Box compounds were diluted in DMSO to a 2 mM concentration and 1 µl manually spotted into transparent, U-bottomed 96-well assay plates (Costar 3367). Chilled Basch Medium 169 (100 µl; containing 100 U/ml penicillin, 100 mg/ml streptomycin and 4% heat-inactivated FBS (Corning Mediatech, New York, USA) was then added to mix the compound and this was followed by another 100 µl of medium containing 40–50 schistosomula. The final concentration of compound and DMSO was 10 µM and 0.5%, respectively. Each compound was tested in duplicate and solvent controls,

containing 0.5% DMSO only, were placed in columns 1 and 12 of each plate. Assay plates were placed into plastic boxes humidified with wet tissue and these were incubated at 37 °C in a 5% CO_2 environment.

Phenotypic changes in the schistosomula were visually recorded at 24, 48 and 72 h using a Zeiss Axiovert A1 inverted microscope (100×) as described [6, 22–24]. Briefly, simple descriptors that describe the effects of compounds on the parasites (changes in shape, motility and density) are employed. To allow for comparisons of compound activity, each descriptor is awarded a 'severity score' of 1 and these are added up to a maximum score of 4. Evidence of degeneracy or death is awarded the maximum score of 4 (raw data shown in Additional file 1: Table S1). Scores were averaged across the duplicate wells.

Life-cycle of *S. mansoni* at the Swiss TPH and preparation of schistosomula

The procedures to maintain the *S. mansoni* (Liberian strain) life-cycle, harvest cercariae and obtain schistosomula for *in vitro* testing are described elsewhere [8]. As detailed previously, the cercariae were mechanically transformed into schistosomula using two syringes connected by a Luer-Lok® connector (B. Braun Melsungen AG, Melsungen, Germany) [8, 18, 25]. Schistosomula were adjusted to a concentration of 50 units/100 µl and incubated for at least 12 h at 37 °C in a 5% CO_2 environment before use [14].

In vitro phenotypic assay of schistosomula at the Swiss TPH

On the day of the first drug assay, the 10 mM stock solutions of the Pathogen Box compounds were diluted (1:10) in Medium 199 (Gibco, USA) supplemented with 5% fetal calf serum (FCS; Bioconcept AG, Allschwil, Switzerland) and an antibiotic cocktail developed previously [26]. The drug plates were then stored at − 20 °C upon further use. In flat-bottom 96-well plates, each compound was tested in duplicate at a concentration of 10 µM. Every test plate included two negative controls wells containing 0.1% DMSO. Assay plates were incubated at 37 °C in a 5% CO_2 environment.

Phenotypic changes were visually recorded (10× magnification) at 24, 48 and 72 h using a previously described scoring method [8, 27]. Briefly, each well was scored using a quarter point descending scale from 3 to 0. The maximum score of 3 was assigned to wells containing schistosomula with normal movement and for which no morphological changes were apparent. A score of 2 was assigned when the overall movement in the well was reduced (or abnormally increased) and when morphological changes became apparent, i.e. increase in granularity, swelling, etc. A score of 1 was applied when almost no movement and more severe morphological alterations were observed. Finally, a score of zero indicated worm death (no movement and a complete loss of integrity). The antischistosomal effect was expressed as a fraction of the average test scores compared to the average score of the negative control wells using Microsoft Excel as (1 − average(test)/average(control)) [8]. For the assay to be considered valid, a minimal average score of 2 was required in the control wells.

Life-cycle of *S. mansoni* at FIOCRUZ and preparation of schistosomula

Cercariae of *S. mansoni* (LE strain) were harvested from *B. glabrata* (Barreiro strain). Schistosomula were obtained by mechanical transformation of cercariae using a protocol adapted from that previously described [19]. Briefly, cercariae were distributed into 50 ml conical tubes and allowed to settle on ice for 60 min. Cercariae were concentrated by centrifugation at 1000×*g* for 3 min at 4 °C, followed by resuspension in Medium 199 (without phenol red; Cat. # M3769, Sigma-Aldrich, Buchs, Switzerland) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA). Cercariae were mechanically transformed by passing them four times through a 22-gauge syringe needle followed by 10 cycles of washing and sedimentation. Schistosomula were distributed in flat-bottom 96-well culture plates at 200 parasites/well in a final volume of 195 µl, and incubated at 37 °C and 5% CO_2 for 24 h in the same medium supplemented with 2% heat-inactivated FBS (GIBCO, Thermo Fisher Scientific).

In vitro phenotypic assay of schistosomula at FIOCRUZ

XTT (sodium-2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H–tetrazolium-5-carboxanilide) was used as an indicator of schistosomula metabolic activity by the reduction of the yellow tetrazolium salt XTT to an orange formazan product [28, 29]. XTT was dissolved in Medium 199 without FBS to prepare a 1 mg/ml solution and the electron coupling reagent, N-methyl dibenzopyrazine methyl sulfate (PMS; Cat. # P9625, Sigma-Aldrich) was dissolved at 0.383 mg/ml in PBS. Both the XTT and PMS solutions were filtered through a 0.2 µm pore size membrane and stored at − 20 °C until use. Pathogen Box compounds were diluted to 400 µM from a 10 mM stock concentration in Medium 199 (without phenol red) in V-bottom 96-well assay plates. Afterwards, 5 µl of the diluted compounds were added to the culture plates that contained 200 parasites/well in 200 µl (10 µM final concentration of compound). Incubations were maintained for 48 h at 37 °C and 5% CO_2. The XTT labeling mixture was prepared by mixing the XTT and PMS solutions in a 50:1 ratio and 40 µl of the mixture

was added to each well of the assay plate. The incubations were continued for a further 24 h at 37 °C and 5% CO_2 and the absorbance at 450 nm (reference wavelength of 690 nm) was determined using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA). Positive and negative controls comprised heat-killed parasites and parasites incubated in the presence of 0.1% DMSO, respectively. All compounds were tested in duplicate in two independent experiments, totaling four wells for each compound. Schistosomula viability was determined using absorbance values applied to the following equation:

$$\%\ \text{Viability} = \frac{(\text{Sample} - \text{Positive control})}{(\text{Negative control} - \text{Positive control})} \times 100$$

where "Sample" is the absorbance measured in each well containing parasites tested with compounds, and "Negative control" and "Positive control" represent the average absorbance measured in each of the respective controls.

Data analysis

The phenotypic scores generated by UCSD and Swiss TPH, and the percentage viability data generated by FIOCRUZ are presented in Additional file 1: Table S2. Active compounds were identified as follows: an UCSD assay score of ≥ 2, a Swiss TPH drug effect of ≥ 0.5 and a viability of $\leq 50\%$ for the FIOCRUZ assay. The data from the three assays were also mathematically transformed (below) to obtain activity values within the same range and thus facilitate direct comparisons (Additional file 1: Table S3). Before running the transformation, the raw data were inspected. For some of the compounds tested in the Swiss TPH assay, negative viability values were obtained when the scores of the test compounds surpassed the scores in the control wells. These were converted to zero to indicate inactivity. Similarly, in the FIOCRUZ assay, compounds inducing an increase over the 100% baseline viability of the DMSO controls were not included in the data transformation process. However, some of these compounds that increased activity over baseline were also associated with antischistosomal activity in at least one of the other two assays and these are discussed below. Finally, because the scoring methods applied were opposite to one another, i.e. for the UCSD and Swiss TPH, the greater the value the more active the compound, whereas for FIOCRUZ, the reverse was the case, the raw data values were inverted to be consistent across all three groups. The data were then transformed in a span range from 0 (inactive) to 1 (most active) using the following equation:

$$\text{Transformed data} = \frac{(x - \text{median})}{(\max(x) - \text{median})}$$

The median was selected as the threshold below which all the compounds were considered inactive. We employed the median instead of the minimum value of each series given that the majority of compounds were inactive. Data analysis was performed using Microsoft Office Excel and Molsoft ICM software [30]. For the transformed data, the Pearson's correlation was calculated between pairs of data sets. The value for the F-distribution was also calculated to determine whether the correlation values occurred by chance or not. A low F-distribution value indicates that there is a low probability that the correlation between activity data occurred by chance. Finally, data for the normalized median activity score at 72 h were generated in R (version 3.6.0) using the *ggplot2* package (version 3.2.0) [31].

Results and discussion

Active and inactive Pathogen Box compounds identified among the three institutions

Schistosoma mansoni schistosomula were screened with the 400 constituent compounds of the MMV Pathogen Box by three institution-specific assays developed by UCSD, the Swiss TPH and FIOCRUZ. The data arising from each assay were then assembled for comparative analysis (Additional file 1). Our study is relevant as, to date, a plethora of schistosomula screening assays employing different methodologies (e.g. ATP and NAD(P) metabolic indicators, DNA intercalation agents such as propidium iodide, and visual- or automated image-based systems) and readouts (e.g. percentage death, EC_{50} value or phenotypic score), have been developed such that the general interpretability of data may be constrained by the particular assay employed [9–11, 24, 28, 32–34].

The main questions to address were the extent to which the data generated are assay-specific and which compounds can be considered common actives or non-actives independent of the assay employed. Table 1 indicates the degree of concordance in the number of actives and non-actives identified across the three assays. The complete dataset is presented in Additional file 1: Table S2.

For the UCSD and Swiss TPH assays, which employ visual interpretations of anti-schistosomal activity as a function of time, there was strong concordance of 87% in identifying compounds as being either active or inactive at 24, 48 and 72 h (Table 1). When comparing the UCSD and Swiss TPH assays with the FIOCRUZ metabolic-based XTT assay at the 72 h time point, agreement remained high, i.e. 83% and 78% between FIOCRUZ and UCSD, and FIOCRUZ and SWISS TPH, respectively. When data of all three groups were compared at 72 h, the concordance was lower but still a robust 74%.

Table 1 Degree of concordance in the number of actives and non-actives identified in the Pathogen Box between the UCSD (U), Swiss TPH (S) and FIOCRUZ (F) assays

Time (h)/ Comparison	U *vs* S	U *vs* F	S *vs* F	U *vs* S *vs* F
24	**87** (349)	na	na	na
48	**87** (348)	na	na	na
72	**87** (347)	**83** (331)	**78** (312)	**74** (295)

Notes: For the times indicated, the percentage (in bold) and number of compounds (in parentheses) that were identified as active or non-active between the institutions are indicated. Comparisons between UCSD and the Swiss TPH were run on a daily basis, whereas comparisons across all three groups were possible at the 72-h time point when the XTT assay (FIOCRUZ) was completed and the data analyzed

Abbreviation: na, not applicable

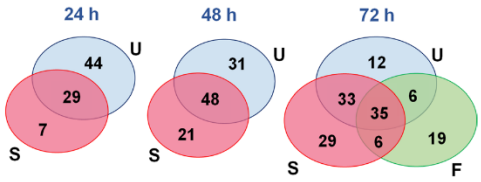

Fig. 1 Active Pathogen Box compounds identified by the three institutions. Venn diagram indicating the number of unique or shared actives identified by the three assays. Comparisons across all three groups were only possible at the 72-h time point when the XTT assay (FIOCRUZ) was completed and the data analyzed. U (blue), S (magenta) and F (green) represent UCSD, Swiss TPH and FIOCRUZ, respectively

This lower score might be attributable to the assay differences and parasite strains. In particular, the single metric XTT assay used by FIOCRUZ measures the oxidation of NAD(P)H as an indicator of viability [29] whereas visual interpretations of activity used by UCSD and the Swiss TPH are more holistic by identifying changes in features such as size, shape, color and motility, relative to DMSO controls.

Because both the UCSD and Swiss TPH assays employed daily observations at 24, 48 and 72 h, it was possible to note some trends for those compounds declared as active. The number of unique actives identified by UCSD decreased over time [44, 31 and 18 compounds at 24, 48 and 72 h (not counting the FIOCRUZ component at 72 h), respectively] (Fig 1; Additional file 1: Table S4). This was partly due to transiently active compounds, whereby changes noted at 24 h were no longer obvious at the later time points. Such compounds have been noted before [6, 35] and involve relatively mild changes in shape and/or motility, and with scores no greater than 2. The second trend was the increasing proportion of actives that was shared with the Swiss TPH assay as a function of time such that by 72 h, 68 active compounds were shared. These shared actives generally involved more progressive, irreversible and degenerative phenotypes (Additional file 1: Table S2). Nonetheless, at 72 h, UCSD had 18 active compounds (of which 10 registered strong scores ≥ 3), that were not identified by the Swiss TPH assay: these may be due to differences in the assay design and/or interpretation, or the parasite strain (NMRI at UCSD and Liberian at the Swiss TPH).

In contrast to the UCSD assay, the number of unique Swiss TPH actives increased as a function of time from seven at 24 h to 35 at 72 h (not counting the FIOCRUZ component at 72 h; Fig. 1; Additional file 1: Table S4). This may be due to differences in the screen assessment methods or parasite strains. The scoring system used by UCSD is based on a range of phenotypic descriptors that encompass shape and color, in addition to motility. Although these parameters are taken into account in the Swiss TPH scoring scale, the emphasis is on motility. A closer examination of the 35 compounds identified as active by the Swiss TPH at 72 h indicates that 27 had borderline or modest scores of between 0.5 and 0.65 (Additional file 1: Table S4). In contrast, and as noted above, those actives that were shared between Swiss TPH and UCSD at 72 h and the earlier time points tended to be more potent, i.e. scores of ≥ 0.75 for Swiss TPH and ≥ 3 for UCSD.

At 72 h, the FIOCRUZ assay identified 66 actives, less than the 86 and 103 actives captured by the UCSD and Swiss TPH assays, respectively (Fig. 1; Additional file 1: Table S2). Of these FIOCRUZ actives, 35 were shared with both other assays (termed 'core actives;' Additional file 1: Table S5), an additional 12 actives were shared with either UCSD (six) or Swiss TPH (six), and 19 were unique. Of the 35 core actives, the majority (29) were strongly active ($\leq$ 30% viability) in the FIOCRUZ assay and were also registered as strong actives in the other two assays (scores ≥ 3 and ≥ 0.75 for UCSD and Swiss TPH, respectively) at 72 h (Additional file 1: Table S5).

As a single-metric assay using the dye XTT, the FIOCRUZ assay is designed to specifically measure metabolic viability via NAD(P)H turnover, whereas the other two assays are based on the observational appraisals of phenotypic effects. Thus, it is anticipatable that the number of actives captured overall by the XTT assay would be less than the observation-based methods as depicted in Fig. 1. Nonetheless, the XTT assay has a robust 74% concordance with the other two assays and, in terms of automation, scalability and stringency, is particularly suitable for high-throughput formats [28]. One possibility for improving the active capture rate of the XTT assay

is to introduce a time component; however, this would require more parasites and compound, hence the current decision to use a single 72 h time point.

The finding of 19 unique XTT hits is intriguing and, apart from the possible influence of strain, the result may indicate the assay's particular ability to identify compounds that decrease the metabolic fitness of the parasite yet are beyond visual detection in either of the other two assays. Indeed, 12 of the 19 compounds decreased viability to less than 25% of the control value (Additional file 1: Table S4). Based on a PubChem search, we note that four of the 19 XTT-active compounds inhibit *Plasmodium falciparum* L-lactate dehydrogenase, which employs NADP(H) to catalyze the interconversion of lactate and pyruvate. Interference of this metabolic pathway in the schistosome would likely be detected by the XTT assay as it could interfere with the parasite's dehydrogenase activity.

Finally, the discussion above related to the XTT assay is based on measuring *a decrease* in metabolic activity from the 100% baseline. When including those compounds that *increase* activity above baseline, arbitrarily > 130%, an additional 34 compounds are identified, and of these, nine compounds are also active in at least one of the other assays (Additional file 1: Table S2). The association between increased metabolic rate and anti-schistosomal activity demonstrated here has not been noted before but is worthy of consideration going forward.

To allow for direct comparisons of the data arising from the three assays, the raw activity data were transformed and ranked on a scale of 0 to 1 from least to most active (Additional file 1: Table S3). The transformed data were used to calculate the Pearson's correlation and the respective value of the F-distribution at the different time points. Robust correlation values of 0.61, 0.63 and 0.68 were recorded at 24, 48 and 72 h, respectively, between the UCSD and Swiss TPH assay data, indicating that most of the active compounds demonstrated comparable activity. As might be anticipated with the XTT assay, the Pearson's correlation values were more moderate when compared to either the UCSD or Swiss TPH assays at 72 h (0.46 and 0.50, respectively).

Activity of reference compounds and consistency with previous data

Included in the Pathogen Box are 26 reference compounds, i.e. compounds known to be active against and/or marketed as drugs for various microbial diseases. As shown in Fig. 2 (also Additional file 1: Table S6), eight of the 26 reference compounds were identified as active in the UCSD and Swiss TPH assays at the 72-h time point. Among these were drugs that had been previously

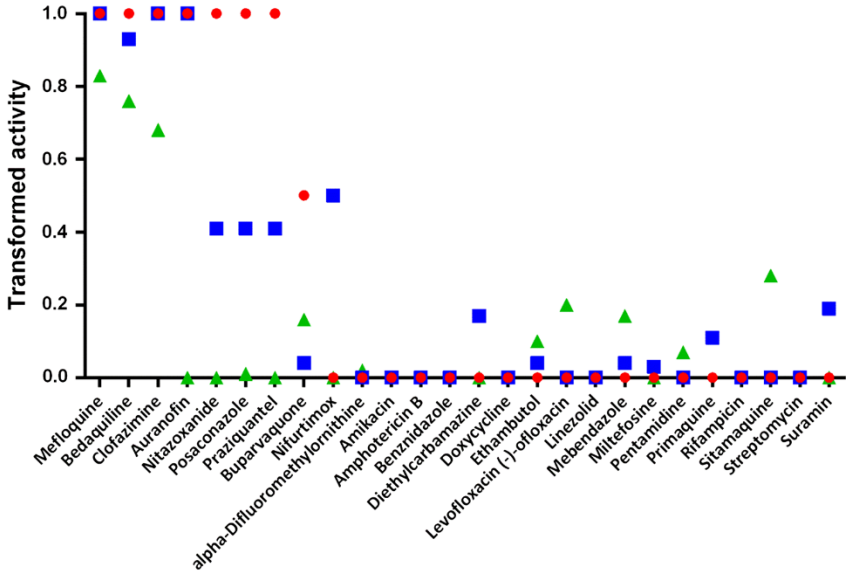

Fig. 2 Activity of 26 reference compounds in the Pathogen Box as measured by each assay. Data were transformed on a scale of 0 to 1 to allow for direct comparisons of the three datasets at the 72-h time point. Data for UCSD (red circles), Swiss TPH (blue squares) and FIOCRUZ (green triangles) are indicated

identified as antischistosomal, namely clofazimine [36], mefloquine [37], auranofin [38, 39] and nitazoxanide [6]. Both groups also had unique actives, namely, buparvaquone (UCSD) and nifurtimox (Swiss TPH). In the Swiss TPH assay, by contrast, PZQ generated a transformed score of 0.4 (raw score of 0.56), i.e. borderline active. The discrepancy in the activity between the two assays in detecting PZQ may be due to parasite strain or scoring methodology.

The FIOCRUZ assay returned three actives among the 26 reference compounds, namely, mefloquine, bedaquilline and clofazimine: each of these was strongly active in the other two assays. PZQ was not active in the XTT assay. In this context, it is pertinent to note that a previously employed CellTiter-Glo assay methodology which utilizes ATP as the metabolic readout did not identify PZQ or oxamniquine, an older marketed drug for treatment of schistosomiasis mansoni, as active against schistosomula [32]. This contrasts with the morphological and motility derangements induced by PZQ as detected here and previously [6, 40, 41]. We speculate that metabolic-based assays like the XTT assay which are designed to measure viability in single cells, are prone to missing important anti-schistosomal chemistries that may not affect cellular vitality/viability *per se*, but have mechanisms of action of particular consequence to complex metazoans, e.g. dysregulation of the neuromuscular system.

Interestingly, among the reference compounds that were inactive in all three assays was miltefosine, which was shown to be active against *S. mansoni* miracidia and cercariae *in vitro*, as well as against eggs, and adult worms *in vitro* and *in vivo* [42–44]. The mechanism of action of miltefosine may depend on the interplay between parasite and host. Thus, El-Faham and colleagues [45] demonstrated that treating parasites with miltefosine enhances serological recognition of defined adult worm surface antigens. The absence of activity here may suggest a stage-specific mode of action, i.e. lacking activity against schistosomula.

Finally, of note, is the inter-assay consistency in the number of shared actives identified here and previously during the assembly of the Pathogen Box in 2015. Specifically, of 13 compounds declared by the MMV as 'schistosomiasis active', nine had been identified as active in the Swiss TPH assay against schistosomula after 72 h (https://www.mmv.org/sites/default/files/uploads/docs/mmv_open/Pathogen_Box_Activity_Biological_Data_Smiles.xlsx). In the present setting and at the same time point, six of those nine actives were confirmed by the Swiss TPH assay and corroborated by the UCSD assay, with the latter identifying one additional compound (Table 2; Additional file 1: Table S7). Lastly, the FIOCRUZ assay identified five of the same six active compounds, plus three additional actives, one of which was shared with UCSD. Overall, therefore, there was solid cross-assay consistency and recall in confirming actives among those compounds previously designated by the MMV as 'schistosomiasis active.'

Actives identified as a function of disease set

Apart from the strong representation from the MMV 'schistosomiasis actives' among the identified hits (Table 2), there were also representations from most of the other 11 sets designated by the MMV as being active against a particular disease (Fig. 3; Additional file 1: Table S8). As

Table 2 Actives among the three assays for 13 compounds that had been designated earlier as 'schistosomiasis active' by the MMV

	UCSD			Swiss TPH			FIOCRUZ (mean ± SD)
MMV ID/Time point (h)	**24**	**48**	**72**	**24**	**48**	**72**	**72**
MMV688761	**4**	**4**	**4**	**1.00**	**1.00**	**1.00**[a]	**− 8.0** ± 2.5
MMV688763	**4**	**4**	**4**	**1.00**	**1.00**	**1.00**[a]	**− 5.8** ± 4.9
MMV688762	**4**	**4**	**4**	**1.00**	**1.00**	**1.00**[a]	**− 3.4** ± 3.7
MMV688768	**3**	**4**	**4**	**1.00**	**1.00**	**1.00**[a]	**6.4** ± 3.3
MMV688178	0	0	**2**	**0.60**	**1.00**	**1.00**[a]	**3.1** ± 8.5
MMV676382	**4**	**4**	**4**	0.45	**0.68**	**0.94**[a]	61.7 ± 11.2
MMV688270	**2**	**4**	**4**	0.05	0.00	0.11[a]	**39.0** ± 24.6
MMV688766	0	0	0	0.15	0.17	0.19	**0.1** ± 0.4
MMV688313	0	**2**	0	0.10	0.17	0.28[a]	**14.7** ± 1.7
MMV688771	1	**2**	0	0.05	0.10	0.16	62.2 ± 7.1
MMV676536	0	0	0	0.10	0.14	0.11[a]	75.5 ± 36.1
MMV688552	0	0	0	0.15	0.17	0.31	117.5 ± 14.6
MMV1198433	0	0	0	0.10	0.06	0.00	86.0 ± 19.9

[a] Compounds that the Swiss TPH had previously determined as 'schistosomiasis active' *vs* schistosomula at 72 h during the assembly of the Pathogen Box. Active compounds are delineated in bold typeface

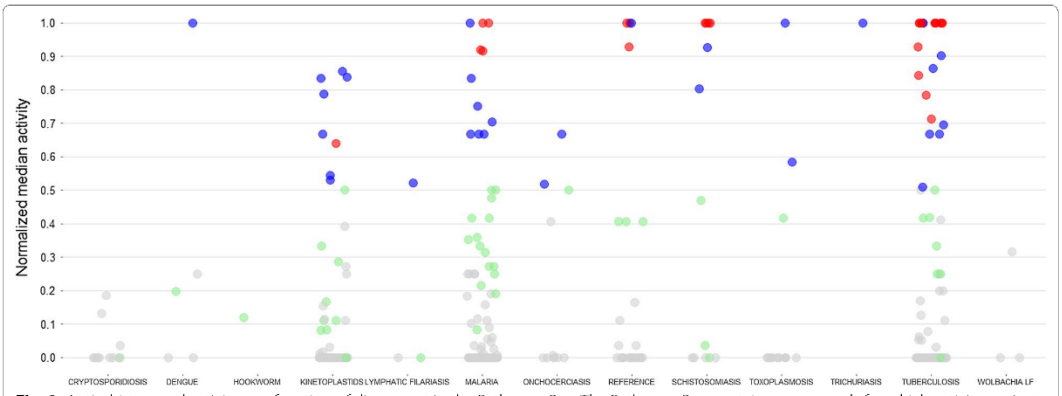

Fig. 3 Antischistosomal activity as a function of disease set in the Pathogen Box. The Pathogen Box contains compounds for which activity against various micro-organisms had been previously determined during the assembly of the collection (disease sets). For each compound (represented as a circle), the normalized median activity score at 72 h was calculated and plotted as a function of disease. A grey circle indicates that a median activity score of < 0.5 was attributed by all of the screening centers, whereas green, blue and red circles indicate that a score ≥ 0.5 was attributed by one, two or all three centers, respectively

examples, one of four dengue compounds, MMV688543, was active, i.e. normalized median activity ≥ 0.5, in both the UCSD and Swiss TPH assays. Among the 70 kinetoplastid compounds, eight (MMV658988, MMV676602, MMV688273, MMV688283, MMV688372, MMV689244, MMV690027 and MMV690102) were active in at least two of the assays. Similar findings were made for 11 of the 125 malaria compounds (MMV001059, MMV020391, MMV020623, MMV022029, MMV022478, MMV023233, MMV023985, MMV024114, MMV024406, MMV663250 and MMV676881), two of the 11 onchocerciasis compounds (MMV671636 and MMV676063), one of the three filariasis compounds (MMV687775) and two of 15 toxoplasmosis compounds (MMV688364 and MMV688417). Among the 116 tuberculosis compounds, 21 were active in two or more assays. Finally, among the 11 cryptosporidiosis, one hookworm, and three *Wolbachia* compound sets, actives in two or more assays were not identified (Fig. 3; Additional file 1: Table S8).

Conclusions

In this study, the Pathogen Box compound set was phenotypically screened with *S. mansoni* schistosomula using assays originating from three different institutions, namely, UCSD, the Swiss TPH and FIOCRUZ. The study's goals were to assess the degree of inter-assay variability and identify a core set of compounds that are active across the three assays performed. Among the inherent variables were parasite strain, culture medium, whether or not the parasites were pre-incubated prior to addition of compounds and the detection system employed. In this context, field isolates of *S. mansoni* with differential susceptibilities PZQ are known [46] and the choice of culture medium can influence the viability of schistosomula [7, 8, 17]. Evaluating the possible influences of these variables on the data, however, was not a goal of the current study. Our data show that concordance in identifying actives and non-actives was greatest between the UCSD and the Swiss TPH which employ visual assessments of phenotypic changes; however, the single-metric XTT assay employed by FIOCRUZ maintained the concordance among all groups at a robust 74%. Each assay identified unique actives, including the finding with the XTT assay that a chemically-induced increase in baseline metabolic rate is associated with antischistosomal activity. Thus, it could be argued that the best strategy to increasing the identification of active compounds is to combine an observation-based approach with the single-metric XTT assay, assuming that the resources necessary are not limiting. Overall, a common core set of 35 active compounds was identified which could be considered for further investigation. In this context, the activity of 24 out of the 35 core actives has already been tested against adult *S. mansoni* [15] with 13 compounds being active on both developmental stages (Additional file 1: Table S9). These particular hits provide starting points for further optimization.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-019-3747-6.

Additional file 1: Table S1. Descriptors and associated severity scores applied by UCSD. **Table S2.** Concordance between the 3 groups for active and inactive compounds. **Table S3.** Data transformation. **Table S4.** Unique hits per group. **Table S5**. Active compounds shared by all three groups ('core actives'). **Table S6**. Transformed activity of the reference compounds. **Table S7**. Comparisons with data previously generated for the same compounds. **Table S8.** Transformed activity by disease set. **Table S9.** Core active compounds compared to activity measured previously by the Swiss TPH against adult *S. mansoni*.

Abbreviations

ATP: adenosine triphosphate; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EC50: half maximal effective concentration; FBS: fetal bovine serum; FCS: fetal calf serum; FIOCRUZ: Fundação Oswaldo Cruz; MMV: Medicines for Malaria Venture; NAD(P): nicotinamide adenine dinucleotide (phosphate); NMRI: Naval Medical Research Institute; PBS: phosphate-buffered saline; PMS: N-methyl dibenzopyrazine methyl sulfate; PZQ: praziquantel; Swiss TPH: Swiss Tropical and Public Health Institute; UCSD: University of California San Diego; XTT: sodium-2,3- bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H–tetrazolium-5-carboxanilide.

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ChemAxon's JChem for Office (Excel; Version 15.1.1900.1773) was used to display the structures shown in Additional file 1: Table S1 (http://www.chemaxon.com).

Authors' contributions

All authors designed the studies and analyzed the data. PHNA, VP, MP, BMS, SM, MP and CRC performed the screening experiments. MM, PHNA, VP, MMM, JK and CRC drafted the first version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

We acknowledge the MMV for designing and supplying the Pathogen Box. Vertebrate maintenance and handling at the UCSD Animal Care Facility were in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at UCSD. The procedures involving laboratory animals at the Swiss TPH were approved by the Canton of Basel-Stadt and met the ethical standards set both by the cantonal and Swiss veterinary authorities (license no. 2070). They were also performed according to the 3Rs principles (http://www.nc3rs.org.uk). At FIOCRUZ, the experiments involving laboratory animals were carried out according to Brazilian national guidelines for the use of animals in scientific research. Animal experiments carried out for this report were approved by the Ethics Commission for Animal Use (CEUA) under the license number LW-32/17.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Chapter 4: Drug repurposing

Chapter 4a

Screening a repurposing library, the Medicines for Malaria Venture Stasis Box, against *Schistosoma mansoni*

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RESEARCH Open Access

Screening a repurposing library, the Medicines for Malaria Venture Stasis Box, against *Schistosoma mansoni*

Valérian Pasche[1,2], Benoît Laleu[3] and Jennifer Keiser[1,2*]

Abstract

Background: The development of new treatments against schistosomiasis is imperative but lacks commercial interest. Drug repurposing represents a suitable strategy to identify potential treatments, which have already unblocked several essential steps along the drug development path, hence reducing costs and timelines. Promoting this approach, the Medicines for Malaria Venture (MMV) recently distributed a drug repurposing library of 400 advanced lead candidates (Stasis Box).

Methods: All 400 compounds were initially tested in vitro against the larval stage of *Schistosoma mansoni* at 10 μM. Hits progressed to screening on adult worms and were further characterised for IC_{50}, cytotoxicity and selectivity. Ten lead compounds were tested in mice harbouring a chronic *S. mansoni* infection.

Results: Eleven of the 37 compounds active on the larval stage were also highly active on adult worms in vitro (IC_{50} = 2.0–7.5 μM). IC_{50} values on adult *S. mansoni* decreased substantially in the presence of albumin (7.5–123.5 μM). Toxicity to L6 and MRC cells was moderate. A moderate worm burden reduction of 51.6% was observed for MMV690534, while the other 9 compounds showed low activity. None of the in vivo results were statistically significant ($P > 0.05$).

Conclusions: Phenotypic screening of advanced lead compounds is a simple and resource-low method to identify novel anthelminthics. None of the promising hits of the Stasis Box identified in vitro against *S. mansoni* yielded acceptable worm burden reductions in vivo, which might be due to the high plasma protein binding. Since the in vitro hits interfere with different drug targets, they might provide a starting point for target based screening and structure-activity relationship studies.

Keywords: Schistosomiasis, Anthelminthics, *Schistosoma mansoni*, Drug discovery

Background

The lack of proper sanitation infrastructure worldwide puts more than 700 million people - mostly children - at risk of acquiring schistosomiasis [1–3]. This tropical parasitosis, transmitted by *Schistosoma* spp. trematodes, causes a wide range of severe chronic morbidities that affect the digestive and the urogenital system [2, 4]. Listed as a neglected tropical disease (NTD), schistosomiasis accounts for more than 2.6 million disability-adjusted life years (DALYs) lost [5]. Schistosomiasis has a considerable socio-economic impact in endemic countries, notably by reducing the attendance at school or work place [6, 7]. Schistosomiasis is almost exclusively controlled by preventive chemotherapy with praziquantel. Safe, affordable and an oral treatment, praziquantel presents many advantages [8]. With the global aim to eliminate the disease as a public health problem in the next decade, formalized by the London Declaration on Neglected Tropical Diseases [9], praziquantel treatment coverage will substantially increase over the next years [3, 10]. A major concern is that, in absence of any alternative on the market or advanced candidates in the drug discovery and development pipeline, such intensified use would result in the emergence of drug resistance [11–13]. Hence, in order to meet the

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long-term goal of elimination, new drug candidates have to be urgently identified [14, 15].

As the return on investment for a new antischistosomal treatment is expected to be very low (or inexistent), drug repurposing is a cost-effective solution to expand the pool of therapeutic candidates. This approach enables the bypassing of certain steps of the development process, which reduces the cost of research and development (R&D) and shortens the "bench to market" period without compromising safety [15, 16]. In this framework and following the same open-access model as the Malaria Box [17] and the Pathogen Box [18], Medicines for Malaria Venture (MMV) selected and compiled a library of 400 compounds. The so-called Stasis Box includes drugs that were stopped at an advanced stage in their clinical development. The reasons for this termination were different for each drug and ranged from lack of efficacy to bankruptcy of the developing company. The availability, the "druglikeness" and the affordability of these molecules were the main criteria used by MMV to build this library. Screening the Stasis Box already identified hits against *Haemonchus contortus* and *Madurella mycetomatis* [19, 20]. It represents therefore a promising and unique repertoire of advanced drugs to test on other organisms particularly those being responsible for neglected tropical or rare diseases. In this study, the activity of the Stasis Box was screened on *Schistosoma mansoni*. These compounds were first tested in vitro on newly transformed schistosomula (NTS). Hits progressed into testing on adult worms and in vitro toxicity assays. In vivo studies were performed with selected lead molecules.

Methods

Media and compounds

The Stasis Box compounds were compiled for MMV by Evotec (Hamburg, Germany) in 96-wells plates as 10 mM solutions and dissolved in 10 µl pure DMSO. The plates were stored at -80 °C until use. Stock solutions (1 mM) in M199 medium were prepared for the in vitro assays. For IC_{50} determination on adult worms, cytotoxicity assays and in vivo studies, MMV690732 and MMV690787 were purchased from Adooq Bioscience (Irvine, USA), MMV690596, MMV690599 and MMV690646 were purchased from Bio-Techne (Minneapolis, USA), MMV690466 and MMV690765 were purchased from SanBio BV/Cayman (Uden, The Netherlands), MMV690684 was purchased from Selleck Chemicals (Houston, USA), MMV690534 and MMV001539 were purchased from Sigma-Aldrich (Buchs, Switzerland). For NTS transformation and maintenance of the parasites, Hank Balanced Salt Solution 1X (HBSS), M199 medium and RPMI 1640 were purchased from Gibco (Waltham MA, USA). Penicillin/Streptomycin 10'000 U/ml and inactivated foetal calf serum (iFCS) were purchased from Bioconcept AG (Allschwil, Switzerland). For cytotoxicity assays, rat skeletal myoblast L6 cells were grown in RPMI supplemented with FCS and L-glutamin (Sigma-Aldrich). Podophyllotoxin (PTT) was purchased from Sigma-Aldrich and stock solutions (5 µg/ml) were prepared in L6 cells medium.

Schistosoma mansoni adult worms and schistosomula

The *S. mansoni* (Liberian strain) life-cycle, is maintained in-house at the Swiss Tropical and Public Health Institute (Swiss TPH), as described before [21]. *Biomphalaria glabrata* were infected with 6 to 8 *S. mansoni* miracidia. They were kept in pond water under natural light, temperature and humidity conditions until the infectious stage, the cercariae, started to shed. The cercariae were mechanically transformed to schistosomula, the NTS, using a procedure adapted from Milligan & Jolly [22]. NTS were incubated (37 °C, 5% CO_2) until use for 12 to maximum 24 h in M199 medium supplemented with FCS and antibiotics. Adult *S. mansoni* of both sexes were collected by dissecting the intestinal veins of mice euthanized 7 weeks post-infection. All the adult worms recovered were incubated for maximum one week until use in RPMI medium supplemented with FCS and penicillin-streptomycin.

In vitro assays

For in vitro screening on NTS, the parasite suspension was adjusted to 50 NTS/100 µl in supplemented M199 medium and added to the drug dilutions in 96-wells plates (Eppendorf AG, Hamburg, Germany). NTS were initially exposed to a drug concentration of 10 µM (0.1% DMSO). Each drug was tested in duplicate.

Hit compounds identified on NTS progressed into testing on adult worms. Females, males and pairs (3 to 5 worms per well) were exposed to the drug dilution in supplemented RPMI medium. The assays were performed in duplicate in 24-wells plates (Eppendorf AG, Hamburg, Germany). IC_{50} values were determined for compounds that demonstrated a high activity against NTS and adults (effect ≥ 75% at 10 µM after 72 h). Each assay on NTS was performed in triplicate with serial drug dilutions (1:2, range: 10–0.16 µM) and repeated once. Similarly, the IC_{50} values on adult worms were determined after incubating the worms for 72 h in serial drug dilutions (1:3, range: 33.3–1.23 µM). Each IC_{50} assay was repeated once. These assays were repeated for the lead compounds in presence of albumin (BSA) at the human physiological concentration of 45 g/l (AlbuMAX II, Gibco). For all in vitro assays (NTS and adults), negative controls containing the highest concentration of DMSO were included. Worms incubated with praziquantel served as positive controls. The parasites were evaluated under an inverted optical microscope over 3 days after drug exposure. Their movement and morphology were assessed and scored as described previously [23, 24].

Cytotoxicity assays

Rat skeletal myoblast L6 cells (2×10^4 cells/ml) were exposed to serial dilutions of the 11 lead compounds (1:3, range: 0.37–90 μM). Podophyllotoxin (PPT, 1:3, range: 2×10^{-4} –0.05 μg/ml) was used as a positive control. After a 72-hour incubation, resazurin dye (Alamar Blue) was added to the plates. Fluorescence and the cytotoxic concentration (CC_{50}) were measured at 530 nm excitation and 590 nm emission wavelength using a SpectraMax M2 (Molecular Device, Sunnyvale CA, USA; Softmax version 5.4.6). Each drug was tested in duplicate and each assay was repeated once.

In vivo studies

Three weeks old NMRI female mice were used (Charles Rivers, Germany) for in vivo drug efficacy studies. The animals were kept in groups of ten with constant access to food and water. After one week of habituation, mice were sub-cutaneously injected a suspension containing 100 *S. mansoni* cercariae in phosphate-buffered saline (PBS). The drugs (200 mg/kg single dose) were administered seven weeks post-infection by oral gavage to groups of 4 mice. They were dissolved in tap water with Tween80/ethanol (10%, 70:30). Untreated mice (n = 10) served as control and were dissected 7 weeks post-infection. Treated mice (n = 40) were euthanized with CO_2 and dissected between 16 to 18 days post-drug administration. The worms were then collected, sexed and counted.

Statistics

For in vitro assays, the average viability scores between replicates (or individual adult worms) were normalised to the controls and converted into percentage activity (or effect). Drug IC_{50} values (for NTS or adult worms) were calculated by computing different dose-effect values using CompuSyn2 software (ComboSyn Inc., 2007). The linear correlation coefficient (r) reflects the experimental fit. In this study, r-values > 0.70 were considered acceptable. The mean IC_{50} were considered for analysis only if the values obtained in each replicate did not differ more than 5.5-fold. The selectivity index (SI) of each drug was calculated by dividing the CC_{50} measured on the L6 cells by the IC_{50} measured on the parasites. For in vivo studies, as previously described [25], the worm burden reduction [%] was determined by comparing the worm burden of treated and untreated mice. For statistical significance a Kruskal-Wallis test was employed (R version 3.2.2).

Results

In vitro studies

Following incubation for 72 hours, 37 of the 400 drug-like compounds screened were active on NTS at 10 μM (effect ≥ 50%), including 16 that were lethal (effect = 100%) and 10 that were highly active (effect ≥ 75%). Seven of these were fast-acting as they demonstrated a high activity on NTS already after 24 hours (Table 1). The compounds with a moderate effect (< 75%) after 72 h were not investigated further, while the 26 most active ones and MMV690466 - that showed a high effect at the 48 hour time point - proceeded to adult testing (Fig. 1). Sixteen compounds showed only a moderate (effect < 75%) activity against adult worms. On the other hand, 7 compounds displayed a high activity against adult worms and 4 were even lethal (Table 1). Of these, 5 compounds were already highly active against the adult worms after one day incubation period at 10 μM. These 11 compounds were selected as in vitro leads. Compound characteristics and structures are presented in Table 2 and Fig. 2. IC_{50} values calculated 72 h post-incubation ranged from 2 to 7.5 μM on adult worms and 0.5 to 7.2 μM on NTS. In the presence of albumin, a strong decrease in activity of the lead compounds on adult worms was observed (Table 3). The highest activities were observed for MMV003452 and MMV690684 revealing IC_{50} values of 7.5 and 7.7 μM, respectively, denoting a 2-fold increase compared to the values observed without albumin supplementation. The mean IC_{50} values of MMV690596, MMV690599 and MMV690787 were between 10 and 16 μM in the presence of albumin (indicating a 5.4-, 6.1- and 4.3-fold increase, respectively compared to the IC_{50} values without albumin). The remaining 6 compounds showed no activity in the presence of albumin (Table 3).

Table 1 Number of hits identified after the initial screen of the Stasis Box at 10 μM

	Evaluation time point		
	24 h	48 h	72 h
NTS hits (effect ≥ 50%)	11	23	37
NTS (effect > 75%)	4	9	10
NTS (lethal)	3	9	16
Adult hits (effect ≥ 50%)	11	9	16
Adult (effect > 75%)	5	4	7
Adult (lethal)	0	2	4

The lead molecules were rather cytotoxic, except for MMV690534 that showed, on both mammalian cell lines acceptable parasite-selectivity (SI > 1). When measured on L6 rat myoblast cells MMV690596, MMV003452, MMV690599 and MMV690684 were slightly above the selectivity cut-off while MMV690732 and MMV690466 were slightly below. Although MMV690466 and MMV001539 were not selective towards the worms when measured with L6 cells, the SI was > 1 when measured on MRC5 cells (Table 3).

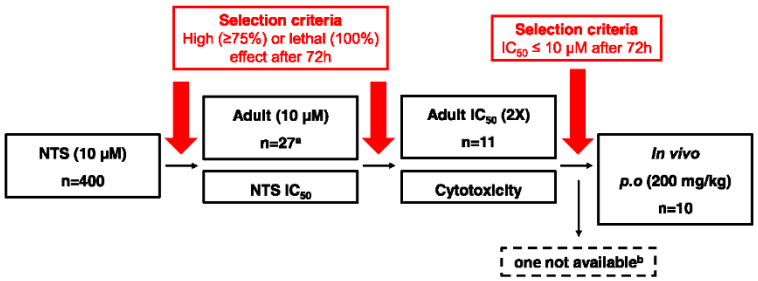

Fig. 1 Workflow of the Stasis Box screening on *S. mansoni* worms. [a] 26 highly effective hits tested on adults and MMV690466 that showed high activity at 48 h. [b] MMV001539 was not available in quantity required for in vivo studies

In vivo activity

All the compounds tested (n = 10) failed to significantly reduce the worm burden in vivo. Eight compounds had no effect on the worm burden in infected mice (worm burden reduction < 36%). Although MMV690534 showed a worm burden reduction slightly above 50%, it was not statistically significant ($P > 0.05$). The mice treated with MMV690646 (Ispinesib) died prematurely and therefore, were not included in the analysis (Table 4).

Discussion

The Stasis Box includes late leads that were abandoned, mainly due to a lack of efficacy against their primary target disease. This library represents therefore a unique repertoire of molecules to test on different organism including *S. mansoni* as the compounds already underwent advanced clinical test phases, which should guarantee satisfactory safety and pharmacological properties.

After screening these drugs in vitro on both larval and adult stages of the parasite, 11 lead molecules revealed

Table 2 Stasis Box lead molecules

MMV ID	CHEMBL ID	Name(s)	Indication	Mechanism of action	Target
MMV690732	CHEMBL3545403	XL-139; BMS-833923	Cancer	Smoothened homolog antagonist	Smoothened homolog
MMV690596	CHEMBL1836102	CGP-71683	Obesity, eating disorder	Neuropeptide receptor antagonist	na
MMV003452	CHEMBL15928	GR-127935	Depression	5-HT 1B/1D receptor antagonist	5-HT 1B and 5-HT 1D receptors
MMV690599	CHEMBL88272	RS-17053	Prostate hyperplasia	Alpha 1 adrenoreceptor antagonist	na
MMV690466	CHEMBL59030	GW-3965	Inflammation, melanoma	Agonist of LXR receptor	na
MMV690787	CHEMBL513909	BI-2536	Cancer	Serine/threonine-protein kinase PLK1 inhibitor	Serine/threonine-protein kinase PLK1
MMV690684	CHEMBL91867	CL-387785	Cancer	Epidermal growth factor receptor (EGFR) receptor antagonist + Tyrosine Kinase (TK) inhibitor	na
MMV690646	CHEMBL2111096	CK0238273; SB-715992-S; Ispinesib	Cancer	Kinesin inhibitor	KIF11
MMV690765	CHEMBL607707	EKB-569; Pelitinib	Cancer	EGFR erbB1 inhibitor	EGFR erbB1
MMV690534	CHEMBL238125	SD-208	Cancer, chronic pulmonary obstruction	TGFb TK inhibitor + receptor antagonist	na
MMV001539	CHEMBL16687	CGS-15943	Ischemia, stroke	Adenosine A2 receptor antagonist	na

Abbreviation: *na* not available

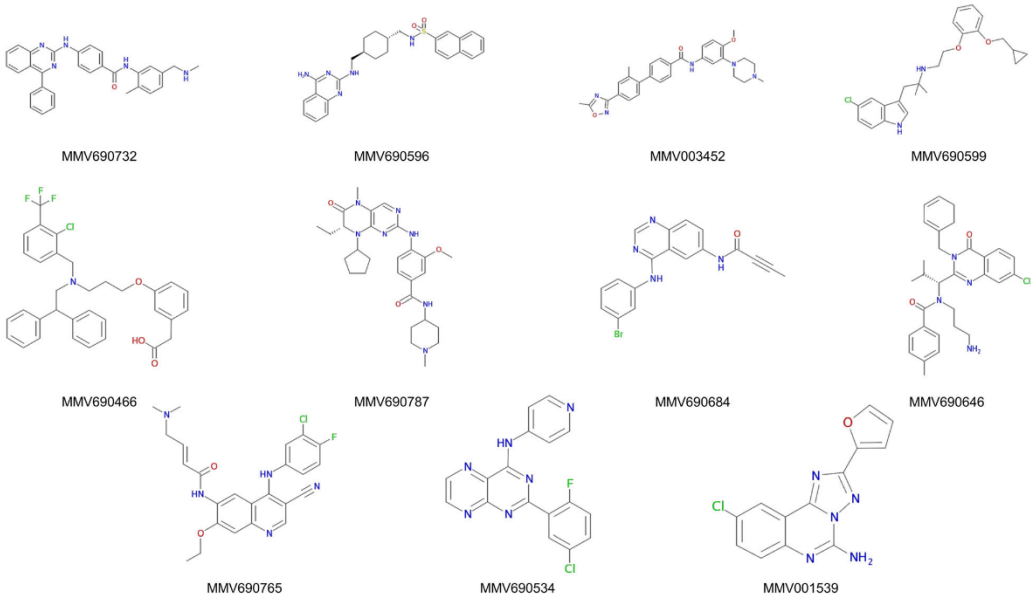

Fig. 2 Chemical structures of the Stasis Box lead molecules. Source: ChEMBL

strong antischistosomal activity. With the exception of MMV690534 (SD-208) and MMV001539 (CGS-15943), the IC_{50} of the compounds measured on adult worms were already below 10 μM after 24 h, hence the compounds were fast acting (Table 3). Speed of action is an important parameter for defining antischistosomal activity as worms will be exposed only very shortly to high mesenteric vein concentrations of the unmetabolised drug [26]. Speed of action was also already taken into account for drug selection and progression in our previous

Table 3 IC_{50} values on NTS and adult *S. mansoni*, toxicity on L6 and MRC5-cells and selectivity of the 11 Stasis Box lead molecules. Cytotoxic concentration (CC_{50}) and selectivity index (SI) are compared to the mean IC_{50} values measured on NTS and adult *S. mansoni* in standard and albumin-enriched medium (45 g/l) over 72 h with 11 Stasis Box lead compounds

Compound MMV ID	NTS IC_{50} (μM)[a]			Adult IC_{50} (μM)[a]			Adult IC_{50} (μM) with albumin[a]			L6 cells		MRC5 cells[d]	
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	CC_{50} (μM)	SI	CC_{50} (μM)	SI
MMV690732	29.5	6.3	3.5	4.9	2.9	2.5	62.8	181.0	54.1	2.3	0.9	1.9	0.7
MMV690596	1.1	1.3	0.6	1.0	2.1	2.0	27.5	14.4	11.0	3.5	1.7	1.9	0.9
MMV003452	4.4	2.2	1.2	1.7	3.0	2.7	22.6	12.0	7.5	3.7	1.4	1.9	0.7
MMV690599	10.5	3.5	1.5	2.5	3.3	2.6	23.6	19.1	15.9	3.4	1.3	2	0.8
MMV690466	14.6	3.8	2.1	2.9	2.3	2.0	18.1[b]	89.7	47.6	1.6	0.8	8.4	4.3
MMV690787	38.4[b]	8.1	6.3	7.9	7.2	3.1	39.5	14.4	13.3	na	na	0.1	0
MMV690684	4.0[c]	3.1[c]	0.7	4.9	4.7	3.7	76.8	14.4[b]	7.7	7.0	1.9	7	1.9
MMV690646	3.0[c]	1.1	0.9	3.7	2.2	2.2	32.0	121.8	33.6	na	na	0.2	0.1
MMV690765	914.4[b]	> 1[c]	0.5[b]	4.2	3.4	2.9	651.7	225.9[b]	123.5[b]	11.8	4.1	0.7	0.3
MMV690534	11.8	14.3[c]	7.2[c]	17.4	19.3	7.5	na	na	na	74.5	15.0	12.4	2.5
MMV001539	12.7	11.0[c]	1.7	28.2	12.3	7.0	nd	nd	nd	2.9	0.4	16	2.1

Abbreviations: *na* not applicable. *nd* not done
[a] *r*-values ranged between 0.7 and 1.0
[b] These values are based on one replicate only because of an *r*-value < 0.70 was obtained for the second replicate
[c] The IC_{50} values obtained in each of the two replicate differed more than 5.5-fold
[d] The CC_{50} values on MRC5 cells were provided by MMV

Table 4 In vivo efficacy of the lead molecules from the Stasis Box. Effect on worm burden of a single 200 mg/kg oral dose of nine lead molecules identified after screening the Stasis Box in vitro administered to mice harbouring a 49-day-old adult *S. mansoni* infection

Compound	Mice tested[n]	Mean worm burden ± SD	WBR (%)
MMV690732	4[1]	47.5 ± 24.3	0
MMV690599	3[1]	44.7 ± 29.6	3.2
MMV690787	4[1]	48.3 ± 21.6	0
MMV003452	4[1]	40.5 ± 17.2	12.2
MMV690596	4[1]	52.0 ± 22.7	0
MMV690684	4[1]	41.5 ± 16.5	10.0
MMV690765	3[1]	84.0 ± 56.4	0
MMV690466	4[2]	10.3 ± 6.9	35.9
MMV690534	4[2]	7.8 ± 7.5	51.6
Control[1]	8	46.1 ± 21.9	
Control[2]	2	16.0 ± 19.8	

[n] Indicates the mice control batch. One mouse died prematurely because of toxic effects of MMV690646 (Ispinesib) and therefore data is not shown

screenings of an FDA library of approved drugs [25] and of a set of oncology drugs [27]. Although the IC_{50} values measured on NTS after 24 h were very different, they all ranged under the 10 μM cut-off 72 h post-incubation.

The high in vitro activity changed in presence of albumin at the human plasma concentration of 45 g/l. The efficacy of each molecule dropped considerably, notably for MMV690732 (XL-139), MMV690466 (GW-3965) and MMV690646 (Ispinesib) that showed high IC_{50} values ranging from 30 to 55 μM 72 h post-drug exposure in the presence of this protein (Table 3). This finding suggests a strong drug-binding effect of albumin. This finding is consistent with the incapacity of all the lead molecules tested in vivo to significantly reduce the worm burden in infected mice. Hence, the lack of efficacy in vivo might have been caused by a strong drug-binding effect of the host plasma proteins reducing therefore the amount of free drug available to kill the parasite. However, other factors that affect pharmacokinetic processes such as drug metabolism might obviously also play a role.

In order to avoid losing advanced drug leads as potential novel antischistosomal drugs, the hit to lead selection criteria in the present work was less strict than in our previous screenings [25, 27]. For this reason and because the Stasis Box drugs were assumed to have an acceptable safety and pharmacokinetic profile (at least for the relevant therapeutic indication), all compounds moved into in vivo testing despite a higher IC_{50} in the presence of albumin. However, plasma protein binding should be considered among other factors (as clearance, safety, exposure) in the screening cascades, as suggested by Gelmedin et al. [28].

Oncology is a privileged source of drugs to repurpose against schistosomiasis and other NTDs, particularly because of their potency to interfere with conserved signalling pathways that are also involved either in the metabolism or the reproduction of the parasite [11, 15, 29]. For instance, the antischistosomal activity of different protein kinase inhibitors (PTK) has been described previously [28, 30–33]. Also many of the hits identified in our study were developed to target intracellular neoplastic pathways (Table 2). However, one of the disadvantages working with anticancer drugs is obviously toxicity. Although the Stasis Box compounds were selected for their "druglikeness", the majority of the lead molecules identified were moderately toxic as demonstrated by our cytotoxicity tests, the data provided by MMV, and the death of the mice treated with MMV690646 (Ispinesib). Nonetheless, the identified oncology late leads could serve as starting point for future studies. For example, structure-activity relationship (SAR) studies on these pharmacophores should be conducted to identify less toxic hits.

Our study identified different lead candidates in vitro, which likely act on different targets in *S. mansoni*. For example, we confirmed the in vitro activity of the Polo-like kinase (Plk) competitive inhibitor BI-2536 (MMV690787) on *S. mansoni* [28, 32, 34] targeting SmPlk1 and hence resulting in reproductive impairment of both sexes. MMV690534 (SD-208) might have impaired the reproductive function of the parasite by interfering with the TGF-beta mediated intracellular signalling pathway notably involved in egg production [28, 35, 36]. Aside from reproduction, other functions in *S. mansoni* may have been negatively impacted. For example, epidermal growth factor receptors (EGFR) might have been targeted by antagonists such as MMV690765 (Pelitinib), while MMV001539 (CGS-15943) might have interacted with purinergic receptors. These few examples illustrate the variety of potential targets in *S. mansoni*, notably for kinase inhibitors. In this study, in vitro tests were assessing parasite phenotype (e.g. motility, shape, or colour) after drug exposure. No parameters related to the reproductive function (e.g. egg count) were recorded, which is a limitation of our study given the importance of protein kinases in reproduction.

Considering the good activity in vitro of some of these compounds, it would be worth testing analogues and initiate SAR studies. As the information openly available on these compounds was scarce, manufacturers should be encouraged to provide more information in the public domains. Functional studies are also essential to better characterize potential drug targets and design more effective and selective drugs.

Conclusions

Despite the fact that no drugs with clear in vivo antischistosomal activity emerged from the Stasis Box

screening on *S. mansoni*, different molecules were identified with promising in vitro activity. Together with a better understanding of their potential drug targets, SAR studies could be conducted, particularly taking into account protein binding and in vivo pharmacokinetics. Our findings confirmed that open access libraries such as the Stasis Box are powerful and yet essential tools for drug discovery on NTDs.

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Availability of data and materials
The data supporting the conclusions of this article are included within the article. Raw data are available on request to the corresponding author.

Authors' contributions
JK and VP designed the studies and analyzed the data. VP performed the experiments and drafted the first version of the manuscript. BL provided information on the compounds and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Animal work was approved by the veterinary cantonal authorities of the Kanton Basel-Stadt (authorization n°2070). All in vivo procedures were performed in respect to the 3R rules and met the standards set by the Swiss federal law and the cantonal regulations on animal experimentation.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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Chapter 4b

Evaluation of emodepside in laboratory models of human intestinal nematode and schistosome infections

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RESEARCH Open Access

Evaluation of emodepside in laboratory models of human intestinal nematode and schistosome infections

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Abstract

Background: Helminthiases are very prevalent worldwide, yet their treatment and control rely on a handful of drugs. Emodepside, a marketed broad-spectrum veterinary anthelminthic with a unique mechanism of action, undergoing development for onchocerciasis is an interesting anthelmintic drug candidate. We tested the *in vitro* and *in vivo* activity of emodepside on nematode species that serve as models for human soil-transmitted helminth infection as well as on schistosomes.

Methods: *In vitro* viability assays were performed over a time course of 72 hours for *Trichuris muris*, *Necator americanus*, *Ancylostoma ceylanicum*, *Heligmosomoides polygyrus*, *Strongyloides ratti*, *Schistosoma mansoni* and *Schistosoma haematobium*. The drug effect was determined by the survival rate for the larvae and by phenotypical scores for the adult worms. Additionally, mice infected with *T. muris* and hamsters harboring hookworm infection (*N. americanus* or *A. ceylanicum*) were administered orally with emodepside at doses ranging from 1.25 to 75 mg/kg. Expelled worms in the feces were counted until 3 days post-drug intake and worms residing in the intestines were collected and counted after dissection.

Results: After 24 hours, emodepside was very active *in vitro* against both larval and adult stages of the nematodes *T. muris*, *A. ceylanicum*, *N. americanus*, *H. polygyrus* and *S. ratti* ($IC_{50} < 4$ µM). The good *in vitro* activity was confirmed *in vivo*. Hamsters infected with the hookworms were cured when administered orally with 2.5 mg/kg of the drug. Emodepside was also highly active *in vivo* against *T. muris* ($ED_{50} = 1.2$ mg/kg). Emodepside was moderately active on schistosomula *in vitro* ($IC_{50} < 8$ µM) 24 h post-drug incubation and its activity on adult *S. mansoni* and *S. haematobium* was low (IC_{50}: 30–50 µM).

Conclusions: Emodepside is highly active against a broad range of nematode species both *in vitro* and *in vivo*. The development of emodepside for treating soil-transmitted helminth infections should be pursued.

Keywords: Emodepside, Drug repurposing, Soil-transmitted helminthiases (STH), Hookworms, *Trichuris* spp., Nematodes, Trematodes, *Schistosoma* spp.

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Background

Helminths affect a fifth of the world population and their associated morbidities include general fatigue, food malabsorption or iron deficiency anemia [1–4]. They are an important public health issue in low and middle income countries, where they enhance the vicious cycle of poverty notably by reducing school attendance and productivity [5, 6]. The most prevalent helminthiases are schistosomiasis (primarily caused by *Schistosoma haematobium*, *S. japonicum* and *S. mansoni*) that affects more than 250 million people and soil-transmitted helminthiases (STH) that account for more than 1.5 billion infected cases worldwide [2, 4, 6, 7]. Infections with the hookworms *Ancylostoma duodenale* and *Necator americanus*, the whipworm *Trichuris trichiura*, the roundworm *Ascaris lumbricoides* and the threadworm *Strongyloides stercoralis* are grouped as soil-transmitted helminths, based on their mode of transmission [8, 9].

Preventive chemotherapy is the strategy of choice, recommended by the World Health Organization (WHO) to control these helminth infections. Schistosomiasis control relies on praziquantel while albendazole, mebendazole, levamisole and pyrantel pamoate are used against STH [7, 10, 11]. A recent meta-analysis showed that all four drugs used against STH have a limited and even decreasing efficacy against the parasites [12]. Also, the recent epidemiological survey from Crellen et al. [13] reported that the efficacy of praziquantel against *S. mansoni* was reduced, likely because of frequent mass drug administration campaigns (MDA). Together with the rising risk of drug resistance due to an intense use of the same drugs and the lack of lead molecules in the development pipeline, the discovery of new anthelmintic treatments is urgent [12, 14, 15].

As the expected return on investment for helminthiases is negligible, drug repurposing represents a sustainable approach and an effective strategy to expand the pool of active molecules, in particular when using veterinary anthelmintics as starting point [16]. Emodepside, is a broad-spectrum veterinary anthelmintic licensed under the name of Profender® and Procox® and is used in combination with praziquantel and toltrazuril, respectively [17]. Its activity has been demonstrated against a wide range of nematodes in the veterinary field [18–25]. Repurposing emodepside for human use started more than ten years ago with preclinical studies against filarial nematodes which may be considered surrogates of human filarial infections [26]. These promising results triggered in 2016 a phase I study with emodepside in healthy volunteers, which was then completed by single and multiple ascending dose studies [17, 27].

Aiming at possibly expanding its range of application in human medicine and in order to broaden previous work on laboratory models, we thoroughly tested emodepside against seven species of helminths *in vitro* and *in vivo* [28–30]. The drug was tested first *in vitro* on both larval and adult stages of *T. muris*, *A. ceylanicum*, *N. americanus*, *S. ratti* and *H. polygyrus*, as well as on *S. mansoni* and *S. haematobium*. The activity of emodepside was next tested *in vivo* in animal models infected *with T. muris*, *A. ceylanicum* and *N. americanus*. The experimental flow for the study on nematodes is presented in Fig. 1.

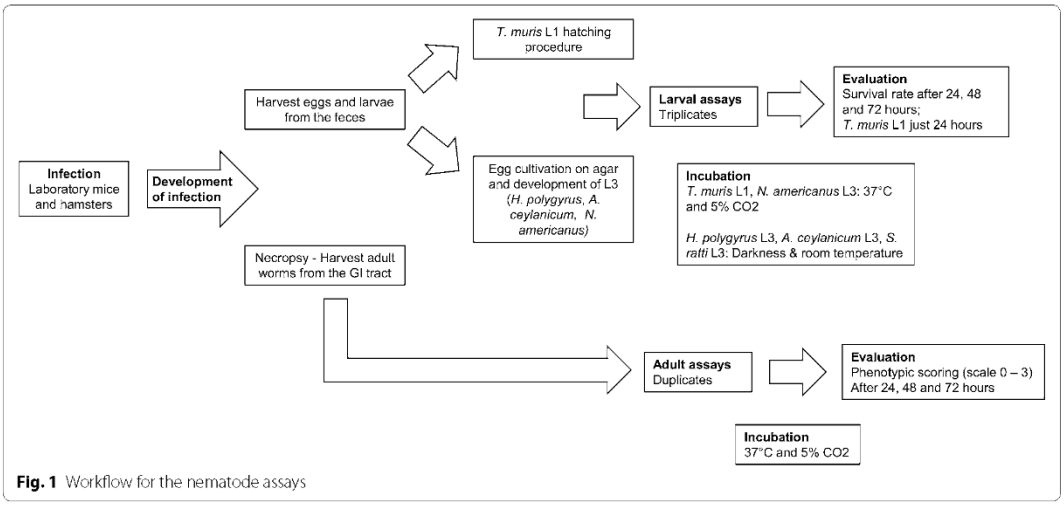

Fig. 1 Workflow for the nematode assays

Methods

Drugs

Emodepside was purified by preparative high-performance liquid chromatography (HPLC) from the commercially available topical solution for cats Profender® (Bayer, Leverkusen, Germany). Profender® was diluted 1:1 (v/v) with acetonitrile (ACN), filtered (0.22 µm) and injected (10 mL) into a preparative HPLC (Varian ProStar system). The purification was performed on a Reprosil® 100 C18, 7 µm, 250 × 40 mm column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with double distilled water, 0.1% Trifluoroacetic acid (TFA) as solvent A and HPLC grade ACN (Buchs, Sigma-Aldrich) as solvent B at 20 ml/min flowrate. The following linear gradient was used: 0 min 20% B; 0.5 min 20% B; 34 min 100% B; 40 min 100% B. Fractions containing emodepside were then combined and lyophilized.

Emodepside's ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker 400 and 500 spectrometers and referenced to residual solvent peaks. LC-MS spectra of the purified compound were measured on an Acquity™ (Waters system) coupled to an Esquire HCT from Bruker (Bremen, Germany) using an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm) at 0.6 ml/min flow with a linear gradient of A (double distilled water 0.1% v/v formic acid) and B (ACN 0.1% v/v formic acid); t = 0 min, 5% B; t = 0.25 min, 5% B; t = 1.5 min, 100% B; t = 2.5 min, 100% B. NMR and MS spectra corresponded to that previously reported for emodepside (Segment solid-phase total synthesis of the anthelmintic cyclooctadepsipeptides PF1022A and emodepside) [31].

Levamisole was purchased in powder from Sigma-Aldrich (Buchs, Switzerland). Stock solutions of emodepside and levamisole (10 mM) were dissolved in pure dimethyl sulfoxide (DMSO, Sigma-Aldrich, Buchs, Switzerland) and stored until use at − 20 °C.

Culture media

RPMI 1640 (Gibco, Waltham MA, USA) medium supplemented with 5% amphotericin B (250 µg/ml, Sigma-Aldrich, Buchs, Switzerland) and 1% penicillin 10,000 U/ml, and streptomycin 10 mg/ml solution (Sigma-Aldrich, Buchs, Switzerland) was used for the assays with *T. muris* adults and stage 1 larvae (L1), *H. polygyrus* adults and supplemented additionally with 10% inactivated fetal calf serum (iFCS; Bioconcept AG, Allschwil, Switzerland) for *T. muris* L1 hatching medium. RPMI 1640 supplemented with 5% amphotericin B (250 µg/ml), 1% penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution and 1% of the antibiotics mixture developed by Mäser et al. [32] was used for the *H. polygyrus* third stage larval (L3) assays. Adult *S. mansoni*, *S. haematobium* and *S. ratti* were incubated in RPMI 1640 medium supplemented with 5% iFCS and 1% penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution. Phosphate-buffered saline (PBS, Sigma-Aldrich, Buchs, Switzerland) supplemented with 1% penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution was used to incubate *S. ratti* L3 and to wash the adult worms. For the *S. mansoni* newly transformed schistosomula (NTS) assays, M199 medium (Gibco, Waltham MA, USA) supplemented with 5% iFCS and a mixture of antibiotics was used for the incubation [33]. *Ancylostoma ceylanicum* and *N. americanus* L3 stages were incubated in Hanks' balanced salt solution (HBSS; Gibco, Waltham MA, USA) supplemented with 10% amphotericin B and 1% penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution. The adult hookworms were kept in HBSS supplemented with 10% iFCS, 5% amphotericin B (250 µg/ml) and 1% penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution.

Laboratory animals

Before the infection, all animals were left one week for acclimation in our facility. Three-week-old male Syrian golden hamsters (Charles River, Sulzfeld, Germany) were orally infected with 150 L3 of *A. ceylanicum* or subcutaneously with 150 *N. americanus* L3. Four-week-old female NMRI mice (Charles Rivers, Sulzfeld, Germany) were used for *S. mansoni* infection and injected subcutaneously with 100 cercariae. The same mice strain was used for growing *H. polygyrus* which were administered orally with 88 L3. Six-week-old female C57BL/6NRj mice (Janvier labs, Le Genest-Saint-Isle, France) were orally infected with 200 embryonated *T. muris* eggs. Three-week-old male Wistar rats (Janvier labs, Le Genest-Saint-Isle, France), were infected subcutaneously with 1300 *S. ratti* L3. Three-week-old male LVG Syrian Golden hamsters (Charles River, USA) were infected with *S. haematobium* cercariae at the Biomedical Research Institute (Atlanta, USA) before being sent to the Swiss Tropical and Public Health Institute.

All animals were kept in polycarbonate cages under environmentally-controlled conditions (temperature: 25 °C, humidity: 70%, 12:12 h light/dark photocycle) and had free access to tap water and rodent food. To guarantee a sustainable infection, dexamethasone (Sigma-Aldrich, Buchs, Switzerland) was supplied in the drinking water until 2 days before treatment for the NMRI mice infected with *H. polygyrus* (0.25 mg/l dexamethasone), the C57BL/6NRj mice infected with *T. muris* (1 mg/l dexamethasone) and the hamsters infected with the hookworms (0.5 mg/l dexamethasone). Animals were killed using the CO_2 method to collect the adult worms for the in vitro studies as described below.

Drug assays

To determine the half maximal inhibitory concentration (IC_{50}) of the drug, each compound and concentration was tested in triplicates in the larval assays and in duplicates in adult worm assays. Parasites incubated in wells containing culture medium and DMSO corresponding to the highest drug concentration, served as negative controls and were included in every *in vitro* assay.

Heligmosomoides polygyrus, S. ratti and *A. ceylanicum* L3 larvae were then kept in the dark and at room temperature for 72 h. *Strongyloides ratti* L3 plates were sealed with Parafilm (Faust AG, Schaffhausen, Switzerland) before incubation. *T. muris* L1, *N. americanus* L3, *S. mansoni* NTS and the adult assays of all seven species were kept in the incubator for 72 h at 37 °C and 5% CO_2.

In vitro tests on N. americanus and A. ceylanicum L3

Necator americanus and *A. ceylanicum* L3 were obtained from infected hamsters by the cultivation of eggs, gained by repeated filtration and centrifugation of the infected feces. The eggs were washed with tap water and cultivated on agar plates protected from light for 8 to 10 days at room temperature. The L3 were then kept in tap water supplemented with 5% amphotericin B (250 µg/ml), 1% penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution and used within 3 weeks. In each well of a 96-well plate (Sarstedt, Nümbrecht, Germany), 30 L3 were exposed to 4 serial dilutions (1:4) ranging from 0.016 µM up to 1 µM emodepside concentrations in a final volume of 200 µl.

In vitro tests on adult N. americanus and A. ceylanicum

Five weeks to six weeks post-infection (p.i.), the worms were collected directly from the hamster's intestines. In a 24-well plate (Sarstedt, Nümbrecht, Germany), 3 to 4 adult worms per well were incubated in 2.5 ml drug solution with 4 different concentrations ranging from 0.005 µM to 0.5 µM for *A. ceylanicum* and with concentrations of 0.01 µM, 0.1 µM and 1 µM for *N. americanus*.

In vitro tests on T. muris L1

Six weeks after the infection of the mice, *T. muris* eggs were gained by filtration of their feces and storage in tap water for three months in the dark. To obtain the first stage larvae, egg hatching was triggered by incubation with *Escherichia coli* (BL21 strain) in hatching medium for 3 to 4 h at 37 °C in a wet chamber. For the assay, the L1 suspended in a total volume of 100 µl medium were placed in each well of a 96-well plate containing 14 emodepside concentrations ranging from 0.098 µM to 100 µM. Wells that contained levamisole (25 µM or 100 µM) served as positive control. The assays were kept in the incubator for 24 hours.

In vitro tests on adult T. muris

Trichuris muris adult worms were collected manually from the intestines of infected mice, 41 days p.i. The drug assays were performed in 24-well plates. In each well, 2 to 3 adult worms were incubated with the drug (1:4 serial dilutions ranging from 0.039 µM to 10 µM) in a final volume of 2.5 ml.

In vitro tests on H. polygyrus L3

The eggs were collected 2 weeks p.i. from mice feces and placed on agar at room temperature for 8 to 10 days in the dark. Forty L3 were exposed to emodepside at 3 different concentrations (0.625 µM, 2.5 µM and 10 µM) in a final volume of 100 µl.

In vitro assay on adult H. polygyrus

Heligmosomoides polygyrus adults were collected 2 weeks p.i. when dissecting mice intestines. In each well of 24-well plates 3 to 4 adult worms were exposed to emodepside (1:2 serial dilutions ranging from 0.125 µM to 1 µM) in 2.5 ml culture medium.

In vitro studies on S. mansoni NTS

Schistosoma mansoni (Liberian strain) cercariae were harvested from infected *Biomphalaria glabrata* snails and were then transformed into NTS [33, 34]. The drug assays and phenotypic screening were performed as described previously [33].

In vitro studies on adult S. mansoni and S. haematobium

Seven weeks p.i. the worms were extracted from the rodent mesenteric veins. Three single flukes or 2 pairs were placed in a final volume of 2.4 ml in each well of a 24-well plate exposed to 1:3 serial dilutions ranging from 3.7 µM up to 33.33 µM emodepside.

In vitro studies on S. ratti L3

The L3 were obtained following the procedure described by Garcia (1998) [35]. Thirty L3 were exposed to emodepside (1:4 serial dilutions ranging from 0.039 µM to 10 µM) in a final volume of 100 µl.

In vitro studies on adult S. ratti

The infected rats were dissected 3 weeks p.i. The intestines of the rats were excised, opened and immerged in PBS supplemented with penicillin/streptomycin. They were incubated for 4 h at 37 °C and 5% CO_2 in order to

detach the nematodes from the intestinal wall. A maximum of 15 worms were then transferred into the wells of 24-well plates and placed in 2 ml culture medium containing emodepside at 6 different concentrations ranging from 50 µM to 0.25 µM.

Evaluation of the assays

The drug effect was evaluated 24, 48 and 72 h post-exposure. For evaluating the L3 assays, the larvae were stimulated if necessary by the addition of 50 µl (*T. muris* L1) to 100 µl (others spp.) hot water (≈ 80 °C) and the percentage of survival was determined by the ratio of moving larvae to the total number of larvae present in the well. The *N. americanus* L3 assay was an exception as the wells were stimulated by vigorous up and down pipetting. The adult worms of each parasite species were scored microscopically based on their phenotype, using a viability scale ranging from 3 to 0 (3: good motility and no morphological changes; 2: low motility and light changes in morphology; 1: very low motility and morphologically impaired; and 0: death). In case the adult worms did not move enough for a clear scoring, they were stimulated with hot water at the last evaluation time-point.

Trichuris muris in vivo studies

C57BL/6NRj mice were *orally* infected with 200 embryonated *T. muris* eggs. At 42 days p.i., the feces were collected and soaked for 1 hour in 0.9% sodium chloride, before the filtered suspension was examined under the microscope to determine the success of the infection. According to their infection intensities, the mice were equally assigned to the different groups. The compound was dissolved in 70:30 Tween 80-ethanol in ultrapure water (10% v/v) and was administered by gavage first at a dose of 75 mg/kg based on results from a previous study [28] followed by lower dosages from 1.25 to 10 mg/kg. Untreated mice served as controls. Until 3 days after drug administration the feces of the mice were examined for expelled worms. Six to seven days post-treatment, the animals were killed, their intestines were dissected, and the adult worms were collected and counted.

Ancylostoma ceylanicum and *N. americanus* in vivo studies

Hamsters were orally infected with 150 *A. ceylanicum* L3 or subcutaneously with 150 *N. americanus* L3. A fecal sample was collected from each hamster, just before treatment. The fecal samples were processed using an in-house sedimentation method to determine the infection intensity of each animal [36]. The different dosage and control groups were composed of hamsters evenly distributed depending on their infection status. *A. ceylanicum* infected hamsters were then treated on day 28 p.i. with a single oral dose of 2.5 mg/kg emodepside and *N. americanus* infected hamsters with a single oral dose of 1.25–10 mg/kg emodepside. Expelled worms were counted from each hamster from the collected feces until 72 h after treatment. One week post-treatment, the hamsters were euthanized and the worms remaining in their intestines were collected and counted.

Data analysis

For the *in vitro* drug sensitivity assays, all viability scores and larval survival counts were averaged across replicates and normalized to the control wells. The effect of emodepside was determined by normalizing the mean parasite survival rate to the control wells. Based on these values, the IC_{50} values were calculated using CompuSyn software (ComboSyn Inc., version 1.0), as well as the *r*-values (the linear correlation coefficient) that reflects the goodness of the fit. For each assay, a minimal *r* value and viability of the controls was required. The detailed selection criteria are presented in Table 1.

To assess the effect of the drug *in vivo*, the mean numbers of living worms recovered in treated animals were compared to the controls. The worm burden reduction (WBR) was calculated as described previously [16]. The worm expulsion rate (WER) was determined by the number of dead worms excreted in the feces during 72 h after treatment, over the total number of worms (alive and dead) recovered during the necropsy. The Kruskal-Wallis test (Statsdirect version 3.1.20) were used to determine statistical significance of WBR at a level of 0.05. The median effective dose (ED_{50}) values were calculated using CompuSyn software (ComboSyn Inc., version 1.0).

Results

In vitro studies

Table 1 and Additional files 1 and 2 summarize the mean IC_{50} values for each helminth species over 3 days post-drug exposure, except for *T. muris* L1 that were assessed after a 24 h incubation period. Emodepside showed IC_{50} values below 1 µM, within 24 h for all nematode species with the exception of *T. muris* L1 that had an IC_{50} of 3.7 µM. The highest drug activity was observed for adult hookworms. After one day of incubation, emodepside was highly active against adult *A. ceylanicum* and *N. americanus* with IC_{50} values below 0.005 µM, which were reduced by half over the incubation period ($IC_{50} < 0.0025$ µM). Decreasing IC_{50} values were also recorded over the 72 h incubation period for the adult worms of every species tested. Against *T. muris* adults emodepside showed an IC_{50} value below 0.3 µM after 24 h of drug exposure. This value decreased below 0.05 µM after another day of incubation. IC_{50} values in the range of 0.2 µM to 0.8 µM were observed for adult *H. polygyrus* and *S. ratti*.

Table 1 Mean IC_{50} values *in vitro* of emodepside tested on larval and adult stages of different helminths

Species	Replicates	No. of parasites per well[a]	24 hours Mean $IC_{50} \pm SD$ (μM)	48 hours Mean $IC_{50} \pm SD$ (μM)	72 hours Mean $IC_{50} \pm SD$ (μM)
T. muris (L1)	9	20–40	3.73 ± 6.54	–	–
T. muris (adults)	2	2–3	0.28 ± 0.15	0.043 ± 0.0089	0.022 ± 0.013
H. polygyrus (L3)	2	30	0.78 ± 0.086	0.9 ± 0.034	0.48 ± 0.05
H. polygyrus (adults)	3	3–4	0.57 ± 0.42	0.21 ± 0.13	0.25 ± 0.16
A. ceylanicum (L3)	2	30	0.14 ± 0.041	0.086 ± 0.08	0.25 ± 0.051
A. ceylanicum (adults)	3	2–3	0.0044 ± 0.0021	0.0015 ± 0.00078	0.0024 ± 0.002
N. americanus (L3)	2	30	0.77 ± 0.52	0.15 ± 0.069	0.083 ± 0.033
N. americanus (adults)	2	2–3	0.0031 ± 0.0011	0.0029 ± 0.0018	0.0021 ± 0.0012
S. ratti (L3)	4	30	0.73 ± 0.5	0.27 ± 0.21	0.25 ± 0.14
S. ratti (adults)	3	5–15	0.75 ± 0.57	0.21 ± 0.29	0.36 ± 0.32
S. mansoni (NTS)	2	100	7.79 ± 1.57	6.92 ± 0.21	2.48 ± 0.78
S. mansoni (adults)	2	2–3	50.4 ± 3.32	37.27 ± 10.47	34.1 ± 9.18
S. haematobium (adults)	2	2–3	40.51 ± 24.96	40.25 ± 6.49	36.73 ± 6.49

[a] Each assay included 2 to 3 wells per concentration/condition

Notes: The inclusion criteria used in our analysis were different for each stage and parasite. Minimal survival rates (larvae) or viability scores (adults and NTS) and IC_{50} r-values considered acceptable were as follows: *T. muris* L1 (survival rate: 60%; R = 0.7), adults (score: 2.5; R = 0.8); *H. polygyrus* L3 (70%; 0.9), adults (1.9; 0.8); *A. ceylanicum* L3 (55%; 0.7), adults (2; 0.7); *N. americanus* L3 (60%; 0.75), adults (2; 0.8); *S. ratti* L3 (60%; 0.75), adults (2; 0.7); *S. mansoni* NTS (2; 0.75), adults (1.5; 0.85); *S. haematobium* adults (2; 0.7)

Abbreviation: SD, standard deviation

IC_{50} values for all nematode L3 ranged from 0.9 μM to 0.08 μM. The IC_{50} values of *S. ratti* and *N. americanus* L3 decreased, while they decreased and increased over the 3 days incubation period for *H. polygyrus* and *A. ceylanicum* larvae, respectively. For the schistosomes *S. mansoni* and *S. haematobium*, IC_{50} values above 30 μM were calculated for adult worms while decreasing IC_{50} from 7.8 μM after 24 h to 2.5 μM after 72 h were observed for *S. mansoni* NTS.

For all the nematode species, the IC_{50} values were higher for the larval stages than for the adult worms. *Strongyloides ratti* was the only species where the difference between the two life-stages was less than 2-fold. The IC_{50} on adult worms was of about twice as high than for the larval stage for *H. polygyrus*, 13 times for *T. muris* and between 30 to 250 times for the hookworms *N. americanus* and *A. ceylanicum*. The exact opposite was observed for *S. mansoni*. At the 24 h and 48 h evaluation time-points, the IC_{50} values on NTS were 5 to 7 times lower than the ones measured on adult *S. mansoni*. When assessed after 3 days, a 13-fold difference was observed between *S. mansoni* NTS and the adult worms.

Emodepside was lethal (100% effect) *in vitro* on *A. ceylanicum* adults, *S. ratti* L3 and both life stages of *N. americanus*. Drug effects above 90% were observed at a concentration of 25 μM emodepside for *T. muris* L1 and at a concentration of 2.5 μM for adult worms. This was also the case for *A. ceylanicum* when incubated at 1 μM (L3) or 0.1 μM (adults) and *N. americanus* at 0.25 μM (L3) or 0.1 μM (adults). Effects of more than 75% were reached at 2.5 μM for *H. polygyrus* L3 and at 0.5 μM for adult worms. Emodepside had an effect above 75% at a concentration of 2.5 μM on *S. ratti* (L3) and showed a similar effect at a 10 times lower concentration when tested on adult worms.

In vivo studies

The worm expulsion rates and worm burden reductions obtained with single-dose, oral emodepside against *T. muris* are summarized in Table 2. A high dose of 75 mg/kg emodepside resulted in complete elimination of all worms. At doses of 10 mg/kg and 2.5 mg/kg worm burden reductions of 85.9% and 69.6% and worm expulsion rates of 62.0% and 60.9%, respectively were observed. The lowest dose tested (1.25 mg/kg) showed low activity, with a worm burden reduction of 73.9% and worm expulsion rate of 5.3%. The worm burden reductions obtained with emodepside (all doses *versus* controls) were statistically significant ($t = 7.18$, $P = 0.0073$). Based on worm burden reductions we calculated an ED_{50} value of 1.2 mg/kg.

Single doses of 10 mg/kg and 5 mg/kg cured all *N. americanus*-infected hamsters. A worm burden reduction of 93.8% and a worm expulsion rate of 87.5% were observed at a dose of 2.5 mg/kg. Moderate activity (worm burden reduction of 43.8% and worm expulsion rate of 40.0%) was observed in *N. americanus*-infected hamsters with the lowest dose tested of 1.25 mg/kg (all doses, $t = 3.52$, $P = 0.06$). An ED_{50} value of 0.5 mg/

Table 2 *In vivo* dose response relationships of emodepside on *A. ceylanicum*, *N. americanus* and *T. muris*

	Dose (mg/kg)	Mean no. of worms ± SD	Worm expulsion rate (%)	Worm burden reduction (%)	*P*-value	ED_{50} (mg/kg)
T. muris						
Emodepside	75[c]	0	100	100	0.007[a]	1.2
	10[c]	18.8 ± 20.8	62.0	85.9		
	2.5[c]	36.5 ± 30.8	60.9	69.6		
	1.25[d]	133 ± 27.9	5.3	73.9		
Control 1[c]		120.8 ± 12.0	0	–		
Control 2[d]		121.7 ± 4.7	0	–		
A. ceylanicum						
Emodepside	2.5	0	100	100	0.014[a]	
Control		21.3 ± 2.6	0.6	–		
N. americanus						
Emodepside	10[c]	0	100	100	0.060[a]	0.5
	5[c]	0	100	100		
	2.5[d]	0.25 ± 0.5	87.5	93.8		
	1.25[d]	2.25 ± 2.3	40.0	43.8		
Control 1[c]		5.5 ± 6.1	5.6	–		
Control 2[d]		4.0 ± 1.4	0	–		

[a] Kruskal-Wallis test was applied to determine statistical significance on worm burden reduction of all doses *versus* controls

[c] Control 1 was used for this dose

[d] Control 2 was used for this dose

kg was determined for emodepside in *N. americanus*-infected hamsters. To confirm that emodepside also acts on *Ancylostoma* spp. the minimum effective dose on *N. americanus* of 2.5 mg/kg was tested in the *A. ceylanicum* hamster model, which resulted in cure of all animals ($t = 6.05$; $P = 0.014$).

Discussion

Given a promising activity against a wide range of resistant worm infections and its unique mode of action, it is worthwhile to evaluate the activity of emodepside against other helminth infections including STH and schistosomiasis. For the first time we thoroughly tested emodepside against a wide range of laboratory models for these diseases.

Both the larval and the adult nematode and schistosome stages were screened phenotypically in presence of emodepside over a time course of 72 hours followed by *in vivo* studies. As emodepside belongs to the group of cyclooctadepsipeptides that are known to be very active against different animal gastrointestinal nematodes and filarial parasites, good antinematicidal activity was expected [20, 30, 37–40]. The drug showed a high efficacy *in vitro* against all the nematode species and was highly effective against the two hookworms (*A. ceylanicum* and *N. americanus*) and the whipworm (*T. muris*). On the contrary, the effect of emodepside on schistosomes, remained only moderate.

We further investigated the efficacy of emodepside *in vivo* on rodents infected with *T. muris*, *A. ceylanicum* and *N. americanus*. Overall, the promising *in vitro* activity of emodepside was confirmed *in vivo*, where the drug demonstrated high worm burden reduction rates, even when administered orally as a low, single dose regimen.

These results were consistent with previous findings. The ED_{50} value obtained *in vivo* for *T. muris* (ED_{50} of 1.2 mg/kg) was very similar to the one reported by Kulke et al. [28]. Our study also confirms the good activity of emodepside *in vitro* against larval and especially adult stages of the nematodes *S. ratti* and *H. polygyrus* that was so far only described *in vivo* [29].

Emodepside performed much better *in vitro* and *in vivo* than albendazole, levamisole and pyrantel pamoate, the standard drugs used against STH infections tested in a previous study [36], where none of the standard drugs showed *in vitro* activity against adult *A. ceylanicum* (Table 3). Moreover, only a moderate *in vitro* efficacy against *T. muris* and *N. americanus* was reached by levamisole and pyrantel pamoate. In contrast, emodepside was very active *in vitro* against the larvae and adult worms of all three species. *In vivo*, albendazole was the only drug that performed as well as emodepside on *A. ceylanicum* infected hamsters (Table 4). While none of

Table 3 Mean IC_{50} values (µg/ml) after 72 hours drug exposure on L3 and adult stages of *A. ceylanicum*, *N. americanus* and *T. muris* of emodepside compared to the ones of albendazole, levamisole and pyrantel pamoate

Species	Mean IC_{50} (µg/ml) after 72 hours of drug incubation			
	Emodepside	Albendazole[a]	Levamisole-HCl[a]	Pyrantel pamoate[a]
T. muris (L1) after 24 h	4.18			
T. muris (L3)	–	≥ 200	33.1	95.5
T. muris (adults)	0.022	≥ 200	16.5	34.1
A. ceylanicum (L3)	0.28	32.40	1.60	90.9
A. ceylanicum (adults)	0.0027	≥ 100	≥ 100	≥ 100
N. americanus (L3)	0.090	≥ 100	0.50	2.0
N. americanus (adults)	0.0024	≥ 100	13.40	7.6

[a] All values for this drug are taken from the study of Tritten et al. [36]

Table 4 *In vivo* dose response relationships of emodepside, albendazole, levamisole and pyrantel pamoate on *A. ceylanicum*, *N. americanus* and *T. muris*

Drug	Dose (mg/kg)	Worm expulsion rate (%)	Worm burden reduction (%)
T. muris			
Emodepside	75	100	100
	10	62.0	85.9
	2.5	60.9	69.6
	1.25	5.3	73.9
Albendazole[a]	600	49.4	20.2
Levamisole-HCl[a]	200	90.5	95.9
Pyrantel pamoate[a]	300	9.4	0
A. ceylanicum			
Emodepside	2.5	100	100
Albendazole[a]	1.25	70.5	87.8
	2.5	100	100
	5	100	100
Levamisole-HCl[a]	10	44.3	60.2
Pyrantel pamoate[a]	10	63.4	87.2
N. americanus			
Emodepside	10	100	100
	5	100	100
	2.5	87.5	100
	1.25	40.0	62.5
Albendazole[a]	10	100	100
	5	69.6	70.8

[a] All values for this drug are taken from the study of Tritten et al. [36]

the three standard drugs cured mice harboring a *T. muris* infection, emodepside was fully active at a concentration of 75 mg/kg.

Although emodepside was also very active *in vitro* on *H. polygyrus* L3 (with IC_{50} values below 1 µM), previous studies reported lower *in vivo* sensitivity of *H. polygyrus* larvae compared to the larval stages of other nematode species. This decreased activity against *H. polygyrus* larvae *in vivo* was explained by their presence burrowed deep into the gastro-intestinal tissues which was likely to protect them from the drug [29, 41]. Aiming at a formulation of emodepside active on all parasite stages, *in vivo* studies should preferably be performed at both early and late stages of infection. Hence future *in vivo* studies should evaluate the activity against the early developmental stages of the nematodes.

In our study, the *in vitro* activity of emodepside against the nematode species was higher in adult worms than in the larvae. Such difference in anthelmintic susceptibility between the early and the late developmental stages of the parasite was reported previously [42–44]. A differential expression of emodepside molecular target(s) between the parasites life-stages or differences in the permeability of the cuticle (or both) may account for it [42, 45]. Moreover, a similar trend was observed *in vivo* in other studies on *Nippostrongylus brasiliensis*, *S. ratti* and *H. polygyrus* [29]. However, for *S. mansoni*, we documented the opposite finding, with revealing lower IC_{50} values than the adults.

We observed in our *in vitro* studies that whereas the morphology of the parasites seemed not affected by the drug, often no motility or pharyngeal pumping movement could be detected. This observation corroborates the suggested mechanism of action of emodepside [30, 37, 46–49]. Although its exact mechanism of action is not fully understood yet, the drug is known to bind to two different targets of the neuromuscular junction, the evolutionary conserved calcium-activated potassium channel slowpoke 1 (SLO-1) and the latrophilin receptors LAT-1/LAT-2 [30, 37, 47, 50, 51]. In nematodes, the over activation of the SLO-1 receptors by emodepside is likely to induce a potassium efflux triggering a hyperpolarization of the neurons that results in a decreased synaptic

transmission and muscle contraction, leading notably to a paralysis of the worm pharynx [37, 42, 47, 50, 52]. The specificity of emodepside towards the nematode channel subunits might account for its lack of efficacy against the trematodes [49].

The *in vitro* assay read-out methods varied among the different parasites and life-stages. While visual scoring of adult worms is generally straightforward for a trained operator, evaluating larval assays was more challenging, especially for *T. muris* and *S. ratti* larval assays. This led to a high variability between the different assays and explained a higher number of replicates reported compared to the other parasites. This finding urges the optimization and development of more accurate assessment methods.

Conclusions

Our study confirms that emodepside represents a promising broad spectrum human anthelmintic drug candidate with intriguing activity against a wide range of nematodes. Since emodepside is already well characterized in veterinary medicine and undergoing clinical development for onchocerciasis, and the activity observed in this study against different nematodes was similar to previous findings on filarial worms [26] this will allow a significant shortcut developing this drug for human STH. A drug development plan should therefore be established to fill the missing gaps required so that emodepside will soon be available for the treatment of both filarial and STH infections.

Additional files

Additional file 1: Figure S1. Mean IC_{50} overtime for the nematode L3.

Additional file 2: Figure S2. Mean IC_{50} overtime for the adult nematodes.

Abbreviations

IC_{50}: half maximal inhibitory concentration; ED_{50}: median effective dose; STH: soil-transmitted helminthiases; WHO: World Health Organization; MDA: mass drug administration; HPLC: high-performance liquid chromatography; ACN: acetonitrile; TFA: trifluoroacetic acid; LC-MS: liquid chromatography-mass spectrometry; NMR: nuclear magnetic resonance; MS: mass spectrometry; DMSO: dimethyl sulfoxide; RPMI: Roswell Park Memorial Institute; L1: stage 1 larvae; L3: stage 3 larvae; iFCS: inactivated fetal calf serum; PBS: phosphate-buffered saline; NTS: newly transformed schistosomula; HBSS: Hanks' balanced salt solution; CO_2: carbon dioxide; p.i.: post-infection; WBR: worm burden reduction; WER: worm expulsion rate; SLO-1: calcium-activated potassium channel slowpoke 1; LAT-1/LAT-2: latrophilin receptors; 3 R: Replacement, Reduction, Refinement.

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Authors' contributions

TK, VP and JK designed the study. TK and VP performed the experiments *in vitro*. CH infected, treated and dissected the animals involved in this study. AN synthetized emodepside powder. TK, VP and JK analyzed the data and wrote the first draft of the manuscript that was revised and completed by IS and AN. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusion of this article are included within the article and its additional files.

Ethics approval and consent to participate

All *in vivo* studies were carried out at the Swiss Tropical Institute (Basel, Switzerland), in accordance with both cantonal (license no. 2070) and Swiss national regulations on animal experimentation and complied with the 3Rs principles.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Chapter 5

Adapting an anticestodal movement-based *in vitro* drug screening method on *S. mansoni* newly-transformed schistosomulae

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This chapter is a working manuscript

Background

Praziquantel as the standard drug in use against schistosomiasis is not optimal for all patients and its large-scale administration as preventive chemotherapy continues to increase the risk of resistance emergence. No new molecule has recently entered the drug development pipeline. The absence of a simple, fully automated screening system is an important limitation for researchers working in antischistomal drug discovery. Building on the work of Ritler and colleagues (2017) that described a movement-based screening method for cestodes, we propose here to adapt this assay for newly transformed schistosomula (NTS) assays.

Results

The method detected the activity of 6 reference compounds (albendazole, clofazimine, ivermectin, mefloquine, MMV665807, and praziquantel) at 0.03 - 10 µM on NTS after 1, 6, 12, 18, 24, 48, and 72 hours of incubation. While no change in motility was observed for albendazole up to 72 h post-incubation, it decreased dose- and time-dependently for all other drugs. After 24 h of drug-incubation, dose-response curves were obtained and the average movement as assessed by the newly adapted method correlated well (R^2 > 50%) with the values obtained by the gold standard method, which is based on phenotypic assessment and scoring.

Conclusions

Here, we provide a proof of principle that the objective, semi-automated system described by Ritler and colleagues (2017) could be adapted and optimized for NTS screening by applying minimal changes to the originally described method.

Keywords: Newly transformed schistosomula, microscope, *S. mansoni*, anthelminthic, drug screening

Background

Schistosomiasis is a parasitosis listed as a neglected tropical disease that infects more than 200 million people around the world (Colley et al., 2014). The primary infection causes an acute form known as “Katayama fever” (Colley et al., 2014; Ross et al., 2007). This generally happens several weeks after the penetration of the free-swimming stage, the cercariae. After losing their tails in the process, they enter the blood circulation. Referred as schistosomulae, they eventually reach the lungs (Colley et al., 2014; McManus et al., 2018). If not appropriately treated, the maturation and the persistence of parasites in the organism followed by the release of their eggs trigger an immunopathological response leading to impairing chronic morbidities (Colley and Secor, 2014; Schwartz and Fallon, 2018). The morbidities associated with schistosomiasis are responsible for 1.9 million disability-adjusted life years (DALYS) and have important socio-economic repercussions (McManus et al., 2018).

The risk of resistance emergence towards praziquantel, the only antischistosomal treatment to date, is already a reality in the veterinary field, and it is enhanced by its large-scale distribution as preventive chemotherapy (Doenhoff et al., 2008; Geerts and Gryseels, 2001; Rose et al., 2015; WHO, 2006). No alternative drug against schistosomiasis has been developed yet and the number of advanced candidates is very scarce. Thus, the identification of new active molecules against schistosomes is urgent and critical for populations at risk, and also to achieve the control and elimination goals aimed at by the international community (Bergquist et al., 2017; Pedrique et al., 2013) .

Early drug discovery against schistosomiasis was limited by the impossibility to cultivate worms *in vitro* and the lack of automated drug screening systems (Geary et al., 2015; Keiser, 2010). Manual evaluation based on the phenotype of adult worms is, until now, the gold standard method to screen for drug new candidates (Lombardo et al., 2019; Ramirez et al., 2007). Despite a recent attempt to cultivate lung-stage schistosomulae (Frahm et al., 2019), culturing schistosomes, as for many other helminths, requires the use of laboratory animals. Due to ethical reasons and the costs associated with the *in vivo* procedures, many laboratories introduced larval stage screening for primary hit selection (Abdulla et al., 2009;

Mansour and Bickle, 2010; Tekwu et al., 2016). However, phenotypic drug assays on the larval stage of *S. mansoni*, referred hereafter as newly transformed schistosomula (NTS), remain time-consuming and subjective.

Recently, Ritler and colleagues (2017) validated a semi-automated motility read-out method for anticestodal drug screening based on *Echinococcus multilocularis* protoscoleces. Given the similar size (*E. multilocularis* protoscoleces: 150–350 µm in length, NTS: 100-150 µm) and the conditions under which either the *E. multilocularis* protoscoleces or *S. mansoni* NTS are usually tested, we explore here the feasibility of applying the same method for early antischistosomal drug discovery.

Method

Parasites, medium and drugs

S. mansoni parasites were bred, maintained and collected as described by Lombardo and colleagues (2019). After mechanical transformation, the NTS were suspended in M199 medium (Gibco, Waltham MA, USA) supplemented with 5 % fetal calf serum (FCS, Bioconcept AG, Allschwil, Switzerland), 100 U/ml penicillin, 10 mg/ml streptomycin (Sigma-Aldrich, Buchs, Switzerland), and a cocktail of antifungals (Mäser et al., 2002). Albendazole, clofazimine, ivermectin, mefloquine, and praziquantel were purchased from Sigma-Aldrich, and MMV665807 from Princeton Bio Molecular Research (Monmouth Junction, NJ, USA). Stock solutions of 10 mM were prepared in pure dimethyl-sulfoxide (DMSO) and stored at -20°C. For each assay, predilutions in supplemented M199 were made in order to obtain drug concentrations of 40 to 0.12 µM in a 1:3 dilution series, and 0.4 % of DMSO. 5 µl of these pre-dilutions were distributed into a 384 well-plate (Greiner bio-one, via Huberlab, Aesch, Switzerland). Then, 15 µl of the NTS suspension containing between 15 and 30 parasites/µl were distributed to each well using a multidrop combi peristaltic pump (Thermo Fisher Scientific, Reinach, Switzerland). Like this, final drug concentrations of 10 to 0.3 µM of each drug in 0.1 % DMSO were reached. Each drug concentration was

tested in six replica, and two wells as negative control, where the parasites were exposed to 0.1% DMSO. Before assay-reading, each plate was covered with a pressure-sensitive seal (Greiner bio-one, via Huberlab) and spin down at 50 x g for 30 seconds. The assay was repeated three times independently.

Assay read-out

Assays were run in a live cell imaging system at 37 °C, where screening plates were incubated and pictures taken at 1, 6, 12, 18, 24, 48, and 72 hours. At each timepoint, two images were taken for each well at a 5 seconds interval by a Nikon TE2000E microscope connected to a Hamatsu ORCA ER camera , and the NIS Elements software Version 4.40 including the additional module JOBS. The difference in pixels between the two images of each well was determined in ImageJ (version 1.49), using the same macro as described by Ritler et al., 2017, and with a grey value threshold of 220. After excluding the minimal and maximal values of each replica, averages of movement in percentage of the DMSO controls and standard deviations were calculated in Microsoft Office Excel (2016, version 1902).

Phenotypical assessment of parasites was performed as described by Lombardo and colleagues (2019). The viability in percentage was calculated by the following formula: Viability = 100 - drug effect (%). Linear correlation analysis was performed in R (version 3.3.2) between phenotypical viability assessment after 24 h and the respective movement indices.

Results

NTS control-incubated in 0.1 % DMSO are stable over 48 hours

Control wells included NTS distributed over the whole 384 well plate and incubated with 0.1 % DMSO. In these wells, the average movement of NTS remained between 3697.4 (± 1673.5) and 4286.0 (± 1596.4) between the first assessment point at 1 h of incubation and after 48 h of incubation. After 72 h of incubation, the movement decreased to 2578.7 (± 2573.4). For these reasons, drug-assessments were performed up to 48 h in the following.

Time-dependent effects of standard compounds against NTS movement

Time-dependent effects of compounds at 10 µM on the movement of NTS are depicted in figure 1. The average movement calculated relative to the DMSO control remained constant and over 100 % for albendazole, indicating no effect of this drug. The same was also observed for praziquantel that induced even higher values of up to 150 %. At 48 h of incubation with praziquantel, the movement decreased to 45.8 ± 12.3. On the contrary, MMV665807 was strongly effective against NTS movement after 1 h of incubation. Both clofazimine and ivermectin induced movement slightly below 150 % that progressively decreased overtime to reach values of 1.5 % after 24 h and below 1 after 48 h. In presence of mefloquine, the movement of NTS was below 50 % after 1 h, followed by a steep decrease that led to absence of any movement after 48 h of drug incubation.

Dose-dependent effects of standard compounds against NTS movement after 24 h

Effects of standard compounds after 24 h of incubation were compared over a concentration series from 10 to 0.03 µM (figure 2). The average movement of NTS incubated with either praziquantel or albendazole stayed above 100 % at all drug concentrations. At 10 µM, all the other drugs reduced motility

to below 2 %, mefloquine was even completely lethal (no movement at all recorded). At 30 µM, movement increased to at 3 µM, and 38.4 % ± 37.6 for MMV66580. At concentrations of 1 µM or lower, there was no more reduction of NTS motility observed with any of the tested compounds.

Automated NTS motility assessment correlates well to manual phenotypic scoring

The average viability of the parasites as assessed by phenotypic screening correlated well with the automatedly recorded relative motility values, with the exception of albendazole (R^2 = -0.32) and praziquantel (R^2 = -0.53). The highest correlation was found for clofazimine (R^2 = 0.84). It was followed by Ivermectin and MMV66580 (R^2 = 0.79), then mefloquine (R^2 = 0.63).

Discussion

With the international effort to control schistosomiasis (and other neglected tropical diseases), and the success of open drug discovery initiatives such as the Medicines for Malaria Venture (MMV) libraries, large compound libraries are more available for antischistosomal screenings. Many laboratories employ a pre-screen on NTS to increase the throughput and overcome costs and ethical issues associated with adult worm testing. However, the respective assessment methods have remained essentially based on visual inspection of the parasite's phenotype (Keiser, 2010; Lombardo et al., 2019; Ramirez et al., 2007). These methods are laborious, therefore, different studies attempted to replace phenotypic scoring by metabolic or real-time monitoring assays (Manneck et al., 2011; Panic et al., 2015a; Peak et al., 2010; Rinaldi et al., 2015; Smout et al., 2010). Image-based automated systems are also under development but for now, phenotypic screening of compounds against NTS seems to be the most straightforward and reliable method for antischistosomal hit identification (Geary et al., 2015; Mansour and Bickle, 2010; Tekwu et al., 2016). Phenotypic screening has the main advantage of being very cost-effective as it requires no special equipment. However, it is time-consuming, and relatively subjective. The anticestodal motility-based screening described by Ritler et al. (2017) employs a simple algorithm that can be applied to any microscope that allows digital images to be taken. Thus, no extremely specialized equipment is needed. For this reason, we applied the same method developed for anticestodal screening by Ritler et al. (2017) to NTS that we distributed to 384 wells, and incubated with various standard compounds. We applied the same device and settings as previously described, with the exception of a shorter time interval between the pictures (5 instead of 10 seconds) and another grey value threshold (220 instead of 230). The motility-based method has the advantage of being an objective, semi-automated method, and it allows to detect also very small movements, which are not visible by sheer eye. In addition, as pictures of every condition are taken for motility calculations, these allow also for morphological comparisons later on. Robust linear correlations between the average movement relative to the DMSO control and the phenotypic viability were above 0.5 for all the drugs except for albendazole and praziquantel that had no

lethal activity on the NTS. The relative movement was increased in presence of praziquantel which could also reflect the increased frequency of spastic contractions usually observed in presence of this drug (Xiao et al., 1985). Testing MMV66580 confirmed its high activity on NTS, even at low concentrations, which was described already in an earlier study (Ingram-Sieber et al., 2014). The already known strong activities of clofazimine and mefloquine against NTS after 24 h of drug-incubation was also confirmed (Panic et al., 2015b; Pasche et al., 2018). Moreover, this study highlighted a strong antischistsomular activity in vitro that has so far not been extensively described.

These preliminary results suggest that this rather simple approach, with minimal changes made to the original method used for anticestodal drug screening, was effective at capturing effects on motility, including specific known features of the tested drugs. We noted that later time-points than 48 h of incubation led to decreased motility, most likely due to the small volumes of medium used and subsequent nutrient depletion. This suggest that earlier time points are better to use in this system and provide a clearer read-out. For optimization, further tests and a complete validation of the method with more drugs and conditions, i.e. DMSO concentrations, temperature etc., would be necessary.

The major limitation of the method described here is that it relies only on parasite movement. Hence, other phenotypes as well as abnormal movement might be missed unlike with visual assessment (Mansour and Bickle, 2010). However, morphological effects can be assessed based on the pictures taken for motility calculation. Automated and semi-automated systems, especially if coupled with image recognition algorithms, are very promising and likely to increase the throughput of antischistosomal drug assays as they would enable a continuous read-out and would not require the presence of personal at specific time-points. They would however still depend on the access to the parasites, which is often the actual limiting factor for antischistosomal drug testing (Lombardo et al., 2019).

Conclusion

Antischistosomal drug discovery is limited by the lack of automated screening devices. Here, we provide a proof of principle study where a semi-automated image-based system similar to the one used in anticestodal drug research could be applied for NTS screening. The method allows objective and medium-throughput screening of compounds against NTS, and at the same time morphological effects are recorded. Although this method should be optimized and validated with larger libraries, it could be used in laboratories with access to a similar device for a first triage of compounds to further test by phenotypical screening.

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Figures

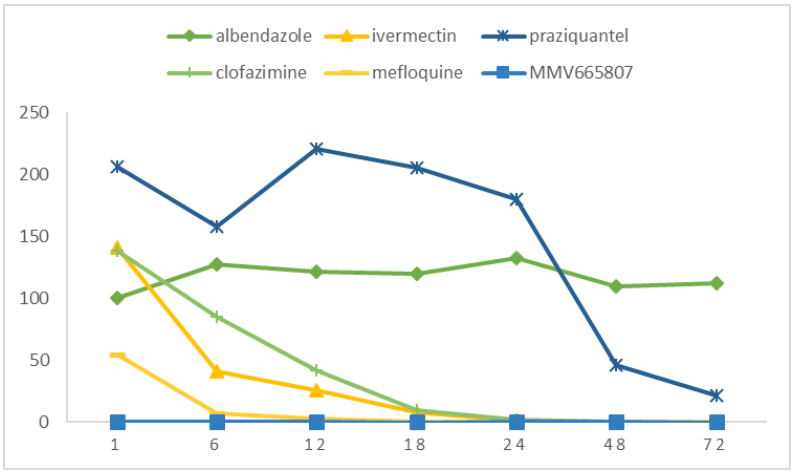

Figure 1: relative movement to the DMSO control (%) over 72 h when the NTS were incubated with 10 µM of the different drugs

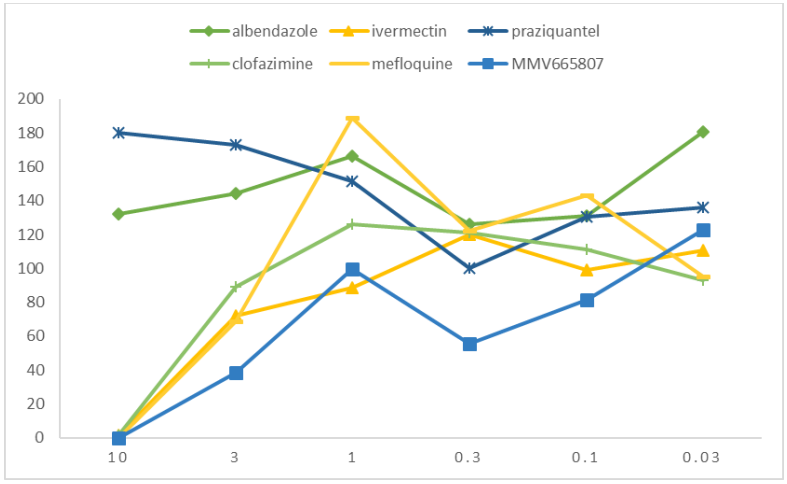

Figure 2: relative movement to the DMSO control (%) at 24 h with different drug concentrations

Chapter 6: General discussion

6.1 Context and principal outcomes

With the recent commitment of the international community, set in stone in 2012 by the London Declaration on neglected tropical diseases (NTDs), preventive chemotherapy has been intensified for schistosomiasis (Anderson et al., 2012; Lo et al., 2017; WHO, 2017). Although controlling the disease in the next years is very unlikely, significant progress has been made (Becker et al., 2018; Kyu et al., 2018). More than 182 million tablets of praziquantel are now distributed each year for preventive chemotherapy (PC). However, this only covers 30% of the needs (WHO, 2017). Production and donation are planned to be increased over the years in order to reach the goal set by the WHO of 75 % coverage for school-aged children (Lo et al., 2017; WHO, 2017). In parallel to this situation, the selective pressure put on praziquantel and the risk of resistance are raising, which is very alarming since decreased activity has been reported complemented by the fact that neither any vaccine nor alternative is currently available (Bergquist et al., 2017; Crellen et al., 2016; Doenhoff et al., 2002; Ismail et al., 1999; Melman et al., 2009). Hence, the development of new antischistosomal treatment is imperative though there is still a long way to go. At the moment, the development pipeline not only lacks clinical candidates but also advanced preclinical leads (Keiser and Utzinger, 2010; Mäder et al., 2018; Panic and Keiser, 2018; Pedrique et al., 2013).

Building on previous studies, the first and main objective of this thesis was to expand the list of active molecules against the schistosomes through two common approaches in drug discovery: the screening of compound libraries and drug repurposing (Abdulla et al., 2009; Cowan and Keiser, 2015; Ingram-Sieber et al., 2014; Panic et al., 2014, 2015b; Sayed et al., 2008).

The work on the Stasis Box – that was composed of drugs to reposition – bridged these two approaches and the screening of that first library on schistosomes led to identify eleven potent *in vitro* leads, mostly anti-neoplastic drugs. Eleven *in vitro* leads were also identified when screening the second library, the Pathogen Box. For these two sets of compounds, the very promising *in vitro* activity of the leads was tempered by their lack of efficacy in *S. mansoni* infected mice. Nevertheless, these leads feed the dry

antischistosomal pipeline with potent scaffolds. Furthermore, a preliminary SAR on three of the leads from the Pathogen Box already provided information regarding the combination of moieties that could potentiate the efficacy of the respective scaffolds, which can be used as a strong basis in further medicinal chemistry programs.

This thesis included two projects under the umbrella of drug repurposing. In addition to testing multiples drugs on one parasite (the schistosomes) in the Stasis Box study, one drug, emodepside, was tested on multiple parasites. This broad-spectrum anthelminthic drug was tested on seven species of medically relevant helminths and models of human infection. Whereas the activity on the schistosomes was almost negligible, emodepside was highly active against the hookworms and the whipworm, both *in vitro* and *in vivo*. With its high-level safety demonstrated in the veterinary field but also in recent human trial(s) on onchocerciasis patients, emodepside is thus an interesting candidate to be repurposed.

In line with the main objective to identify new antischistosomal drug candidates, I contributed in the elaboration of a protocol which, for the first time, unifies the methods for culturing schistosomes in a laboratory with the ones used in early drug discovery. This work was based on our routine drug screening workflow that involves both *in vitro* and *in vivo* procedures. In this protocol, the assessment of the parasites *in vitro* was particularly emphasized and detailed as whole parasite phenotypic assessment is still the gold standard method to screen drugs on the schistosomes. Hereafter, I will develop more on the advantages, limitations and potential improvements of the phenotypic screenings in antischistosomal drug discovery.

The WHO Special Programme for Research and Training in Tropical Diseases (TDR) recommends drug assays with 72 h follow-up on adult worms (Ramirez et al., 2007). However, larval drug assays are often included as a prior selection step when screening large batches of compounds because of the important financial and ethical costs raised by the use of laboratory animals to grow the adult schistosomes (Ramirez et al., 2007; Tekwu et al., 2016). The screenings performed in the framework of this thesis made no exception and almost a thousand molecules were pre-screened on NTS. Furthermore, another

objective of this thesis was to compare the outcomes of the Pathogen Box larval screening with the results of two other institutions that tested the same library. An overall agreement of 74% was found between the 3 laboratories even though they used different parasite strains, methodologies and hit selection criteria. In contrast to this “manual” approach, I tested an image-based anticestodal drug screening device recently validated by the VetSuisse Faculty from the University of Bern (Ritler et al., 2017). Completing the last objective defined for this thesis, the comparison led to promising results. With very minor changes to the protocol designed for drug assays on cestode protoscolesces, a preliminary experiment with six reference drugs was performed and this method seemed to correlate rather well with the standard visual method after 24 h. To go further, an extensive validation process could assess its complete reliability and its relevance in antischistosomal drug screenings.

The introduction of this thesis presented a list of objectives that all chapters intended to meet. Specific aspects of each of these objectives were thus already discussed in the relevant chapters (figure 3). In the light of the variety and number of compounds screened in addition to the particular focus on methodology in this thesis, I will first discuss the different challenges faced in the current antischistosomal screening cascade. Secondly, I will critically review the proposed alternatives and finally suggest potential improvements. The relevance of phenotypic assessment and NTS assays will be particularly emphasized. Peculiar aspects of anthelminthic drug discovery will then be considered in the light of my experience working with repurposed drugs and open access compound libraries.

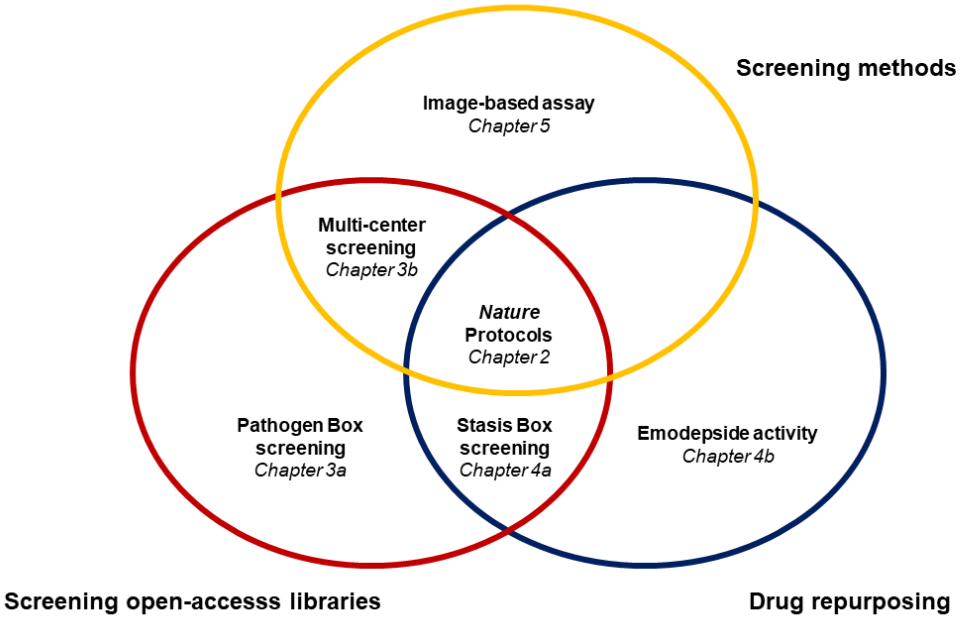

Figure 3: Synopsis of the different aspects of anthelminthic drug discovery explored in this thesis

6.2 Screening large libraries: lessons learned and perspectives

6.2.1 Different sets and selection criteria

In this thesis, two main sets of compounds were tested for their antischistosomal activity. While the Pathogen Box was composed of molecules that were selected for their drug-like properties and their efficacy *in vitro* against one or more infectious disease agents, the other set, the Stasis Box was composed of advanced drug candidates that where withdrawn from their initial development pipeline, mainly because of a lack of efficacy against their primary target.

The Pathogen Box provided more active compounds on NTS, with an effect ≥ 50% on the NTS (n=144) than the Stasis Box (n=37). While for the Stasis Box, the compounds that displayed an effect of 75% and above after 72 h were considered as hits, with the Pathogen Box, it was decided to select only the fast acting ones as done, for example, in a previous study (Cowan and Keiser, 2015).

The criteria chosen for the hit to lead selection were then different between the two libraries. The first reason for this difference was that not all the same information on the compounds was openly available. Information relative to physicochemical properties, toxicity and pharmacokinetic parameters were given openly for almost all the compounds in the Pathogen Box, whereas the information on the Stasis Box compounds was confidential. Indeed, besides the information concerning the eleven leads shown in chapter 4a, no other information could be obtained on the Stasis Box compound.

The second reason for applying different selection criteria was that the compounds from the Stasis Box were already developed drugs, therefore they were assumed to have already satisfactory drug-like profile. Thus, more chances for slower acting drugs were given for the Stasis Box because of their advanced stage of development. Although the general workflow described in chapter 2 was followed in all our previous studies, selection criteria where slightly different each time (Cowan and Keiser, 2015; Ingram-Sieber et al., 2014; Panic et al., 2015b).

In the protocol we developed (chapter 2), neither specific activity threshold nor time-point – within the 3 days recommended – was suggested for initial screening on NTS or adult worms. Hence, the choice should be made by the researchers depending on the singularity of the set of compounds tested, their screening capacity in further steps or their study goals.

6.2.2 Improving the in vitro *to* in vivo *translation*

On the almost thousand compounds screened in the framework of this thesis, more than 20 lead molecules emerged from the screening of the MMV “boxes”. They were highly active *in vitro* on NTS and adult schistosomes but failed to significantly reduce the worm burden in infected mice. Although these failures in the *in vitro* to *in vivo* translation occur very often and contribute to the attrition process inherent to drug development, improvements should be made in the drug screening cascade (Keiser, 2010). Better

prediction should be made to limit the risk of failure as well as the financial and ethical costs associated with *in vivo* testing.

Upstream selection might be made thanks to the Lipinski's rule of 5 that was proposed as a rule of thumb to select orally bioavailable compounds (Lipinski et al., 2001). This is particularly suitable when only minimal information on the test compounds is available. Different software can also be used to classify and prioritize compounds based on their structure and moieties, e.g. DataWarrior (Cowan, 2015; Sander et al., 2015).

Another possibility is the addition of elements to the culture medium to resemble, as much as possible, to the physiological conditions (Cowan, 2015; Gelmedin et al., 2015). The introduction of protein binding assays is a rather simple way to select drug candidates at an early stage. Worms can be incubated with plasma proteins such as albumin or α-1 acid glycoprotein (Cowan and Keiser, 2015; Gelmedin et al., 2015). For example, imatinib, a very potent antischistosomal *in vitro* lead and tyrosine kinase inhibitor, failed *in vivo*. This loss of activity was attributed to a strong plasma protein binding effect that was partially reverted in a competition assay with erythromycin (Beckmann et al., 2014; Gelmedin et al., 2015).

In the Stasis Box study, the albumin binding assays were performed after testing the leads *in vivo* . For most of the compounds, the IC_{50}s were drastically increased in presence of albumin. Protein binding was then the only hypothesis tested to explain the decrease of efficacy in animal models. Subsequently, during the Pathogen Box screening, protein binding was tested prior to the *in vivo* studies. In this case, no notable change in the IC_{50} was observed, therefore no candidate was eliminated on this criterion. Nevertheless, given their simplicity, the use of protein binding assays should be systematized (Cowan, 2015).

Depending on the nature of the compounds, the incubation with other molecules or physiological components may potentiate their activity. For instance, synthetic peroxides such as the ozonides were often tested in presence of hemin, hemoglobin or red blood cells as their hypothesized mechanism of

action is likely to involve the action of ferrous porphyrins (Creek et al., 2008; Ingram et al., 2012; Leonidova et al., 2016; Meshnick, 2002).

Permeability and metabolic stability assays (PAMPA, caco-2-cells, liver microsomes etc.) are more complex tools to provide information on the drug-likeness of a molecule. These standardized methods have been implemented with success in our laboratory (Cowan, 2015). However, they might be more relevant to be employed when characterizing batches of *de novo* molecules than in the screening of open access libraries for which this information is generally available, even partially.

Also, with the advent of *in silico* tools, better predictions and a more targeted approach might be possible for drug candidates thanks to computational approaches, i.e virtual screening, chemoinformatic etc. (Calixto et al., 2018; Neves et al., 2016). As highlighted in chapter 4a and in previous studies, kinases are a pit of potential drug targets in schistosomes. Interestingly, pelitinib and Bi-2536 from the Stasis Box were also identified thanks to an online application inferring *S. haematobium* proteome (Stroehlein et al., 2018). However, the predicted targets were not the same as the ones proposed in chapter 4a. We can suspect the drugs may act on several targets, which would then explain their significant activity.

Although *in silico* tools are unlikely to completely replace experimental tests in drug discovery and need to be improved (Neves et al., 2016), they already identified promising molecules and targets (Mafud et al., 2016; Stroehlein et al., 2018). Therefore, in the short term, they should progressively be integrated into the screening workflow, but given their technicality and the specific skills they might require, they should both foster and benefit from more collaboration between laboratories.

6.2.3 Phenotypic screening

Regardless of the promising developments discussed above, screening for antischistosomal candidates with a reliable and recognized method is more than ever necessary. For now, whole parasite phenotypic screenings, which require no specific equipment, are the most widespread and the gold standard method.

Yet, the major limitation of this approach is its rather low throughput, mainly because the assessment is done visually by one or a handful of operators. In addition to this lack of automation, the subjectivity of the phenotypic screenings has encouraged the development of alternative screening methods based on fluorescence, luminescence, metabolism, impedance or temperature (Aguiar et al., 2017; Chawla et al., 2018; Manneck et al., 2011; Panic et al., 2015a). Although some of these methods were able to identify hits quite efficiently, they did not perform as accurately as the visual observation in detecting sublethal or particular phenotypes (e.g. tegumental damages), which might explain the poor correlation sometimes observed with the standard method (Manneck et al., 2011; Panic et al., 2015a, 2015b). Automated and semi-automated image read-out systems represent therefore an engaging alternative. Different systems, even with specific software interface, already exist for other helminths (Geary et al., 2015; Preston et al., 2015). Their main disadvantage is that they are essentially based on motility as the main proxy of the parasite viability. Thus, similarly to the methods mentioned above, subtle phenotypes might be missed. Indeed, this was a major limitation of the system described in chapter 5. More advanced systems involving image-recognition algorithms and machine learning are under development and are likely to produce more accurate results (Asarnow et al., 2015; Singh et al., 2018).

The subjectivity of the visual phenotypic assessment has been repeated as a mantra in the literature and raised as the critical limitation of this approach. Rather undeniable, I will argue here that subjectivity should not be completely confounded with the integration, interpretation and contextualization process of the observations made by the operator during phenotypic screenings. In this, both human eyes and mind are rather unbeatable. In the multi-center study presented in this thesis, we observed a better concordance between the laboratories using the visual approach over the one using a metabolic assay. Moreover, phenotypic screenings are normally performed according to strict criteria with defined scores (Keiser, 2010; Lombardo et al., 2019; Ramirez et al., 2007).

Despite the fact that this phenotypic approach has undeniable limitations and that alternative methods would certainly increase the quality of the data and the screening throughput, it successfully identified

most of the current lead candidates and should continue to be considered as a robust method, even if it is further improved by technology.

6.2.4 Larval assays

The NTS, the mechanically-transformed schistosomula, were given a central place in this thesis. Their assessment was particularly emphasized in chapter 2. They were also used in the screening cascade of the two MMV boxes (chapters 3a and 4a) and were tested in the image-based device described in chapter 5. All methods confirmed that NTS assays are reliable models to test compounds on and efficient/accurate predictors of drug activity (Tekwu et al., 2016). One of the principal advantages of the NTS is that they can be obtained in higher quantity than the adult worms and require significantly less infected rodents, which makes their use extremely relevant under the scope of the 3Rs principles. Nonetheless, obtaining a sufficient yield was always a struggle and a highly critical limiting factor in my screening work. More globally, parasite yield should be taken into consideration when developing higher throughput screening systems and motivate more research towards the development of novel cultivation methods. Ultimately, a closed egg-to-egg system should resolve not only the problems of access to the parasites but also overcome the challenges inherent to animal experimentation (Geary et al., 2015). On that note, a recent study described the cultivation of juveniles schistosomes from mechanically transformed schistosomula and their use in drug assay. Still far from an ideal egg-to-egg system, it provides a first step in that direction (Frahm et al., 2019). In the meantime, a pragmatic and rather simple approach to increase the number of parasite would be the development of a drug assay using the eggs, that can be harvested by thousands from the livers of infected rodents (Dinguirard et al., 2018; Lombardo et al., 2019).

6.2.5 Antischistosomal screening cascade, a step into the future

New ways of screening drugs on schistosomes are heavily researched and very promising systems are already available, all with their strength and limitations. Further research is of course needed, I will therefore not argue in favor of one or another system to replace phenotypic screenings as gold standard method. Instead, I would suggest adopting pragmatic integrated approaches (Figure 4), that include technological and information sharing between laboratories, as well as the progressive inclusion of more predictive methods – i.e. chemoinformatic – or biotechnological devices – i.e. microfluidic systems and (multi)organ(s)-on-a-chip (Neves et al., 2016; Njoroge et al., 2014; Skardal et al., 2016). Multiplying such complementary approaches could let more room to serendipity, that has been, until know, a rather valuable identifier of drugs (Geary et al., 2015).

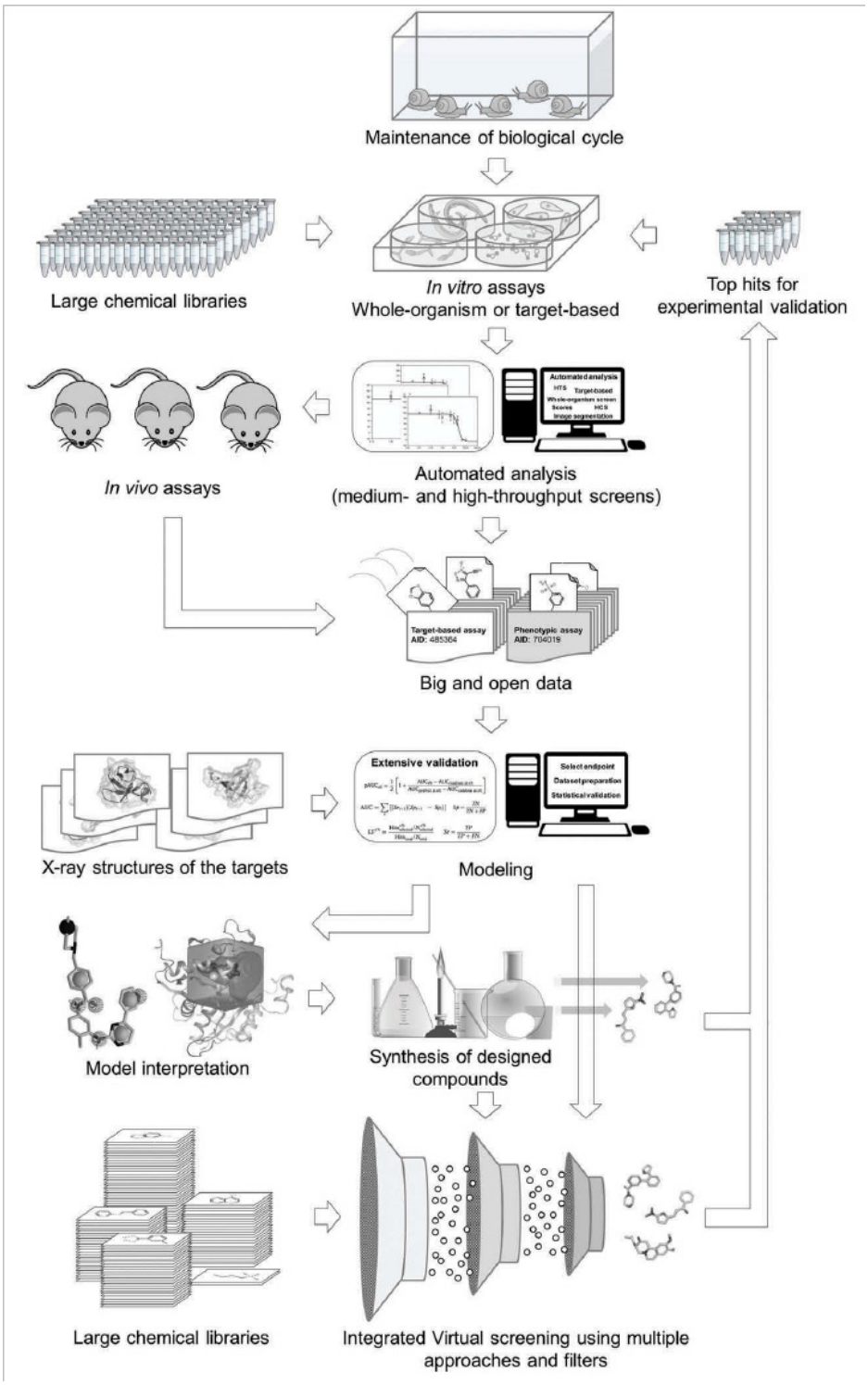

Figure 4: A model of an integrated screening approach in early antischistosomal development (adapted from Neves et al., 2016).

6.3 Considerations on anthelmintic early drug development

6.3.1 Open access compound libraries

Antischistosomal and more broadly anthelmintic drug discovery relies on highly specific requirements as illustrated by the challenges in cultivating, testing and assessing the parasites in drug assays as evoked above. Also, despite a regained interest from the international community and more resources allocated to this purpose, anthelminthic drug discovery remains a neglected area of research with low commercial interest. Thereby, alternative strategies to the “traditional” R. & D. model were developed with, notably, the involvement of public-private partnerships.

Since large compound libraries from the pharmaceutical industry are generally inaccessible to researchers in public institutions, the contribution of MMV was central to this thesis since it provided all the compounds that were tested. Aiming to fill this research gap, the Malaria Box was made available in 2012 to stimulate drug discovery in diseases of poverty (Voorhis et al., 2016). New initiatives such as the Pathogen Box and the very recent launch of the Pandemic Response Box (https://www.mmv.org/mmv-open/pandemic-response-box) confirmed that these open access compound libraries are now an essential tool, fostering not only the identification of hits but also more collaboration. This was the case in chapter 3b where the Pathogen Box served as a platform for methodological comparison. Although the MMV boxes are free of charge and undeniably provide researchers with interesting molecules, they are still far from an ideal open access model. Even if efforts are made, like the DMPK data of the Pathogen Box compounds that was made available in the public domain, accessing information regarding the compounds was difficult, in the Stasis Box especially. A better collaboration between chemists and companies participating in the development of these libraries would be required. Sharing information between laboratories should also be encouraged. One way would be to develop data repository and online tools as it is now the case in the malaria field (Robertson et al., 2014; Wells et al., 2015). With schistosomiasis, collaborative efforts in early drug development is likely to be even more challenging as,

for now, it has neither any dedicated PDP nor it is included in the portfolio of an existing organization (Panic and Keiser, 2018).

As already mentioned above, the Pandemic Response Box from MMV will alternatively provide an interesting set of antibacterial, antiviral and antifungal drugs and drug-like molecules to test on schistosomes. The composition of the library being already available, I would recommend an upstream prioritization of the compounds based on their chemical structure and the data available.

6.3.2 Drug repurposing

Everything old is new again! This adage may not apply only to vintage clothing but also to drug development. Indeed, drug repurposing is a well-known strategy that enables to decrease R.& D. costs by finding new application to generally failed or old drugs. In the context of research funding scarcity, this is a privileged strategy in the NTD world (Panic et al., 2014). It was also at the root of two projects conducted in this thesis.

First, the Stasis Box was a set of drugs originally developed for various application and whose development was interrupted at advanced stages. Our study completes the screening of an FDA-approved drugs and a set of anti-cancer drugs also tested in our laboratory. It also provides new material for further SAR analysis and optimization to develop safe drug candidates better adapted to oral administration (Cowan and Keiser, 2015; Panic et al., 2015b).

The aim of the second project was to elaborate the necessary basis to expand the application of the veterinary drug emodepside to the human use as the pharmacopeia against soil-transmitted helminthiases now relies mainly on the benzimidazoles (albendazole and mebendazole) and ivermectin. Levamisole and pyrantel pamoate can also be used but are not included in PC (Jourdan et al., 2018). As for praziquantel, most of these drugs were repurposed from the veterinary field and present several drawbacks. Some of these drugs lack efficacy or are simply contraindicated. For instance, while

albendazole severely lacks activity against *T. trichiura* infections, ivermectin can be deadly if administered to patients co-infected by *Loa loa* (Jourdan et al., 2018; Schulz et al., 2018). Furthermore, the emergence of drug resistance adds a huge challenge to the current situation, since resistance is already widespread in livestock animals (Kaplan and Vidyashankar, 2012; Rose et al., 2015). In order to complete the modest anti-STH arsenal, moxidectin, tribendimidine and oxantel pamoate have recently been tested in randomized trials but their use still needs to be optimized (Barda et al., 2018; Keiser et al., 2014; Moser et al., 2017; Schulz et al., 2018). Hence, there is undeniably room for more candidates. Together with encouraging safety data from two human trials (Kuesel, 2016), the evaluation of emodepside *in vitro* and *in vivo* on seven species of medically relevant helminths confirmed its potential of becoming a very promising broad-spectrum anthelmintic for human use. However, because emodepside is already largely used in the veterinary field, developing and testing analogues would be an additional step to prevent or at least limit the emergence of resistance.

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Conclusion

The five objectives defined for this thesis all aimed at addressing different challenges in early anthelmintic drug discovery, with a particular emphasis on schistosomiasis.

The Pathogen Box and the Stasis Box enabled to test a rare diversity of molecules on the schistosomes. The screening of these two libraries helped to identify more than twenty *in vitro* leads. This positive activity unfortunately did not translate *in vivo*. For these leads, further characterization and optimization should be pursued, especially since some of them were drugs already well-advanced in their clinical development. The development of open access initiatives, similar to the two “boxes” screened in the context of this thesis, should be highly supported as such resources expand the number of active molecular scaffolds and the chances to discover new promising compounds for NTDs.

The antischistosomal screening cascade should also be improved. I would recommend the inclusion of more predictive methods in the screening cascade to refine the hit selection process. I would suggest applying an integrated approach, using existing methods in a complementary way. On that note, I advise to intensify research in the development of efficient culturing methods, as parasite yield is critical to increase the throughput of assays, no matter which screening technic is used.

Of all the molecules tested in the framework of this thesis, emodepside represents the one with the highest potential. Its repurposing process into a broad-spectrum human anthelmintic should therefore be a priority.

Curriculum vitae

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Scientific experience

Since Jan. 2020	**Clinical Research Associate** **CHUV,** Lausanne**,** Switzerland
Apr. 2019 to Aug. 2019	**Visiting Researcher** **Université de Montreal**, Faculty of Pharmacy, Canada
Jan. 2016 to Apr 2019	**Researcher (Ph.D. candidate)** **Swiss Tropical and Public Health Institute**, Basel, Switzerland
Oct. 2014 to May 2015	**Research Assistant** **Transfusion Interrégionale CRS**, Épalinges, Switzerland
Feb. to Jul. 2012	**Research Assistant** **Natural History Museum**, Neuchâtel, Switzerland

Education

2019	**Ph.D. in Microbiology** – Swiss Tropical and Public Health Institute, Universität Basel, Switzerland
2014	**M.Sc. Biology of Parasites and Behavioral Ecology** – Université de Neuchâtel, Switzerland
2012	**B.Sc. Pluri-disciplinary in Biology and Ethnology** – Université de Neuchâtel, Switzerland

Additional trainings

Feb. 2020 (2 d.)	**Introduction to Regulatory Affairs** – BNF, Lausanne, Switzerland
Jan. 2018 (2 d.)	**Good Clinical Practice (ICH GCP)** – Swiss TPH, Basel, Switzerland
Jul. 2017 (30 h.)	**Global Health Summer School**: "Innovation and Access to Medicines" – IS Global, Barcelona, Spain
Oct. 2014 (5 d.)	**Certificate of "non-violent management of conflicts"** – IFK, Luzern, Switzerland
Sept. 2013 (5 d.)	**Introductory Course in Laboratory Animal Science** – RESAL, Geneva, Switzerland

Languages

French – Native language

English – C1 (Full professional proficiency)

German and Italian– B1 (Basic level)

Computer skills

MS-Office	■■■■■
R, R studio	■■■□□
DataWarrior	■■■□□

Scientific achievements

Peer-reviewed publications

Thirion, Daniel J. G., **Valérian Pasche**, Elias Matouk, Amélie Marsot. (2020) **"Amikacin Nomogram for treatment of adult cystic fibrosis exacerbations based on an external evaluation of a population pharmacokinetics model"** *Pediatric Pulmonology*

Maccesi, Martina[#], Pedro H. N. Aguiar[#], **Valérian Pasche**[#], Melody Padilla, Brian M. Suzuki, Sandro Montefusco, Ruben Abagyan, Jennifer Keiser, Marina M. Mourão, Conor R. Caffrey. (2019) **"Multi-center screening of the Pathogen Box collection for schistosomiasis drug discovery"** *Parasites and Vectors* [#] equal contribution

Thirion, Daniel J. G., **Valérian Pasche**, Amélie Marsot. (2019) **"What is the recommended amikacin dosing for cystic fibrosis patients with acute pulmonary exacerbations?"** *Pediatric Pulmonology*

Karpstein, Tanja, **Valérian Pasche**, Cécile Häberli, Ivan Scandale, Anna Neodo, Jennifer Keiser. (2019) **"Evaluation of emodepside in laboratory models of human intestinal nematode and schistosome infections"** *Parasites and Vectors*

Lombardo, Flavio[#], **Valérian Pasche**[#], Gordana Panic, Yvette Endriss, and Jennifer Keiser. (2019) **"Early drug discovery on *Schistosoma mansoni*: from life cycle maintenance to drug sensitivity assays"** *Nature Protocols* [#] equal contribution

Pasche, Valérian, Benoît Laleu, and Jennifer Keiser. (2019) **"Early antischistosomal leads identified from *in vitro* and *in vivo* screening of the Medicines for Malaria Venture Pathogen Box"** *ACS Infectious Diseases*

Pasche, Valérian, Benoît Laleu, and Jennifer Keiser. (2018) **"Screening a repurposing library, the Medicines for Malaria Venture Stasis Box, against *Schistosoma mansoni*"** *Parasites & Vectors*

Contribution to scientific conferences

- **Screening open access libraries for early antischistosomal drug discovery: new leads, old challenges**

  Poster – SSI, SSHH, SSTMP and SSTTM Joint Annual Meeting, Interlaken, Switzerland, Sept. 2018

- **Ozonides mechanism of action in *S. mansoni***

  Oral presentation – SSTMP Student meeting, Schwarzenberg, Switzerland, Nov. 2017

- **Screening the MMV Pathogen Box on schistosomula: first steps, first hits**

  Oral presentation – SSTMP Student meeting, Grindelwald, Switzerland, Nov. 2016

Teaching and mentoring

Swiss TPH, Universität Basel, Switzerland

Apr.2017 to Feb. 2019	Co-supervisor of a M.Sc. student in Infection Biology who graduated with honors
Dec. 2016, 2017, 2018	Teaching Assistant in the bloc-course "Infection Biology" given to 3rd year B.Sc. Biology students (36 hours/year)

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Oct. to Nov. 2013	Teaching Assistant in the course "Introduction to statistics using R" given to 3rd year B.Sc. Biology students (3 hours/week)