Advancing drug discovery and treatment evaluation for schistosomiasis: improved screening, drug repurposing and rescuing, and the metabonomics of praziquantel treatment

Inaguraldissertation

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Summary

Schistosomiasis is a widespread chronic inflammatory disease caused by a parasitic blood fluke of the *Schistosoma* genus, primarily by *S. mansoni*, *S. haematobium* and *S. japonicum*. More than 200 million people in low-resource rural dwellings of the tropics and sub-tropics are affected, 90% of which are in Africa and many of which are children. The chronic disease is characterized by anemia, abdominal pain, bloody stool or urine, portal hypertension, swelling and degradation of the liver, spleen, intestines or the urogenital organs, caused by hypersensitivity reactions to eggs that become lodged in visceral organs.

The main strategy for control endorsed by the World Health Organization is morbidity reduction by mass administration of the only drug available, praziquantel. The treatment is safe and effective but carries important drawbacks such as its lack of efficacy against juvenile worm stages and its child-unfriendly bitter taste. Importantly, the reliance on a single drug to treat millions is unwise and treatment alternatives are required. Yet schistosomiasis falls into the category of neglected tropical diseases, so called because their treatment and drug candidate arsenal is marginal compared to their public health impact.

This PhD thesis had two major aims. The first was to mitigate this need for novel antischistosomal drug candidates by exploring automated *in vitro* drug screening systems and by invoking drug repurposing, rescuing and re-designing strategies to fill the pipeline. The second was to enhance our understanding of schistosomiasis and its treatment with praziquantel in pre-school and school-aged children with a clinical dose-finding trial and complementary metabonomic investigations.

In testing 11 fluorescence/luminescence-based viability/cytotoxicity markers, resazurin, Vybrant® and CellTiter-Glo® presented as markers that correlated with *S. mansoni* larval stage viability. Of these, CellTiter-Glo® could determine IC_{50} values for some standard drugs in the range of microscopically assessed values and was 100% accurate in identifying hits from a small sub-set of a screen.

An evaluation of a new calorimetry device, the calScreener™, showed that heat flow and heat flow fluctuation signals from single adult stage worms of two nematodes

could be used to monitor worm viability over time. However, this did not extend to *S. mansoni* adult stage nor larval stage worms.

A literature review of drug repurposing anthelmintic *in vivo* and clinical trial candidates detailed the continued reliance on veterinary drug discovery as a source for new compounds, largely for nematodes. Antimalarials were found to be an important contributor of antischistosomal drug candidates, though additional sources, such as anticancer drugs are increasingly in the foreground.

To expand repurposing sources, we screened an open-source library of 1600 FDA approved compounds against *S. mansoni in vitro* and *in vivo*. Our screen identified 121 hits against Newly Transformed Schistosomula (NTS, the larval stage) at a concentration of 10 µM and 36 active compounds on the adult stage at a concentration of 33 µM. Despite needing to discard many compounds for pharmacokinetic or toxicity reasons, 11 compounds were tested further *in vivo*, with doramectin (10 mg/kg) and clofazimine (400 mg/kg) showing significant worm burden reductions of 60.1% and 82.7%, respectively.

In seeking to revive a pre-praziquantel era antischistosomal candidate, Ro 13-3978 was characterized for its activity against *S. mansoni*. *In vitro*, it displayed only minor activity against adult *S. mansoni*. *In vivo* it was more potent than praziquantel with an ED_{50} of 14.6 and 138.9 mg/kg against adult and juvenile stage worms respectively. Hepatic shift and SEM studies of ex-vivo worms showed minimal activity within the first 24 h but by 48 h were worms are shunted to the liver and the tegument thoroughly damaged. Further investigations with immunohistochemistry on histological sections of treated versus non-treated mice indicated significant recruitment of macrophages and B cells to the worm at 24 h post-treatment and additional recruitment of T cells and neutrophils at 48 h post treatment.

Oxamniquine was re-designed by synthesizing organometallic derivatives. Three compounds- a ferrocenyl, a ruthenocenyl and a benzyl derivative- presented potent *in vitro* activity against adult *S. mansoni* worms, reducing their viability by > 75% within 4 to 7 h after exposure. This contrasted with oxamniquine, which presents no activity *in vitro*. In *S. mansoni*-infected mice, a 100 mg/kg dose of the derivatives reduced worm burden by 76 to 93%, comparable to the worm burden reduction of oxamniquine at the same dose.

Summary

Our randomized controlled dose-finding clinical trial of praziquantel in school-aged and pre-school aged children infected with *S. mansoni* in southern Côte d'Ivoire showed slightly lower efficacy of praziquantel in pre-school versus school-aged children. When administered either a placebo or 20, 40, 60 mg/kg praziquantel, cure rates for pre-schoolers were 37.1, 62.2, 72.2 and 71.4%, respectively, as compared to those for school-aged children which were 11.9, 30.4, 68.8 and 82.9%, respectively. An E_{max} model predicted an egg reduction rate of 99% with 65 mg/kg praziquantel for school-aged children while for pre-schoolers, this was not in range.

Metabonomic analyses of urine samples from this same clinical trial revealed that heavily infected children could be differentiated from non-infected children prior to treatment, indicating potential urinary biomarkers of morbidity. Treatment with praziquantel produced a plethora of metabolic responses 24h after treatment, which differed between pre-school and school aged children, which may be linked to the diferring treatment success rates between the two groups. Most metabolites were correlated to energy, liver and gut microbial metabolisms.

In conclusion, we present CellTiter Glo® as a possible hit/no hit pre-screening tool for *in vitro* assays. Moreover, our FDA library screen proposes additional sources of drug indications from which antischistosomal compounds and scaffolds could be sourced. Meanwhile Ro 13-3978 is an efficacious antischistosomal, active against multiple stages of infection, and presents as an excellent pre-clinical candidate worthy of further investigations. The oxamniquine derivatives described elucidate a successful drug re-design strategy using organometallic derivatization. Finally, praziquantel treatment itself has been better characterized for an increasingly important demographic population, pre-school aged children, and illustrated through a systemic metabolic lens.

Table of Abbreviations

ADME	Absorption, Distribution, Metabolism, Eliminations
DnDi	Drugs for Neglected Disease initiative
ED_{50}	Median effective dose
EIS	Electrical impedance spectroscopy
FDA	Food and Drug Administration
^{1}H NMR	Nuclear magnetic resonance
HPLC	High-performance liquid chromatography
HTS	High-Throughput Screening
IC_{50}	Drug concentration required to inhibit parasite viability by 50%
iFCS	Inactivated fetal calf serum
MFQ	mefloquine
MOA	Mode of action
MW	Molecular weight
NTDs	Neglected Tropical Diseases
NTS	Newly Transformed Schistosomula
OPLS/ OPLS-DA	Orthogonal partial least squares/ OPLS- direct anaylsis
OXA	Oxamniquine
PCA	Principle Component Analysis
PK PD	Pharmacokinetic/ Pharmacodynamic
PZQ	praziquantel
PPP	Public Private Partnership
SI	Selectivity Index
SAR	Structure Activity Relationship
WASH	Water, Sanitation and Hygiene
WHO	World Health Organization
WB	Worm burden
WBR	Worm burden reduction

Chapter 1

Introduction

1.1 Epidemiology and global disease burden of schistosomiasis

Schistosomiasis is a disease of tremendous global public health concern. It is widely distributed throughout the tropics and sub-tropics and found mostly in poor rural areas, (Adenowo et al., 2015; WHO, 2016a). The causative agents are parasitic flatworms of the genus *Schistosoma: S. mansoni, S. haematobium* and *S. japonicum* are the main culprits, whereas *S. mekongi, S. intercalatum* and *S. guineensis* are less often implicated (Gryseels, 2012). There are two main forms of the disease. Intestinal schistosomiasis is caused mainly by *S. mansoni* and *S. japonicum*, whereas the urinary form is caused only by *S. haematobium*, which nonetheless accounts for 64% of infections (Hotez et al., 2006).

Throughout 78 countries, around 779 million people live at risk of infection, 85% of which are living in Africa (Steinmann et al., 2006a). With over 200 million people infected, resulting in 2.6 million DALYs lost, schistosomiasis is third only to malaria in terms of global disease burden associated with a parasitic disease (Kassebaum et al., 2016).

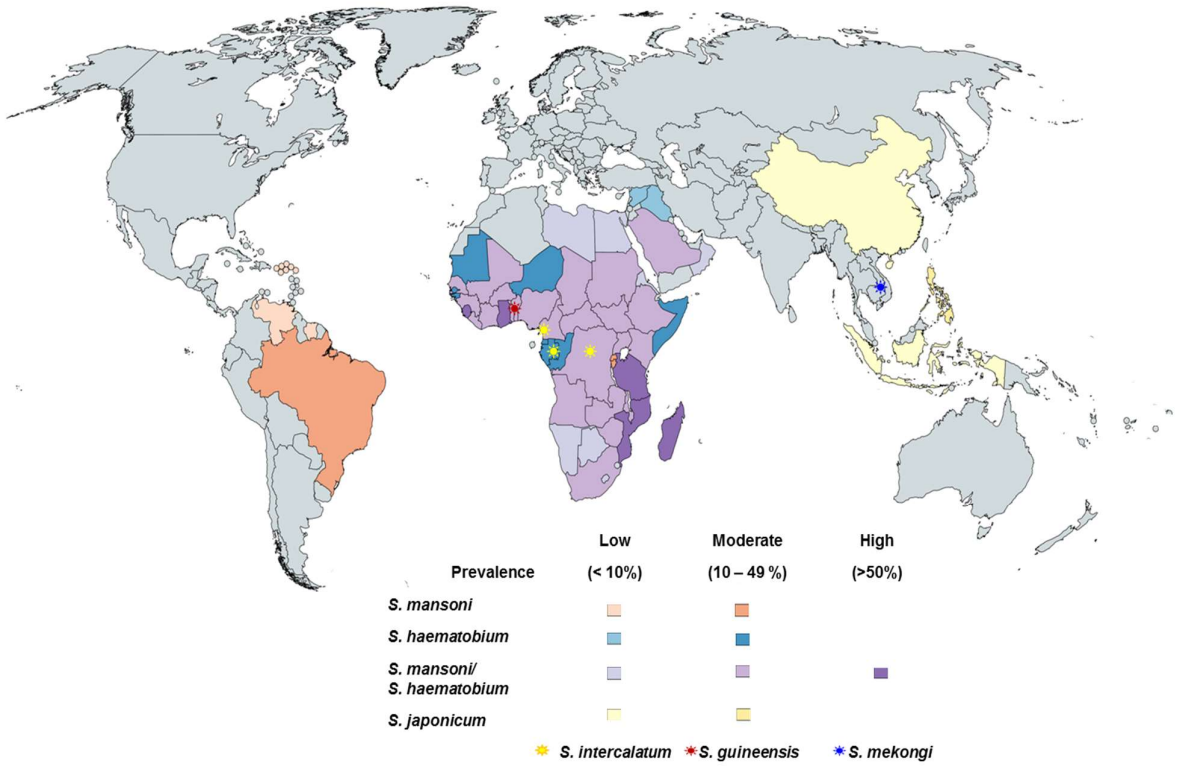

Figure 1 Global distribution of schistosomiasis according to species and prevalence. (Adapted from Colley et al., 2014 and WHO, 2012).

Schistosomiasis is a water-borne disease whereby transmission occurs by exposure to contaminated freshwater (Gryseels et al., 2006). Distribution is centered around significant and minor fresh water bodies and can often be focal. Consequently, development and management of water resources, such as dams and irrigation systems, are a significant risk factor for schistosomiasis (Steinmann et al., 2006a). Moreover, those most exposed to contaminated waters, such as rice farmers, fisherman or mothers and children that bathe and wash in rivers, are most at risk, though some acquisition of immunity is apparent in adulthood (Colley and Secor, 2014; Ismail et al., 2014; Steinmann et al., 2006b). Importantly, provision of safe water and human waste disposal infrastructure as well as safe hygienic practices are protective against this disease (Grimes et al., 2015).

1.2 Biology and lifecycle

Schistosoma worms are digenetic trematodes- parasitic flatworms with two sexes, a syncytial tegument and a ventral and oral sucker (Olson et al., 2003). The adult worms are about 1-2 cm long, complete with a blind digestive tract, reproductive organs and a primitive neuromuscular system. Sexual diphormism is very evident: the female is long and thin with a tegumental texture akin to an elephant trunk whereas the male is wide with a tegument patterned by distinct tubercules (Hockley, 1973; Humans, 2012). The female resides in the gynocephoral canal of the male and they remain in a monogamous state of copulation for years inside the host, where they produce tens to thousands of eggs per day (Cheever et al., 1994).

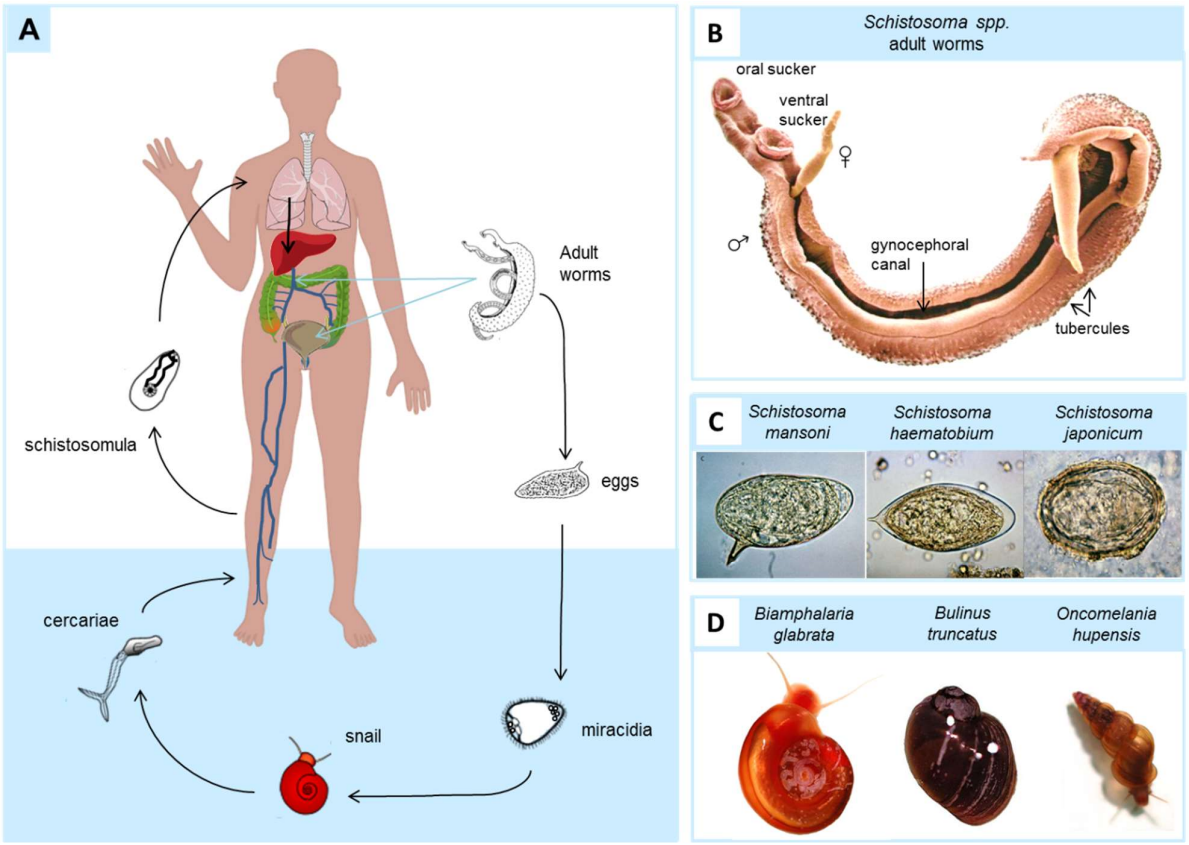

Figure 2 (A) Life cycle of *Schistosoma spp.* (B) Adult worm morphology. (C) Species-specific eggs and their (D) intermediate snail hosts. (Adapted from: CDC, 2012; Gouvras, 2014; Gray et al., 2011; Lewis et al., 2008; Wikimedia Commons, 2015)

Humans become infected when they come into contact with freshwater bodies infested with cercariae, the free-swimming infectious stage of *Schistosoma spp.* The cercariae burrow through the host skin by using the propulsive force of their flagella, in combination with secreting serine proteases from acetabular glands that digest skin proteins (Ligasová et al., 2011; Salter et al., 2000). Penetration takes anywhere from 2-24 hours and in the process, the tail breaks off (McKerrow and Salter, 2002). The remaining schistosomula locate blood vessels and circulate in the blood until they reach the lungs, where they reside in the pulmonary capillaries and enter the juvenile stage. At around 4 to 6 weeks post-infection, the worms migrate to the liver where they will sexually mature to their adult form, mate, and relocate to the veins of

the mesenteries, or in the case of urogenital schistosomiasis, vesicular veins of the bladder or urogenital organs (Gryseels, 2012). There they reside for an average of 2 to 5 years, employing a multitude of immune evasion mechanism to protect from the hostile humoral environment (Gryseels et al., 2006; Pearce and MacDonald, 2002). The tens to thousands of eggs they shed trigger Th2-type immune responses, which the eggs co-opt in order to assist their migration through the intestinal or bladder walls (Pearce, 2005). They are thus expelled with the feces (intestinal schistosomiasis) or urine (urogenital schistosomiasis). When eggs come into contact with fresh water, they hatch into aquatic free-swimming forms called miracidia, which locate their intermediate species-specific snail host via chemotactic and chemokinetic mechanisms (Macinnis et al., 1974). In the snails, they undergo multiple rounds of asexual reproduction through mother and daughter sporocyst stages. In a matter of four to six weeks, the snails start to shed cercariae, the infective form of the parasite (Doughty, 1996). Cercariae are viable for one to three days and in this time, they actively pursue their definite host also using phototaxis and chemotaxis (McKerrow and Salter, 2002).

1.3 Pathology

Within hours of penetration through the skin by the cercariae, a rash characterized by maculopapular lesions, known as “swimmer’s itch” may appear. In temperate zones, this can also be caused by avian cercariae wrongly attacking the wrong host, but does not lead to infection (Bourée and Caumes, 2004).

Schistosomiasis follows an acute, then a chronic phase. The acute phase, called Katayama syndrome, presents as general flu-like symptoms: fever, myalgia, fatigue, nausea, pain in the abdomen, coughing, eosinophilia and urticaria are but a few (Jesus et al., 2002; Ross et al., 2007). Symptoms are thought to be the result of hypersensitivity reactions to the migrating schistosomula (Ross et al., 2002). Notably, in endemic populations, both swimmer’s itch and the acute phase are often absent, though both heavy re-infections and re-infection with *S. japonicum* can elicit strong acute reactions (Ross et al., 2002; Zhou et al., 2005). Because it can come several weeks to several months after infection, and because of its unspecific presentation, it is the phase most likely to be misdiagnosed by travel physicians (Ross et al., 2007).

Most of the morbidity associated with schistosomiasis is attributable to the chronic phase of the disease which is in turn attributable to eggs produced by the adult worms (Murray et al., 2012). If left untreated, the adult worms continue to shed eggs daily, only half of which are expelled by the feces or urine. The other half are either swept by circulating blood or are unsuccessful in their migration and consequently become lodged in proximal organs (Colley et al., 2014). These eggs elicit strong Th2-type immune responses, encapsulating the eggs into granulomas and eventually resulting in fibrosis and sometimes necrosis of the tissue (Fairfax et al., 2012). Overtime, this leads to chronic inflammation, swelling and deterioration of affected organs. The severity of the pathology is thus highly correlated with infection intensity, but variation in host genetics and immune responses may play a role (Colley et al., 2014; Pearce and MacDonald, 2002; Russell et al., 2015).

In gastrointestinal schistosomiasis, the inflamed intestinal wall may be plagued by hyperplasia, abscesses and polyp formation, which cause alternating constipation and diarrhea, bloody stools and gastric pain. Overtime, severe infection may lead to colonic or rectal stenosis (Gray et al., 2011). Inflammation, hardening granulatomous lesions and calcified collagen deposits result in hepatomegaly and liver cirrhosis. Obstruction of blood flow, portal hypertension and anemia follow (Barsoum et al., 2013). Portal hypertension itself can provoke dilation of sub-mucosal veins in the esophagus, leading to lethal ascites and hematemesis (Richter et al., 1998).

In the case of urinary schistosomiasis, the lesions in the bladder cause hematuria and dysuria, apparent early at two to three months post-infection (King and Bertsch, 2013). Overtime, calcifications and polyps in the bladder wall lead to downstream consequences such as obstructive uropathy (usually concomitant with bacterial superinfection and renal dysfunction) or squamous-cell carcinoma of the bladder (Gray et al., 2011). Female genital schistosomiasis is due to eggs trapped in various organs of the genital tract, which can also lead to cancers and has strong and often neglected consequences for female reproductive health (Kjetland et al., 2012).

Neuroschistosomiasis is a rare but severe disease caused by ectopic migration of eggs or worms to areas of the central nervous system (Carod-Artal, 2008). Meningitis and epilepsy as well as other central nervous system disorders manifest and are mostly associated with *S. japonicum* infection (Ross et al., 2012).

Over the long term, schistosomiasis is associated with malnutrition, particularly iron deficiency, growth stunting and cognitive inhibition (King, 2007). Due to its slow but debilitating long term effects, it is one of the diseases that is said to perpetuate the poverty trap (Hotez et al., 2009).

1.4 Diagnosis

Clinical diagnosis of schistosomiasis relies on a combination of medical history and sign and symptom evaluation and confirmation with laboratory tests. The current gold standard for detecting *Schistosoma* infections is by microscopic examination of stools, for intestinal schistosomiasis, or urine, for urinary schistosomiasis, for presence of species-specific eggs (Gray et al., 2011). In hospital settings, the direct thick smear along with the formalin ether concentration method are often used (Utzinger et al., 2010). Epidemiological surveys require more quantitative methods. For urinary schistosomiasis, a simple urine syringe filtration and microscopic evaluation of the filter permits a quantification of eggs per 10 ml urine. For intestinal schistosomiasis, the most widely employed method is the Kato-Katz thick smear, differentiated from the direct smear by a platform that allows for easy mounting of a specific amount of stool and thus the quantification of eggs per gram feces (Katz et al., 1972). Notably, concomitant helminth infections can also be detected in this manner. However, both the filtration and Kato-Katz methods are vulnerable to day-to-day egg output variation and are insensitive to low intensity infections. Multiple sampling and read-outs are therefore recommended (Booth et al., 2003; Ebrahim et al., 1997; Knopp et al., 2013, p. 2013; Lamberton et al., 2014). The FLOTAC and its subsequent, field-friendly Mini-FLOTAC employ egg-flotation methods and allow for sampling of a larger quantity of stool sample. They appear to have a superior sensitivity over the above-mentioned methods, with a detection limit of 10 eggs per gram (EPG) (Barda et al., 2013; Glinz et al., 2010).

For the accurate mapping and impact monitoring of control programs, sensitive point-of-care (POC) dipstick diagnostics are desired (Utzinger et al., 2015). Microhematuria- detecting reagent strips for the detection of *S. haematobium* (King and Bertsch, 2013) and the POC-CCA (circulating cathodic antigen) for the detection of *S. mansoni, S. japonicum* and *S. mekongi* infections (Stothard et al., 2006; van Lieshout et al., 2000) have shown superior sensitivity over the above-described microscopic methods (Coulibaly et al., 2011; Danso-Appiah et al., 2016; van Dam et

al., 2015b, 2015a). A new generation of monoclonal antibody based “up-converting phosphor-lateral flow” (UCP-LF) CAA tests can even detect single-worm *S. mansoni* infections (Corstjens et al., 2008), are able to detect *S. japonicum* infections with 10-fold higher sensitivity than microscopic methods (van Dam et al., 2015b) and were found to be more sensitive than urine filtration for the detection of *S. haematobium.*

Serological tests for the detection of circulating antibodies against *Schistosoma* antigens include several different enzyme-linked immunosorbent assays (ELISAs), indirect hemagglutination assays (IHAs), and immunofluorescent-antibody tests (IFATs) which can detect either adult worm antigens (AWA), soluble egg antigens (SEA) or cercarial antigens (CA) (Kinkel et al., 2012). These tests are generally not suitable for use in endemic populations, as they are not able to discriminate between active infection versus previous exposure. They can be useful as clinical tools for testing potentially infected travelers. However, the degree of sensitivity and specificity can greatly vary from test to test and as a result, using at least two tests is recommended (Doenhoff et al., 2004).

Finally, the vast array of molecular techniques, such as PCR, for the detection of parasite DNA are generally the most specific and sensitive but are far less suitable in endemic countries due to the costly equipment and specialized staff required (Verweij and Stensvold, 2014). However, the exciting developments in loop-mediated isothermal amplification (LAMP) technology could render these techniques accessible to resource-constrained areas (Utzinger et al., 2015).

1.5 Control strategies and treatment

Despite many interesting pre-clinical and clinical developments, a vaccine for schistosomiasis will likely not be within reach soon (Tebeje et al., 2016). An optimal control strategy would include vector control (e.g. molluscicide spraying), health education, hygiene and sanitation measures (e.g. Water Sanitation and Hygiene (WASH)) and anthelmintic treatment (Inobaya et al., 2014). However, as the former can be difficult to sustainably implement and measure, the mainstay of the WHO control strategy is regular preventative chemotherapy for morbidity reduction (Hotez et al., 2007).

Praziquantel, introduced in the 1970s by Bayer, is the drug of choice used in preventative chemotherapy programs. It is a broad anthelmintic, active against all

species of *Schistosoma* infecting humans as well as many cestodes. It has been a very important drug as its safety, efficacy and ease of use (single oral dose: 40 mg/kg) has made it possible to treat millions of people worldwide. Nonetheless, it carries drawbacks. First it is a racemic tablet, where the S- praziquantel enantiomer is fairly inactive (Meister et al., 2014). Second, it is not effective against the juvenile stage, meaning developing infections are not treated during mass drug administration campaigns (Pica-Mattoccia and Cioli, 2004, p.; Wu et al., 2011). Finally, it is literally a very bitter pill to swallow, which can make it difficult to administer effective doses to young children.

There are several studies hinting at the mode of action of praziquantel but no definite smoking gun (Wu et al., 2011). Though it is known that praziquantel disrupts Ca^{2+} homeostasis in adult worms, the actual molecular target has remained elusive (Angelucci et al., 2007; Greenberg, 2005). Evidence points to praziquantel binding to beta sub-units of voltage-gated Ca^{2+}channels as the target (Kohn et al., 2003; Pica-Mattoccia et al., 2007). However, direct interaction of praziquantel with channel subunits has never been observed (Angelucci et al., 2007). Moreover, additional targets have also been explored such as glutathione s-transferase, adenosine receptors and surface-membrane actin (Angelucci et al., 2007; McTigue et al., 1995; Tallima and El Ridi, 2007).

Oxamniquine was introduced around the same time as praziquantel, but in contrast, it is only active against *S. mansoni*, and no other helminths. For a long time, it was used as the mainstay of schistosomiasis control in Brazil. However, its use was halted in the 1990s as resistance to the drug could already be demonstrated in the early 1970s (Coura and Amaral, 2004; Webster et al., 2014). Since then, the drug has been obsolete. However, it's mechanism of action, unlike that of praziquantel, is very well characterized (Valentim et al., 2013). Oxamniquine is a prodrug that binds to an endogenous *S. mansoni*-specific sulfotransferase. In conjugation with 3'phosphoadenosine 5'phosphosulfate (PAPS), it is sulfonated to an unstable intermediate which degrades into an electrophilic molecule that alkylates proximate DNA, proteins and other macromolecules (Pica-Mattoccia et al., 2006).

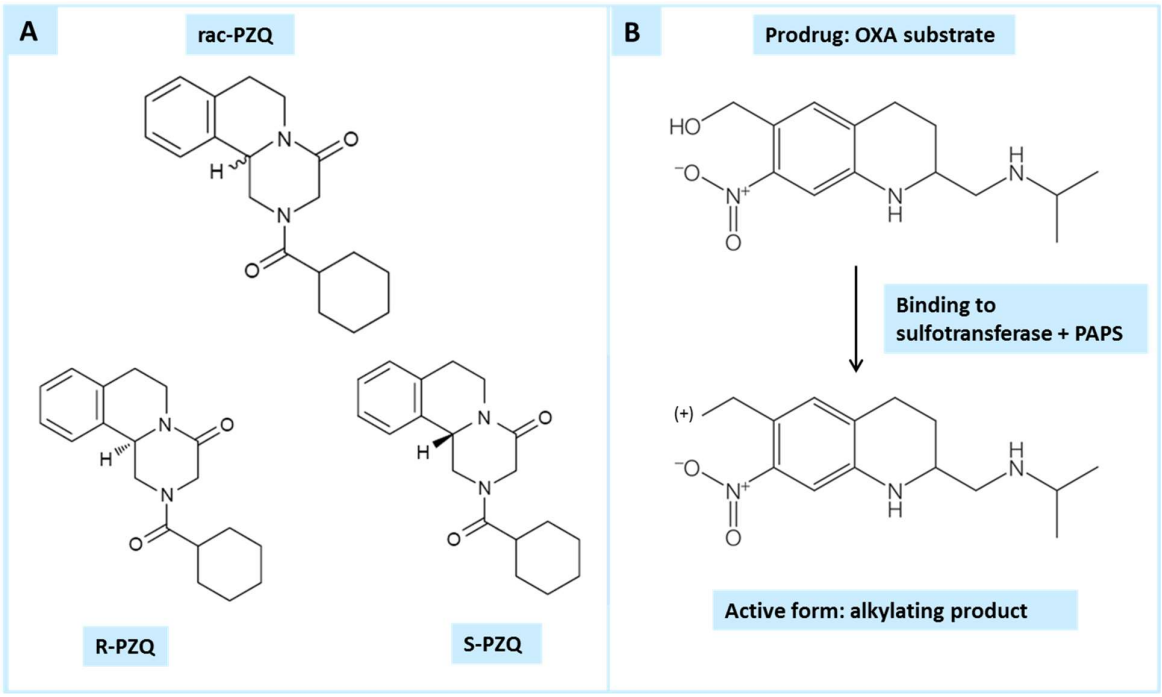

Figure 3 (A) Praziquantel (PZQ) and its enantiomers. (B) Oxamniquine (OXA) prodrug and active form.

1.6 Need for new treatments

In 2014, more than 258 million people were estimated to be in need of treatment, about half of which were children and 91% of which were living in the African region (WHO, 2016b). However, only 21% of these received preventative chemotherapy and only 58% of endemic countries reported on coverage (WHO, 2016b). In 2012, the World Health Assembly adopted resolution WHA65.1, which called for increased investment in schistosomiasis control and support for countries to eliminate the disease (WHO, 2012). Along with clean water and hygiene interventions, vector control and health education, treatment is targeted to expand to 235 million people by 2018. Increased treatment coverage is required to reach this ambitious goal but raises concerns of drug resistance in the face of increased selective pressure. It is therefore imminent that treatment alternatives be developed.

Challenges to anthelmintic drug development are abundant. A hallmark of NTDs is that they are generally poorly funded even despite their significant public health impact and need for new and better drugs. In fact, according to a 2015 G-FINDER report, less than 1% of global R&D funding was spent on schistosomiasis, where only $ 3.3 million was spent on drug development (Moran et al., 2015). As a direct result,

many components of the antischistosomal drug discovery process are paltry or outdated.

To begin with, industry-standard target-based drug screening approaches are not yet feasible. It is not yet possible to maintain neither a completely *in vitro* life cycle nor clonal cultures. Despite the sequencing of the 3 most important *Schistosoma* genomes and employment of *in silico* and *C. elegans* pre-screening approaches, drug targets continue to be poorly defined (Berriman et al., 2009; Keiser, 2015; Neves et al., 2015; Young et al., 2012; Zhou et al., 2009). This is partly because, aside from sporadic RNAi knockout studies, well established gene manipulation methods are lacking to validate suspected targets (Guidi et al., 2015). It is possible that future applications of CRISPR/Cas systems might aid in this goal (Jurberg and Brindley, 2015).

That being said, the majority of drugs approved by the FDA in the last decade have been identified by whole-organism and not target-based screens (Keiser, 2012). Thus the current gold standard for *in vitro* drug screening for schistosomiasis continues to be phenotypic microscopic evaluation of whole adult worms (Ramirez et al., 2007). This requires infection of mice or hamsters and their subsequent euthanization in order to extract the adult worms. This is not only animal unfriendly, it is also costly and time consuming (Keiser, 2010). As a direct result, the use of larval-stage worms as a pre-screening tool has been popularized. Larval stage worms can be easily obtained by mechanically transforming cercariae that are shed from infected snails, providing more worm material for drug screens with cheaper, faster and more ethical methods. However, microscopic assay evaluation is still a laborious and subjective bottleneck (Paveley and Bickle, 2013; Peak and Hoffmann, 2011).

In the last decade, much has been done to attempt screening automation, albeit with mixed results. Fluorescent dyes, such as Alamar Blue or propidium iodide/fluorescein diacetate were shown to correlate with worm viability but still required a copious use of larvae. They can also only be used for endpoint assessments and fail to measure dose-response effects for some important drugs (Mansour and Bickle, 2010; Peak et al., 2010). Microcalorimetry instrumentation could measure real-time heat flow as a proxy for adult worm viability, but was not sensitive enough for larval-stage worms, an issue also observed with the xCelligence impedance-based system (Manneck et al., 2011; Rinaldi et al., 2015). An image-based automated microscopic system was

described as a label-free method to evaluate helminth viability based on both morphology and motility (Paveley et al., 2012). Though usable for a yes/no screen, it has not yet been adopted for high-throughput dose-response assays and simultaneous real-time monitoring.

In Chapter 2a and 2b, investigations of further fluorescence/luminescence-based drug assays and the use of a novel microcalorimetry device for assessing worm viability, respectively, are described.

1.7 Drug rescuing and repurposing

Another important bottleneck to drug discovery and development, for any disease, is the enormous cost and high risk of failure, in particular during the clinical phases of drug development. This clearly manifests itself at the end of the pipeline: in 2010, the number of new chemical entities (NCE) was 50% lower than 5 years before (Paul et al., 2010). A 2013 assessment shows an even bleaker picture for NTDs, with no NCEs approved since the turn of the millennium (Pedrique et al., 2013). Drug repurposing, which is the application of on-the-market drugs or compounds in development for new indications, provides a means of circumventing these costs (Chong and Sullivan, 2007). In most cases, the pre-clinical and clinical safety testing has already been conducted; hence a repurposed drug candidate can often be accelerated to Phase 2 or 3 efficacy studies (Figure 4). Moreover, additional information, such as chemical, pharmacokinetic and analogue properties have already been determined and are usually available (Oprea et al., 2011).

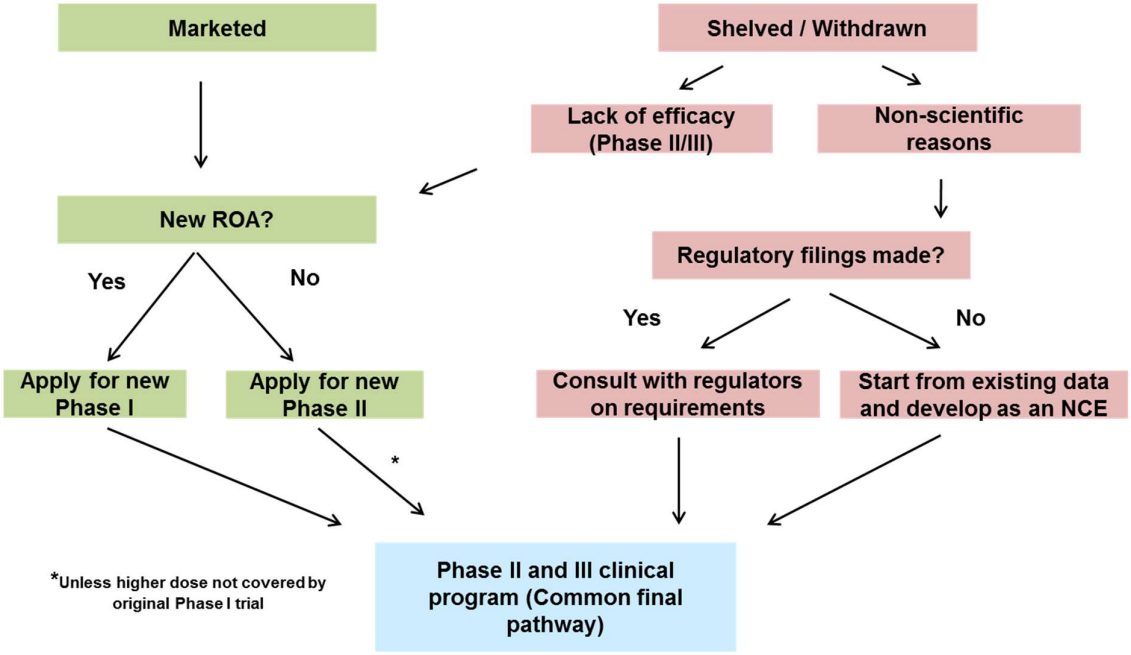

Figure 4. Pathways to drug repurposing for marketed and shelved or withdrawn drugs. NCE= new chemical entity; ROA= route of administration (Adapted from Mucke, 2010)

Anthelmintic drug discovery has already benefited much from this strategy, as many anti-nematodal drugs used for human infection have been repurposed from the veterinary field (Caffrey, 2012). Antischistosomal drug discovery has particularly profited from the antimalarial pipeline. Artemisinin and its derivatives, for example, have demonstrated potent activity against the juvenile stage of *Schistosoma spp. in vivo* (Keiser, 2012), and prophylactic and synergistic effects with praziquantel in clinical trials (Liu et al., 2011; Utzinger et al., 2000). These promising revelations prompted investigations of "artemisinin-inspired" synthetic peroxide analogues as well as screens of an open-access library of antimalarial candidates, culminating to the elucidation of several promising pre-clinical candidates (Ingram-Sieber et al., 2014; Keiser et al., 2012; Xiao et al., 2011).

In Chapter 3a, the anthelmintic drug repurposing landscape is reviewed in detail. Chapter 3b presents our repurposing efforts with a screening of an FDA-approved library of 1600 drugs.

Just as on-the market drugs can be repurposed, failed lead candidates can be rescued. Famous examples include the shelved anti-cancer treatment, azido-

thymidine, which was later rescued for HIV treatment and prevention or the toxic thalidomide, originally indicated as a sedative and later re-employed for multiple myeloma (Reed, 2016; Singhal et al., 1999). Drug rescuing will likely continue to be an important source of new drug candidates, as demonstrated by the National Institutes of Health (NIH) National Center for Advancing Translational Sciences announcement of a significant drug repurposing initiative (Allison, 2012). In the case of schistosomiasis, rescuing old leads may prove to be a fruitful strategy, particularly because there are excellent antischistosomal candidates that were dropped once praziquantel's dominance had clearly been established. In Chapter 4a and 4b, we describe the potent activity of Ro 13-3978, an antischistosomal candidate pursued in Roche and dropped at the pre-clinical phase (Keiser et al., 2010).

Finally, it is also possible to leverage the known mechanism of action of an old or obsolete drug and remodel it to obtain more potent or less toxic analogues. For example, Filho and colleagues synthesized three oxamniquine derivatives which showed excellent activity against *S. mansoni in vivo.* Unfortunately, this was accompanied by higher toxicity and follow up derivatives were less potent (Filho et al., 2007, 2002). On the other hand, organometalic derivatization of compounds, especially ferrocene derivatives, has previously yielded promising antibacterial, anticancer and antimalarial candidates, with, for example, ferroquine already entering clinical trials (Hess et al., 2015). Moreover, chromium praziquantel derivatives have exhibited *in vitro* antischistosomal activity in the nanomolar range (Patra et al., 2013). In line with this successful strategy, we present potent *in vitro* and *in vivo* efficacy of novel organometallic derivatives of oxamniquine in Chapter 4c.

1.8 Dynamics of schistosomiasis and praziquantel treatment in children

As plans coalesce to place schistosomiasis on the road to elimination, it will be important to treat all those affected. It was previously thought that pre-school aged were not often exposed to sources of schistosome infections, yet epidemiological surveys from east and west sub-Saharan regions report prevalences of 14 – 86% in preschoolers (Ekpo et al., 2012; Stothard et al., 2011). Currently, praziquantel is used off-label to treat very young children diagnosed with schistosomiasis at the standard dose used for school-aged children and adults (40 mg/kg), but this is just a back-

calculated extrapolation (Coulibaly et al., 2012). Proper dose-finding clinical trials for children younger than 4 years old have never been conducted (Keiser et al., 2011). Differences in drug digestion, absorption, metabolism and elimination in young children could mean that the praziquantel pharmacokinetic profile is quite different for this group, requiring different dosing regimens (Kearns et al., 2003). In recognition of this need, the Pediatric Praziquantel Consortium was thus formed with the aim of creating a single-dose pediatric formulation of praziquantel. Aside from removing the problematic bitter taste and the potentially inactive –S- enantiomer from the pill, an evidence base is needed to determine the effective dose. In Chapter 5a, we present a dose-finding study for praziquantel in pre-school age and school age children that attempts to provide this.

1.10 Metabonomics to study schistosomiasis infection and treatment

Though much is known about the organ and tissue level effects of schistosomiasis, increased insight into disease pathology could be extrapolated from molecular system-level analyses. Helminth “omics” is an ever-expanding field which promises to bring novel insight into host-parasite interactions and pharmacodynamics (Gaze et al., 2014; Nahum et al., 2012; Nóbrega de Sousa et al., 2013; Wang and Hu, 2014). Metabonomics is a relatively young field that is complimentary to other omics approaches. It is defined as the quantitative measurement of the metabolic responses of living systems to patho- physiological stimuli (Lindon et al., 2007; Nicholson et al., 2002). The development of powerful spectroscopic technologies such as ^{1}H nuclear magnetic resonance (NMR), ultraperformance liquid chromatography (UPLC) or gas chromatography (GC) coupled with mass spectroscopy (MS) and capillary electrophoresis coupled with ultra violet spectroscopic detection (CE-UV), have allowed for identification of metabolites from multiple tissues and fluids at high resolution ((Nicholson et al., 1999)). However, such data is generally noisy, incomplete and contains copious inter-correlated variables per biological sample (Trygg and Lundstedt, 2007). Important developments in chemometrics, namely advances in pattern recognition, spectral alignment and pre-processing, and supervised and unsupervised multivariate statistics have allowed for systematic analysis of inter-group differences and therefore an “omics” approach to analysis (Nicholson et al., 1999; Trygg, 2002; Veselkov et al., 2009).

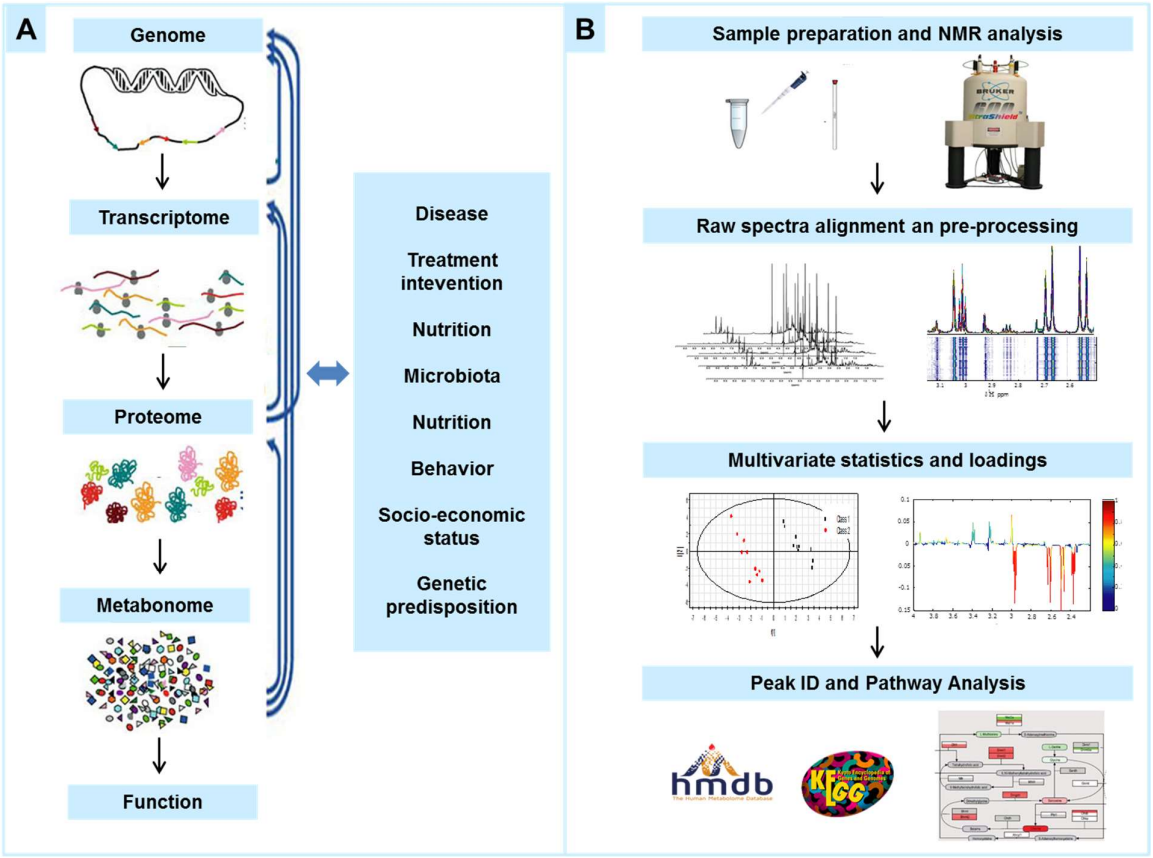

Figure 5 (A) Metabonomics is complimentary to other "omics" approaches to study host responses to disease and other stimuli. (B) Workflow for NMR-based metabonomic analysis. (Adapted from Goodacre, 2005; Veselkov et al., 2009)

Metabolic profiling of human biofluids has previously been employed to identify biomarkers of infectious as well as non-infectious diseases and to reveal drug-response metabolic effects of treatment (Rozen et al., 2005). The former can be a source of novel diagnostics; the latter can provide insight into drug pharmacodynamics and pharmacokinetics, mechanisms responsible for individual variation in drug response and identify markers of treatment efficacy (Kaddurah-Daouk et al., 2008; Lindon et al., 2004)

Metabonomic studies of nematode and trematode rodent infection models have already brought interesting insights into the host-parasite interactions and system-level dynamics of disease. Experiments with *Echinostoma caproni, Fasciola hepatica, Necator americanus and S. japonicum* rodent infection models show species-specific disturbances in metabolites related to energy metabolism (glycolysis, TCA cycle),

amino acid depletion, inflammation and microbial-mammalian co-metabolites, in infected versus non-infected animals (Saric et al., 2009, 2010, Wang et al., 2009, 2010; Wu et al., 2010). ^{1}H NMR profiling analyses *S. mansoni*-mouse models exhibited metabolite alterations indicative of glycolysis stimulation, TCA cycle suppression and amino acid metabolism alterations indicative of liver damage. Moreover, alterations in microbial-mammalian co-metabolites, such as hippurate, 4-cresol glucuronide and phenylacetylglycine, mirrored those observed in the above-mentioned studies, while a significant reduction in urinary keto-acids was specific to the *S. mansoni* infection (Wang et al., 2004). A subsequent study using CE confirmed these findings, while detecting additional biomarkers of infection (García-Pérez et al., 2008). A deeper investigation using magic angle spinning (MAS) NMR and multiple compartments showed the above-described metabolite alterations could be associated with inflammation and fibrosis in multiple organs, but also revealed metabolites associated with osmotic dysfunction in the kidneys, even while alterations were not detectable at the macroscopic level (Li et al., 2009).

Despite these advances, metabonomic investigations of helminth infections in humans are rare. An initial attempt to profile 500 individuals with multiparasitism were not successful in revealing species-specific biomarkers, likely due to the large variability in age, genetic and behavioral differences, nutritional and socio-economic status and too much variation in type and intensity of helminth infections (Singer et al., 2007). Nonetheless, an investigation of onchocerciasis patients revealed 14 infection-associated biomarkers (Denery et al., 2010) and later tyramine was identified as a diagnostic biomarker for *Onchocerca volvulus* infections (Globisch et al., 2013). Balog and colleagues looked at metabolic profiles of *S. mansoni*-infected adults and children and were able to characterize some discriminatory biomarkers of infection in adults (Balog et al., 2011). In Chapter 5b, we demonstrate how metabonomics can be used to study *S. mansoni* infection and its treatment in two important demographic groups: pre-school-aged and school-aged children.

1.10 Aim and objectives

The state of the antischistosomal drug development pipeline understates the urgency and impact of schistosomiasis. The lack of a vaccine and the wide-spread

dissemination of the only drug available to treat this important neglected tropical disease render schistosomiasis control vulnerable to rapid re-infection and potential drug resistance. New developments in drug assay technologies show potential to update the compound screening process to more objective and higher-throughput methods, but they still need improvement in accuracy, resolution and efficiency. However, even if identification of pre-clinical candidates is accelerated, *de novo* drug discovery is becoming increasingly costly and time-consuming. Therefore, drug repurposing and drug rescuing remain as important sources of potential novel antischistosomals. Meanwhile, there is still much to be understood about systemic impacts of this disease and its treatment with praziquantel in the demographic that is most affected- children.

In this light, this PhD project had two main aims. The first aim was to bolster the drug discovery pipeline for schistosomiasis by investigating alternate *in vitro* screening systems and repurposing and rescuing old drugs into potential antischistosomals. The second aim was to expand our understanding of the pharmacodynamics of schistosomiasis and praziquantel treatment.

This was pursued with the following specific objectives:

1. To investigate an automated medium-throughput drug screening assay for *Schistosoma mansoni.*

2. To review the drug repurposing landscape and identify potential antischistosomal compounds from a library of FDA approved drugs.

3. To characterize the antischistosomal activity of the 3-arylhydantoin Ro 13-3978.

4. To evaluate the *in vitro* and *in vivo* properties of novel organometalic oxamniquine derivatives.

5. To elucidate the metabolic effects of schistosomiasis infection and its treatment with praziquantel in children.

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Chapter 2

Investigations of novel automated *in vitro* screening assays

Chapter 2a

Fluorescence/luminescence-based markers for the assessment of *Schistosoma mansoni* schistosomula drug assays

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Abstract

Background: Schistosomiasis is responsible for a tremendous public health burden, yet only a single drug, praziquantel, is available. New antischistosomal treatments should therefore be developed. The accuracy, speed and objectivity of *in vitro* drug screening depend on the assay read-out. Microscopy is still the current gold standard and is in need of updating to an automated format. The aim of the present study was to investigate a panel of fluorescence/luminescence dyes for their applicability as viability markers in drug sensitivity assays for *Schistosoma mansoni* schistosomula.

Methods: A search for available viability and cytotoxicity marker assays and dyes was carried out and a short-list of the most interesting candidates was created. The selected kits and dyes were tested on *S. mansoni* Newly Transformed Schistosomula (NTS), first to assess whether they correlate with parasite viability, with comparatively low background noise, and to optimise assay conditions. Markers fulfilling these criteria were then tested in a dose–response drug assay using standard and experimental drugs and those for which an IC_{50} value could be accurately and reproducibly calculated were also tested on a subset of a compound library to determine their hit-identification accuracy.

Results: Of the 11 markers selected for testing, resazurin, Vybrant® and CellTiter-Glo® correlated best with NTS viability, produced signals ≥ 3-fold stronger than background noise and revealed a significant signal-to-NTS concentration relationship. Of these, CellTiter-Glo® could be used to accurately determine IC_{50} values for antischistosomals. Use of CellTiter-Glo® in a compound subset screen identified 100 % of hits that were identified using standard microscopic evaluation.

Conclusion: This study presents a comprehensive overview of the utility of colorimetric markers in drug screening. Our study demonstrates that it is difficult to develop a simple, cheap "just add" colorimetric marker-based drug assay for the larval stage of *S. mansoni*. CellTiter-Glo® can likely be used for endpoint go/no go screens and potentially for drug dose–response studies.

Keywords: *Schistosoma mansoni*, Newly Transformed Schistosomula, Drug sensitivity assay, Fluorescence, Luminescence, Viability marker, Cytotoxicity marker

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Background

Schistosomiasis, causing 3.3 million DALYs lost, is one of the most important Neglected Tropical Diseases (NTDs) [1]. It is a water-borne trematodiasis caused by any of six *Schistosoma* species that parasitise humans: *Schistosoma haematobium, S. mansoni, S. japonicum, S. mekongi, S. guineensis* and *S. intercalatum*- the former 3 being most common [2, 3]. Schistosomiasis is prevalent mainly in rural areas of poor sanitation, with the majority of cases occurring in Sub-Saharan Africa [3, 4]. The bottom line of the WHO schistosomiasis control programme is morbidity reduction via preventative chemotherapy [3]. Only a single drug- praziquantel- is used to treat millions of people annually and its coverage is projected to reach 235 million people by 2018, which raises concerns of increasing drug pressure [5, 6]. Moreover, although praziquantel is a safe and cheap therapy effective on the adult stage of the disease, it is inactive against the juvenile stage [6, 7]. Therefore, new chemotherapies are desperately needed.

In the past, standard operating procedures (e.g. established at TDR-designated compound screening centers) relied on adult worms incubated with the candidate drugs for 72 hours, after which their viability is assessed microscopically [8]. This method requires the intensive use of mice (as no *in vitro* life cycles currently exist), is time consuming and low-throughput. Thus recently, the use of Newly Transformed Schistosomula (NTS) has been popularised as a higher-throughput screen [9–12]. Nonetheless, assessing parasite viability via microscopic read-out is slow and subjective and is therefore a bottleneck for high-throughput screening. Consequently, with recent advancements in automated technologies, a number of assay read-out alternatives have been attempted with varying degrees of success [9]. These methods include the measurement of parasite mobility over time via electrical impedence with xCELLigence [13], isothermal microcalorimetry [14] and automated image-based Bayesian classification [15]. On the other hand, using dye-based assays that can be read by an automatic plate reader would be a simpler, cheaper, more practical and more trainable read-out alternative, requiring little extra equipment or software. Three fluorescent and one luminescent assay reagent have been studied on *S. mansoni* NTS to date: the Alamar Blue® viability assay (resazurin), the fluorescein diacetate/ propidium iodide fluorescent multiplex assay and a fluorometric L-lactate assay (fluorescent) and CellTiter-Glo® (luciferase) as the luminescent assay. In more detail, Alamar Blue® could discriminate between live and dead NTS after 7 days of incubation with standard drugs but not for earlier time-points and it could not be used to measure dose–response drug effects [11]. The fluorescein diacetate/ propidium iodide assay is a duplex viability and cytoxicity assay where fluorescein diacetate stains live NTS and propidium iodide stains dead NTS [16]. This assay was successful in that it could be used to calculate an LD_{50} value for auranofin, an antirheumatic agent that is active against *S. mansoni*, and could distinguish between dead and alive NTS for several standard drugs. However, practical issues, such as the requirement of a rinsing step and the need for a high number of NTS, as well as its questionable ability to determine dose-dependent effects for all standard drugs, were elements that could be improved upon. Howe and colleagues investigated lactate, a byproduct of glycolysis known to be secreted via aquaglyceroporins from NTS and adult worms, as a possible marker for viability. They too were able to generate dose–response curves for some but not all standard drugs using a commercial L-lactate kit [17]. Nonetheless, the procedure requires removing the supernatant from the drug assay (without aspirating the NTS) and then diluting it to an acceptable fluorescence range as needed, rendering it less than high-throughput. More recently, Lalli and colleagues (2015) validated the use of the commercial luminescence-based cell viability kit, CellTiter-Glo®, in an *in vitro* assay using *S. mansoni* NTS and adult worms [18]. Here, however, a precise multi-drop dispenser was required to ensure an exact number of NTS was present in each well. Hence, although the investigation of marker-dye based assays has been a popular pursuit, the aim of a simple, inexpensive and precise dye that does not require much additional equipment or analysis has not entirely been met.

In the present study we sought to identify an easy-to-use, "just-add" viability or cytotoxicity marker assay that can accurately determine the viability of NTS in a drug sensitivity screen. We therefore reviewed the literature for previous use of dyes and markers on *S. mansoni* for their potential use as viability or cytotoxicity markers in an automated drug assay. We also researched the market for commercially available viability and cytotoxicity kits and dyes that could in theory be adapted for use in an *S. mansoni* NTS drug assay. Eleven markers, resazurin (the active component of Alamar Blue®), OmniCathepsin™, CellTiter-Glo®, Vybrant®, CytoTox- ONE™, LIVE/DEAD® viability/cytotoxicity kit-, ApoTox-Glo™, CellTox™ Green Cytotoxicity Assay, DAPI, Hoechst 33258 (bis-Benzimide) and FluoForte® Calcium Assay with diverse modes of action were selected. All markers were tested to elucidate whether wells containing NTS could produce signals significantly stronger than wells containing medium only, a significant signal-to-NTS concentration relationship and if differential signals between live and dead NTS could be observed. For the three markers resazurin, Vybrant® and CellTiter-Glo® meeting this criteria, dose–response drug assays were conducted. CellTiter-Glo® was further validated on a 25-compound subset of a compound library of FDA-approved drugs.

Methods

Literature search

A recent review on approaches to measuring helminth viability [9] served as a starting point to investigate dyes and methods that had already been attempted to measure *S. mansoni* viability. Each marker listed in the publication was checked to see how it was used on *S. mansoni*. Specifically, we looked if the named marker had already been used on NTS in a multi-well assay. In parallel, a simple Google and PubMed search was conducted in order to identify commercial viability/cytotoxicity kits available on the market or markers and dyes that are not normally used to measure viability but could be used to measure *S. mansoni* viability in theory. Terms used were "colorimetric viability markers", "colorimetric viability assays", "viability assays", "fluorescent viability markers", "cytotoxicity assays", "cytotoxicity marker assays", "*Schistosoma mansoni* cytotoxicity", "*Schistosoma mansoni* viability" and "*Schistosoma mansoni* assay". With the aim of identifying desirable candidate markers for NTS, the following primary exclusion criteria were applied: (i) active component should not be one that has already been identified as ineffective at measuring viability in NTS (based on published or unpublished data); (ii) must not rely on cellular replication since NTS do not replicate or grow very fast; (iii) must not require additional rinsing steps since NTS do not fix to the bottom of well plates. In addition, it was desirable that the marker did not also measure bacteria and fungi viability and would not be too costly.

Media, chemicals and drugs

Medium 199 was purchased from Gibco (Basel, Switzerland), inactivated foetal calf serum (iFCS) was obtained from Connectorate AG (Dietikon, Switzerland) and a mixture of penicillin-streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin) was purchased from Sigma-Aldrich (Buchs, Switzerland).

CellTiter-Glo®, ApoTox-Glo™, CellTox™ Green Cytotoxicity Assay and CytoTox-ONE™ were purchased from Promega. The Vybrant® Cytotoxicity Assay Kit and the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells were purchased from Molecular Probes (Invitrogen), whereas FluoForte® Calcium Assay kit was acquired from Enzo Life Sciences Inc. DAPI (4′, 6-Diamidino-2-phenylindole dihydrochloride) and Hoechst 33258 were purchased from Sigma-Aldrich and stock solutions of 5 mg/ml were constituted by dissolving the substrate in dH_2O and DMSO respectively. The OmniCathepsin™ reagent was prepared by dissolving OmniCathepsin™ substrate (Enzo Life Sciences) in DMSO solution at a concentration of 10 mM. The resazurin dye was constituted by dissolving resazurin sodium salt (Sigma) in 1x PBS solution at a concentration of 125 mg/l. All markers were stored at −20 °C until use except DAPI and Hoechst 33258, which were stored at 4 °C.

Praziquantel, and mefloquine were purchased from Sigma-Aldrich (Buchs, Switzerland), and oxamniquine was donated by Dr. Quentin Bickle. Drug stock solutions were made by dissolving the compounds in DMSO (dimethyl sulfoxide, Fluka, Buchs, Switzerland) at a concentration of 10 mM and were stored at −20 °C until use.

Transformation of S. mansoni cercariae into Newly Transformed Schistosomula (NTS)

For the transformation of cercariae into schistosomula, a cercarial suspension was collected from *S. mansoni*-infected *Biomphalaria glabrata* snails and subjected to a mechanical *in vitro* transformation described previously [12]. The NTS were then placed in warm culture medium: Medium 199 supplemented with 5 % iFCS and 1 % penicillin-streptomycin mixture and incubated for 24 hours at 37° C and 5 % CO_2 until use.

Signal correlation to NTS concentration assays and exposure time assays

Assays were set up to measure whether incubation with the selected marker yielded a significant 3:1 signal-to-background (S/B) ratio. In addition, viability markers should present a linear curve between the signal (fluorescence or luminescence) and increasing concentrations of live NTS, but a poor signal and no relationship when incubated with dead NTS and vice-versa when cytotoxicity markers are used. Thus for each marker, an assay with increasing concentrations of NTS/well (20, 40, 60, 80, 100, 200, 300 and 400 NTS/well), of live and dead NTS was set up. To measure signal strength in correlation to exposure time to the marker, the NTS concentration assays were measured at multiple time-points (Additional file 1: Table S1). In addition, because it was noted for Cell-Tox™ Green that it mattered for signal intensity how NTS were killed, a variety of substances were tested to kill the NTS for the potential cytotoxicity markers, the FluoForte® Calcium Assay, DAPI and Hoechst 33258 (Additional file 1: Table S1).

The total well volume, including the marker, was between 100 and 250 μl. Control wells contained culture medium only (supplemented Medium 199). Altogether, 2 to 3 trials of duplicates/triplicates were performed for each marker. Marker-specific methods (the amount of marker added, the plate incubation times and the excitation/emission (Ex/Em)) are summarised in Additional file 1: Table S1. Spectra were determined by the commercially published protocols or in the case of resazurin, Hoechst 33258 and DAPI, from previous publications [11, 19–21]. Luminescence or fluorescence was read using the SpectraMax® M2 Multi-Mode Microplate Reader (Molecular Devices).

For OmniCathepsin™, preliminary assays were done to determine the optimal concentration of the dye. LIVE/DEAD® and ApoTox-Glo™ are duplex assays, and thus the plates were scanned twice, each time at the Ex/Em spectra specified for each dye (Additional file 1: Table S1).

Since LIVE/DEAD®, CellTox™ Green Cytotoxicity, DAPI and Hoechst 33258 are fluorescence markers that stain components within the NTS themselves, read-outs were complemented by confirmation of NTS staining via fluorescence microscopy. From the assay, 20 μl of live and dead NTS suspensions were placed on glass slides with cover slips. Inspection was conducted using the Leica DM5000B upright microscope. The L5 filter was used to view objects stained by calcein from the LIVE/DEAD® kit and CellTox™ Green Cytotoxicity Assay cyanine dye; the Texas Red (TR) filter was used to view objects stained by EthD-1. The CY3 filter was used for DAPI and the A4 filter for Hoechst 33258. Imaging was possible via the microscope camera, which was connected to Leica Application Suite 2.4.0 imaging software.

Drug sensitivity assay

Markers that showed an S/B of 3:1 and a strong signal-to-NTS concentration relationship were selected for testing with two standard and one experimental drug to assess if they could be used to determine IC_{50} values in a dose–response drug sensitivity assay. Drugs were serially diluted to fit a range of previously reported IC_{50} values [22, 23]. The dilution series were as follows: 20, 10, 5, 2.5, 1.3 and 0.7 μM for praziquantel and mefloquine; and 240, 120, 60, 30, 15, 7.5 and 3.7 μM for oxamniquine.

The drug assays were set up according to manufacturer protocols using 200 NTS and an incubation time of 24 hours with resazurin, 15 min with Cell-Titer-Glo® and 70 min with Vybrant®. Assays were evaluated at various time-points. Each SpectraMax® read-out was accompanied by a microscopic read-out in order to compare the two methods. Microscopic assessment of viability was done by observing the NTS morphology and motility and by assigning the NTS viability scores as follows: 0 = dead; 1 = both slower movement and damage to tegument or severely impaired movement or severely damaged tegument; 2 = slow movement or notable damage to tegument; 3 = lively movement and undamaged tegument. IC_{50} values, which describe the drug concentration at which worm viability is inhibited by 50 % as scored by the viability scare, were calculated as described below using both read-outs and compared.

Assay validation

To confirm that CellTiter-Glo® could be reliably used in a drug screen, a subset ($n = 25$) of a previously screened library of FDA approved compounds [24] was chosen for re-screening with CellTiter-Glo®. Compounds were selected by randomly picking from the "hit" and "not hit" lists, such that the hit rate in the assay would be ~20-25 %. The compounds were screened on NTS at 10 μM and CellTiter-Glo® was added as previously described. Each compound was tested in duplicate twice, along with an NTS-free blank for background measurement. Assays were assessed by both a microscopic evaluation and the SpectraMax® scan. Hit compounds were defined as those compounds that achieved ≥ 75 % reduction in viability (calculated using the viability score described above) for the microscopic evaluation and ≥ 75 % reduction in fluorescence signal relative to live controls. Dead controls were included in the assay to calculate the Z' factor for each plate.

Statistical analysis

Averages, standard deviations and S/B ratios were calculated and graphs generated with Microsoft Office Excel 2013. Dose–response drug sensitivity assays using the markers were read both by the SpectraMax® and manually via microscopic read-out. From the SpectraMax® read-outs, the IC_{50} values were calculated in SoftMax® Pro. From the microscopic read-out, the IC_{50} values were calculated with the help of CompuSyn® (2006). The Z' factor was calculated according to the formula described by Zhang *et al.*, where a score ≥ 0.5 is considered excellent [25].

Results

Literature search

A substantial number of dyes have been tested on the different stages of *S. mansoni* and many of them specifically on the NTS stage (Additional file 2: Table S2). Many dyes, however, were not suitable for automated drug sensitivity assay read-outs, the reasons for which are presented in Additional file 2. Our search identified 2 viability, 3 cytotoxicity, 2 multiplex and 4 "experimental" markers for further testing. Their features/mechanism of action are summarised in Additional file 2: Table S2.

Signal to NTS concentration assays and exposure time assays

Viability markers

For all assays, optimal incubation parameters and key results are summarised in Table 1.

CellTiter-Glo® assay results showed a strong correlation between live NTS number and luminescence signal ($R^2 = 0.98$) (Fig. 1a). The signals were strongest at 15 min incubation time with the reagent, after which the signal strength decreased. A S/B ratio of at least 3:1 was observed from 100 or more NTS/well. Dead NTS did yield a luminescence signal but comparable to that of the background luminescence.

Table 1 Summary of marker optimizations and results

Marker Type	Marker Assay	Optimal incubation time	S:B ratio ≥ 3:1?	Correlation to NTS viability/cytotoxicity	Selected for drug assay testing?	Justification
Viability Markers	CellTiter-Glo®	15 min	Yes, with ≥ 100 NTS	Strong	Yes	Met criteria
	Resazurin	24 hours	Yes, with ≥ 200 NTS	Strong	Yes	Met criteria
Cytotoxicity Markers	Vybrant®	70 min	Almost, with ≥ 300 NTS	Strong up to 300 NTS	Yes	Met criteria
	CytoTox-ONE	2 hours	No	Strong	No	S:B ratio low, large standard deviations between data points
	CellTox™ Green Cytotoxicity Assay	24 hours	Yes, with ≥ 400 NTS	Strong with ≥ 400 NTS	No	Viable NTS died within 24 hours of exposure to dye; strong signals from completely lysed cells only
Multiplex Markers	LIVE/DEAD® Viability/ Cytotoxicity Kit	Does not exist	No	Poor for both live and dead NTS	No	Poor correlation to NTS viability/ cytotoxicity
	ApoTox-Glo™	Does not exist	No	Strong for live-cell marker, poor for dead-cell marker	No	Markers induced spazzing and death of NTS after 6 hours
Experimental Markers	OmniCathepsin™	2 hours (with 10 μM marker concentration)	No	Good: Differential signals for live vs. dead observed	No	S:B ratio too low, not enough difference between live and dead NTS
	FluoForte® Calcium Assay	1.5 hours with ≥ 200 dead NTS	Yes, with ≥ 200 dead NTS (lysed only)	Poor: strong staining for completely lysed cells only	No	Stains only completely lysed cells
	DAPI	15 min (with 10 μg/ml dye concentration)	No	Good: stained many dead NTS phenotypes	No	Background fluorescence too high, signals too low
	Hoechst 33258	15 min (with 1 μg/ml dye concentration)	No	Good: stained many dead NTS phenotypes	No	Background fluorescence too high, signals too low

Resazurin presented a strong linear relationship with live NTS concentration ($R^2 = 0.98$) but revealed large standard deviations (Fig. 1b). The signals, S/B ratio and R-squared values were highest after 24 hours of incubation. The minimum number of live NTS that gave at least a 3:1 S/B ratio was 200 NTS. The fluorescence signal for dead NTS was low (183.6 ± 40.3 RFU at 300 dead NTS when subtracted from background) and there was not a strong correlation between number of dead NTS and the fluorescence signal ($R^2 = 0.003$).

Cytotoxicity markers

Dead NTS incubated with Vybrant® yielded a strong signal-to-dead-NTS concentration correlation up to 300 dead NTS ($R^2 = 0.95$), after which the signal plateaued. The highest S/B ratio was obtained after 70 min with 300 dead NTS per well (S/B = 2.78:1), though this fell just below the 3:1 cut-off. The signals for live NTS were comparable to background levels (Fig. 2a).

A strong correlation between dead NTS concentration and fluorescence signal ($R^2 = 0.97$) was observed for CytoTox-ONE™, however, with very large standard deviations for each data point. Live NTS signals were comparable to background levels but background levels for CytoTox-ONE™ were very large (6605 RFU) (Fig. 2b). As a result, the S/B ratio was always at around 1:1 regardless of dead NTS concentration or exposure time to CytoTox-ONE™.

CellTox™ Green showed a linear relationship between fluorescence and number of dead NTS per well, exhibiting a significant signal at a concentration from 400 NTS and onwards at 24 hours (Fig. 2c). Fluorescence microscopy after 4 hours incubation with the marker showed that the dye was effectively penetrating the cell Additional file 3: Figure S1(E, F), though the signal intensity and relationship linearity measures are significant from 24 hours after addition of reagent (S/B >3:1). Additionally, live NTS measured by this reagent gave low fluorescence signals (S/B <2:1). However, after 24 hours of exposure to CellTox™ Green, live NTS viability was severely diminished and hence could not be used as reliable controls at this time-point measurement. An important point is that only lysed NTS, and not heat, EtOH 10 %, DMSO 25 %, praziquantel nor mefloquine – killed NTS, showed significant values compared with the control wells.

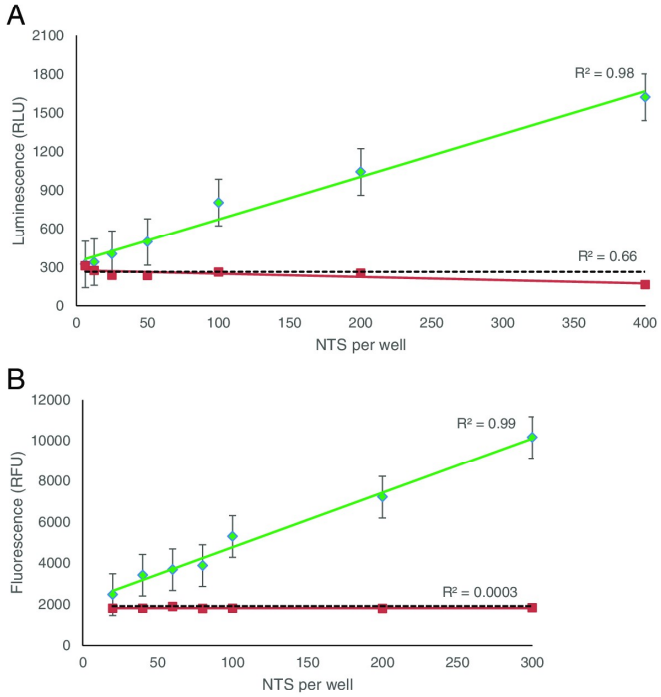

Fig. 1 Correlation to NTS concentration for viability markers **a** CellTiter-Glo® and **b** resazurin. Green lines represent data from live NTS and red lines represent data from dead NTS. The dotted line represents the background signal from medium-only wells. Curves are shown for the optimal incubation time for each marker: 15 min for Cell-Titer Glo and 24 hours for resazurin.

Multiplex assays

Using the LIVE/DEAD® Viability/Cytotoxicity Kit, measurements with calcein showed weak signals and a poor correlation with live NTS concentration (Fig. 3a). However, microscopic images demonstrate that at 45 minutes after exposure to the dyes, the NTS are stained properly and discriminately (Additional file 3: Figure S1(A-D)). Additionally, signals from dead control NTS were very close to the range of the live NTS signals. Meanwhile, signals generated from dead NTS scanned by the EthD-1 Ex/Em spectra yielded an extremely low signal and only a moderate linear correlation (Fig. 3b). The negative controls, however, also showed poor signals and linearity. Measurements with calcein using as many as 1000 NTS showed an improved signal correlation with NTS numbers ($R^2 = 0.89$ at 4 hours incubation time) but nonetheless a low fluorescence signal overall (Additional file 4: Figure S2(A)). Measurements with EthD-1 showed no significant improvement in signal and the signal even plateaued at higher NTS concentrations (Additional file 4: Figure S2(B)). The S/B ratio was less than 3 for both calcein and EthD-1 at all time-points.

ApoTox-Glo™ also presented as a poor multiplex assay. The AFC reagent yielded a strong linear relationship with live NTS concentrations and high signal levels (Fig. 3c) but only after 6 hours of exposure to the marker, when microscopic evaluation revealed spastic NTS. By 24 hours, all NTS were dead. The bis-AAF-R110 reagent yielded a very poor signal and NTS concentration correlation with dead NTS (Fig. 3d). Paradoxically, the signal correlation to increasing live NTS concentrations was strong ($R^2 = 0.99$).

Experimental reagents

As the experimental dyes selected for this work have not yet been used as viability or cytotoxicity markers, no standard protocol for reagent concentration or suggested reagent exposure time existed. Hence, initial experiments were conducted to determine these parameters, summarised in Table 1. Thereafter, experiments were conducted to determine if the reagent could produce differential signals for live versus dead NTS.

Using the OmniCathepsin™ assay, high fluorescence signals were yielded by live NTS (Fig. 4a). The signals for live NTS were notably higher than for dead NTS,

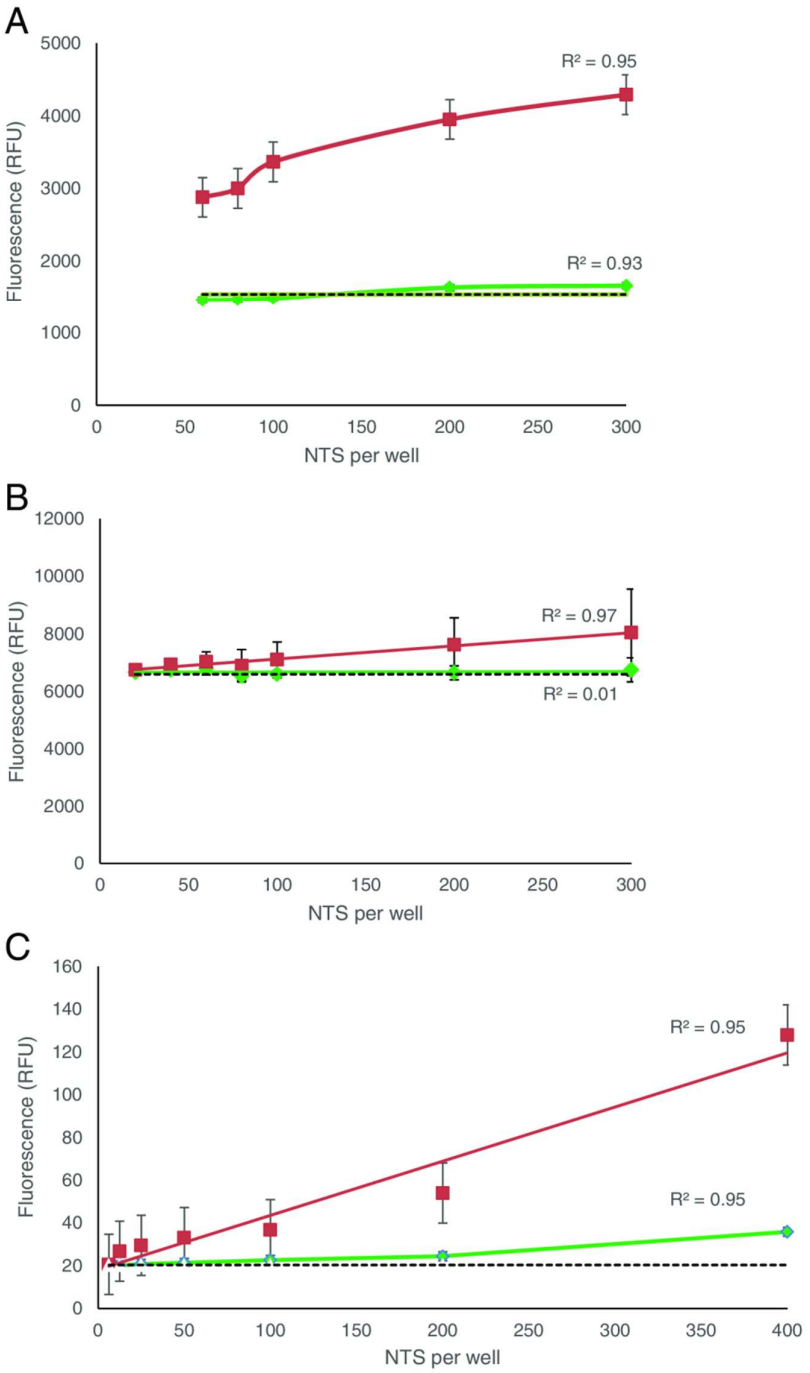

Fig. 2 Correlation to NTS concentration for cytotoxicity markers **a** Vybrant®, **b** CytoTox-ONE™ and **c** CellTox™ Green. Green lines represent data from live NTS and red lines represent data from dead NTS. The dotted line represents the background signal from medium-only wells. Curves are shown for the optimal incubation time for each marker: 70 min for Vybrant®, 2 hours for CytoTox-ONE and 24 hours for CellTox™ Green.

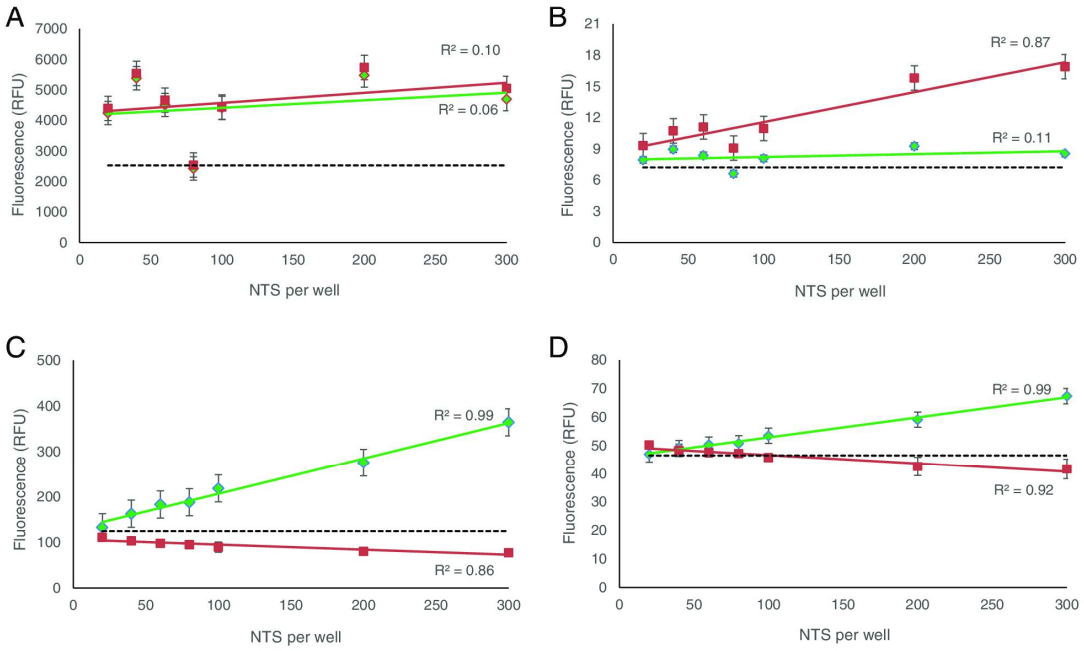

Fig. 3 Correlation to NTS concentration for multiplex markers **a** and **b** LIVE/DEAD® Viability/Cytotoxicity Kit and **c** and **d** ApoTox-Glo™. Since these kits contained both a viability and cytotoxicity reagent, a separate scan was done for the viability reagent (**a**) for LIVE/DEAD® and (**c**) for ApoTox-Glo™ and for the cytotoxicity agent (**b**) for LIVE/DEAD® and (**d**) for ApoTox-Glo™. The green curve is for live NTS, the red is for dead NTS and the dotted line is for background signals. The graphs presented correspond to the optimal incubation time for each marker, which is 24 hours for both LIVE/DEAD® and ApoTox-Glo™.

however, high background signals interfered with the assay, and neither significant differences between live and dead NTS signals, nor a 3:1 S/B ratio could be achieved.

Incubation of dead NTS with FluoForte® Calcium Assay produced significant signal intensity in comparison with live NTS (Fig. 4b). However, this was only the case for completely lysed NTS. NTS killed by heat, EtOH 10 % or DMSO 25 % did not produce high signals. Since no drug completely lyses NTS, further investigations with this assay were discontinued.

For both DAPI and Hoechst 33258, the S/B ratio did not reach the 3:1 threshold, though a difference was observed between the fluorescence values of live versus lysed or mefloquine-killed NTS (Figs 4c and d). When observed with fluorescence microscopy, both dyes stained dead NTS at their determined optimal incubation times and did not stain control live NTS (Additional file 3: Figure S1(E-J)).

Since high background signals were a notable problem for many of these stains, assays with Omnicathepsin™, DAPI and Hoechst 33258 were set up in order to assess the effect of phenol red, iFCS and medium type on the background fluorescence values (Additional file 5: Figure S3). Indeed, both iFCS and phenol red contribute to high background noise though most of it is attributable to presence of iFCS. However, it is not possible for NTS to remain viable without iFCS, hence removing it from the medium or decreasing the concentration are not viable options for a 72 hour drug assay.

Drug sensitivity assays

Resazurin, Cell-Titer Glo®, and Vybrant® were selected for further testing in a drug sensitivity assay using standard drugs. An automatic read-out with resazurin did not produce dose-dependent fluorescence values and thus IC_{50} values could not be calculated (Table 2). Vybrant® did not yield IC_{50} values for praziquantel and oxamniquine and gave an IC_{50} for mefloquine that was higher than microscopically derived values. In the case of CellTiter-Glo®, an automatic read-out did not produce sufficient dose-dependent values to calculate an IC_{50} for praziquantel. The IC_{50} calculated for oxamniquine was higher than the microscopically determined one, though oxamniquine IC_{50} values fluctuated widely even between microscopic read-outs. Nonetheless, the IC_{50}

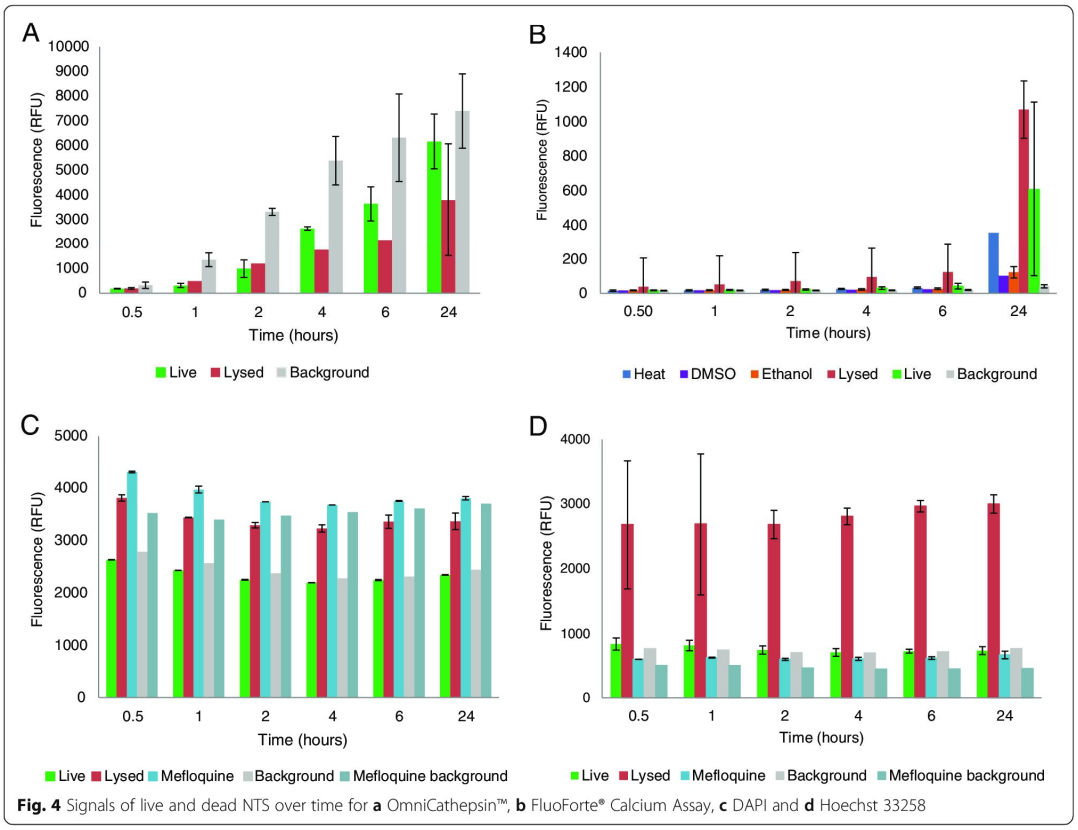

Fig. 4 Signals of live and dead NTS over time for **a** OmniCathepsin™, **b** FluoForte® Calcium Assay, **c** DAPI and **d** Hoechst 33258

value obtained for mefloquine was consistent with the microscopic read-out. Furthermore, a single concentration (10 μM) drug screen with CellTiter-Glo® revealed that of the 25 compounds screened, 100 % of positives were identified as such, and 100 % of negatives were also identified as such (Table 3). The plate Z' scores were ≥ 0.6.

Discussion

Concerns about increasing drug pressure due to the exclusive use of praziquantel underscore the need for new treatments [26]. The growing availability of new technologies, partnerships and open source drug discovery present great opportunities to screen more compounds for their antischistosomal activities, but to take advantage of them, effective, inexpensive and practical *in vitro* methods are required.

The aim of this research was to study a plethora of fluorescent or luminescent viability and cytotoxicity markers with various modes of action. In doing so, our hope was to develop a simple, novel and automated high-throughput *in vitro* drug sensitivity assay, as well as to elucidate types of colorimetric markers that are suitable for larval helminth screens.

Table 2 IC_{50} values generated by resazurin, CellTiter-Glo® and Vybrant® compared to microscopic values

	Resazurin		CellTiter-Glo®		Vybrant®	
Drug	IC_{50} SpectraMax (μM)	IC_{50} Microscopic Readout (μM)	IC_{50} SpectraMax (μM)	IC_{50} Microscopic Readout (μM)	IC_{50} SpectraMax (μM)	IC_{50} Microscopic Readout (μM)
Praziquantel	not calculable	1.7 ± 1.5	not calculable	1.3 ± 0.9	not calculable	2.4 ± 1.2
Mefloquine	not calculable	2.6 ± 1.1	4.9 ± 2.9	2.4 ± 1.3	7.9 ± 1.7	1.7 ± 0.3
Oxamniquine	not calculable	135.1 ± 128.8	194.5 ± 52.6	87.7 ± 49.7	not calculable	20.3 ± 9.5

Table 3 CellTiter-Glo® and microscopic evaluation of a small subset ($n = 25$) of an FDA-approved compound library. Values of % reduced viability of compound-treated NTS relative to the controls were based on average luminescence for CellTiter-Glo® (subtracted from background) and on average viability scores for the microscopic evaluation.

	% reduced viability relative to controls	
Compound	CellTiter-Glo®	Microscopic Evaluation
acemetacin	0	11.1
benzalkonium chloride	94.9	100
cefpodoxime proxetil	0	0
clofazimine	85.2	100
clofibric acid	0	0
docosanol	0	0
docusate sodium	0	0
ecamsule triethanolamine	0	0
eletriptan hydrobromide	0	0
etidronate disodium	0	0
flumazenil	0.5	0
lomerazine hydrochloride	78.6	100
methylbenzethonium chloride	84.5	100
nicardipine hydrochloride	76.8	88.9
perhexiline maleate	94.5	100
piperperone	0	0
pipemidic acid	0	0
quinine ethyl carbonate	0	0
ritodrine hydrochloride	0	0
saccharin	0	0
spiramycin	0	0
sulpiride	0	0
tamoxifen citrate	85.4	100
teicoplanin	0	0
tetramizole hydrochloride	0	0

For most of the tested markers, with the exception of LIVE/DEAD®, the signals tended to correlate well with an increased concentration of NTS (live NTS for viability markers and dead NTS for cytotoxicity markers). Moreover, the signals were usually differential between live and dead NTS for markers that reacted with NTS components that could be found in the medium, excluding CytoTox-ONE™ for which the difference was not at all significant. An issue for the markers that relied on staining NTS themselves was that signals were usually far too low to differentiate between live and dead NTS. Indeed, for markers that stained DNA of membrane-damaged NTS, only complete cell lysis would provide sufficiently high fluorescence signals. Since no drug completely lyses NTS, markers with this limitation could not be considered for further testing. In the case of LIVE/DEAD®, we attempted to increase the signal with a higher concentration of NTS and a smaller surface area to scan (using a 384-well plate), which was sufficient to render the signals differential between live and dead NTS (Additional file 4: Figure S2). However, the number of NTS required (1000 NTS) to achieve this and to negate the background noise is far too high to make for a realistically higher-throughput assay.

The largest issue that prevented markers from proceeding to further tests was that the background signals were far too high, meaning the S/B ratio could not exceed 3:1, our minimum standard. Issues with background signals were briefly explored in separate studies with culture medium (Additional file 5: Figure S3). The medium type and the presence of phenol red in the medium did tend to affect the signal, but this depended on the fluorescence marker used. The most notable impact on the signal, however, resulted from presence of iFCS in the medium, where removing it altogether would have the greatest impact on reducing background fluorescence. Nonetheless, previous studies have demonstrated that this is not a realistic option if NTS are to remain viable throughout the duration of a 24 or 72 hour drug assay [27].

Despite the above-described obstacles, resazurin, CellTiter-Glo® and Vybrant® could be tested in a drug-response assay. While a read-out with resazurin did not correlate at all with microscopic findings (signals were not differential enough to produce a curve), IC_{50} values could be generated using CellTiter-Glo® and Vybrant® that were close to the microscopic values for mefloquine. However, assays with Vybrant® could not produce a curve for oxamniquine. No marker gave signals that could be used to generate an IC_{50} value for praziquantel.

In the case of oxamniquine, the not entirely reproducible IC_{50} values are not altogether surprising since it is not highly active *in vitro* and even microscopic evaluations yield highly variable IC_{50} values. In contrast, microscopic evaluations for praziquantel usually do result in a typical dose–response curve. The lack of a dose–response relationship for praziquantel on NTS was observed also in previous studies using fluorescence/luminescent markers [9, 11, 18], and also when isothermal microcalorimetry was used [14]. It has been suggested that this is because praziquantel does not induce NTS death, which is true, but it does very severely damage them and reduce their motility, which should be reflected by reduced signals in such markers. It is therefore conceivable that while praziquantel does induce extensive damage, it also results in high enzyme or ion channel activity, which interacts with the viability markers and produces high signals.

Our comprehensive overview and studies are placed in context of previous studies with fluorescence and luminescence based NTS viability assays. Because of the Alamar

Blue® experiments conducted by Mansour *et al.*, [11] and resazurin experiments with *S. haematobium* [23] that did not yield promising results at the level of drug assay applicability, we did not expect resazurin to perform well as a marker and this was corroborated in our study. Our results mirror those of Mansour *et al.* [11], where the signal-to-NTS concentration tests shows promising results (albeit only after 24 hours of incubation with the marker), but the resazurin marker failed to generate dose-dependent viability curves and to distinguish between live and dead NTS after 72 hours of drug exposure.

The fluorescein diacetate/ propidium iodide assay published by Peak *et al.* is novel in that a duplex assay allows for simultaneous assessment of viability and cytotoxicity for *S. mansoni* adults and NTS [16]. The LIVE/DEAD® viability/cytotoxicity kit assay tested in our study functions in much the same way. Although Peak and colleagues had better success in obtaining concordant signals from both dyes, the fundamental issue with such markers in the end is the high number of NTS required to assess viability in a drug sensitivity assay, which reduces their throughput. Indeed, Lalli *et al.* [18] showed in their study that CellTiter-Glo® provides a far more sensitive assessment of parasite viability and, considering that it measures ATP production (which is theoretically abundant) after all the NTS have been completely lysed by the reagent, this is not surprising. In our study we also showed that CellTiter-Glo® can be used to assess drug dose–response effects. Furthermore, a screen of a 25 compound subset of an FDA-approved library showed 100 % correspondence between microscopic evaluation and CellTiter-Glo® with regards to hit identification, though all the active drugs in this screen were completely schistocidal.

In contrast to the study by Lalli and colleagues [18], we did see that if one wants to include compounds that damage but may not necessarily kill the worm as hits, the assay becomes less sensitive (as was shown with, for example, praziquantel). This lack of sensitivity could be due to variability in NTS concentration- if a well contains 100 NTS +/− 20 NTS, this already presents +/−20 % deviation in NTS concentration and in corresponding signal. Large signal variability might, therefore, impede the measurement of fine gradients of reduced viability. This might be the reason for large amounts of NTS used in previous studies [16] or why Lalli and colleagues [18] used a multi-drop sorter to dispense a more specific number of worms within the assay. Thus for a sensitive marker-based assay, much care needs to be undertaken to reduce NTS number variability.

Conclusion

In summary, by testing a myriad of colorimetric markers with diverse mechanisms of action, we conclude that due to large fluctuations in signals, likely due to low numbers of NTS that are sensitive to variation in NTS concentration and viability, and high background noise, it is difficult to develop a simple, cheap "just add" colorimetric marker-based drug assay for the larval stage of *S. mansoni.* Markers that stain NTS themselves require a very large number of worms, and markers that assess elements spilled into the medium may require either a very specific number of worms or the removal of the assay supernatant in order to yield high and uniform signals. We could, however, confirm that CellTiter-Glo® may be used as a pre-screening tool in determining live and dead NTS in single drug concentration and potentially in dose–response assays.

Additional files

Additional file 1: Table S1. Marker-specific methods used in this study. (DOCX 17 kb)

Additional file 2: Table S2. Markers identified from literature review. (DOCX 34 kb)

Additional file 3: Figure S1. Microscopic verification of staining. In the LIVE/DEAD® kit, calcein stains live NTS green (A, B) and EthD-1 stains dead cells red (C,D) already at 45 minutes. CellTox Green stains lysed NTS (E, F) already at 4 hours. Both Hoechst 33258 (G, H) and DAPI (I,J) stain even mefloquine-killed NTS well. (PPTX 1849 kb)

Additional file 4: Figure S2. Fluorescence signals for live (green) and dead (red) NTS from a scan for the calcein reagent (A) and EthD-1 reagent (B) of LIVE/DEAD®. A 384-well plate assay was used and measured at various time-points- the curves shown here are for the 4-hour time-point. The background here is already subtracted (G,H). (PPTX 172 kb)

Additional file 5: Figure S3. Fluorescence generated from culture medium as measured by (A) Omnicathepsin, (B) DAPI and (C) Hoechst 33258. Since our standard culture medium could also contribute to high fluorescence, RPMI medium was also tested. Graphs presented here correspond to optimal marker concentrations and incubation times are indicated in the main text. (PPTX 79 kb)

Competing interests

The authors declare that they have no competing interests.

Author's contributions

GP, DF, KIS and JK designed the studies. GP and DF carried out the experiments. GP wrote the first draft of the manuscript and DF helped to draft it. DF, KIS and JK revised the manuscript. All authors read and approved the final version of the manuscript.

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Chapter 2b

A novel isothermal microcalorimetry tool to assess drug effects on Ancylostoma ceylanicum and Necator americanus

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A novel isothermal microcalorimetry tool to assess drug effects on *Ancylostoma ceylanicum* and *Necator americanus*

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Abstract Soil-transmitted helminths, which affect the poorest communities, worldwide cause a range of symptoms and morbidity, yet few treatment options are available and drug resistance is a concern. To improve and accelerate anthelminthic drug discovery, novel drug screening tools such as isothermal microcalorimetry (IMC) have been tested with great potential. In this study, we used a novel microcalorimeter, the calScreener™, to study the viability on the hookworms *Necator americanus* and *Ancylostoma ceylanicum* as well as the whipworm *Trichuris muris.* Significant heat flow signals could be obtained with already one adult worm per channel for all three species. High-amplitude oscillations were observed for the hookworms; however, adult *T. muris* showed a twofold heat flow decrease during the first 24 h. Antinematodal effects of ivermectin and levamisole at 1, 10, and 100 μg/ml were evaluated on adult *N. americanus* and *A. ceylanicum*. Levamisole-treated hookworms showed a decline in heat flow and oscillation amplitude in a dose-response manner. Heat flow for ivermectin-treated hookworms increased proportionally with increased concentrations of ivermectin, though the wavelet analysis showed an opposite trend as observed by flatter wavelets. In conclusion, the calScreener™ is an excellent tool to study drug effects on intestinal hookworms at the adult worm stage as it offers a lower detection limit than other IMC devices and the possibility to monitor worm viability online.

Keywords Drug screening · Drug discovery · Hookworm · Isothermal microcalorimetry · Whipworm

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Introduction

Soil-transmitted helminth (STH) infections, including whipworm (*Trichuris trichuria)*, roundworm (*Ascaris lumbricoides*), and hookworms (*Ancylostoma duodenale*, *Necator americanus*) infections belong to the so-called neglected tropical disease group and are widely distributed all around the world, mainly in tropical and sub-tropical regions. According to recent estimates, 5.2 million disability-adjusted life year (DALYs) are lost globally every year due to intestinal helminthiases and 1.45 billion people are infected with at least one species of intestinal nematode worldwide (Murray et al. 2012; Pullan et al. 2014). Treatment of STH infections is mainly based on two old anthelminthic drugs, namely albendazole and mebendazole, which vary in efficacy and spectrum of activity (Keiser and Utzinger 2008). These drugs are widely used in preventive chemotherapy programs, and the shortage of new anthelminthic drugs generates concerns in the scientific community regarding emergence of drug-resistant isotypes of parasites infecting humans (Keiser and Utzinger 2010).

Current drug screening methods for nematodes are mainly based on standard motility assays which rely on microscopic evaluation of the parasites. Motility assays have limitations due to their subjectivity, the need for an experienced observer,

and especially their low throughput. Several methods including calorimetric measurements have therefore been tested in the last years in order to improve anthelminthic drug screening (Smout et al. 2010; Silbereisen et al. 2011; Tritten et al. 2012; Wimmersberger et al. 2013).

Isothermal microcalorimetry (IMC) is a technique used to measure heat flow at a constant temperature within rates that are in the μW range (Braissant et al. 2010; Braissant and Daniels 2011). Calorimetry has been used to study diverse biological processes as summarized in a recent review (Braissant et al. 2010); the real-time evaluation of cultured cells (Braissant and Daniels 2011), quantification of small amorphous contents in pharmaceutical products (Gaisford 2012), and bacterial characterization in terms of growth and metabolism (Zaharia et al. 2013) are just a few examples. In the field of parasitology, several helminth species, namely *Trichuris muris* (adults) (Silbereisen et al. 2011), *Ancylostoma ceylanicum* (L3 and adults) (Tritten et al. 2012), *Fasciola hepatica* (juvenile and adults) (Keiser et al. 2013), and *Schistosoma mansoni* (Manneck et al. 2011), have recently been studied with this method using a TAM 48 (TA Instruments, New Castle, DE). In these studies, the advantages of using calorimetric methods to assess worm viability and drug response were evident; compared to the motility standard assay used for drug screening, IMC showed improved sensitivity, accuracy, and objectivity, as well as the ability to monitor worm viability online. In addition the "random oscillations" in the heat flow pattern observed were shown to correlate well with motor activity of the tested worm (Manneck et al. 2011). Nonetheless, certain limitations were also observed, namely the necessity for sterile materials, the long equilibration times, and the large sample size required.

Recently, a novel 48-channel isothermal microcalorimeter, calScreener™, (SymCel Sverige AB, Kista, Sweden) has been developed (Braissant et al. 2015). Considering the previous successes as well as the limitations observed with the TAM 48, we investigated the calScreener™ for evaluating the viability of the three nematodes *T. muris*, *A. ceylanicum*, and *N. americanus* and assessed in vitro responses to two standard antinematodal drugs, levamisole and ivermectin. We selected these drugs for our studies since they were used in previous experiments with the former IMC and have excellent in vitro activity (Richards et al. 1995; Utzinger and Keiser 2004; Silbereisen et al. 2011; Tritten et al. 2012). The calScreener™ has a well plate design with much smaller vials made of titanium. As a consequence of the low mass and the high thermal conductivity of the microcalorimetric vials of the calScreener™, the time constant of the instrument is much lower and its sensitivity to motor activity-generated heat is expected to be higher (Braissant et al. 2015). Our preliminary test with *Schistosoma* (Braissant et al. 2015) showed an increased sensitivity compared to other instruments hinting that it might be possible to get accurate data with single worms, decreasing the need to sacrifice hosts.

Materials and methods

Animals and parasites

For studies on adult *T. muris*, C57BL/10ScSnOla female mice (3 weeks old) were acquired (Harlan Laboratories, Blackthorn, England) and allowed to acclimatize for 1 week in macrolon cages in groups not exceeding 10 mice. About 4 days before the infection, their drinking water was supplemented with 1 mg/l dexamethasone (water-soluble dexamethasone, Sigma-Aldrich). Each mouse was infected orally with around 200 *T. muris* eggs, and at 41 days post-infection, the mice were euthanized, the gut was dissected, and adult *T. muris* worms were manually collected.

The hookworms *A. ceylanicum* and *N. americanus* were maintained in male Syrian golden hamsters (Charles River; Sulzfeld, Germany). The hamsters (3 weeks old) were kept in macrolon cages in groups of 5 and allowed to acclimatize for 1 week. Their drinking water was supplemented with 0.5 mg/l water-soluble dexamethasone 4 days before infection. The hamsters were then infected orally with approximately 150 *A. ceylanicum* L3 larvae or subcutaneously with 150 *N. americanus* L3 larvae. Respectively, at 3 and 5 weeks post-infection, the hamsters infected with *A. ceylanicum* or *N. americanus* were euthanized, the gut dissected, and the adult worms were collected.

The room containing both hamster and mice cages was kept under environmentally controlled conditions at 25 °C, 70 % humidity, and a 12-hour light/dark cycle according to the Swiss Animal Welfare guidelines. The rodents had free access to water and food pellets.

Drugs and media

Hookworm medium was prepared by adding 10 % amphotericin B (250 μg/ml) and 1 % penicillin/streptomycin (10,000 U/ml penicillin/100 μg/ml streptomycin) to Hank's Balanced Salt Solution (HBSS) (all purchased from Sigma-Aldrich) supplemented with 10 % iFCS (Connectorate, AG). *T. muris* medium was prepared using 10.44 g RPMI 1640, 5 g albumax H (both from LuBio Science, Luzern, Switzerland), 5.94 g HEPES (Sigma-Aldrich), and 2.1 g sodium bicarbonate (Merck, Zug, Switzerland) in 1 l deionized water containing 5 % amphotericin B and 1 % penicillin-streptomycin. Levamisole and ivermectin were purchased from Sigma-Aldrich (Buchs, Switzerland). Stocks solutions of 10 mg/ml were prepared for both drugs in 100 % (*v*/*v*) dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at −20 °C until use. Further dilutions were prepared using the parasite's corresponding media.

Microcalorimeter

Heat measurements were performed using a pre-production instrument calScreener™ microcalorimeter (SymCel Sverige AB, Stockholm, Sweden) with its corresponding 48-well plate (calPlate™) as previously described (Braissant et al. 2015). Each well consists of a screw-capped titanium vial in which a maximum of 300 μl media can be added. Data was collected with the corresponding calView™ software (Version 1.0.28.0, © 2014 SymCel Sverige AB). For our assays, the machine was set and calibrated at 37 °C. General handling and device manipulation were done according to the manufacturer's recommendations.

Correlation of heat flow to worm viability and limit of detection

For the three species studied, initial experiments were set up in order to ascertain that the calorimeter was able to detect and record the heat flow, to define the least number of adult worms required and to determine the worms survival time in the calPlate™. All media was filtered with a 0.20-μm nanopore filter (Sarstedt AG) before use, and the titanium cylinders were autoclaved at 121 °C for 20 min. In each well, one to three helminths were used along with 300 μl of sterile medium. Wells containing culture medium only and/or wells containing two to four dead worms served as the negative controls. Additionally, 16 of the 48 wells of the calPlate™ served as reference wells, in which culture medium was placed. Once the assays were set up, the titanium cylinders were covered with the lid and closed with a torque screwdriver set to 60 cNm. Each assay was required to pass through two equilibration stations within the calorimeter (15–20 min each) after which the plate was pushed to the final station for measurement. Each experiment was performed once in duplicates or triplicates. All the assays were run for a duration of at least 120 h. At the end of each experiment, the worm viability was verified microscopically.

Drug sensitivity assay

Two worms per well were incubated with 1, 10, and 100 μg/ml of ivermectin and levamisole. For each assay, the wells containing dead worms and the wells containing medium only served as controls. CalPlate™ handling and assay measurement were conducted as described above. Each drug assay was performed once in duplicates or triplicates for a duration of 120 h, and worm viability was checked microscopically at the end of the experiment.

Data collection and statistical analysis

The sample's heat flow was continuously recorded at one data point every 75–100 s by calView™ software. The heat flow and the motor activity derived from the observed random oscillation in the signal were analyzed using R (R Core Team 2011) and the R Studio (© 2009–2013 RStudio, Inc.) interface. Microsoft Excel 2013 (Microsoft® Office Professional Plus 2013) was used to calculate statistical significance between the samples using an unpaired *t* test. The motor activity was extracted from the raw signal using a wavelet smoothing provided by the R waveslim package (Gençay et al. 2002). Higher frequency wavelets were discarded as they reflected mostly electronic noise. Equilibration at the final station was set as heat flow variations ≤0.5 μW/h. The time of worms' death was defined as the presence of both of the following conditions: (1) the recorded heat flow was equal or less than the average background heat flow from the medium or dead parasite wells and (2) no motor activity (i.e., random oscillations extracted using wavelet smoothing) were observed for at least 6 h. In order to correct for variations between wells, a plate containing culture media and 10 % formaldehyde (to fix any potential enzymatic reactions) was measured for 24 h and the resulting signals were used for baseline correction for the subsequent experiments for the medium of the same type.

Results

Correlation of heat flow to worm viability and limit of detection

For the three helminth species studied, the time required to reach signal equilibration was on average around 2 h (range 1–4 h) (data not shown). The wells containing culture medium only and dead worms showed average low signals from −1.088 to 0.007 μW, and no significant difference between these samples was observed ($p>0.05$). As a result, from this point forward, the medium wells were selected for background signal correction. Figure 1 shows the thermogenic profiles of one to three *T. muris* (a), *N. americanus* (b), and *A. ceylanicum* (c) adult worms over time.

T. muris

For the whipworm *T. muris*, the following average heat flow signals were obtained as measured at 12 h: 12.22 μW (±1.20 μW) for one adult worm, 16.61 μW (±7.85 μW) for two worms, and 16.57 μW (±6.21 μW) for three worms (Fig. 1a). For the channels with three worms, the heat flow drastically decreased, revealing only around 50 % of the initial value after 24 h of incubation. The channels containing two

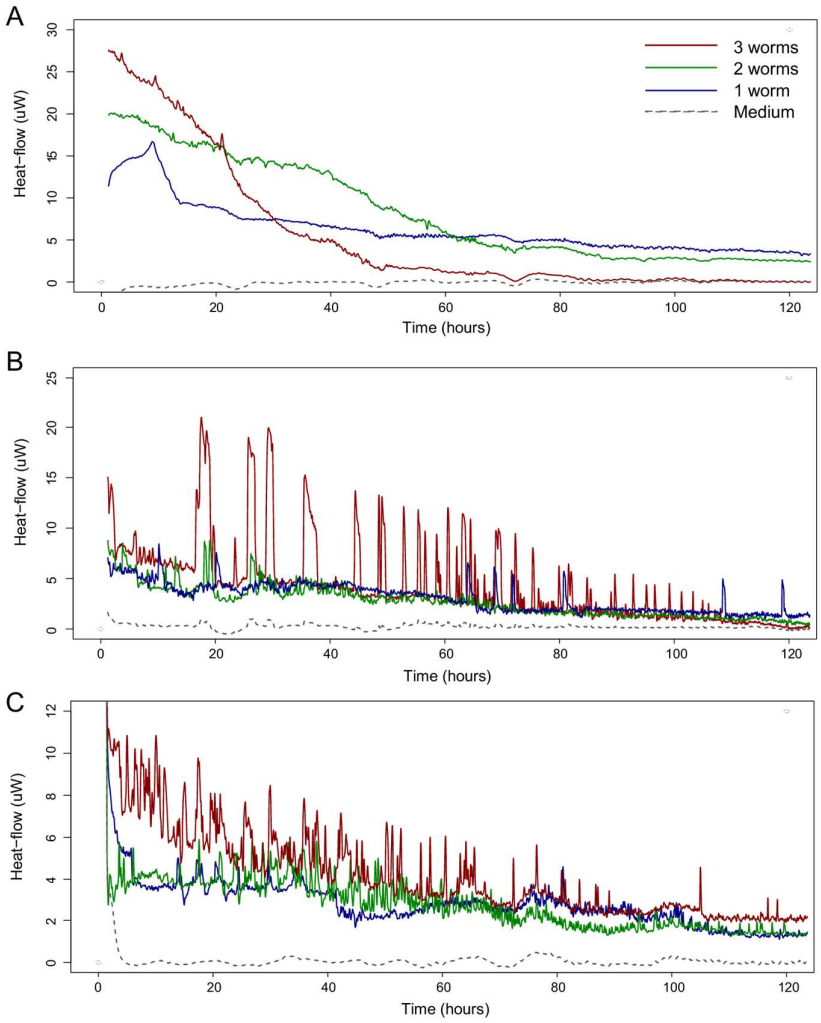

Fig. 1 Heat flow curves of one to three adult worms of **a** *T. muris*, **b** *N. americanus*, and **c** *A. ceylanicum* incubated for 120 h at 37 °C in culture media

worms demonstrated a less drastic but still more than twofold decrease in heat flow within 48 h, while the heat flow from single worm-containing wells fell by 5 μW within the first 12 h, followed by a steady decline over the remainder of the 120-h period. Random oscillations were from 0.20 μW for one worm to 0.40 μW for two worms and 1.2 μW for three worms. At 120 h when the experiment was finished, all the worms were dead as confirmed by microscopy.

N. americanus and *A. ceylanicum*

The hookworm *N. americanus* showed heat flow values from 4.13 μW (±0.33 μW) for one adult worm to 4.57 μW (±0.54 μW) for two worms and 6.53 μW (±0.82 μW) for three adult worms measured at 12 h (Fig. 1b). Similar observations were made for *A. ceylanicum*, where the thermogenic profile was in the same range: one worm (3.47 μW, ±0.11 μW), two worms (3.86 μW, ±0.11 μW), and three worms (6.28 μW, ±0.38 μW) at 12 h (Fig. 1c). Both hookworms showed large motility-related random oscillations; however, *N. americanus* wavelets were higher compared to *A. ceylanicum*: 7 μW for three worms (*A. ceylanicum* 2.7 μW), 2.6 μW (1.8 μW) for two worms, and 2.7 μW (1.4 μW) for one worm. Both parasites showed a slow but progressive decrease in

the heat flow from the beginning of the tests after the equilibration was reached. At the end of the experiments, both hookworm species were still alive; correspondingly, their heat flow curves never completely diminished to background signal levels.

Drug assays

Drug assays were conducted using the hookworms *N. americanus* and *A. ceylanicum*. Based on our initial experiments described above, we used two worms per well instead of only one worm for increased significance and to take into account variations that could occur with only one worm. *T. muris* drug assays were not conducted since the parasite's heat flow drastically decreased after 24 and 48 h for two and three worms per well, respectively, as described above.

N. americanus

Effect of levamisole and ivermectin on heat flow

Compared to the controls, the heat flow of all treated worms was significantly lower ($p<0.01$) following incubation with both drugs at all concentrations. Initial heat flow signals recorded after equilibration when incubated with 1, 10, and 100 μg/ml ivermectin were 10.39, 9.89, and 9.55 μW, respectively. Twelve-hour post-incubation assessment revealed heat flow reductions of 70, 64, and 53 %, correspondingly. Heat reductions of 89, 90, and 84 were calculated for worms incubated at these concentrations 72 h post-incubation (Fig. 2a). For levamisole-treated parasites, the heat flow recorded after equilibration were 10.07, 7.29, and 7.52 μW for concentrations of 1, 10, and 100 μg/ml, respectively. A significant decline was observed with

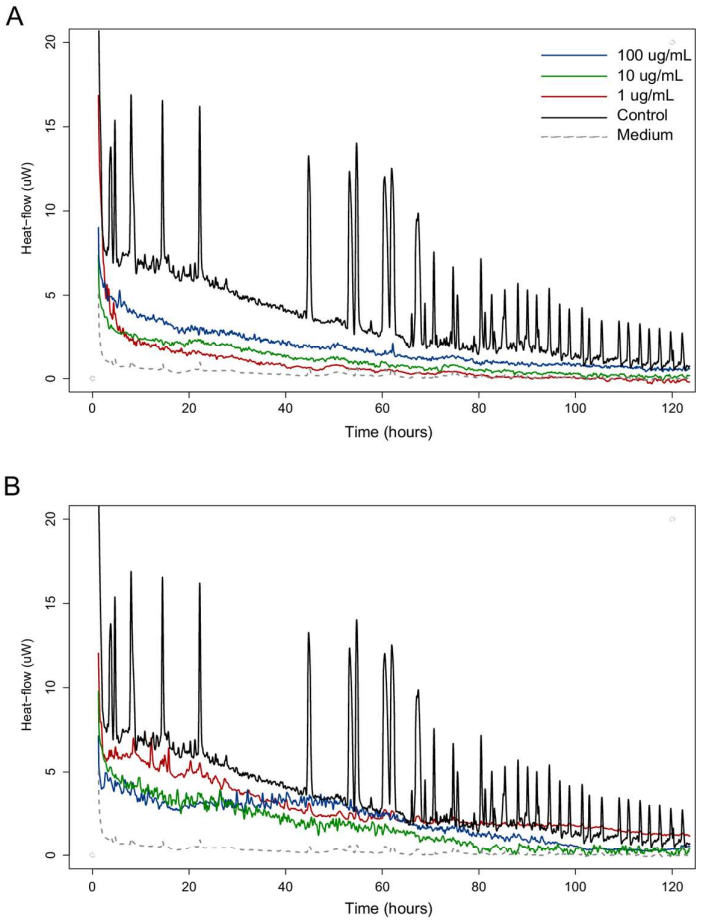

Fig. 2 Heat flow curves of two *N. americanus* adult worms incubated with 1, 10, 100 μg/ml ivermectin (**a**) and levamisole (**b**)

reductions of 52, 35, and 61 % calculated for the respective concentrations when measured at 12 h. Seventy-two-hour post-incubation, the heat flow reductions were 92, 76, and 100 % for *N. americanus* treated with 1, 10, and 100 μg/ml levamisole, respectively (Fig. 2b).

Effect of levamisole and ivermectin on motor activity

Figure 3 shows the wavelet decomposition (motor activity) of two *N. americanus* adult worms from a single control well (Fig. 3a) and incubated with 10 μg/ml ivermectin (Fig. 3b) and with 10 μg/ml levamisole (Fig. 3c). The worms in the control wells presented amplitude lengths from 7.5 to 5.9 μW at 24 h, 4.5 to 6 μW at 48 h, and 3.8 to 4 μW at 72 h (Fig. 3a). A progressive decrease in amplitude length was evident along the experiment. Ivermectin-treated parasites displayed flattened and stable small oscillations of ≤0.6 μW at all concentrations tested, whereas levamisole-treated worms gave noise amplitudes of <1.2 μW that became even shorter after 60 h post-incubation. The dead worms produced amplitude lengths <0.04 μW.

A. ceylanicum

Effect of levamisole and ivermectin on heat flow

The thermogenic profiles for the ivermectin- and levamisole-treated worms followed similar trends for all concentrations. A decrease of 73 and 67 % in the heat flow was observed 12 h post-treatment for 10 and 100 μg/ml ivermectin, respectively. The worms treated with 10 and 100 μg/ml levamisole showed a heat flow reduction of 85 and 88 %, respectively, at this time point. Seventy-two hours post-incubation, the signals from drug-containing wells reached the baseline.

Effect of levamisole and ivermectin on motor activity

Compared to the controls (initial oscillations up to 0.5 μW) (Fig. S1A), oscillations from the levamisole-treated parasites showed decreased amplitude lengths from the beginning (≤0.25 μW) of the experiment up to 48 h when amplitudes reached the level of the background signal. As observed with *N. americanus,* when exposed to ivermectin (both 10 and 100 μg/ml), the motility-related oscillations were flattened and close to the background signal with very small variations ≤0.3 μW even from the beginning of the experiment

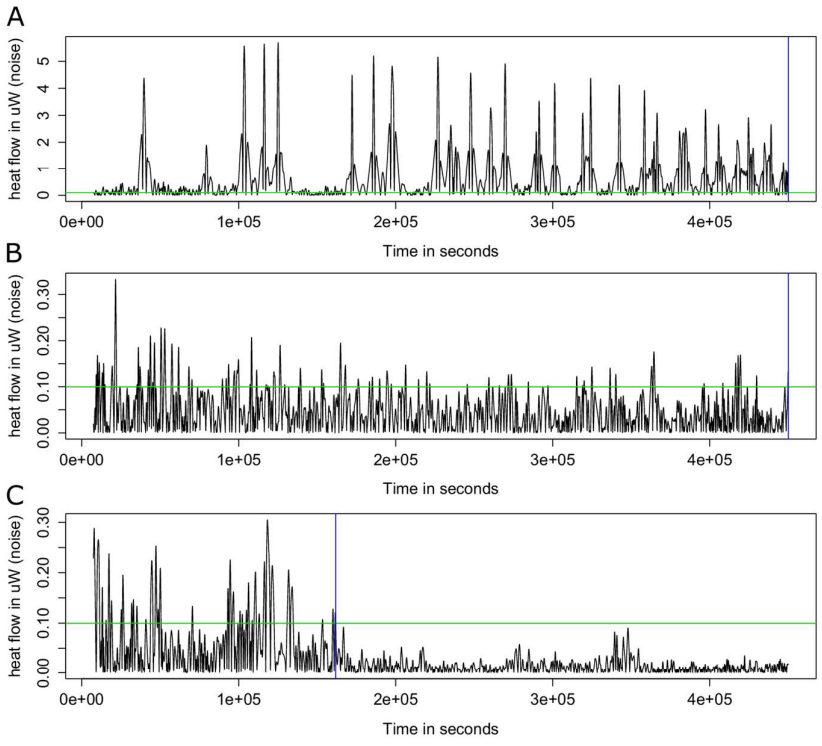

Fig. 3 Wavelet decomposition (motor activity) of two *N. americanus* untreated adult worms (**a**), two *N. americanus* incubated with 10 μg/ml ivermectin (**b**), and two *N. americanus* incubated with 10 μg/ml levamisole (**c**). The *horizontal lines* correspond to the thresholds (1.0 μW for the noise amplitude) and the *vertical lines* represents the last motor activity observed above this threshold

(Fig. S1B). Figure S1C presents the signal of *A. ceylanicum* treated with 10 μg/ml levamisole with initial oscillations up to 0.20 μW that drastically decreased after 14 h post-incubation and became flatterer and stable around 38 h.

Discussion

The work described presents one aspect of the continuous efforts to improve STH drug sensitivity assays with the ultimate aim of accelerating the provision of lead compounds to the drug development pipeline. As previously stated, whole-organism microscopic viability evaluation is the current "gold standard" for in vitro testing of the anthelminthic properties of new or known compounds. This is a low-throughput method that has many disadvantages, namely the subjectivity of the results, the intensive labor involved, and the requirement for trained and experienced personnel (Gill et al. 1991; Kotze et al. 2004; Tritten et al. 2012). The utility of microcalorimetry as a tool to study the effects of drugs using diverse parasites has been described earlier (Manneck et al. 2011; Keiser et al. 2013): the drug onset of action can be easily determined by constant real-time monitoring and drug effects that are not related to motility or morphology can be observed indirectly by the heat flow decrease, reflecting the worm's metabolic activity. Moreover, a clear advantage of using such an automated system is that such monitoring can be simply done online, instead of manually checking the worm viability at various time points with a microscope. Considering the previous success of measuring *A. ceylanicum* and *T. muris* with calorimetric methods (Silbereisen et al. 2011; Tritten et al. 2012) as well as the limitations of the previous model, the calScreener™ was seen as a worthwhile improved calorimetric method for us to test. Table 1 shows a summary of the main characteristics of the calScreener™ compared with the TAM 48 and the microscopic evaluation.

Our initial tests aimed to investigate if heat flow signals correlate with worm viability. For all three worms species, heat flow and random oscillation signals were much higher than background or dead worms' signals and increased when more worms were present in the well (an indirect measure of viability). Moreover, the number of worms required to reach signals significantly higher than background was minimal compared to previous experiments using the TAM 48 (Silbereisen et al. 2011; Tritten et al. 2012). For example, a signal as high as 8.8 μW was observed for one adult *T. muris*. For comparison, in a previous study using the TAM 48, a signal of 1.7 μW was recorded with six adult worms (Silbereisen et al. 2011). A similar result was observed for *A. ceylanicum:* in our study, one adult hookworm achieved

Table 1 Comparison of key features between drug assays using calScreener™, TAM 48, and microscopy (gold standard)

	calScreener™	TAM 48	Microscopy
Costs	Medium	High	Low
Data analysis	Complex	Complex	Simple
Drug addition	Does not presently allow an injection system	Allows an injection system	Manually added at any time
Drug onset of action	Can be precisely determined	Can be precisely determined	Needs multiple observation time points to determine; not precise
Measurements	Real-time continuous measurements	Real-time continuous measurements	Requires well-trained technician each time
Measurable parameters	Heat flow (metabolic activity) and random oscillations (motility)	Heat flow (metabolic activity) and random oscillations (motility)	Visual assessment of motility and morphological damage
Measurable dose-response and IC_{50}	Possible at any time point	Possible at any time point	Possible at time point measured
Objectivity	Automated objective measurements	Automated objective measurements	Manual, prone to subjectivity
Sample size required			
A. ceylanicum	2 worms	8 worms	3–4 worms
N. americanus	2 worms	Not done	3–4 worms
T. muris	2 worms[a]	>6 worms	3–4 worms
Sensitivity	Detection limit better than 50 nW[b]	Detection limit better than 100 nW[c]	Less sensitive to non-visible drug effects
Throughput	32 samples per measurement	48 samples per measurement	Limited only by worm availability and capacity of reader
Worm stages that can be studied	Adult stage only	Adult stage only	Both larval and adult stage

[a] Up to 24 hours only, since viability of worms will decrease considerably afterwards

[b] As described at the provider's web site (http://www.symcel.se/products.html)

[c] As described at the provider's web site (http://www.tainstruments.com/main.aspx?siteid=11&id=216&n=1)

higher heat flow values than eight adult worms in an earlier study using the TAM 48 (Tritten et al. 2012). This is not unexpected as the heat capacity of the calScreener™ vials filled with 0.3 ml of water would be circa 2.44 $J*K^{-1}$. In comparison, the heat capacity of a TAM 48 glass vial filled 3 ml of water and closed by an aluminum cap would be circa 17.28 $J*K^{-1}$. As a result, for a similar event (i.e., one movement of the worm), the temperature of the calScreener™ vial would increase much more resulting in a higher electrical signal from the Peltier. Ideally, both signals should be compared after being corrected by the Tian equation (Jesús et al. 2011) compensating for the time constant of the calorimeter that depends on the heat capacity of the samples.

Presently, adult stage worms are still extracted from infected rodents, and drug assays using six to eight worms per well are not efficient. Moreover, the use of a small number of worms allows to better adhere to the 3R principles of animal testing (Flecknell 2002), as fewer mice would need to be sacrificed.

Though the smaller sample volumes per ampoule (3.0 ml vs. 300 μl) and the heat capacity result in an increase of sensitivity, one disadvantage is that it appeared to affect *T. muris* viability. We observed that the heat flow (and therefore the viability) of *T. muris* adults decreased considerably within the first 24 h of the experiments, which is of course not ideal for 72-h drug assays. Furthermore, the observed decrease was faster with a higher number of worms (Fig. 1a). This could be due to the limited nutrients and gas exchange in the restricted volume of the media. Nonetheless, the hookworms (*A. duodenale*, *N. americanus*) assayed here all remained viable throughout the duration of all experiments, and especially long enough to run a standard 72-h drug assay.

The drug effects of high doses of levamisole and ivermectin observed in our study were very similar to those observed in the former study with *A. ceylanicum* (Tritten et al. 2012), in that treatment with both resulted in an almost immediate and severe reduction in the amplitude of the motility-related wavelet for both *A. ceylanicum* and *N. americanus*. This occurred for all ivermectin doses; a similar but not so pronounced effect was observed with levamisole, demonstrating the potent and immediate effects of the drugs. Heat flow values for levamisole-treated worms demonstrated predictable dose-responses: higher doses of levamisole resulted in stronger and faster reductions in random oscillations and heat flow. However, the heat flow reductions resulting from ivermectin-treated worms actually slightly decreased with increasing ivermectin concentrations, both in the *N. americanus* and *A. ceylanicum* assays. Although higher heat flow should indicate better worm viability, we could confirm, both by checking microscopically and by the wavelet analysis, that the ivermectin-treated worms were indeed dead. This cannot be explained by intrinsic properties of ivermectin, as signals from ivermectin-containing culture media with no worms were comparable to blanks (data not shown). It might be possible that the drug's mode of action also results in slight exothermic processes which are then increased with higher doses: ivermectin binds to glutamate-gated chloride channels of invertebrates resulting in long-lasting hyperpolarization or depolarization of the neuron or muscle cells and consequently paralysis and death (Wolstenholme and Rogers 2005). Interestingly, the IMC profile of another helminthic ion channel-blocker, praziquantel, was similar when Manneck et al. studied the use of IMC on *S. mansoni* (Manneck et al. 2011), in that the heat curves produced were high and not indicative of an active drug, but the wavelet analysis showed immediate action.

Standard methods for drug screening have been formerly described for both hookworms: egg hatching, larval, and adult worm viability (motility and morphology) (Treger et al. 2014) but to our knowledge, it is the first time that the viability and the exposure to levamisole and ivermectin of *N. americanus* were assessed using IMC. Aside from demonstrating that IMC can be used as an excellent drug discovery tool, we were also able to gain an interesting biological insight using this method. Although *N. americanus* and *A. ceylanicum* look morphologically similar, our experiments show that they possess different calorimetric and motility profiles. *N. americanus* appears to be more active with measurably bigger motility wave oscillations than *A. ceylanicum*, and it would be erroneous to expect that they would behave similarly in the same drug assay. Hence, lead compounds should necessarily be tested separately on each species.

Some important drawbacks of the tool should, however, be taken into consideration. First, the closed cylinders do not allow the use of an injection system to add the test compound after the samples are loaded (and the pre-drug signal is recorded). Rather, the drugs have to be added at the beginning of the experiment as it can take up to 1 hour to load samples and 30–40 min to equilibrate the plate in the machine, the first 1.5 h of data could not be used. In our case, due to the sample preparation and loading time, the exact time of death of the parasites could not be precisely determined, as we worked with very fast-acting drugs. In this sense, a model with an injection system would be desirable so that worm viability could be conveniently recorded before and after drug addition. On the other hand, this could be mediated by recording baseline viability, removing the plate, adding the drugs, and measuring, which is at worst as time consuming as the time it takes for the current calorimeters with injection systems to equilibrate (Manneck et al. 2011) after injection.

The second point is an issue with any microcalorimeter vis a vis measuring worm viability: the compulsory requirement for the sterility of the materials. As adult worms are usually extracted from the gut of the rodent, sterility is not always

possible and signals from the contamination might overpower those from the worms. However, since bacterial and fungal growth show signature curves, one can quickly spot them and abort the assay. Third, at present, only adult worm viability can be assessed, whereas attempts to measure larval stage viability were not so successful (data not shown). This is likely due to the very low metabolic activity noted for larval stage worms—even with microscopic assessment adding hot water to stimulate movement of the worms is required.

In conclusion, our study confirmed that isothermal calorimetry is an excellent methodology to study adult stage hookworm viability and drug effects over time. Worm viability can be tracked continuously and remotely using both heat flow and random oscillation parameters. The calScreener™ as a novel calorimeter offers a lower detection limit requiring a small number of worms, rendering drug discovery efforts using IMC more feasible and practical.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Ethical approval Experiments were approved by the veterinary authorities of the Canton Basel-Stadt (permit no. 2070) based on Swiss cantonal and national regulations.

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Chapter 3

Drug repurposing for schistosomiasis

Chapter 3a

Repurposing drugs for the treatment and control of helminth infections

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Invited Review

Repurposing drugs for the treatment and control of helminth infections

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Cestodes

ABSTRACT

Helminth infections are responsible for a considerable public health burden, yet the current drug armamentarium is small. Given the high cost of drug discovery and development, the high failure rates and the long duration to develop novel treatments, drug repurposing circumvents these obstacles by finding new uses for compounds other than those they were initially intended to treat. In the present review, we summarize *in vivo* and clinical trial findings testing clinical candidates and marketed drugs against schistosomes, food-borne trematodes, soil-transmitted helminths, *Strongyloides stercoralis*, the major human filariases lymphatic filariasis and onchocerciasis, taeniasis, neurocysticercosis and echinococcosis. While expanding the applications of broad-spectrum or veterinary anthelmintics continues to fuel alternative treatment options, antimalarials, antibiotics, antiprotozoals and anticancer agents appear to be producing fruitful results as well. The trematodes and nematodes continue to be most investigated, while cestodal drug discovery will need to be accelerated. The most clinically advanced drug candidates include the artemisinins and mefloquine against schistosomiasis, tribendimidine against liver flukes, oxantel pamoate against trichuriasis, and doxycycline against filariasis. Preclinical studies indicate a handful of promising future candidates, and are beginning to elucidate the broad-spectrum activity of some currently used anthelmintics. Challenges and opportunities are further discussed.

Contents

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1. Introduction

Helminth infections caused by roundworms (nematodes) and flatworms (platyhelminths) comprise the greatest group of the neglected tropical diseases (NTDs) (Hotez et al., 2006). An estimated 11.5 million disability adjusted life years (DALYs) are attributed to intestinal nematode infections, schistosomiasis, lymphatic filariasis, onchocerciasis, food-borne trematodiasis, cysticercosis and echinococcosis (Murray et al., 2012). Most of the burden of these diseases results from disability (rather than premature death), influencing school attendance, child development and overall economic productivity, thus resulting in disease driven poverty traps (Hotez et al., 2006). In a recent special issue of the Disease Clinics of North America (Zumla and Keiser, 2012), cestode infestations (Brunetti and White, 2012), schistosomiasis (Gryseels, 2012), food-borne trematodiases (Fürst et al., 2012), filariases (Knopp et al., 2012a) and soil-transmitted helminthiases (Knopp et al., 2012b) were presented in great detail and hence for background information on these diseases, the reader is referred to these excellent publications.

Preventive chemotherapy is the strategy of choice to control schistosomiasis, soil-transmitted helminthiases, lymphatic filariasis, onchocerciasis and food-borne trematodiases yet as emphasized in the following sections of this review, limited tools are available to treat these infections. In addition, for many of the available drugs, resistance is a threat since it has already developed in veterinary medicine and many of the drugs have an imperfect activity profile. Yet clear targets have been set to eliminate and control several of these diseases (http://www.unitingtocombatntds.org/downloads/press/london_declaration_on_ntds.pdf). Hence research and development (R&D) to find the next generation of anthelmintics is indispensable. However, a recent systematic assessment of databases of drug regulatory authorities and the World Health Organization (WHO) as well as clinical trial registries revealed a dry drug pipeline for NTDs, supported by the fact that no new chemical entity had been approved for these diseases in the past decade (Pedrique et al., 2013).

Drug repurposing (also termed re-profiling, re-tasking, therapeutic switching or drug repositioning) is the process of developing new indications for existing, failed or abandoned drugs or advanced clinical candidates (Sekhon, 2013). Drug repurposing is a useful strategy to accelerate the drug development process due to lower costs, reduced risk and decreased time to market due to availability of preclinical data (Padhy and Gupta, 2011). This enables not only pharmaceutical companies but also public-sector researchers to engage in drug discovery and development efforts (O'Connor and Roth, 2005), and hence might result in treatment options for diseases almost exclusively addressed by public sector researchers, such as the neglected tropical diseases. Over the past years, a variety of drug-repurposing initiatives have been launched with particular attention to neglected tropical and rare diseases (Allarakhia, 2013), hence it is likely that these efforts will bear fruit in the next years.

The aim of the present article is to highlight the status of drug repurposing for neglected helminth diseases. The focus is on the state-of-the-art treatments and how drug repurposing has been supplying the drug development pipeline for schistosomiasis, infections with major food-borne trematodes, *Fasciola* spp., *Opisthorchis* spp. and *Clonorchis sinensis*, soil-transmitted helminthiasis, *Strongyloides stercoralis*, the major human filariasis, lymphatic filariasis and onchocerciasis, taeniasis, neurocysticercosis and echinococcosis.

Our review complements a recent article by Andrews and colleagues which summarized antiprotozoal drug repurposing for major parasitic protozoal diseases including malaria, trypanosomiasis, and leishmaniasis (Andrews et al., 2014).

2. Schistosomiasis and food-borne trematodiases

2.1. Current treatment

Since its discovery in the 1970s, praziquantel has replaced many other drugs as the sole treatment for a range of helmintic infections. Praziquantel is effective against all three major species of *Schistosoma* (*S. mansoni*, *S. haematobium* and *S. japonicum*), and is the standard treatment against *C. sinensis*, *Opisthorchis viverrini* and *Opisthorchis felineus*, and intestinal flukes (Keiser and Utzinger, 2004; Utzinger and Keiser, 2004). It is administered orally, is safe, and highly effective.

As mentioned above, preventive chemotherapy is the strategy of choice to control schistosomiasis (WHO, 2006). This program is expected to expand ten-fold to include the treatment of 235 million people by 2018 (Knopp et al., 2013) which, on the one hand may significantly drive down morbidity and transmission but, on the other hand, would exacerbate drug pressure, likely resulting in resistance to praziquantel (Caffrey, 2007). Furthermore, praziquantel still has deficiencies: it is inactive against the juvenile stage of *Schistosoma* spp. and its (S)-enantiomer is inactive, a problem because at the moment, it is not separated from the (R)-enantiomer in production and hence the tablets are large and bitter (Stothard et al., 2013). Therefore, there is great motivation to seek alternative medications.

In the case of fascioliasis, triclabendazole is the treatment of choice (Keiser et al., 2005). Triclabendazole is safe and effective against both the human and veterinary forms of the disease. However, there are two major setbacks: in veterinary medicine, triclabendazole resistance has already been documented (Brennan et al., 2007). Additionally, triclabendazole is not registered for use in many nations, and is therefore not always available for the treatment of human fascioliasis (Keiser et al., 2005).

2.2. Repurposed drugs

2.2.1. Antimalarials and their derivatives

The largest anthelmintic drug repurposing success story by far has been the application of antimalarials against a wide variety of trematode infections, as well as other broader applications.

Investigations of the use of antimalarials against *Schistosoma* spp. and the liver flukes are based on the rationale that these flukes have the same blood-feeding characteristic as *Plasmodium* spp., and therefore share heme degradation mechanisms (Toh et al., 2010). If antimalarials target this pathway, they may also be effective against blood-digesting flukes. However, antimalarials have shown activity also against the non-blood feeding intestinal flukes (Ingram et al., 2012b), not only indicating their broad-spectrum activity, but also hinting at other mechanisms of action. As emphasized earlier, the use of antimalarials in de-worming programs should be eyed with caution, as this could accelerate resistance to these drugs. Nevertheless, since malaria and schistosome infections largely overlap geographically, the wide use of antimalarials might be beneficial in co-infected patients (Keiser and Utzinger, 2012).

2.2.1.1. Artemisinins. Artemisia annua (*quinghao*) has been used for thousands of years in Chinese medicine for a wide range of ailments, yet its antimalarial properties did not become known to the wider world until the 1970s, despite its prevalence as a common herb (Tu, 2011). Artemisinin, the plant's active ingredient, is a sesquiterpene lactone, with a characteristic peroxide bridge that is widely believed to be the active pharmacophore. Though potent, its short half-life, poor bioavailability and the reliance on plant cultivation spurred a search for semi-synthetic and completely synthetic alternatives (Ho et al., 2014). The most widely used artemisinin-derivatives are the first-generation semi-synthetics artesunate and artemether. Artemisinin-based therapies have shown great efficacy against *Schistosoma* spp., *Fasciola* spp., *Opisthorchis* spp. and *C. sinensis*, as summarized in previous reviews (Keiser and Utzinger, 2007b, 2012; Utzinger et al., 2007). Briefly, artemether, artesunate and dihydroartemisinin (the active metabolite of all artemisinin compounds) share common antischistosomal therapeutic characteristics. *In vivo* studies have demonstrated that these artemisinins are highly effective against juvenile infections and only moderately effective against adult infections (Keiser and Utzinger, 2012; Li et al., 2014). This observation suggests that these drugs particularly show clinical benefit when used as prophylactic treatments. Indeed, as calculated by a recent meta-analysis by Liu and colleagues, administration of multiple low doses of artemether or artesunate over a 1–2 week period achieved a protection rate of 65–97% against schistosomiasis japonicum (Liu et al., 2011). In addition, a decreased risk was observed using artemether for the prevention of *S. mansoni* and *S. haematobium* infections (Utzinger et al. 2000; N'Goran et al. 2003). Contradictory findings were obtained when these agents were used in the treatment of chronic infections. While some studies reported high efficacy, low to moderate cure rates (CRs) were observed in other trials (Keiser and Utzinger, 2007a). However, overall the therapeutic effect of artesunate, artemether or artemisinin based combination therapy (ACTs) against schistosomiasis was inferior to praziquantel in clinical trials (Keiser and Utzinger, 2007a; Utzinger et al., 2010). Finally, the artemisinins often showed synergistic effects with praziquantel in clinical trials, as elucidated by two meta-analyses (Liu et al., 2011; Perez del Villar et al., 2012).

The artemisinins have also been thoroughly characterized against food-borne trematodes in *in vivo* studies. Artemether and artesunate were both found to be very effective against the liver flukes *Fasciola hepatica* and *C. sinensis*, as well as the intestinal fluke *Echinostoma caproni* in the rodent infection models, achieving complete worm clearance (Keiser et al., 2006a,c,d), though lower worm burden reductions (WBRs) were achieved in the *O. viverrini* hamster infection model (66% and 78% for artemether and artesunate, respectively, administered as a single oral dose of 400 mg/kg) (Keiser et al., 2006d). Artemether and artesunate administered to sheep naturally infected with *F. hepatica* also achieved significant WBRs (91.3% at a dose of 140 mg/kg intramuscular (i.m.) and 91.9% at a dose of 40 mg/kg i.m. respectively), though embryotoxicity and toxicity were of potential concern (Keiser et al., 2008a, 2010e).

Studies with the artemisinins have progressed to clinical trials in *Fasciola*-infected patients: in central Vietnam, patients subjected to a 10-day treatment course with artesunate, administered daily at a dose of 4 mg/kg, were less likely to report abdominal pain at hospital discharge than patients treated with a standard oral dose of 10 mg/kg triclabendazole. However, 3 months post-treatment, clinical and serological response rates were lower among artesunate recipients compared to triclabendazole-treated patients (Hien et al., 2008). This result mirrors an exploratory Phase 2 trial conducted in Egypt, where chronically infected patients treated with 6×80 mg and 3×200 mg artemether only achieved CRs of 35 and 6%, respectively (Keiser et al., 2011). Finally, low CR and egg reduction rate (ERR) of 4% and 32% were also observed after administration of artesunate (10 mg/kg in 3 split doses) to school-aged children infected with *O. viverrini* (Soukhathammavong et al., 2011).

2.2.1.2. Synthetic peroxides. The success of the semi-synthetic artemisinins along with their pharmacologic draw-backs motivated the creation of fully synthetic derivatives in antimalarial drug discovery, the most investigated of these being the synthetic ozonides. The antimalarial OZ439 is currently in Phase 2 clinical trials (Wells, 2013). Two synthetic trioxolanes in particular have been widely investigated as potential anthelmintics- OZ78 and OZ288- whose trematocidal activity has been reviewed by Keiser and Utzinger (2007b). The review highlights the major *in vivo* studies which reveal interesting properties of these two compounds. First, their activity is comparable to those of the artemisinins and they exhibit the same increased *in vivo* activity against juvenile *S. mansoni* as compared to adult worms in mice and in hamsters. Second, in *S. mansoni in vivo* models, the adult WBR is affected by the rodent model, achieving only moderate WBRs in the mouse but high WBRs in the hamster model. However follow-up studies indicated similarly significant activity of OZ78 against *S. japonicum* juvenile and adult infection in hamsters, mice and rabbits, and of OZ277 in hamsters (Xiao et al., 2011, 2012). Interestingly, the ozonide OZ418 was recently found to be active against both juvenile and adult worms in the mouse *S. mansoni* infection model, and the adult *S. haematobium* hamster infection model (Keiser et al., 2012a), as well as moderately active against 7 day-old and adult *S. japonicum*- infected mice (Xue et al. 2014)- a result which shows that further modifications of the aryl ozonides can yield promising compounds.

With regard to the food-borne trematodes, *in vivo* activity of OZ78 against the liver flukes *F. hepatica* and *C. sinensis* and the intestinal fluke *E. caproni* is very high but studies with *O. viverrini* showed no activity at all (Keiser and Utzinger, 2007c). Importantly, in follow-up studies, OZ78 was found to be effective against triclabendazole-resistant *F. hepatica* (100% WBR at oral dose of 100 mg/kg) (Keiser et al., 2007a). Oddly, the high activity of the OZs against *F. hepatica* could not be repeated in naturally-infected sheep, regardless of the administration route (Keiser et al., 2010b; Meister et al., 2013). This discrepancy could potentially be explained by pharmacokinetic differences observed in rat and sheep, as discussed by Meister and colleagues (Meister et al., 2013).

Where OZ78 failed to treat *F. hepatica* in sheep, MT04 succeeded. MT04 is another synthetic peroxide, identified in the search for more effective analogues, which was found to have a 92% WBR and a 99% ERR in naturally infected sheep (Zhao et al., 2010; Wang et al., 2011; Meister et al., 2013).

Table 1
Trematocidal *in vivo* drug candidates.

			Adult infection		Juvenile infection		
Parasite	Drug	Host animal	Dose (mg/kg)	WBR (%)	Dose (mg/kg)	WBR (%)	References
Schistosoma mansoni	Dihydroartemisinin	Mouse	3 × 200–400	60–70	3 × 200–400	89–90	Li et al. (2012)
	OZ78	Mouse	400	52	200	95	Xiao et al. (2007)
		Hamster	200	85	100	83	Xiao et al. (2007)
	OZ288	Mouse	400	0	200	82	Xiao et al. (2007)
		Hamster	200	72	100	84	Xiao et al. (2007)
	OZ418	Mouse	400	96	200	100	Keiser et al. (2012a)
	Tribendimidine	Mouse	400	0	n.d.	n.d.	Keiser et al. (2007b)
	Imatinib	Mouse	3 × 1000	0	n.d.	n.d.	Katz et al. (2013)
	Miltefosine	Mouse	5 × 20	95	5 × 20	76	Eissa et al. (2011a)
	Nilutamide	Mouse	400	85	50–400	5–36	Keiser et al. (2010d)
	BTP-iso	Mouse	300	55	n.d.	n.d.	El Bialy et al. (2013)
	Clorsulon	Mouse	1–3 × 5	88–98	n.d.	n.d.	Mossallam et al. (2007)
	Anisomycin	Mouse	100	0	n.d.	n.d.	Abdulla et al. (2009)
	Lasalocid sodium	Mouse	100	41–44	n.d.	n.d.	Abdulla et al. (2009)
	Diffractic acid	Mouse	10–40	0	n.d.	n.d.	Abdulla et al. (2009)
	Gamboic acid	Mouse	100	0	n.d.	n.d.	Abdulla et al. (2009)
	Niclosamide	Mouse	100	0	n.d.	n.d.	Abdulla et al. (2009)
	Rafoxanide	Mouse	50	50–56	n.d.	n.d.	Abdulla et al. (2009)
Schistosoma haematobium	OZ418	Hamster	400	86	n.d.	n.d.	Keiser et al. (2012a)
Schistosoma japonicum	Dihydroartemisinin	Mouse	300	61	300	65	Li et al. (2011)
	OZ78	Hamster	200	70–94	200	73–81	Xiao et al. (2011)
		Rabbit	15	423	n.d.	n.d.	Xiao et al. (2012)
		Mouse	200–600	67–80	400	75	Xiao et al. (2012)
Fasciola hepatica	OZ78	Rat	100	100	100	100	Keiser et al. (2006b)
		Sheep	50–100	0	n.d.	n.d.	Keiser et al. (2010b), Meister et al. (2013)
	MT04	Sheep	100	92	n.d.	n.d.	Meister et al. (2013)
	Tribendimidine	Rats	800	0	n.d.	n.d.	Keiser et al. (2007b)
Clonorchis sinensis	OZ78	Rat	300	99	300	79	Keiser and Utzinger (2007a)
	Tribendimidine	Rat	150	98	n.d.	n.d.	Keiser et al. (2007b)
Opisthorchis viverrini	OZ78	Hamster	600	77	n.d.	n.d.	Keiser and Utzinger (2007a)
	Tribendimidine	Hamster	400	63	n.d.	n.d.	Keiser et al. (2007b)

WBR indicates worm burden reduction in relation to untreated control animals. n.d. denotes a lack of data.

2.2.1.3. Mefloquine. Mefloquine is a 4-quinolinemethanol whose discovery as an antimalarial dates to around the same time as the artemisinins. Its antischistosomal activity was revealed in 2008 and was thoroughly covered in a review by Xiao et al. (2013). This review highlights some excellent antischistosomal properties of mefloquine. First, it is active against all three major *Schistosoma* spp. and against both the juvenile and adult stages, a characteristic that neither praziquantel nor the artemisinins possess. Second, it causes severe morphological damage to the fluke and acts independently of the host immune response. Third, initial results from clinical trials were promising; mefloquine- artesunate achieved an ERR of 95% against *S. haematobium* in school-aged children (Keiser et al., 2010c). In addition, pregnant women who used mefloquine as intermittent preventive treatment for the prevention of malaria showed significantly higher CRs and ERRs against *S. haematobium* than women treated with sulfadoxine-pyrimethamine (Basra et al., 2013). Moreover, mefloquine-related arylmethanols also show antischistosomal activity *in vivo* (Ingram et al., 2012a).

The effectiveness of mefloquine against food-borne trematodes was briefly reviewed by Keiser et al. (2010a). This review highlighted that mefloquine is not active against *F. hepatica* nor *C. sinensis*, but interestingly, mefloquine was active against juvenile and adult *O. viverini* in a hamster infection model, achieving a WBR of 89% and 96% respectively, following a single oral dose of 300 mg/kg (Keiser et al., 2009a). However, mefloquine failed to produce an effect against *O. viverini* in clinical trials (Soukhathammavong et al., 2011). In a later study, mefloquine yielded significant results against *C. sinensis* infection in a rat model (when given at multiple dosages) and *Paragonimus westermani* infection in a dog model, but the results were not better than a treatment with praziquantel (Xiao et al., 2010).

2.2.2. Tribendimidine

Also originating from China, tribendimidine is a Chinese anthelmintic drug which has a similar activity profile against the soil-transmitted helminths as albendazole (high activity against *Ascaris lumbricoides* and hookworms but only moderate activity against *Trichuris trichiura*) (Xiao et al., 2013). Besides its broad range of antinematodal activity, tribendimidine has demonstrated trematocidal activity *in vivo* against *C. sinensis* and *O. viverrini* (Keiser et al., 2010a; Xiao et al., 2013). A recent randomized open-label trial in Guangxi, PR China presented moderate CRs (44% following a single dose of 400 mg/kg and 58% following 400 mg/kg administered once daily for 3 days) and high ERR (98–99%), similar to praziquantel. Tribendimidine compared favorably to praziquantel with regards to appearance of adverse events (Qian et al., 2013). Similarly, tribendimidine cured 19 of 24 patients infected with *O. viverrini* and achieved an ERR of 99% (Soukhathammavong et al., 2011). Further studies with tribendimidine are currently ongoing (e.g. dose-finding, pharmacokinetic studies) in *O. viverrini*-infected patients in Laos with the ultimate goal of developing an alternative opisthorchicidal drug.

To offset eventual resistance to praziquantel, using tribendimidine in combination with praziquantel is an attractive option. However, a recent study showed that praziquantel-tribendimidine administered to *O. viverrini*-infected hamsters, though achieving synergistic effects *in vitro*, had antagonistic effects in the hamster murine model (Keiser et al., 2013a). Further studies are required to shed light on this phenomenon. In contrast, in the *C. sinensis*

rat model, tribendimidine (12.5–50 mg/kg) showed synergistic interactions with 150 mg/kg praziquantel (Keiser et al., 2009b).

2.2.3. Other compounds

The most recent *in vivo* antitrematodal candidates come from a range of sources and fields, including cancer research, though expanding veterinary drug applications continues to be popular. The discoveries are described below and summarized in Table 1.

2.2.3.1. S. mansoni. A (well-funded) cancer drug pipeline provides a cornucopia of compounds. Dissous and Grevelding (2011) recently explored the potential of repurposing cancer drugs, specifically protein kinase inhibitors, towards schistosomiasis. The principal rationale was the identification of select protein kinases as being essential to schistosome development and the subsequent identification of certain cancer drugs as inhibitors of these kinases. However, most of the drugs discussed were only inhibitors of egg development and had little effect on the adult worms themselves. The one exception was imatinib (Gleevec), a successful anti-leukemia agent, which was shown to be lethal to adult worms *in vitro* at high concentrations (Beckman and Grevelding, 2010). However, imatinib was ineffective in an *S. mansoni* infection model (Table 1) (Katz et al., 2013) which is not surprising, as the drug is highly bound to alpha-1-acid glycoprotein (Soo et al. 2010).

Nonetheless, two anticancer agents have shown promising *in vivo* activity. Miltefosine, an alkylphospholipid, was initially developed to combat cutaneous metastasis of mammary carcinomas (van Blitterswijk and Verheij, 2013). As discussed in a complementary review by Andrews et al. (2014), it has also recently been licensed to treat leishmaniasis- a protozoan that also has cutaneous manifestations, though this drug treats both the visceral and cutaneous forms (Dorlo et al., 2012). Recently, it has shown *in vivo* activity in the *S. mansoni* mouse model at various stages of infection. Administered at 20 mg/kg for five consecutive days, commencing either on the day of infection (schistosomula stage), 21 days p.i. or 42 days p.i., miltefosine showed WBRs of 91%, 76% and 95%, respectively (Eissa et al., 2011a). A recent study by Eissa and colleagues also demonstrated its ovicidal, larvicidal and molluscicidal activity and its *in vitro* activity against *S. haematobium* adult worms (Eissa et al., 2011b). Interestingly, our own studies have shown that miltefosine was only moderately active against newly transformed schistosomula (NTS) of *S. mansoni* (unpublished findings).

The antiandrogen, nilutamide, blocks the binding of testosterone to the androgen receptor and was initially marketed for the treatment of metastatic prostate cancer (Akaza, 2011). Recently, we have tested it in the *S. mansoni* mouse model since related hydantoin derivatives studied at Hoffmann-La Roche had intriguing antischistosomal properties (Link and Stohler, 1984) – a single 400 mg/kg oral dose achieved a WBR of 85% and an even higher WBR was observed when given in combination with praziquantel (Keiser et al., 2010d). However, nilutamide did display a stage specific effect – it was only moderately active against the juvenile stage (Keiser et al., 2010d).

The advantages and disadvantages of using cancer drugs in the treatment of helmintic diseases should be carefully weighed. On the one hand, there is a great deal of effort to minimize toxic effects of cancer drugs due to their long treatment course. If a cancer drug is deemed safe when administered over a long period, there is a good chance it is also safe as an anthelmintic, where treatment is annual or twice annual (if part of regular preventive chemotherapy programs) but short (i.e. single dose). However, due to the severity and life threatening state of many cancers, the benefit of taking the drugs may outweigh potential severe adverse events. Thus, just because a drug is approved for cancer treatment, does not mean it is without significant adverse events. Yet a drug used in preventive chemotherapy programs must have an excellent tolerability profile.

Extending the application of veterinary anthelmintics continues to be a source of human anthelmintic candidates. Abdulla et al. (2009) showed that rafoxanide, a salicylanilide derivative and a veterinary anthelmintic, caused a 50–56% WBR in mice at a single oral dose of 50 mg/kg. This compound was identified from a screen of a commercially available FDA-approved library. This same screen also identified anisomycin, lasalocid sodium, diffractic acid, gamboic acid and niclosamide as hits for *in vivo* testing in the *S. mansoni* mouse model. Unfortunately, these drugs failed to reduce worm burden *in vivo,* the exception being lasalocid sodium which caused a WBR of 41–44% when administered at 100 mg/kg orally. Rafoxanide was not originally in the library, but due to niclosamide's excellent *in vitro* profile (but poor *in vivo* results), rafoxanide was tested as well, as it is structurally related to niclosamide. Clorsulon (a veterinary flukicidal drug) caused a WBR in *S. mansoni*-infected mice of 88%, 96% and 98% when treated with either a single, double or triple dose of 5 mg/kg one week apart starting from the 4th week postinfection (Mossallam et al., 2007). Yet in our own studies only moderate activity against NTS were observed *in vitro* when the NTS were incubated with 10 μM clorsulon for 72 h (unpublished observations).

Another re-purposing strategy is to increase the anthelmintic spectrum of activity through slight modification of the molecule. For example, BTP-Iso, a novel benzimidazole (in the same family as triclabendazole) was recently investigated as an antischistosomal. At a dose of 300 mg/kg given to *S. mansoni*-infected mice, BTP-Iso showed statistically significant higher reductions ($p < 0.01$) in female (77%), male (35%), and total worms (55%) in comparison with the control group (El Bialy et al., 2013).

2.2.3.2. F. hepatica. Two fasciocidal drugs were moved directly into clinical testing, without previous publications of *in vivo* studies. In Iran, 46 patients who were positive for *Fasciola* 3 months after triclabendazole treatment were given 1.5 g/day metronidazole orally for three weeks. Two months after end of therapy, stool exams became negative in 35 patients (81%), of which 31 patients (72%) presented both negative serology and stool exams. At 12 months, 28 patients were examined and all were negative (Mansour-Ghanaei et al., 2003).

Nitazoxanide, a thiazolide derivative and a pyruvate ferredoxin oxireductase inhibitor, has a broad spectrum of activity. The US Food and Drug Administration (FDA) approved nitazoxanide in 2002 for the treatment of diarrhea caused by *Cryptosporidium* species and *Giardia intestinalis* in pediatric patients 1–11 years of age, and in 2004 for its use in adults (Hemphill et al., 2006). Two clinical trials elucidated nitazoxanide's fasciocidal effects. In northern Peru, a double-blind placebo-controlled trial showed moderate effects in children and adults: a 7-day course of nitazoxanide resulted in CRs of 60% in adults and 40% in children (Favennec et al., 2003). In the Atlixco municipality in Mexico, a study carried out in 50 Mexican schoolchildren showed nitazoxanide to be effective against light *F. hepatica* infections. Children diagnosed with fascioliasis were administered nitazoxanide at 7.5 mg/kg body weight, every 12 hours over seven days. The efficacy against fascioliasis was 94% and 100% after first and second treatment courses, respectively. Its efficacy was also very high against protozoan and intestinal helminths (Zumaquero-Rios et al., 2013). However, this second study did not have a control group, and therefore could not be compared to a placebo or treatment with triclabendazole. Given these somewhat contradictory findings, larger randomized control trials should be conducted to elucidate the role of nitazoxanide in the treatment of fascioliasis.

Myrrh has been marketed as an antischistosomal, though its actual effect is controversial (Abdul-Ghani et al., 2009; Yakoot,

2010). Despite a 2004 field trial in Egypt, showing a CR of 88% and 94% CR, 2 and 3 months after treatment (Abo-Madyan et al., 2004), the overall consensus based on bench and field publications is that it is ineffective as a fasciocidal agent (Botros et al., 2009; Keiser et al., 2010a).

In the veterinary field, the benzimidazoles are often used as broad-spectrum anthelmintics, as discussed further in the soil-transmitted helminths section below. In livestock, the use of oxfendazole has previously been restricted to the treatment of roundworm, strongyloides and pinworm infection. However, very recently, it was demonstrated that it has potent anti-flukicidal activity: 100% WBR was achieved after a single oral dose of 30 mg/kg was given to *F. hepatica* infected pigs (Ortiz et al. 2014).

3. Soil-transmitted helminthiasis and strongyloides

3.1. Current treatment

The two benzimidazoles, albendazole and mebendazole, as well as pyrantel pamoate and levamisole are the four available treatments against soil-transmitted helminths, marketed between 1966 (pyrantel pamoate) and 1980 (albendazole) (Keiser and Utzinger, 2010; WHO, 2013). These drugs are widely used (in 2010, 328 million children were treated with albendazole or mebendazole (Anonymous, 2012)) since, as mentioned, the current strategy to control morbidity due to the most common soil-transmitted helminths (*A. lumbricoides*, *T. trichiura* and the hookworm species *Ancylostoma duodenale* and *Necator americanus*) is by regular treatment of at risk populations (WHO, 2006, 2011). The limitations of these drugs are widely known. Briefly, using a single dose regimen they all are very efficacious against *A. lumbricoides*, yet for the treatment of hookworm infections only albendazole produces satisfying CRs, while all drugs achieve poor CRs against infections with *T. trichiura* (Keiser and Utzinger, 2008). As no new anthelmintic drugs are on the horizon, repurposing of existing drugs is an essential strategy to improve treatment of soil-transmitted helminths, particularly *T. trichiura* infections (Keiser and Utzinger, 2008; Olliaro et al., 2011).

The current drug of choice against *S. stercoralis* infections is ivermectin, which is widely used in mass drug administration programs to combat lymphatic filariasis (Knopp et al., 2012a). A single dose of 200 μg/kg results in a CR of 88% against *S. stercoralis* (Keiser and Utzinger, 2010). The drug has the disadvantages that it is not licensed for treating *S. stercoralis* infections in several countries (e.g. Switzerland, Germany, Great Britain), that the ideal dosage schedule of ivermectin has yet to be evaluated, and that it can cause harmful and dangerous treatment effects in individuals co-infected with *Loa loa* which is an insect-borne filarial infection (Bisoffi et al., 2013; Greaves et al., 2013). People not tolerating ivermectin can alternatively use albendazole, which is inferior compared to ivermectin regarding ERRs and CRs; however, it still achieves satisfactory efficacy (Keiser and Utzinger, 2010; Knopp et al., 2012a). In addition, mebendazole achieves CRs and ERRs of 67–98% against *S. stercoralis* when given in multiple doses (Musgrave et al., 1979; Shikiya et al., 1990, 1991; Zaha et al., 2000). Of note, even though ivermectin is not listed as an essential drug against soil-transmitted helminths (WHO, 2013), several studies have shown that by combining ivermectin with the standard drug albendazole or mebendazole, the efficacy against soil-transmitted helminths (especially against *T. trichiura*) can be improved significantly (a summary of trials is presented in Keiser et al. (2012b)).

3.2. Repurposed drugs

3.2.1. Drugs evaluated in clinical testing

3.2.1.1. Nitazoxanide. Nitazoxanide, mentioned above for its protozoan and fasciocidal properties, showed (in manufacturer-financed trials) very high CRs against *A. lumbricoides*, *T. trichiura*, *S. stercoralis* and even against *A. duodenale* when the drug was given in multiple doses (Romero Cabello et al., 1997; Abaza et al., 1998; Juan et al., 2002; Diaz et al., 2003). The drug also demonstrated superior activity than the standard drugs in *in vitro* screening against *Trichuris muris* (Tritten et al., 2012). However, a recent clinical trial using a single dose of 1000 mg nitazoxanide could not confirm the initial results obtained in earlier trials and in the laboratory. Nitazoxanide achieved low CRs and ERRs (7% and 13%, respectively) against *T. trichiura* in school-aged children on Pemba, Tanzania. Additionally the drug was slightly less well tolerated compared to standard treatment and hence nitazoxanide was not recommended as an alternative treatment against soil-transmitted helminthiasis (Speich et al., 2012). Of note, within the same trial, the efficacy of a single dose nitazoxanide (1000 mg) against intestinal protozoa was also only moderate (Speich et al., 2013).

3.2.1.2. Oxantel pamoate. Oxantel pamoate, a veterinary drug which was introduced on the market in 1974, revealed high efficacy against *T. trichiura* in a number of exploratory trials (Garcia, 1976; Lee et al., 1976; Lee and Lim, 1978). The drug was only revisited recently, more than three decades later. Laboratory studies demonstrated a significantly higher activity of oxantel pamoate compared to the benzimidazoles, levamisole or pyrantel pamoate against *T. muris in vitro* and *in vivo* (Keiser et al., 2013b). A randomized controlled double blind trial recently confirmed the high efficacy of oxantel pamoate (20 mg/kg) against *T. trichiura* (26% CR and 93% ERR). Hence oxantel pamoate-albendazole, due to its broad spectrum of activity could be useful in the control of soil-transmitted helminthiasis (Speich et al., 2014). Further studies have been planned with the ultimate goal of elucidating the potential of adding oxantel pamoate to the current drug armamentarium.

3.2.1.3. Tribendimidine. A large phase 2 trial in Côte d'Ivoire confirmed the excellent activity of tribendimidine against hookworm and *A. lumbricoides* (N'Goran et al. under review). Following promising results with tribendimidine against *Strongyloides ratti* in the rat model (Keiser et al., 2008b) an exploratory trial in China documented a CR of 55% in patients infected with *S. stercoralis* (Steinmann et al., 2008).

3.2.1.4. The benzimidazoles. The benzimidazoles are a large family, widely used in veterinary medicine, and not only include albendazole and mebendazole (and the fasciocidal drug triclabendazole mentioned earlier) but also flubendazole, fenbendazole, oxfendazole, thiabendazole and oxibendazole (Olliaro et al., 2011). In addition, oxfendazole and cambendazole are promising broad spectrum benzimidazoles used in veterinary medicine, which have not yet been tested in humans. The benzimidazoles have a broad spectrum of activity including whipworms, roundworms and hookworms. For example, multiple doses of thiabendazole have similarly high efficacy against *S. stercoralis* as compared to ivermectin, however the drug was less tolerated (Gann et al., 1994; Zaha et al., 2000; Bisoffi et al., 2011). Clinical trials conducted with flubendazole, fenbendazole, oxibendazole and thiabendazole against soil-transmitted helminths and *S. stercoralis* are summarized in Table 2.

3.2.2. Compounds in early clinical testing and potential veterinary drugs

An excellent summary of potential drug development candidates for human soil-transmitted helminthiasis has recently been provided by Olliaro and colleagues (Olliaro et al., 2011).

Four promising compound classes are worth highlighting. First, emodepside is a cyclic depsipeptide and a broad-spectrum veterinary anthelmintic used for companion animal gastrointestinal

Table 2
Drugs tested in clinical trials against soil-transmitted helminths (See below-mentioned references for further information).

Drug tested	Number of patients	Dose	Cure rate	Egg reduction rate	Reference
Oxantel pamoate	*T. trichiura*: 12-122	10-25 mg/kg	*T. trichiura*: 57-93%	*T. trichiura*: 90-96%	(Lee et al., 1976)
	A. lumbricoides: 53 *T. trichiura*: 10-26	10-20 mg/kg	*A. lumbricoides*: 0% *T. trichiura*: 77-100%		(Garcia, 1976)
	79 *A. lumbricoides* 114 *T. trichiura* 113 Hookworm	20 mg/kg	*A. lumbricoides*: 10% *T. trichiura*: 26% Hookworm: 11%	*A. lumbricoides*: 28% *T. trichiura*: 93% Hookworm: 39%	(Speich et al., 2014)
Nitazoxanide	*A. lumbricoides*: 33-144 *T. trichiura*: 9-86	7.5 mg/kg (500 mg to adults, 200 mg to children under 12 years) every 12 hours for 3 consecutive days	*A. lumbricoides*: 48-100% *T. trichiura*: 56-78%	*A. lumbricoides*: 99.7-100% *T. trichiura*: 99.5-99.6%	(Romero Cabello et al., 1997)
	A. lumbricoides: 155 *T. trichiura*: 29 Hookworm: 46 *S. stercoralis*: 36	500 mg to adults, 200 mg to children 4 to 11 years, and children 1 to 3 years 100 mg every 12 hours for 3 consecutive days	*A. lumbricoides*: 95% *T. trichiura*: 86% Hookworm: 96% *S. stercoralis*: 94%		(Abaza et al., 1998)
	A. lumbricoides: 35 *T. trichiura*: 18 *S. stercoralis*: 6	200 mg to children 4 to 11 years, and 100 mg to children 1 to 3 years every 12 hours for 3 consecutive days	*A. lumbricoides*: 89% *T. trichiura*: 89% *S. stercoralis*: 83%	*A. lumbricoides*: 99.9% *T. trichiura*: 99.8%	(Juan et al., 2002)
	A. lumbricoides: 8 *T. trichiura*: 136 Hookworm: 12	1000 mg single dose	*A. lumbricoides*: 63% *T. trichiura*: 7% Hookworm: 67%	*T. trichiura*: 13%	(Speich et al., 2012)
Oxibendazole	*A. lumbricoides*: 196 *T. trichiura*: 178 Hookworm: 340	15 mg/kg/day for 3 days	*A. lumbricoides*: 93-98% *T. trichiura*: 67-71% Hookworm: 70-81%	Hookworm: 98-99%	(Huang et al., 1990)
Fenbendazole	*A. lumbricoides*: 2-7 *T. trichiura*: 6-17 Hookworm: 5-31	1 g and 1.5 g as single doses and 2 x 500 mg over 24 hours	*A. lumbricoides*: 60-100% *T. trichiura*: 65-100% Hookworm: 8-26%		(Bruch and Haas, 1976)
	A. lumbricoides: 2 *T. trichiura*: 14 Hookworm: 18 *S. stercoralis*: 14	6 x 600 mg each 12 hours	*A. lumbricoides*: 100% *T. trichiura*: 93% Hookworm: 89% *S. stercoralis*: 29%		(Sanchez-Carrillo and Beltran-Hernandez, 1977)
	A. lumbricoides: 31 *T. trichiura*: 28 Hookworm: 18	30-50 mg/kg	*A. lumbricoides*: 84% *T. trichiura*: 29% Hookworm: 83%	*A. lumbricoides*: 97% *T. trichiura*: 38% Hookworm: 82%	(Rim et al., 1981)
Flubendazole	*A. lumbricoides*: 3-5 *T. trichiura*: 4-16 Hookworm: 27-60	2 x 300 mg (spaced by 12 or 24 hours)	*A. lumbricoides*: 100% *T. trichiura*: 38-100% Hookworm: 30-82%	*A. lumbricoides*: 100% *T. trichiura*: 43-100% Hookworm: 88-96%	(Bunnag et al., 1980)
	T. trichiura : 19	100 mg twice a day for 3 days	*T. trichiura*: 89%	*T. trichiura* : >99%	(Yangco et al., 1981)
	A. lumbricoides: 33-47 *T. trichiura*: 32-52	200-600 mg, 300 x 2 mg	*A. lumbricoides*: 90-97% *T. trichiura*: 17-65%	*A. lumbricoides*: 98.7-99.6% *T. trichiura*: 91-95%	(Kan, 1983)
	A. lumbricoides: 43-47	500 mg or 200 mg	*A. lumbricoides*: 86-89%	*A. lumbricoides*: 97-98%	(de Silva et
	T. trichiura: 43-47		*T. trichiura*: 19-26%	*T. trichiura*: 42-54%	al., 1984)
Thiabendazole	*S. stercoralis*: 103	25 mg/kg twice a day for 3 days	*S. stercoralis*: 79%		(A.A. et al., 2003)
	S. stercoralis: 92	25 mg/kg twice a day for 2 days	*S. stercoralis*: 52%		(Bisoffi et al., 2011)
	S. stercoralis: 19	50 mg/kg/day twice a day for 3 days	*S. stercoralis*: 89%		(Gann et al., 1994)

nematode infections (Harder et al., 2003; Geary and Mackenzie, 2011). It showed high activity against *Trichuris* spp., *Ancylostoma* spp. and other nematodes in *in vitro* and *in vivo* studies (Harder and von Samson-Himmelstjerna, 2002; Altreuther et al., 2009). Moreover, the closely related PF1022A was potent in clearing *T. muris* infections in mice at various stages of infection (Kulke et al. 2014). *In vivo* studies on *Haemonchus contortus* have shown that it has a different mode of action than the benzimidazoles and ivermectin (von Samson-Himmelstjerna et al., 2005) hence the drug will likely be active against parasites resistant to the benzimidazoles and ivermectin.

Second, doramectin which belongs to the avermectins, showed high efficacy against *S. ransomi*, *S. papillosus*, *T. suis* and *A. suum* in pigs (Stewart et al., 1996; Yawzinski et al., 1997) as well as against *Trichuris* spp. in cattle (Jones et al., 1993; Saeki et al., 1995). Doramectin showed even more favorable results against nematodes compared to ivermectin, which could be explained by its lower clearance and higher bioavailability (Lumaret et al., 2012). The closely related milbemycin family is also a potent drug class. Milbemycin oxime has proven to be efficacious against *Ascaris* spp., hookworm and *Trichuris* spp. in cats and dogs (Blagburn et al., 1992; Catton and Van Schalkwyk, 2003). On the other hand only low ERRs were found against *T. trichiura* in baboons (Reichard et al., 2007).

Third, moxidectin is another commercially available broad-spectrum antiparasitic from the veterinary field (Reinemeyer and Cleale, 2002). It is a semisynthetic methoxime derivative of naturally occurring nemadectin with a novel mode of action (Awasthi et al., 2013). Moxidectin showed high efficacy in several *in vivo* studies (i.e. in cattle, swine, sheep) against *Ascaris* spp., *Trichuris* spp. and *Strongyloides* spp. (Bauer and Conraths, 1994; Coles et al., 1994; Stewart et al., 1999; Reinemeyer and Cleale, 2002; Ranjan and DeLay, 2004; Lyons et al., 2006).

Finally, from a completely different medical arena, cyclosporine A is an immunosuppressive agent which is commonly used for organ transplantation (Cohen et al., 1984). Its antischistosomal properties were first described in 1981 (Bueding et al., 1981) followed by studies against *S. stercoralis* in dogs and *S. ratti* in rats (Armson et al., 1995). Treatment with cyclosporine A seems to be of benefit in cases of opportunistic, possibly life threatening infections of *S. stercoralis* activated by immunosuppression (Schad, 1986).

4. Filariasis

4.1. Current treatment

Though filariasis is a collection of diseases, the three major control programs target lymphatic filariasis (Global Program to Eliminate Lymphatic Filariasis) or onchocerciasis (African Program for Onchocerciasis Control and the Onchocerciasis Elimination

Program for the Americas), and a major cornerstone of all three programs is mass drug administration. Regardless of the disease, the recommended treatments are limited: ivermectin, albendazole and diethylcarbamazine (DEC), ivermectin being the most widely recommended drug. The use and status of these drugs, including their limitations, have recently been reviewed by Katiyar and Singh (2011). Briefly, resistance to ivermectin has already been documented and its administration to onchocerciasis patients co-infected with loaiasis results in severe adverse events (Kamgno et al., 2007). Albendazole's efficacy is heavily debated and DEC is contraindicated in patients with onchocerciasis (Katiyar and Singh, 2011). Additionally, the treatment regimens required for these drugs in order to fully stop transmission are long: according to current control guidelines, ivermectin or DEC-albendazole need to be administered once a year for 5 years to interrupt LF transmission (Anonymous, 2009, 2014). The long treatment schedule is partly because all three drugs predominantly act on microfilaria only (the larvae shed by the adult worms), with DEC showing some adult stage activity (Dreyer et al., 2006). This latter point is problematic, as the adult stage still survives in the host, ready to shed more microfilaria once the drugs are absent from the host, continuing to cause pathology in onchocerciasis patients and also continuing the transmission cycle. Thus, new drug candidates should necessarily target adult worms as well. Additionally, as all filariasis-causing nematodes (except *L. loa*) carry with them the endosymbiotic *Wolbachia* bacteria necessary for the parasite's development, efforts have been made to target these bacteria.

4.2. Drugs targeting Wolbachia

Amongst the new antifilarial drugs, doxycycline is the most studied. It is a broad-spectrum antibacterial and antiprotozoal whose effects against *Wolbachia* are considered a breakthrough in antifilarial treatment (Katiyar and Singh, 2011). In onchocerciasis patients, a treatment regimen of 100 mg of doxycycline per day for 6 weeks slowly eliminated *Wolbachia* and resulted in long-term embryogenesis block of the adult worms (Hoerauf et al., 2003). The death of *Wolbachia* also leads to macrofilaricidal effects, as adults worms extracted from LF patients treated with 200 mg of doxycycline per day for 4 weeks were found to be either sterile or dead (Debrah et al., 2007). Findings by Taylor and colleagues also showed macrofilaricidal effects in LF patients, though the same level of potency could not be observed in onchocerciasis patients (Taylor et al., 2005; Hoerauf et al., 2008b). In addition, doxycycline is potentially microfilaricidal as well, as shown in LF patients treated with 100–200 mg per day for 6 weeks (Supali et al., 2008). Doxycycline, given prior to antifilarial treatment, appears to be more effective in suppressing microfilaremia in bancroftian filariasis over the long term than the standard treatments alone, while also resulting in fewer severe adverse events (Turner et al., 2006). LF symptoms could also be reduced by doxycycline, regardless of active filarial infection as demonstrated in a trial in Ghana (Mand et al., 2012). This symptomatic alleviation also extends to LF patients who presented with hydrocele (Debrah et al., 2009).

The effects of doxycycline administered in combination with the standard drugs have also been characterized. For example, a combination of doxycycline and albendazole was far more effective at suppressing circulating microfilaria than doxycycline or albendazole alone (nearly 100% suppression vs. 69% and 89%, respectively when checked at day 365 post-treatment) and even succeeded in completely clearing 42% of patients of microfilaremia (Gayen et al., 2013). Different trials also assessed doxycycline/ivermectin and found that the combination was more effective than ivermectin or doxycycline alone (Masud et al., 2009; Turner et al., 2010). Similarly, a 21-day course of doxycycline at 200 mg administered orally to LF patients in Orissa, India, followed by a single 6 mg/kg dose of DEC was effective at completely clearing microfiladermia. Interestingly, the co-administration of doxycycline followed by DEC was also effective at clearing adult worm nests, and less effective when the doxycycline treatment was shortened to 10 days (Mand et al., 2009).

Doxycycline was even found to be effective against *Mansonella perstans*, one of two agents that cause serous cavity filariasis. In a randomized open-label trial in Mali, 97% and 75% of *M. perstans* patients treated with 200 mg doxycycline for 6 weeks were found to be amicrofilaremic at 12 and 36 months post-treatment, respectively. This finding is very significant as the current standard treatments are not effective against *M. perstans* (Coulibaly et al., 2009).

Last but not least, given that *L. loa* is the only filarial nematode that does not possess *Wolbachia* endosymbionts, the use of *Wolbachia*-targeting antibiotics is a possible safe alternative for the treatment of this disease. Doxycycline was already shown to be safe in onchocerciasis patients co-infected with loaisis (Wanji et al., 2009; Turner et al., 2010), but its long treatment course leaves room for short-course therapy innovations.

Considering the importance of a potent anti-*Wolbachia* treatment, there is ongoing research to also elucidate the effects of other potent broad-spectrum antibiotics on *Wolbachia*. Rifampicin was found to be effective in reducing *Wolbachia* bacteria, as well as embryogenesis and microfilaria production in a small number of onchocerciasis patients, though not as efficiently as doxycycline (Specht et al., 2008). However a short-course therapy of 5 days rifampicin was documented to be completely ineffective (Richards et al., 2007). Meanwhile azithromycin was observed to be only marginally effective against *Wolbachia* and showed no effect on the worms in onchocerciasis patients in Ghana (Hoerauf et al., 2008a). In another study, oxytetracycline (the parent compound of doxycycline) had macrofilaricidal properties in cattle infected with *Onchocerca ochengi*, however this success has yet to be translated to human treatment (Nfon et al., 2007).

4.3. Drugs targeting the worms

As mentioned above, drugs effective against the filarial adult stage should be investigated with high priority. The potential of the benzimidazole flubendazole, a veterinary drug used to treat gastrointestinal nematode infection, as a pre-clinical macrofilaricide candidate for preventive treatment of onchocerciasis and lymphatic filariasis is currently being studied in a project lead by the Drugs for Neglected Diseases *initiative* (DND*i*). These investigations have been reviewed by Mackenzie and Geary (2011). In the late 1980s and early 1990s, flubendazole was shown to be macrofilaricidal against a variety of filariases-causing agents *in vivo* and even in a clinical trial for onchocerciasis (Dominguez-Vazquez et al., 1983; Van Kerckhoven and Kumar, 1988; Franz et al., 1990). Interestingly, it has no microfilaricidal effects (Van Kerckhoven and Kumar, 1988), which indicates that it could be safe for *L. loa* co-infected patients as well. The drug is, however, very poorly orally absorbed and hence the current focus of the project lead by DND*i* is to improve the drug's bioavailability. A new cyclodextrin formulation is currently being investigated with mixed results: while it achieved higher plasma concentrations when administered orally to *Echinococcus granulosus*-infected mice, no significant pharmacokinetic effects were observed when it was administered to sheep via the intraruminal nor the intraabomasal route (Ceballos et al., 2009, 2012).

Nonetheless, other drugs on the horizon continue to target microfilaria while also having some effects on the adult stage. Unsurprisingly, moxidectin has shown potent microfilaricidal effects and long term sterilization of females. Furthermore, early-stage clinical trials have shown that it is safe and well tolerated in humans in doses of 3–36 mg and has a longer half-life than

ivermectin (Cotreau et al., 2003; Awadzi et al. 2014). It is being evaluated in ongoing Phase 2 and 3 clinical trials via a partnership between APOC, TDR and Wyeth (Taylor et al., 2009; Babalola, 2011). Nonetheless, tolerability studies are needed to confirm that moxidectin is safe in patients co-infected with *L. loa* and whether the drug is efficacious against ivermectin-resistant filaria.

Other anthelmintics tested include the above mentioned cyclic depsipeptides, emodepside and PF1022A, and levamisole. Emodepside showed potent microfilaricidal effects and even some macrofilaricidal effects (but not against *Brugia malayi*) *in vivo* (Zahner et al., 2001), hence there is great interest in the potential of emodepside as a novel antifilarial (http://r4d.dfid.gov.uk/PDF/Outputs/DNDI/evolutions_of_dndi_portfolio.pdf). With regards to levamisole, though previous studies from the 1980s showed it had no significant treatment effect for LF or onchocerciasis patients (Awadzi et al., 1982; McMahon, 1981), the drug was revisited in a clinical trial in 2004 where levamisole was given alone (2.5 mg/kg) or co-administered with ivermectin or albendazole. Here as well, it proved to be ineffective, alone or in combination (Awadzi et al., 2004).

Nitazoxanide, mentioned earlier in this review, and its metabolite tizoxanide were tested against *B. malayi* worms *in vitro* and *in vivo*. The drugs were lethal to adult worms at 20 μg/ml and significantly decreased microfilaria release at 5 μg/ml. However, they were ineffective against *Wolbachia* and eventually were shown to be ineffective in clearing the worms *in vivo* (Rao et al., 2009).

Considering the success of antimalarials against trematodes, as well as the co-endemicity of malaria and many helmintic diseases, it is always tempting to test antimalarials on other worms as well. Recently, an open randomized control trial investigated the effects of the antimalarials quinine, chloroquine, amodiaquine and artesunate, based on previous *in vivo* successes or case studies, on patients infected with *L. loa*. However, none of the treatments were found to be effective (Kamgno et al., 2010).

5. Cestode infections

5.1. Current treatment

Niclosamide, originally developed as a molluscicide, was introduced in 1959 as the first synthetic drug against taeniasis, characterized by an excellent therapeutic index and high efficacy (WHO/FAO/OIE, 2005). It is a poorly water soluble halogenated salicylanilide with a low bioavailability, which is an advantage since a systemic action is not desired (Pearson and Hewlett, 1985; WHO/FAO/OIE, 2005). Approximately one decade later, in 1972, the taenicidal activity of praziquantel, an acylated isoquinoline-pyranzine, was discovered jointly by Bayer AG and E. Merck (Andrews et al., 1983). Both drugs are still the recommended first line treatments against intestinal cestodes including *Taenia solium*, *Taenia saginata*, *Diphyllobothrium* spp. and *Hymenolepis nana* (WHO/FAO/OIE, 2005). Praziquantel, which is well absorbed from the intestine, presents additional activity against *T. solium* cysticerci located in the host's tissues (i.e. central nervous system, subcutaneous tissue, and the eye). A single oral dose of praziquantel is highly effective against taeniasis; nevertheless multiple treatment courses over 2 weeks are recommended for uncomplicated cysticercosis. Albendazole, is also recommended for the treatment of (neuro)-cysticercosis and is generally more active than praziquantel (Sotelo et al., 1988, 1990; Takayanagui and Jardim, 1992; WHO/FAO/OIE, 2005). Regardless, therapy for neurocysticercosis needs to be individualized depending on the patient's state of disease. Anthelmintics, surgery, and symptomatic treatment with analgesics, corticosteroid drugs, and antiepileptic drugs, to relieve headache, perilesional inflammation and seizures, are the main pillars of the therapeutic approach (Carpio, 2002; Garcia et al., 2011).

Surgical resection of the entire parasitic lesion is indicated in all operable cases of cystic and alveolar echinococcosis. Puncture-aspiration-injection-re-aspiration (PAIR) treatment is applied in inoperable cases of cystic echinococcosis: cysts are punctured and exposed to protoscolicidal substances (i.e. ethanol 95%) for at least a quarter of an hour (WHO, 1996). Chemotherapy is key to preventing secondary echinococcosis and to reducing the risk of re-occurrence following surgery (Brunetti et al., 2010). Mebendazole and albendazole are both recommended for the treatment of echinococcosis. The albendazole dose regimen is more convenient because its phase-1 metabolite, albendazole sulfoxide, also exhibits metacestodicidal properties and thus the drug disposition is greater than for mebendazole (Ingold et al., 1999). Praziquantel itself possesses less pronounced activity against echinococcosis than albendazole. Combination treatment of praziquantel and albendazole seems sensible, because both drugs display a different mechanism of action and praziquantel increases the bioavailability of albendazole sulfoxide when the two are given together (Cobo et al., 1998; Kern, 2006; Garcia et al., 2011). Nonetheless, insufficient clinical data are available to provide treatment recommendations for this combination (Pawłowski, 2001; Bygott and Chiodini, 2009).

5.2. Repurposed drugs

5.2.1. Taeniasis and neurocysticercosis

The food and drug administration (FDA) approved nitazoxanide, mentioned several times earlier in this review, as an antiprotozoal agent against *Cryptosporidium* species and *Giardia intestinalis* in 2002 (Rossignol, 2009). Its chemical structure stems from the taenicide niclosamide and therefore, unsurprisingly, activity was reported against *T. saginata* and *H. nana* infections in the 1980s (Table 3) (Rossignol and Maisonneuve, 1984). Hence, nitazoxanide is not a genuine example of a repurposed drug for taeniasis; still its strong taenicidal activity makes it attractive for off-label use in cases where patients do not respond to praziquantel or niclosamide (Vermund et al., 1986; Lateef et al., 2008). Similarly, quinacrine (mepacrine), which is an ancient antimalarial agent and antigiardial drug, can be used to treat niclosamide-tolerant *T. saginata* infections (Gardner et al., 1996; Koul et al., 2000).

The recent promising results achieved with tribendimidine against *O. viverrini* and *C. sinensis* liver fluke infections (see above) inspired further exploration of its anthelmintic activity profile against cestodes. In an open-label randomized clinical trial, including 15 patients infected with *Taenia* spp., a single oral dose of 200 mg (5–14 year old children) or 400 mg ($\geqslant$15 years) tribendimidine resulted in a CR of 67%. Due to diagnostic challenges and the relatively small numbers of patient enrolled in the study, the authors pointed out that the observed cestocidal effect of tribendimidine should rather be recognized as an indication of possible activity rather than as a proof-of-concept (Steinmann et al., 2008). A distinct anticestodal potential of tribendimidine was demonstrated in mice infected with *Hymenolepis microstoma*, where a triple dose of 50 mg/kg tribendimidine, given over three consecutive days, achieved a WBR of more than 95% (Kulke et al., 2012). No cestocidal effect was observed however, when mice were treated with deacetylated amidantel. This result is somehow unexpected as deacetylated amidantel is supposed to be the major active metabolite of tribendimidine, hence further studies are required (Xiao et al., 2009; Yuan et al., 2010).

Paromomycin (Humatin®) is an aminoglycoside antibiotic with activity against gram-negative and various gram-positive bacteria. It is marketed to decrease bacterial load in the gut in hepatic coma, and for the treatment of amoebiasis and giardiasis. Recently, it has been licensed in India for leishmaniasis therapy (Davidson et al., 2009). In the 1960s the taenicidal activity of paromomycin was

discovered by chance in the course of amoebiasis treatments, where co-infected patients shed segments of *T. saginata* (Salem and el-Allaf, 1969). In a follow-up clinical study it was demonstrated that daily treatments for 1–3 days with 30–50 mg/kg paromomycin resulted in a success rate of 89–100% against *T. saginata*. In addition, an efficacy of approximately 90% was observed against *H. nana* following a 30 mg/kg single or double dose of paromomycin (Salem and el-Allaf, 1969).

Tamoxifen is a first generation selective estrogen receptor modulator (SERM), which acts as an antagonist on estrogen receptors in breast tissue, while possessing an agonistic effect in other tissues. It is used as an adjuvant for breast cancer and the palliative treatment of metastatic or locally advanced mamma carcinoma (den Hollander et al., 2013). It has been demonstrated that estradiol plays an essential role in regulating the asexual reproduction of *Taenia crassiceps* cysticerci in mice, possibly by interfering with the host's cellular immune response (Terrazas et al., 1994), and as a result, tamoxifen was tested against *T. crassiceps* cysticerci. Tamoxifen reduced the parasite load by 80% and 50% in female and male mice, respectively. In addition, loss of motility and reduced reproduction could be demonstrated *in vitro* (Vargas-Villavicencio et al., 2007). A follow-up study showed that tamoxifen decreased the intestinal establishment of *T. solium* in hamsters by approximately 70%. Moreover, tamoxifen induced morphological changes: the recovered tapeworms appeared like scolices without strobilar development and consequently showed an 80% reduction in length (Escobedo et al., 2013). Yet the taenicidal and cysticidal potential of tamoxifen has not been evaluated in humans and livestock.

Metrifonate is an organophosphorous compound applied as an insecticide and veterinary anthelmintic. In humans it has been, for example, for the treatment of schistosomiasis (Holmstedt et al., 1978). A case report published in 1981 about a man with a heavy cutaneous cysticercosis infection, described that two treatment courses of metrifonate at 10 mg/kg/day for six days were able to clear about 75% of the nodules and reduced the size of the remaining ones. Nevertheless, the observed adverse drug events caused by metrifonate's unspecific cholinesterase inhibition were so strong that atropine had to be given to relieve the symptoms (Tschen et al., 1981).

5.2.2. Echinococcosis

In vitro and *in vivo* studies indicate that *Echinococcus multilocularis* and *E. granulosus* can be included in the broad activity spectrum of nitazoxanide. *In vitro* treatment of *E. multilocularis* metacestodes with nitazoxanide lead to morphological and ultrastructure alterations and inhibited larval growth, while *E. granulosus* protoscoleces and metacestodes suffered deleterious effects as well (Stettler et al., 2003; Walker et al., 2004; Reuter et al., 2006). Subsequent *in vivo* experiments showed that a combination of nitazoxanide and albendazole reduced parasite weight by about 4 times in the murine model (Stettler et al., 2004). A good effect was observed in patients with disseminated cystic echinococcosis on affected soft tissue, muscles, or viscera, who received nitazoxanide additionally to the standard albendazole chemotherapy (with or without praziquantel). Nevertheless, nitazoxanide shows no effect on chronic and extensive body lesions (Perez-Molina et al., 2011).

A 3-month treatment course with the anticancer drugs daunorubicin, idarubicin, cytarabine, and fludarabine did not affect the size and contents of the echinococcus cysts in a patient suffering from acute leukemia and cystic echinococcosis of the liver. The antifungal agent amphotericin B was additionally applied within the chemotherapy schedule, but did not show parasitocidal activity against *E. granulosus* cysts either (Ali et al., 2005). However, *in vitro* data suggest that amphotericin B effectively inhibits the growth of *E. multilocularis* metacestodes (Reuter et al., 2003b). Amphotericin B is also able to halt parasite growth in *E. multilocularis*-infected patients and might therefore play a role as salvage treatment, when resistance or intolerance to benzimidazoles occurs (Reuter et al., 2003a).

The anthelmintic agent ivermectin was tested against *E. multilocularis* in hamsters with intraperitoneal inoculation of protoscolices, and in rats with transportal inoculation of protoscolices. The drug showed no effect in either case (Inaoka et al., 1987). In addition, *in vitro* results confirmed that ivermectin is also ineffective against *E. multilocularis* larvae (Reuter et al., 2006).

The spectrum of activity of antimalarials, such as mefloquine or the artemisinins, was also assessed against echinococcosis. Mefloquine presented a dose dependent activity against metacestodes *in vitro*, although a reduction of the parasite weight in *E. multilocularis*-infected mice was only achieved by intraperitoneal mefloquine administration and not with oral treatments (Kuster et al., 2011). Artesunate and dihydroartemisinin, however, had no effect against *E. multilocularis in vivo* (Spicher et al., 2008), despite excellent *in vitro* activity. Furthermore, *in vitro* incubation of *E. multilocularis* larvae with artemether did not induce destruction of parasite vesicles (Reuter et al., 2006).

Table 3
Drugs tested in clinical trials against the major cestodes (*Taenia* and *Echinococcus*).

Drug	Parasite	Number of patients	Dose	Outcome	References
Nitazoxanide	*T. saginata*	22	25 mg/kg	100% CR	Rossignol and Maisonneuve (1984)
	H. nana	18	50 mg/kg	100% CR	
	T. saginata	18 children 34 adults (niclosamide and praziquantel-resistant infections)	20 mg/kg p.o. (children 5–14 years); 500 mg twice daily for 3 days p.o. (> 14 years)	98% CR	Lateef et al. (2008)
	Echinococcus spp. (in combination with albendazole)	5	500 mg/12 hours for 3–24 months	40% improvement (2 patients improved)	Perez-Molina et al. (2011)
Mepacrine (quinacrine)	*T. saginata*	86	1 g p.o. or i.g.	94%	Koul et al. (2000)
Tribendimidine	*Taenia* spp.	15	200 mg p.o. single dose (children 5–14 years) 400 mg p.o. single dose (⩾15 years)	67% CR	Steinmann et al. (2008)
Paramomycin	*T. saginata*	145	1–5 days with 30–50 mg/kg	89–100% CR	Salem and el-Allaf (1969)
	H. nana	49	30 mg/kg single or double dose	90% CR	
Chlorhexidine gluconate	*E. granulosis*	30	0.04% in intracystic injection	100% death of cystic protoscolices	Topcu et al. (2009)

p.o denotes oral treatment, i.g. denotes treatment via a nasogastric tube, CR stands for "cure rate".

Ethanol (70–95%), hypertonic saline (15–30%) and cetrimide solution (0.5%) are considered to be scolicidal agents for PAIR treatment with relatively low risk according to the WHO (1996). Efforts were made to discover more potent scolicidal agents to reduce the recurrence rate following PAIR and surgical interventions. For instance, chlorhexidine gluconate, which is a commonly available, inexpensive, and non-toxic antiseptic agent, proved to be highly active at a concentration of 0.04% following intracystic injection (Topcu et al., 2009). Moreover, *in vitro* data confirm that intracystic albendazole sulfoxide injections at concentrations of 50–100 μg/ml are highly scolicidal and additional studies in rabbits indicate that this treatment lacks biliary and systemic toxicity (Erzurumlu et al., 1998; Adas et al., 2009). Recently, a water-soluble albendazole sulfoxide salt was developed (Bayverm®-PI, Bayer HealthCare), which enabled the preparation of a liquid dosing form and henceforth systemic and even intracystic injections. Importantly, systemic application circumvents the low oral absorption rate of albendazole and significantly elevates drug disposition (Lanusse et al., 1998). A proof of concept trial in sheep infected with *E. granulosus* ($n = 7$) was performed, which demonstrated that intracystic instillation without reaspiration of albendazole sulfoxide significantly decreased cyst size and lead to death of all protoscoleces and daughter cysts 6 months post-injection (Deger et al., 2000). Further studies in animals and human subjects are warranted to evaluate if this therapeutic approach could substitute the current lengthy oral albendazole therapy.

Albendazole and mebendazole are currently the recommended drugs for chemotherapy of echinococcosis; hence it is reasonable that other benzimidazole derivatives are investigated. For instance flubendazole induced 90% reduction in cyst weight compared to untreated mice infected with *E. granulosus*. This is particularly remarkable, as lower quantities of flubendazole were detected in plasma and within the cysts compared to albendazole treatments, suggesting a higher potency of flubendazole (Ceballos et al., 2011). Finally, it could be demonstrated that oxfendazole (60 mg/kg) alone and in combination with praziquantel was able to decrease protoscolex viability and reduce the diameter of liver and lung cysts in sheep naturally infected with *E. granulosus* following short-term treatment regimens (⩽6 weeks) (Gavidia et al., 2010). Notably, triclabendazole has shown potency *in vitro* and it would be interesting to see if it is as successful as the other benzimidazoles *in vivo* (Richter et al., 2013).

6. Conclusion

We have summarized drug repurposing efforts against a wide array of helminth infections. Drug repurposing is an important strategy, in particular for these neglected tropical diseases since drug discovery and development has languished in this field. Moreover, the time, effort and resources saved renders drug repurposing a smart and ethical choice, as it can result in faster and more affordable access to new medicines.

Several dozen compounds were identified by systematically searching the literature and presented in our review. This rather low number mirrors the challenges helminth drug discovery efforts still face. *In vitro* screens are often low throughput and cumbersome, as described for the *T. muris* assay (Wimmersberger et al., 2013). Furthermore, we lack adequate animal models; prominent examples are the two most important human filarials *O. volvulus* and *Wuchereria bancrofti*, and the problematic *L. loa*. As such, it is currently not possible to test drugs on these species directly *in vivo* and most testing is conducted with infection models from analogous animal filariasis infections, such as *O. ochengi* in cattle (Geary and Mackenzie, 2011).

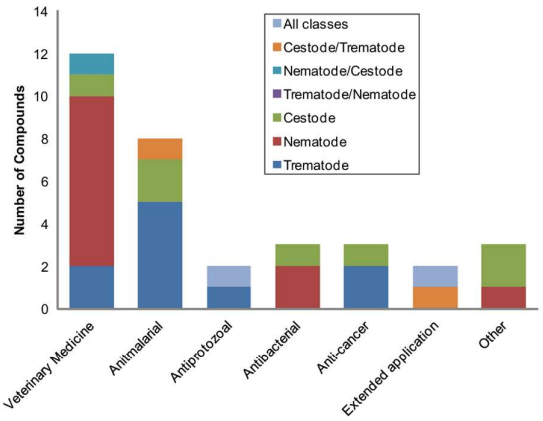

Figure 1. Sources of compounds for anthelmintic repurposing. A summary of the fields from which the repurposed drugs discussed in this review are sourced. Only successful drugs and drug candidates were included. "Extended applications" refers to drugs that were already anthelmintics but whose activity was discovered to extend to a broader range of helminth species.

Interestingly, only a few compound classes have been investigated and several compounds have been studied against different helminth species. Antimalarial and anticancer compounds were most widely studied, though most of the successful anthelmintic compounds still come from the antimalarial and veterinary fields. Though most repurposed drugs still come from veterinary anthelmintic R & D, gradually compounds from fields such as antiprotozoal and anticancer research are being increasingly investigated (Fig. 1). As mentioned in this review these drug classes were not randomly selected but rather based on biological features (flukes have the same blood-feeding characteristic as *Plasmodium* spp.) and possible drug targets (identification of select protein kinases as being essential to schistosome development) of these parasites. With the availability of genomes, e.g. *B. malayi* or the *L. loa* genome, it is the hope of many that compound libraries containing drugs' known modes of action can be matched *in silico* against helminth-specific gene targets, therefore providing potential drug candidates. In addition, unsurprisingly the benzimidazole class of compounds has been studied extensively. However, given that there is known cross-resistance between the various benzimidazoles in animal health, it is worth highlighting that a good clinical candidate arising from this group would rather replace albendazole and mebendazole than serve as backup.

Overall, disappointingly few compounds have been pursued into clinical testing, which does little to alleviate the dry anthelmintic pipelines. A notable exception however is the trichuricidal drug oxantel pamoate.

As public–private partnerships continue to expand, new compounds and compound libraries have been made openly accessible. For example, recently the Medicines for Malaria Venture made available an open-source library consisting of 200 drug-like and 200 probe-like compounds, which was screened against *S. mansoni in vitro* and *in vivo*. Though the *in vivo* results were moderate at best, it did provide two potential new scaffolds to of interest: the N,N′-diarylurea and 2,3-dianilinoquinoxaline derivatives (Ingram-Sieber et al., 2014). This is an example illustrating that closer collaborations between different research fields would be of great benefit in the field of drug discovery and development for helminth diseases. More of such collaborations are needed to take bench findings to the field and to turn field successes into affordable and useable medicines.

Conflict of interest

The authors state that they present no conflict of interest in writing this review.

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Chapter 3b

Activity profile of an FDA-approved compound library against *Schistosoma mansoni*

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RESEARCH ARTICLE

Activity Profile of an FDA-Approved Compound Library against *Schistosoma mansoni*

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Abstract

Background

As plans to expand mass drug treatment campaigns to fight schistosomiasis form, worries about reliance on praziquantel as the sole available treatment motivate the investigation for novel antischistosomal compounds. Drug repurposing might be an inexpensive and effective source of novel antischistosomal leads.

Methodology

1600 FDA approved compounds were first assayed against *Schistosoma mansoni* schistosomula at a concentration of 10 μM. Active compounds identified from this screen were advanced to the adult worm screen at 33.33 μM, followed by hit characterization. Leads with complementary pharmacokinetic and toxicity profiles were then selected for *in vivo* studies.

Principal Findings

The *in vitro* screen identified 121 and 36 compounds active against the schistosomula and adult stage, respectively. Further, *in vitro* characterization and comparison with already available pharmacokinetic and toxicity data identified 11 *in vivo* candidates. Doramectin (10 mg/kg) and clofazimine (400 mg/kg) were found to be active *in vivo* with worm burden reductions of 60.1% and 82.7%, respectively.

Conclusions/Significance

The work presented here expands the knowledge of antischistosomal properties of already approved compounds and underscores variations observed between target-based and phenotypic approaches and among laboratories. The two *in vivo*-active drugs identified in this study, doramectin and clofazimine are widely available and present as novel drug classes as starting points for further investigation.

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Author Summary

For a disease of large global health importance, schistosomiasis has a disproportionally small treatment tool box- only praziquantel is used to treat all 3 major forms of the disease. While drug discovery can be a long, laborious and expensive process, especially for an under-funded neglected disease such as schistosomiasis, drug recycling (also termed repositioning or repurposing) can bypass some of the development processes and offset the costs. We conducted a drug screening project of 1600 FDA-approved compounds from a very diverse set of indications against *Schistosoma mansoni*. The full 1600 compounds were first screened *in vitro* against the larval stage of the worm, of which 121 drugs were identified as active. These hits were then screened on the adult stages of the worm *in vitro* where 36 of these hits were also found to be active on the adult stage. The safety and pharmacokinetic profiles of hit compounds were then compared to their *in vitro* activity and 11 compounds were chosen for studies in mice. Of these, clofazimine and doramectin were found to be moderately active, and present new antischistosomal scaffolds with which further investigations can be pursued. Our findings are placed in context with results obtained from previous *in vitro* and *in silico* chemogenomics work and agreements and disagreements discussed.

Introduction

Worldwide, schistosomiasis continues to affect the health and quality of life of millions, causing 3.3 million Disability-Adjusted Life-Years lost [1]. Most of the burden is contained in the tropics, mostly in Sub-Saharan Africa, where it disproportionally affects children in poor rural areas [2]. The three principal causative agents are *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum*. Infection with any of these three species, when left untreated, results in chronic inflammation which slowly develops into swelling, fibrosis and necrosis of the tissues of intestinal organs, the liver or the bladder, as well as a range of other symptoms which gradually impair the host physiologically and even cognitively [3,4].

The World Health Organisation (WHO) places morbidity control as a priority for treating schistosomiasis via preventative chemotherapy in the form of mass drug administration campaigns. Treatment targeted at high risk groups, mainly school-aged children, interrupts advancement to the cumulative damage of chronic stages, which causes most of the disease burden [5]. To date, this is seen as the most cost-effective strategy, as interruption of transmission is very difficult, costly and subject to many factors, and vaccine development is still far out of reach [6,7]. Yet of the 207 million people infected annually, in 2012 only 35 million received treatment at a given time [4]. Therefore, it has become essential to expand mass treatment campaigns. Indeed, as many as 235 million children are targeted to receive treatment by 2018 [8].

Nonetheless, we still rely on praziquantel as the sole treatment and the expanded use of this drug, while positively reducing morbidity, would also increase the potential for praziquantel resistance [9,10]. Regardless of expansion plans, reliance on one single drug for mass treating a population is dangerous. The international community has repeatedly stated the need for new medication, since the drug discovery and development pipeline is dry [11]. Earlier, we reviewed the ways in which drug repurposing is aiding helminth drug discovery [12] and highlighted several clinical success stories such as antimalarials for the treatment of schistosomiasis. Drug repurposing (or repositioning) is the development of new indications from existing, failed or abandoned drugs and offers some obvious benefits: researchers can piggy-back off the

availability of pre-clinical data, saving time and costs, making more informed decisions on hit-to-lead identification and ultimately decreasing the time it takes to bring a drug to market [13,14].

In the framework of a Gates-funded drug discovery project, overseen by the Drugs for Neglected Diseases initiative (DND*i*), different libraries, including a library of 1600 FDA approved compounds with diverse classes and initial applications, were screened on *Schistosoma mansoni*. Abdulla and colleagues had previously evaluated a similar library of 2160 compounds on a Puerto Rican strain of *S. mansoni*, finding many *in vitro*-active compounds but no strong *in vivo*-active candidates [15]. Considering our past experience with strain and hit cut-off differences [16], we were encouraged to screen the above-mentioned 1600 compound FDA library in the hopes of identifying strong candidates to test *in vivo* and to compare our findings.

In more detail, the full 1600 compound library was initially assayed on newly transformed schistosomula (NTS- the larval stage). Compounds that reduced NTS viability by 75% were further tested on adult worms and their activity was compared to their existing pharmacokinetic and toxicity profiles before initiating studies in a mouse-*S. mansoni* infection model. Finally, we compare our results with findings reported from the above-mentioned screen by Abdulla et al. and a recent target-based chemogenomics screen of a dataset of 2,114 proteins by Neves et al. [17], and discuss overlaps and contradictions.

Materials and Methods

Compounds and Media

The FDA Pharmakon compound library was purchased from MicroSource Discovery Systems, Inc. (USA). Compounds were delivered in microplates (10 mM, dissolved in DMSO) and kept at -80°C until use. For *in vivo* studies, flunarizine hydrochloride, pimozide, nicardipine hydrochloride, oxethazaine, menadione, clofazimine, doramectin and metitepine mesylate were purchased from Sigma-Aldrich (Buchs, Switzerland) and fendiline hydrochloride, manidipine hydrochloride and lomerizine hydrochloride were purchased from Santa-Cruz Biotechnology (California, USA). Hanks Balanced Salt Solution (HBSS) was obtained from Gibco (Lucerne, Switzerland). Culture medium components for NTS and adult worms were obtained as follows: Medium 199 RPMI 1640 and penicillin (100 U/ml) and streptomycin (100 μg/ml) were purchased from Lubioscience (Lucerne, Switzerland) whereas inactivated fetal calf serum (iFCS) was purchased from Connectorate AG (Dietikon, Switzerland).

Schistosoma mansoni Larval *In Vitro* Assay

NTS were obtained using a transformation method described previously [18]. Briefly, cercariae (Liberian strain) were harvested from infected intermediate host snails (*Biomphalaria glabrata*) after several hours' exposure to light. The collected cercarial suspension was cooled, centrifuged and pipetted, and vortexed vigorously in HBSS to remove the tails. The suspension was rinsed in cool HBSS to remove the tails and the resulting NTS suspension was adjusted to a concentration of 100 NTS per 50 μl in NTS culture medium (Medium 199 supplemented with 5% iFCS and 1% penicillin/streptomycin). The NTS suspension was then incubated at 37°C, 5% CO_2 in ambient air for 24 hours. Drugs were first tested at a concentration of 10 μM on NTS. The worms were incubated in culture medium and the test compounds in a 96-well plate in triplicate for 72 hours. Thereafter, they were assessed microscopically using a viability scale previously described [16], which scores the morphology and motility of the NTS (3 = motile, no changes to morphology; 2 = reduced motility and/or some damage to tegument noted; 1 = severe reduction to motility and/or damage to tegument observed; 0 = dead). Hits were

characterized as compounds that achieved an average viability score of 0.5 or less (corresponds to NTS viability of $\leq$ 25%).

In Vitro Drug Sensitivity Assay on *Schistosoma mansoni* Adult Worms

Compounds identified as hits in the NTS drug assay were further tested on adult worms. Mice were infected as detailed in the *in vivo* studies section below and the infection was allowed to mature for 7 weeks. Mice were then euthanized with CO_2 and their intestinal apparatus was dissected. Worms were collected from the hepatic portal and mesenteric veins and subsequently rinsed and stored in culture medium (RPMI supplemented with 5% iFCS and 1% penicillin/streptomycin) at 37°C, 5% CO_2 until use. In a 24-well plate, 2–4 worm pairs were placed in culture medium and 33.33 μM of the test compound for 72 hours, 2 wells per compound. Effects were assessed microscopically with the same viability scale used for NTS and again, compounds that achieved an average score of 0.5 or less but after 24 hours were considered as hits. For further hit characterization, the IC_{50} values were determined in an adult worm dose-response assay (33.33, 11.11, 3.70, 1.43 and 0.41 μM drug concentration) at 1, 2, 4, 7, 10, 24, 48 and 72 hours post-drug incubation.

In Vivo Studies in the *S. mansoni* Mouse Model

For *in vivo* studies, female 3-week old NMRI mice were used. Mice were purchased from Charles River (Sulzfeld, Germany) and allowed to adapt under controlled conditions (temperature ca. 22°C; humidity ca. 50%; 12-hour light and dark cycle; free access to rodent diet and water) for one week. Thereafter, they were infected subcutaneously with approximately 100 *S. mansoni* cercariae (obtained as described above). Seven weeks post-infection, 4 mice were assigned to each drug treatment, while 8 mice were left untreated to serve as controls. Compounds were prepared in a 70:30 Tween/EtOH mixture dissolved in dH_2O (10%). Available compound toxicity data was used to guide the dosing regimen. Compound doses were adjusted to the mouse weight and were administered orally. Three weeks post-treatment, mice were killed by the CO_2 method and dissected, and the worms were sexed and counted. Mean worm burdens of treated mice were compared to the mean worm burden of untreated animals and worm burden reductions were calculated.

Ethics Statement

In vivo studies were conducted at the Swiss TPH, Basel, and approved by the veterinary authorities of the Canton Basel-Stadt (permit no. 2070) based on Swiss cantonal (Verordnung Veterinäramt Basel-Stadt) and national regulations (the Swiss animal protection law (Tierschutzgesetz).

Statistics

For *in vitro* assays, the viability scores were averaged across replicates and normalized to control-well viability scores using Microsoft Office Excel (2010). IC_{50} values were computed using CompuSyn2 (ComboSyn Inc., 2007) by converting viability scores into effect scores for each drug concentration.

The worm burden (WB) of treated mice was calculated and compared with the worm burden of control mice in order to obtain the worm burden reduction (WBR), calculated as

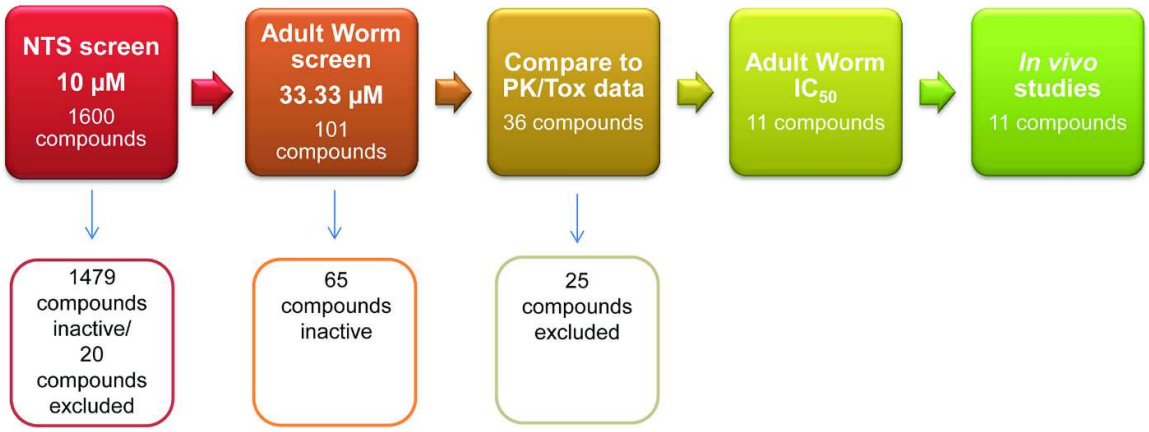

Fig 1. *In vitro* screening cascade of the FDA Pharmakon library against *S. mansoni* NTS and adult worms.

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follows:

$$\mathrm{WBR}\ (\%) = 100\% - (100\% / \mathrm{WB}_{\mathrm{control}} \times \mathrm{WB}_{\mathrm{treatment}})$$

Statistical comparison was done using the Kruskal Wallis Test and the Mann Whitney U test at a significance level of $p < 0.05$.

Results

In Vitro Activity on NTS and Adult *S. mansoni*

The overall screening cascade is presented in Fig 1.

Of the 1600 compounds screened on NTS, 121 compounds (summarized by indication in Table 1) showed activity at a concentration of 10 µM after 72 hours. Of these, 57 compounds killed the NTS completely within 72 hours of exposure and 64 compounds damaged the NTS severely (viability score ≤ 0.5, corresponding to a viability of $\leq 25\%$) within the same time frame. After a quick scan of the hits, 20 compounds were excluded due to their known high toxicity (e.g. colchicine) or because their activity against *S. mansoni* has already been described (e.g. mefloquine) (Table 1).

From the NTS screen, therefore 101 compounds qualified for testing on *S. mansoni* adult worms, at a single high concentration of 33.33 µM. Of these, 36 compounds were found to be active 24 hours post-incubation: the compounds induced death of the worms or a 75% reduction in their viability (a final viability score of ≤ 0.5). However, of the 36 active compounds, 25 were excluded following a closer review for the following reasons: 8 were excluded due to known toxicity in humans, 9 were indicated for topical use only, 5 had been described to have toxicity in rodents (low LD_{50} values), 2 were excluded due to past or current studies conducted on *S. mansoni in vivo* models (niclosamide studied by Abdulla et al. (15); tamoxifen studied by Cowan et al, submitted for publication), and 1 was rejected due to its poor absorption (Table 2).

The remaining 11 compounds were further characterized with an IC_{50} determination assay at various time-points (Fig 2 and S1 Table). Already after 2 hours incubation, most compounds (except pimozide, doramectin, clofazimine and flunarazine hydrochloride) exhibited IC_{50}

Table 1. 121 Hit compounds at NTS screening stage.

Antibacterial	Cefdinir*; Chloroxine; Clofoctol*; Gentian violet; Hexachlorophene; Lasalocid sodium; Monensin sodium*; Narasin; Natamycin; Pyrithione zinc; Salinomycin sodium*; Sulfanitran; Thonzonium bromide;	**Antipsychotic**	Aripiprazole; Chlorprothixene hydrochloride*; Fluphenazine hydrochloride; Metitepine mesylate; Perphenazine; Pimozide; Prochlorperazine edisylate; Sertraline hydrochloride (anti-depressant); Thioridazine hydrochloride; Trifluoperazine hydrochloride; Triflupromazine hydrochloride*;
Antifungal	Broxaldine; Butoconazole; Candicidin; Ciclopirox olamine; Econazole nitrate; Hexetidine; Itraconazole hydrochloride; Miconazole nitrate; Oxyquinoline sulfate; Phenylmercuric acetate; Piroctone olamine; Sulbentine; Sulconazole nitrate; Terbinafine hydrochloride; Thiram;	**Antihistamine/ anti-inflammatory**	Azelastine hydrochloride; Betamethasone sodium phosphate (also immunosuppressant); Cepharanthine; Cinnarazine; Escin; Montelukast sodium; Promethazine hydrochloride*; Terfenadine;
Antiprotozoal	Mefloquine**; Nifursol; Primaquine phosphate; Pyronaridine tetraphosphate;	**Statins/ Anti-diabetic/ coronary disease**	Fluvastatin; Lovastatin; Metformin hydrochloride (Diabetic); Orlistat; Perhexiline maleate
Anthelminthic	Abamectin; Antimony potassium tartrate trihydrate**; Doramectin; Eprinomectin; Hycanthone**; Moxidectin; Niclosamide; Oltipraz**; Praziquantel**; Pyrvinium pamoate; Quinacrine hydrochloride; Selamectin; Trichlorfon**	**Antihypertensive/ vasodilator**	Amlodipine besylate; Fendiline hydrochloride; Flunarizine hydrochloride; Lomerizine hydrochloride; Manidipine hydrochloride; Nicardipine hydrochloride; Prazosin hydrochloride*; Reserpine; Suloctidil; Vinpocetine
Antiinfective/ antiseptic	Acriflavinium hydrochloride; Benzalkonium chloride; Benzoxiquine; Bronopol; Cetrimonium bromide; Mepartricin; Methylbenzethonium chloride; Nifuroxazide; Thimerosal	**Cholinergic/ Anasthetic/ Cardiac stimmulant**	Atracurium besylate; Inamrinone; Neostigmine bromide*; Oxethazaine; Physostigmine salicylate*; Proscillaridin; Pyridostigmine bromide
Anticancer	Arsenic trioxide diethanolamine salt; Tamoxifen citrate; Toremiphene citrate;	**Other**	Acamprosate calcium; Amsacrine; Chlormadinone acetate; Clofazimine; Clomiphene citrate**; Colchicine*; Cyclosporine**; Digitoxin*; Erythrosine sodium; Idebenone; Iodipamide; Menadione; Methylene blue; Mifepristone; Octisalate; Podofilox; Protoporphyrin ix; Riboflavin; Riboflavin 5-phosphate sodium; Securinine; Tenylidone; Tolonium chloride; Tolperisone hydrochloride

Compounds were tested at a concentration of 10 μM and hits were defined as compounds for which the NTS scored $\leq$ 0.5 on our viability scale at 72 hours post-exposure.

*Indicates compound excluded due to toxicity concerns.

**Indicates compound that has already been well-characterized on *Schistosoma spp*. and hence was excluded.

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values below 10 μM, and by the 10 hour time-point, IC_{50} values for these compounds ranged from 1.73–7.80 μM. The fastest acting compounds were nicardipine hydrochloride and oxethazaine, exhibiting IC_{50} values of 2.67 and 2.95 μM respectively already at 1 hour post-incubation. Meanwhile, doramectin and clofazimine were the slowest acting, with IC_{50} values of 16.92 and 20.72 μM respectively at 4 hours post-exposure. Between 24 and 72 hours post-exposure, IC_{50} values did not vary greatly between drugs, ranging between 1.34 to 4.17 μM, except for pimozide which jumped to 8.78 μM at 24 hours and declined to 3.46 μM by the 72-hour time-point.

These timed IC_{50} values were compared to available pharmacokinetic data, and all 11 compounds were selected as good *in vivo* candidates. As previously mentioned, the *in vitro* data and available pharmacokinetic and rodent toxicity data were also used to guide the maximum possible single oral dose regimens.

Table 2. Compounds active on adult *S. mansoni* at a concentration of 33.33 μM at 24 hours.

Indication	Compound	Exclusion
Antibacterial	Pyrithione zinc	Topical
	Gentian violet	Topical
	Hexachlorophene	Topical
	Thonzonium bromide	Topical
	Narasin	Toxicity in rodent
Antifungal	Miconazole nitrate	Topical
	Hexetidine	Topical
	Phenylmercuric acetate	Toxic
Anthelminthic	Doramectin	
	Abamectin	Toxicity in rodent
	Eprinomectin	Toxicity in rodent
	Pyrvinium pamoate	Poor absorption
	Niclosamide	Being studied
	Selamectin	Topical
Anti-infective/antiseptic	Methylbenzethonium chloride**	Topical
	Cetrimonium bromide	Topical
	Thimerosal	Toxic
Anticancer	Arsenic trioxide diethanolamine salt	Toxic
	Tamoxifen citrate	Being studied
Antipsychotic	Pimozide	
	Metitepine mesylate	
Antihistamine/ anti-inflammatory	Manidipine hydrochloride	
	Terfenadine	Toxic
Statins/ Anti-diabetic/ coronary disease	Perhexiline maleate	Toxic
Antihypertensive/ vasodilator	Fendiline hydrochloride	
	Flunarizine hydrochloride	
	Lomerizine hydrochloride	
	Nicardipine hydrochloride	
	Suloctidil	Hepatotoxic
	Amlodipine besylate	Toxicity in rodent
Cholinergic/ Anesthetic/ Cardiac stimulant	Proscillaridin	Toxicity in rodent
	Oxethazaine	
Other	Menadione	
	Clofazamine	
	Tenylidone	Topical
	Securinine	Toxic

Activity was defined as scoring an average of ≤ 0.5 on the viability scale. The reason for exclusion is also listed and the data is based on compound material safety data sheets, FDA documents and previous publications.

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In Vivo Studies in the *S. mansoni* Mouse Model

Dosing, worm burden and worm burden reductions of the 11 compounds tested are presented in Table 3.

Metitepine mesylate proved to be toxic to mice at each dose tested (400, 200, 100 and 50 mg/kg, one mouse tested per dose and observed) and further investigation with this compound was ceased. Doramectin exerted a moderate worm burden reduction (60.1%) and clofazimine

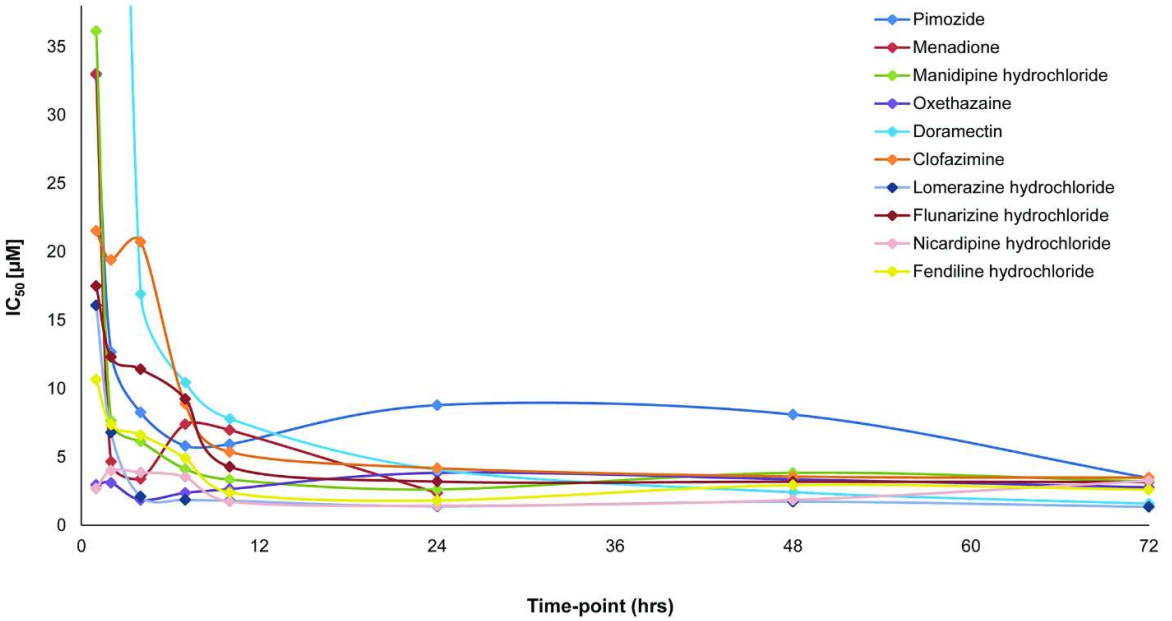

Fig 2. IC_{50} values of compounds selected for *in vivo* testing.

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Table 3. Worm burden reductions of *S.mansoni*-infected mice treated with *in vitro*-hit FDA Pharmakon compounds.

			Worm Burden		Worm Burden Reduction (%)	
Compound	**Dose (mg/kg)**	**No. of mice**	**Female**	**Total**	**Female**	**Total**
Control Batch 1	Untreated	8	24.1	50.1	-	-
Control Batch 2**	Untreated	8	14.8	31.3	-	-
Clofazimine	400	3*	3.7	8.7	80.8	82.7
Clofazimine**	200	4	19.8	38.5	0	0
Doramectin	10	4	10.3	20.0	62.5	60.1
Fendeline hydrochloride	100	3*	34.0	68.7	0	0
Flunarizine hydrochloride	200	4	18.0	36.8	27.9	26.7
Lomerizine hydrochloride	200	4	24.8	52.5	0	0
Manidipine hydrochloride	100	4	19.3	36.3	34.6	27.7
Menadione	400	4	27.3	53.3	0.0	0
Metitepine mesylate	All doses toxic- study stopped	N/A	N/A	N/A	N/A	N/A
Nicardipine hydrochloride	200	3*	12.3	25.3	50.0	49.5
Oxethazaine	200	3*	16.3	32.3	38.5	35.5
Pimozide	100	4	13.0	25.3	52.6	49.5

*Indicates that the 4th mouse of this group died prematurely.

** WBR for clofazimine (200 mg/kg) was calculated based on worm burden of Control Batch 2.

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caused a high, however not statistically significant worm burden reduction (82.7%). In a follow-up *in vivo* study, a dose 200 mg/kg clofazimine was tested in 4 *S. mansoni*-infected NMRI mice. Lowering the dose, however, resulted in a complete lack of efficacy (0% female and total WBR). Pimozide and nicardipine hydrochloride also demonstrated some efficacy (49.5% WBR for both), whereas mild WBRs were observed for flunarizine hydrochloride (26.7% WBR), oxethazaine (35.5% WBR) and manidipine hydrochloride (27.7% WBR). Lomerizine hydrochloride, fendeline hydrochloride and menadione lacked *in vivo* activity.

Discussion

The advent of praziquantel in the 1970s was a great milestone for the control of schistosomiasis in that finally, a safe, cheap and effective drug became available that could be used to treat millions in cost-effective preventive chemotherapy campaigns. Unfortunately, the success of praziquantel also resulted in many labs and firms choosing to drop further investigations on their leads [19]. This, along with inadequate attention and funding has rendered the antischistosomal arsenal dangerously dependent on a single drug [10].

As *de novo* drug discovery becomes increasingly expensive, drug repurposing, on the other hand, has shown to bear fruit in the antischistosomal drug discovery field as well as others with fewer resources involved [12,20,21]. By screening a library of well-characterized compounds, it was our hope to identify new drugs or drug classes that could be explored further in pre-clinical development. An initial screen against NTS revealed a hit rate of ~7.6% and encompassed a wide range of compound indications including antipsychotics, antibiotics, antifungals, antihistamines, antihypertensives and even vitamin precursors and metabolites (Table 1). Results of this work in part mirrored the screen conducted by Abdulla and colleagues (mentioned earlier), in that the variety of active compounds also ranged across a large spectrum of indications [15]. However, in comparing our NTS hits, it was interesting to note that although there was some overlap, there were numerous incongruences as well (S2 Table and Fig 3A). Of the 121 NTS hits we identified in our library, 69 of the compounds were also found in their library, but of these 69, only 25 were identified as hits (36% overlap). Conversely, of the 105 hits identified in the library of Abdulla and colleagues, 55 were found in our library and of those, 25 were characterized as hits (45%). These inconsistencies are likely a combination of differences in drug concentrations used, time of evaluation post-drug exposure and screen cut-off filters for hit identification, which indicates that these factors can greatly influence the outcome of a screen. Indeed, a closer inspection of hits identified by Abdulla et al. and missed in our library revealed that most of these "missed compounds" had some effect on our NTS but not enough to reach the cut-off threshold. Nonetheless, it may also be possible that strain differences result in differing drug susceptibilities (Abdulla et al. used a Puerto Rican strain, whereas ours was a Liberian strain). Indeed, Ingram-Sieber et al. observed similar differential sensitivities from their screen of MMV Malaria Box compounds conducted by two independent labs using exactly these two strains [16]. Bearing these incongruences in mind, it might be useful to start a discussion on a possible need for standardization and replication.

Recently, Neves et al, took advantage of the published genome and transcriptome of *S. mansoni* [22] as well as public drug databases to conduct an *in silico* screen of compounds with known targets that theoretically match targets found in the *S. mansoni* genome and transcriptome [17]. When we compared their hits to our NTS hits, a moderate overlap was observed (S2 Table and Fig 3B). In more detail, of the 162 compounds described to match to an *S. mansoni* target *in silico* (115 compounds they describe as novel along 47 with compounds for which some activity has already been described), 102 were present in our library, and of these 102, 19 were deemed as active, corresponding to a 19% overlap. The fact that the *in silico* prediction

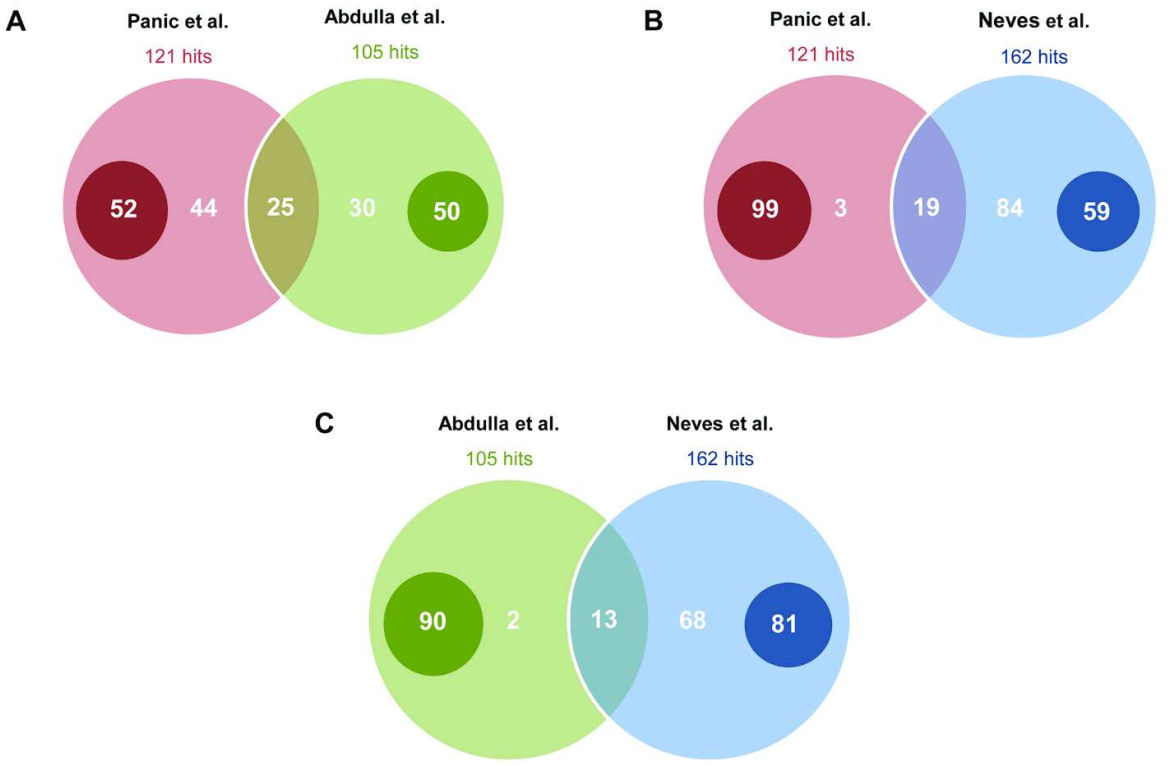

Fig 3. Venn diagrams representing overlaps between: (A) our NTS screen and that of Abdulla et al., (B) our NTS screen and *in silico* hits from Neves et al. and (C) NTS hits from Abdulla et al. and hits from Neves et al. The small dark circles within each large circle represent the number of compounds that were not present in the comparator's library.

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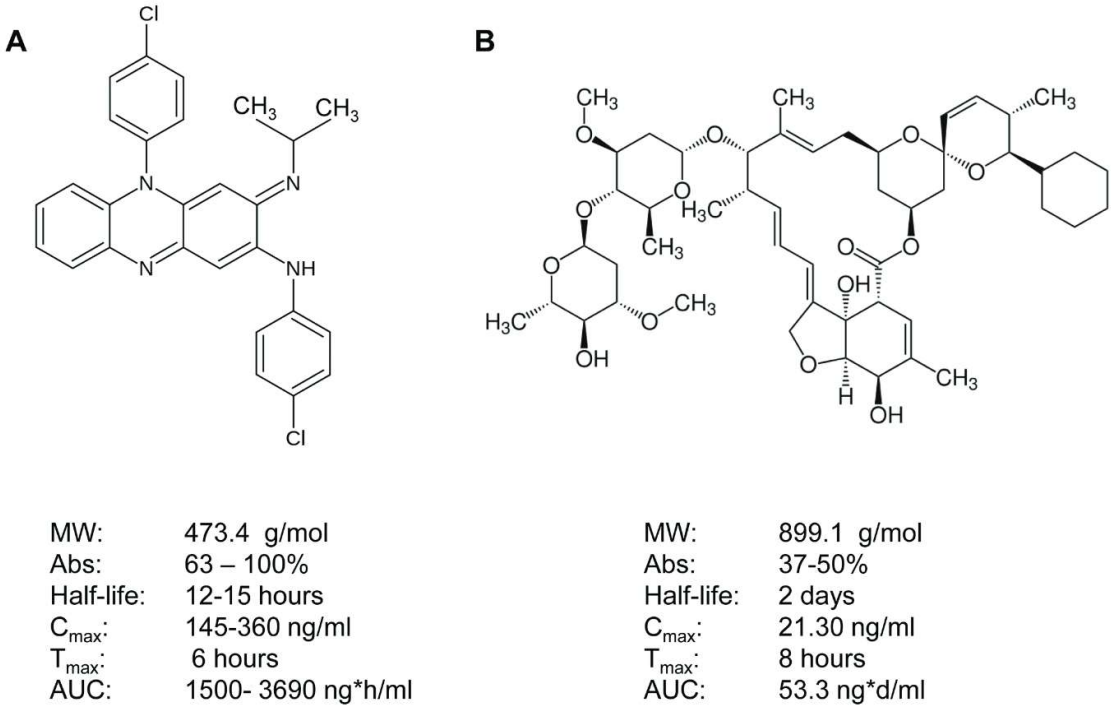
did not strongly match our *in vitro* hits, doesn't necessarily mean the *in silico* hits are incorrect: they could be differentially active on other life stages (ex. juvenile), active *in vivo*, at a higher concentration, or, as we saw with Abdulla and colleagues, in a different screen with a different strain. Nonetheless, it does hint that target-based approaches still require further development.

In this screen we identified doramectin and clofazimine as two moderately active compounds against *S. mansoni* in an NMRI mouse infection model (Fig 4).

The activity of doramectin, though not statistically significant, is nonetheless surprising: studies in *S. mansoni*-infected mice with the highly related ivermectin showed minimal efficacy when administered as a single oral dose of 25 mg/kg [23]. Moreover, clinical trials with ivermectin showed little efficacy against intestinal and urinary schistosomiasis [24]. However, doramectin is reported to have more favorable pharmacokinetic properties, which could account for its higher *in vivo* efficacy in our study [25]. Doramectin has not been previously studied in humans and therefore would need substantial efforts to register the drug for human medicine. However, the closely related moxidectin has shown a moderate effect against *S. mansoni* in preliminary clinical studies [26]. Moxidectin was active against NTS and moderately active against the adult stage worm in our screen, and hence may be worthy of further investigation.



MW:	473.4 g/mol	MW:	899.1 g/mol
Abs:	63 – 100%	Abs:	37-50%
Half-life:	12-15 hours	Half-life:	2 days
C_{max}:	145-360 ng/ml	C_{max}:	21.30 ng/ml
T_{max}:	6 hours	T_{max}:	8 hours
AUC:	1500- 3690 ng*h/ml	AUC:	53.3 ng*d/ml

Fig 4. Structures and pharmacokinetic data of *in vivo*-active clofazimine (A) and doramectin (B). Clofazimine data is based on a single oral dose of 200 mg give to healthy male volunteers [45]. Doramectin data is based on a single oral dose of 200 μg/kg administered to horses [46].

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Clofazimine is originally indicated for treatment against leprosy and is on the WHO Model List of Essential Medicines [27]. It is a fat-soluble iminophenazine dye that has demonstrated immunosuppressive properties, including inhibition of macrophages, neutrophil motility, lymphocyte transformation and mitogen-induced PBMC formation [28–30]. Recently, clofazimine was also identified as a promising preclinical anti-trypanosomal agent in an *in silico* screen of marketed drugs [31]. The authors noted that the compound was also effective in inhibiting epimastigote proliferation *in vitro* and in reducing parasitaemia levels in a murine infection model at a dose of 20 mg/kg. In our study, the initial dose of 400 mg/kg clofazimine showed a moderately high WBR (82.7%), while lowering the dose to 200 mg/kg was not effective. Considering the long half-life of clofazimine (12–15 hours) as well as its reportedly good absorption (60–100%), we deemed it unnecessary to study the effects of multiple dosing. Currently, off-label use of clofazimine is highly discouraged by the WHO, as it is the first line of treatment against leprosy and there are legitimate fears of drug resistance [32]. Nonetheless, it may be worth exploring the antischistosomal activities of related structures.

With the advent of the *S. mansoni* genome, there have been attempts to incorporate rational drug screening in the antischistosomal drug discovery process. Recently, the observation that fatty acids play a major role in schistosome development, fecundity and tegument construction has spurred researches to investigate the potentials of cholesterol-lowering statins [33–36]. Consequently, Rojo-Arreola and colleagues evaluated six statin compounds, atorvastatin,

fluvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin, all targeting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) of the eukaryotic mevalonate pathway on *S. mansoni* NTS and adult worms. All drugs were found to be active *in vitro* and targeted SmHMGR [37]. In our own screen, fluvastatin and lovastatin were found to be active on NTS but inactive on adult worms. Atorvastatin and simvastatin were inactive already at the NTS stage and pravastatin and rosuvastatin were not present in our library. The contrasting results could be partly attributed to the stricter cut-off parameters used in our screen (shorter drug incubation time, only severe reduction in viability considered) or could also be due to strain differences (the use a Puerto Rican strain). *In vivo* tests would be required to say anything substantial about the potential of statins as antischistosomals.

Considering rational *S. mansoni* drug targets, it was interesting to note that none of the calcium channel blockers such as fendeline hydrochloride or flunarizine hydrochloride showed a potent *in vivo* effect. In light of the notion that praziquantel's mode of action is highly suspected to be due its disruption of Ca2+ homeostasis, the idea that known calcium channel blockers could be effective against *S. mansoni in vivo* is not too far-fetched [38,39]. That being said, these drugs are often used to treat chronic human disorders such as hypertension, migraines or allergies, meaning they also bind to human receptors. The fact that these drugs were very potent *in vitro* but not *in vivo* could be attributable to many factors such as drug metabolism or protein binding, but competition between host and parasite receptors might play a role. This could be a general drawback to repurposing drugs with known human receptor targets for use against parasite infections.

Indeed, while drug repurposing can potentially reduce the time and costs of the drug discovery process, its limitations should also be carefully considered and are already observable in our study. Although compound libraries intended for new indication screens often contain already marketed drugs, their safety window may not necessarily be acceptable for schistosomiasis treatment and preventative chemotherapy. Drug repurposing is a popular strategy for an array of diseases, some of which a narrow safety window is acceptable due to the nature of the disease [40,41]. Compounds chosen for development against schistosomiasis, however, must have an excellent safety profile, as they will be very widely used in preventive chemotherapy campaigns mainly targeted towards children [42]. Indeed, of the 36 adult worm hits, it was disappointing to note that 25 of these compounds were unsuitable for testing, notably due to documented severe side-effects, restriction to topical use or low LD_{50} values in mice. Some compounds, for example terfenadine, had even been withdrawn from FDA approval or were no longer marketed [43]. It would be favorable if further libraries of already known compounds would be strictly composed of drugs currently on the market with a good safety profile, in order for a real drug repurposing effort to be possible.

A further major challenge to drug repurposing might be the difficulty to develop a dose regimen in humans that provides plasma exposure in the range of the *in vitro* IC_{50} concentration, which tends to be high for helminths. Hence the chance that safety, pharmacokinetics and pharmacological action will match for a very different indication is uncertain. With these limitations in mind, the NTD community should not rely on drug repurposing alone as a drug discovery strategy. Nonetheless, it is a very worthy venture: as previously stated, many of the anthelmintics used today were repurposed from veterinary applications [12]. Moreover, 90% of the drugs available today have secondary indications, showing that repurposing continues to be a popular strategy both for academia and industry [44]. Importantly, each marketed drug very likely has a library of analogues behind it, with which structure activity relationship for a hit expansion program can certainly be envisaged. Access to these analogues would facilitate an optimization program aiming at the identification of preclinical candidates.

In conclusion, by screening a library of 1600 well characterized compounds, we have identified dozens of compounds active against *S. mansoni in vitro*. Many of the compounds safety or pharmacokinetic profiles rendered them unfavorable for further exploration. Nonetheless, of the 11 compounds screened *in vivo*, we identified two compounds with moderate to high activities with which further investigations may result in novel compound class treatments.

Supporting Information

S1 Table. Table of $1C_{50}$ values over exposure time of compounds selected for *in vivo* testing (complement to Fig 2).
(DOCX)

S2 Table. Comparison of NTS hits between our data (Panic et al.), that of Abdulla et al. (2009) and general *in silico* hits from Neves et al. (2015). Green indicates a hit, red a non-hit and grey means it was lacking from the library.
(DOCX)

Author Contributions

Conceived and designed the experiments: GP JK. Performed the experiments: GP MV. Analyzed the data: GP MV. Contributed reagents/materials/analysis tools: IS. Wrote the paper: GP IS JK.

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Chapter 4

Rescuing and redesigning old antischistosomals

Chapter 4a

Aryl hydantoin Ro 13-3978, a broad-spectrum antischistosomal

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Aryl hydantoin Ro 13-3978, a broad-spectrum antischistosomal

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Objectives: Praziquantel is the only drug available for the treatment of schistosomiasis and the state of the exhausted drug discovery pipeline is alarming. We restarted investigations on the abandoned antischistosomal Ro 13-3978, an aryl hydantoin discovered in the early 1980s by Hoffmann La-Roche.

Methods: Newly transformed schistosomula and adult *Schistosoma mansoni* were studied in the presence of Ro 13-3978 *in vitro*. The metabolic stability of Ro 13-3978 was determined *in vitro* using human and mouse liver S9 fractions. Dose–response relationship, stage specificity, hepatic shift and scanning electron microscopy studies were carried out in *S. mansoni*-infected mice. In addition, efficacy experiments were conducted in rodents infected with *Echinostoma caproni* and *Fasciola hepatica* as well as in *S. mansoni*-infected immunocompromised nude (*Foxn1*^{nu}) mice.

Results: Ro 13-3978 showed minor *in vitro* activity and no damage to the tegument was found. No cytotoxicity was detected for Ro 13-3978. Ro 13-3978 was metabolically stable. ED_{50} values of 138.9 and 14.6 mg/kg were calculated for the treatment of juvenile and adult *S. mansoni* infections, respectively, with a single oral dose of Ro 13-3978. SEM studies revealed severe damage to the worms 48 h post-treatment of infected mice. A single oral dose of Ro 13-3978 (100 mg/kg) administered to *S. mansoni*-infected (*Foxn1*^{nu}) mice reduced the worm burden by 88%. Ro 13-3978 was not active against *E. caproni* and *F. hepatica in vivo*.

Conclusions: Ro 13-3978 has excellent antischistosomal properties *in vivo*. Structure–activity relationship studies with the aryl hydantoins have been launched in order to elucidate active pharmacophores, further investigate the mechanism of action and to identify a derivative with minimal antiandrogenic effects.

Keywords: schistosomiasis, *Schistosoma mansoni*, chemotherapy, drug discovery

Introduction

Schistosomiasis is a waterborne parasitic infection caused by six different trematode species, with *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum* being responsible for the largest public health burden.[1,2] The disease gives rise to a persistent chronic disorder in endemic areas, resulting in common disabling complications such as anaemia, growth stunting, cognitive impairment and decreased aerobic capacity.[2,3] Using the most recent disability-adjusted life year (DALY) metrics,[4] an estimated 3.3 million DALYs have been attributed to schistosomiasis. To reduce the chronic health burden, millions of school-age children are treated each year in the framework of 'preventive chemotherapy' programmes with praziquantel.[5] Praziquantel is a safe and effective drug against the chronic stage of the disease; however, it is the only commercially available drug for the treatment and control of schistosomiasis.[5,6] Since the introduction of praziquantel several decades ago, drug discovery and development for this neglected tropical disease have been minimal and no backup drug is therefore available should praziquantel-resistant parasites evolve.[6]

In the early 1980s, Hoffmann La-Roche discovered the antischistosomal aryl hydantoin Ro 13-3978. The compound, a close structural analogue of the androgen receptor antagonist nilutamide, is a distant chemical cousin of the nitrothiazole, imidazolidinone niridazole[7] (Figure 1), an obsolete schistosomicide, but is otherwise chemically unrelated to known antischistosomal drugs. We recently demonstrated that a single 400 mg/kg dose of nilutamide achieved a high worm burden reduction (WBR) in *S. mansoni*-infected mice.[8] Furthermore, we showed that there is no correlation between antischistosomal activity and androgen receptor interaction for nilutamide, Ro 13-3978 and closely related aryl hydantoins.[9] Drug discovery efforts by Hoffmann La-Roche revealed that Ro 13-3978 was equal to or more effective

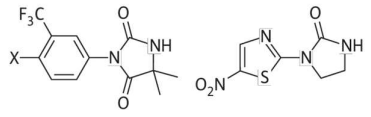

Figure 1. Structures of Ro 13-3978, nilutamide and niridazole.

than praziquantel in different *in vivo* schistosome animal models; thus the compound was recommended for clinical testing.[10] However, despite its remarkable and broad-spectrum activity, Ro 13-3978 was not further investigated.

The aim of the present study was to draw attention to the largely forgotten Ro 13-3978 by a thorough re-examination of its antischistosomal properties. *In vitro* studies with Ro 13-3978 were conducted against newly transformed schistosomula (NTS) and adult *S. mansoni*. Cytotoxicity studies were carried out using rat skeletal myoblast L6 cells. The dose–response relationship and stage specificity were evaluated in *S. mansoni*-infected mice. To begin to understand the pharmacokinetic/pharmacodynamic profile and mechanism of action of Ro 13-3978, metabolic stability, hepatic shift and scanning electron microscopy (SEM) studies and efficacy experiments in immunocompromised mice and mice treated with 1-aminobenzotriazole, a non-specific inhibitor of cytochrome P450, were conducted.

Materials and methods

Animals and parasites

In vivo experiments were carried out at the Swiss Tropical and Public Health Institute (Basel, Switzerland), in accordance with Swiss national and cantonal regulations on animal welfare (permission no. 2070). Female mice (NMRI strain, $n=86$; weight ~20–22 g) and female rats ($n=6$; weight ~80 g) were purchased from Charles River, Germany. Ten NMRI nude mice (weight ~18–20 g) were purchased from Harlan, the Netherlands. Rodents were kept under environmentally controlled conditions (temperature ~25°C; humidity ~70%; 12 h light and 12 h dark cycle) and acclimatized for 1 week before infection. The animals had free access to water and rodent diet.

Cercariae of *S. mansoni* and *Echinostoma caproni* were obtained from infected intermediate host snails (*Biomphalaria glabrata*) as described previously.[11] *Fasciola hepatica* metacercariae were purchased from Baldwin Aquatics, USA.

Compounds

Ro 13-3978 was synthesized as previously described.[10,12] For *in vitro* antischistosomal studies, compounds were dissolved in DMSO (Fluka, Buchs, Switzerland) to obtain 10 mg/mL stock solutions. For *in vivo* studies, compounds were suspended in 7% (v/v) Tween 80 (Fluka, Buchs, Switzerland) and 3% (v/v) ethanol before oral administration to rodents (10 mL/kg).

In vitro studies

NTS

S. mansoni cercariae were harvested from infected snails and mechanically transformed to NTS as described earlier.[13] An NTS suspension at a concentration of 100 NTS per 50 μL was prepared using Medium 199 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% inactivated FCS (iFCS; Connectorate AG, Dietikon, Switzerland) and 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen). NTS suspensions were incubated (37°C, 5% CO_2) for at least 24 h before experiments to ensure that conversion into schistosomula had been completed. NTS were incubated with 100 μg/mL Ro 13-3978 for 72 h at 37°C, 5% CO_2. The experiment was conducted in triplicate and repeated once. The highest concentration of DMSO served as a control. NTS were evaluated by microscopic readout (Carl Zeiss, Germany, magnification ×80) using a viability scale scoring death, changes in motility, viability and morphological alterations.[13]

Adult S. mansoni

Adult schistosomes were removed by picking from the hepatic portal system and mesenteric veins of mice that had been infected with 100 *S. mansoni* cercariae 49 days earlier. The worms were washed and kept in RPMI 1640 culture medium (Invitrogen) supplemented with antibiotics and 5% iFCS at 37°C in an atmosphere of 5% CO_2 until use. In one experiment, the medium was supplemented with a freshly prepared haemin solution (8%) or 2% (v/v) human red blood cells (RBCs: blood group AB, Rh positive) to test whether haemoglobin degradation is involved in the mechanism of action.[14] Worms were incubated in the presence of 25–100 μg/mL Ro 13-3978 for up to 96 h. Phenotypes were monitored daily, scoring motility, viability and morphological alterations under an inverted microscope (Carl Zeiss, Germany, magnification ×80), using the viability scale mentioned above. In addition, membrane damage was checked by a Trypan Blue exclusion assay: Ro 13-3978-treated, live control and dead control worms (killed using a cell lysis buffer) were rinsed three times with non-enriched RPMI medium, then exposed to a solution of 50 μL of Trypan Blue (Invitrogen) in 500 μL of non-enriched RPMI for 10 min. Worms were then returned to the culture medium and checked for Trypan Blue staining. All experiments with adult *S. mansoni* were conducted in duplicate and repeated once.

Cytotoxicity studies on rat skeletal myoblast L6 cells

Cytotoxicity studies were conducted as described elsewhere.[15] Briefly, rat skeletal myoblast L6 cells were seeded in 96-well plates (2×10^3 cells/well) (BD Falcon, USA) using RPMI 1640 medium supplemented with 10% iFCS and 1.7 μM L-glutamine (Sigma-Aldrich, Buchs, Switzerland). Following adhesion of the cells for 24 h at 37°C and 5% CO_2, the IC_{50} of Ro 13-3978 was determined using concentrations of 0.12, 0.37, 1.11, 3.33, 10, 30 and 90 μM. Podophyllotoxin (Sigma-Aldrich) served as positive control. At 70 h post-incubation, 10 μL of resazurin dye (Sigma-Aldrich) was added and the plates incubated for another 2 h. The plates were then read using a SpectraMax M2 (Molecular Devices) plate reader with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The cytotoxicity experiment was conducted in duplicate and repeated three times.

Metabolic stability experiments

The *in vitro* metabolic stability of Ro 13-3978 was determined using human and mouse liver S9 fractions (XenoTech, LLC, Lenexa, KS, USA) following standard protocols.[16,17] Briefly, Ro 13-3978 (2 mM in 0.1% methanol) was premixed with NADPH (1 mM), saccharolactone (5 mM), uridine 5′-diphospho-glucuronic acid (1 mM) and 3′-phosphoadenosin-5′-phosphosulphate (0.1 mM) in potassium phosphate buffer (100 mM, pH 7.4) at 37°C. Reactions were initiated by adding S9 fractions at 1 mg/mL protein concentration in final incubation volumes of 100 μL and were then quenched by adding 100 μL of ice-cold methanol at 0, 5, 10, 15, 30, 45, 60, 90 and 120 min, followed by centrifugation at 17000 **g** for 10 min. Ten microlitres of the supernatants were then analysed by LC-MS/MS

using an Acquity UPLC-BEH Shield RP_{18} column (2.1×100 mm, 1.7 mm; Waters, Milford, MA, USA) with a 2:3 0.1% acetic acid:methanol isocratic mobile phase at a flow rate of 0.25 mL/min. MS/MS analyses were performed in negative electrospray ionization mode; specific detection of Ro 13-3978 was performed by monitoring the transition 288.9→177.9 m/z. For the detection of potential Ro 13-3978 metabolites, multiple reaction monitoring–information-dependent acquisition-enhanced product ion, neutral loss, precursor ion and enhanced MS scans were used. 7-Hydroxycoumarin and testosterone were used as control substrates for phase I and phase II metabolism.

In vivo studies

E. caproni

Mice were infected orally with 20–25 metacercarial cysts of *E. caproni*. Fourteen days post-infection, four mice were treated with a single oral 100 mg/kg Ro 13-3978 dose. Four untreated mice served as controls. One week post-treatment, mice were killed and dissected, and all worms present in the intestines counted.

F. hepatica

Six rats were infected with 20–25 *F. hepatica* metacercariae. Twelve weeks post-infection, three rats were treated with a single oral 100 mg/kg Ro 13-3978 dose while three rats remained untreated. One week post-treatment, livers and bile ducts were examined for flukes and worm burdens were calculated.

S. mansoni

To study the dose–response relationship of Ro 13-3978 in adult *S. mansoni* infections, groups of three to five NMRI mice were treated orally in a single experiment with single doses of Ro 13-3978 (12.5, 25, 50 and 100 mg/kg) at 49 days post-infection. To test whether metabolism contributes to the activity of Ro 13-3978, one group of mice ($n=5$) infected with adult *S. mansoni* was orally treated with 50 mg/kg 1-aminobenzotriazole (Sigma-Aldrich) followed by 100 mg/kg Ro 13-3978, 2 h later. To assess whether a potential interaction of Ro 13-3978 with the immune response plays a role in its antischistosomal efficacy, immunocompromised NMRI nude mice ($n=5$) were treated with a single 100 mg/kg oral dose of Ro 13-3978, 49 days post-infection.

Mice (four to five mice per group) harbouring juvenile infections (established 3 weeks post-infection) were treated orally in a single experiment with doses of Ro 13-3978 (25, 50, 100 and 200 mg/kg).

For the stage-specificity experiment, groups of four mice were treated with a single oral 100 mg/kg Ro 13-3978 dose, 2 and 1 day prior to infection with *S. mansoni* cercariae, on infection day and on days 7, 14, 22, 28, 35, 42 and 49 post-infection.

Untreated mice served as controls in all experiments. At day 21 post-treatment (days 21–49 for the stage-specificity experiment), animals were killed by the CO_2 method and dissected. Worms were removed by picking, then sexed and counted as previously described.[18]

Finally, the hepatic shift was investigated as follows. Four mice infected with adult schistosomes were orally treated with 100 mg/kg Ro 13-3978 and after 4, 8, 28 and 48 h, one mouse was euthanized and dissected. All worms in the mesenteric veins, hepatic portal veins and pressed liver were counted.

SEM studies

SEM studies were conducted for both *in vitro* and *in vivo* Ro 13-3978-treated worms. *In vitro* worms were incubated as described above with 25–100 μg/mL Ro 13-3978 for 72 h. For the *in vivo* studies, two mice were orally treated with 100 mg/kg Ro 13-3978 and dissected at 24 and 48 h post-treatment. Worms were extracted from the mesenteric veins and liver as described above, rinsed twice in PBS, and fixed in 1 mL 2.5% glutaraldehyde for 3–24 h at room temperature. Subsequently, samples were dehydrated by incubating the worms for 30 min in ascending ethanol concentrations of 30, 50, 70, 90 and 100%. The worms were critically point-dried (Bomar SPC-900), mounted on aluminium stubs and sputter-coated with gold of 20 nm particle size (Baltec Med 020). Samples were visualized using a high-resolution SEM accelerating voltage of 5 kV (Philips XL30 ESEM). Control worms (untreated; Figure 4a–d) were prepared and visualized in the same manner.

Statistics

Parasite viability values of treated and untreated NTS and adult schistosomes obtained from microscopic evaluation were averaged (means±SD)

Table 1. Dose–response relationship of Ro 13-3978 in mice harbouring juvenile and adult *S. mansoni*

Stage of infection	Dosage (mg/kg)	No. of mice investigated	No. of mice cured	Mean number of worms (SD)					Total WBR (%)	*P* value	Female WBR (%)	*P* value
				liver	mesenteric veins	total	males	females				
	control 1	8	—	1.0 (1.5)	24.8 (8.0)	25.9 (7.4)	11.1 (4.2)	14.8 (3.2)	—	—	—	—
	control 2	9	—	1.3 (1.9)	24.0 (5.5)	25.3 (5.3)	13.0 (2.6)	12.3 (3.4)	—	—	—	—
	control 3	9		0.9 (1.2)	16.6 (5.2)	17.4 (5.1)	8.6 (3.3)	8.9 (3.4)	—	—	—	—
	control 4	10		1.9 (1.8)	26.0 (13.8)	27.9 (14.4)	15.4 (8.1)	12.5 (7.0)	—	—	—	—
Juvenile	50[1]	4	0	0 (0)	27.3 (11.8)	27.3 (11.8)	10.3 (4.3)	17.0 (7.8)	0	0.005	0	0.01
	100[3a]	4	0	2.3 (2.1)	4.0 (3.6)	6.3 (4.3)	2.5 (0.6)	3.8 (3.9)	63.8		57.3	
	200[2a]	5	0	1.0 (1.0)	2.0 (1.6)	3.0 (1.9)	1.2 (0.8)	1.8 (1.3)	88.1		85.4	
Adult	12.5[1]	4	0	0.5 (1.0)	15.8 (11.6)	16.3 (12.4)	6.5 (4.1)	9.8 (8.8)	37.1	<0.001	33.8	<0.001
	25[1]	4	1	0.3 (0.5)	4.3 (5.4)	4.5 (5.3)	2.3 (2.6)	2.3 (2.6)	82.6		84.5	
	50[3]	4	1	0.3 (0.5)	3.5 (2.6)	3.8 (2.8)	0 (0)	3.8 (2.8)	78.2		57.3	
	100[4a]	4	2	0.3 (0.5)	1.3 (1.5)	1.5 (1.9)	0 (0)	1.5 (1.9)	94.6		88.0	

[a]Data reproduced from Wang *et al.*,[9] superscripts refer to the respective control group (1–4).

using Microsoft Excel software. For *in vivo* studies, WBRs were determined by calculating the percentage reduction in mean worm burdens of the treatment groups relative to the untreated mice and the Kruskal–Wallis test was used to test for significance ($P \leq 0.5$; StatsDirect statistical software, version 2.7.2.; StatsDirect Ltd, UK). The 50% effective dose values (ED_{50}) were calculated using CompuSyn software (Version 3.0.1, 2007; ComboSyn Inc., USA).

Results

In vitro studies

Ro 13-3978 was tested against adult *S. mansoni* and NTS *in vitro*. Ro 13-3978 at 100 μg/mL moderately reduced the motility of both the schistosomula and adults at 24, 48 and 72 h post-incubation. Incubation with 25 or 50 μg/mL Ro 13-3978 produced the same result, although the slowed motility became pronounced only at ≥72 h. Occasional spasms were also observed in the adult worms. Both microscopic evaluation and the Trypan Blue exclusion test indicated no damage to the tegument. A similar behaviour of worms was observed in the presence of haemin. The addition of 2% RBC to the medium resulted in the death of worms at 100 μg/mL, but at lower concentrations (25 and 50 μg/mL) worms were still alive 72 h post-incubation with only slightly decreased motility. No cytotoxicity was detected for Ro 13-3978 (IC_{50} >90 μM).

Metabolic stability of Ro 13-3978

Ro 13-3978 was stable (>95%) in both human and mouse liver S9 fractions for up to 120 min, and no metabolites were detected using LC-MS/MS analysis. In contrast, under the same conditions, more than 65% of the positive controls 7-hydroxycoumarin (glucuronidation and sulphation) and testosterone (CYP hydroxylation) had been consumed within 30 min incubation (data not shown).

Dose–response relationship against juvenile and adult S. mansoni in vivo

In contrast to the marginal *in vitro* activity of Ro 13-3978, single oral doses of this aryl hydantoin showed high *in vivo* antischistosomal efficacies. Total and female WBRs following treatment of juvenile *S. mansoni* infections (22 days post-infection) with 50–200 mg/kg Ro 13-3978 and of adult *S. mansoni* infections (49 days post-infection) with 12.5–100 mg/kg Ro 13-3978 are summarized in Table 1. Adult *S. mansoni* infections were more susceptible to the compound than juvenile infections. Total and female WBRs of 88.2% and 85.4%, respectively, were observed with 200 mg/kg Ro 13-3978 against juvenile infections. In mice infected with adult *S. mansoni*, a single 100 mg/kg oral dose of Ro 13-3978 reduced total and female worm burden by 94.6% and 88.0%. From these data, ED_{50} values of 138.9 and 14.6 mg/kg were calculated for treatment with Ro 13-3978 of mice harbouring 22 and 49 day-old *S. mansoni* infections, respectively.

Stage specificity of single oral 100 mg/kg Ro 13-3978 in mice harbouring S. mansoni

Given the lower activity of Ro 13-3978 against 22 day-old compared with 49 day-old *S. mansoni*, the entire stage specificity of

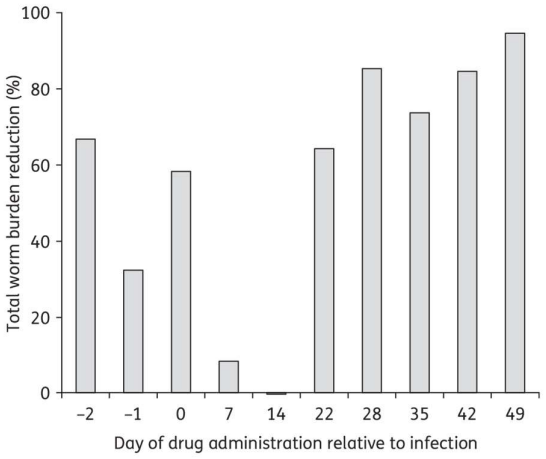

Figure 2. Stage specificity of single oral 100 mg/kg Ro 13-3978 in mice harbouring *S. mansoni*. Groups of four mice were treated 2 and 1 day prior to infection with *S. mansoni* cercariae, on infection day and on days 7, 14, 22, 28, 35, 42 and 49 post-infection. Worm burden reduction was evaluated 21–49 days post-treatment.

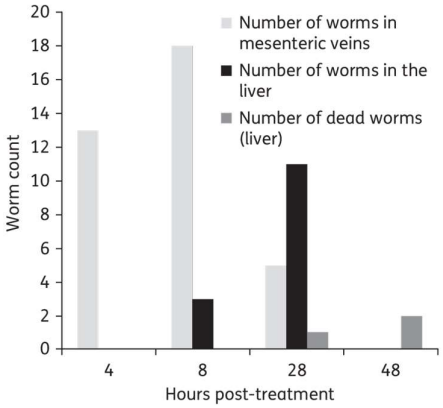

Figure 3. Hepatic shift observed in mice harbouring *S. mansoni* following 100 mg/kg Ro 13-3978. One mouse was dissected at 4, 8, 28 or 48 h post-treatment and worms were counted in the liver and mesenteric veins. Light grey bars, number of worms alive in the mesenteric veins; black bars, number of worms alive in the liver; and dark grey bars, number of dead worms found in the liver.

Ro 13-3978 was tested in mice. Mice were treated with Ro 13-3978 1 and 2 days prior to infection, on infection day and on days 7, 14, 22, 28, 35, 42 and 49 post-infection (Figure 2). Seven and 14 day-old *S. mansoni* were not affected by the compound. Moderate WBRs (32.4%–66.8%) were observed in mice treated with 100 mg/kg Ro 13-3978 before infection. High activities (64.3%–94.6%) were documented in mice treated with Ro 13-3978 in weekly intervals from day 22 onwards.

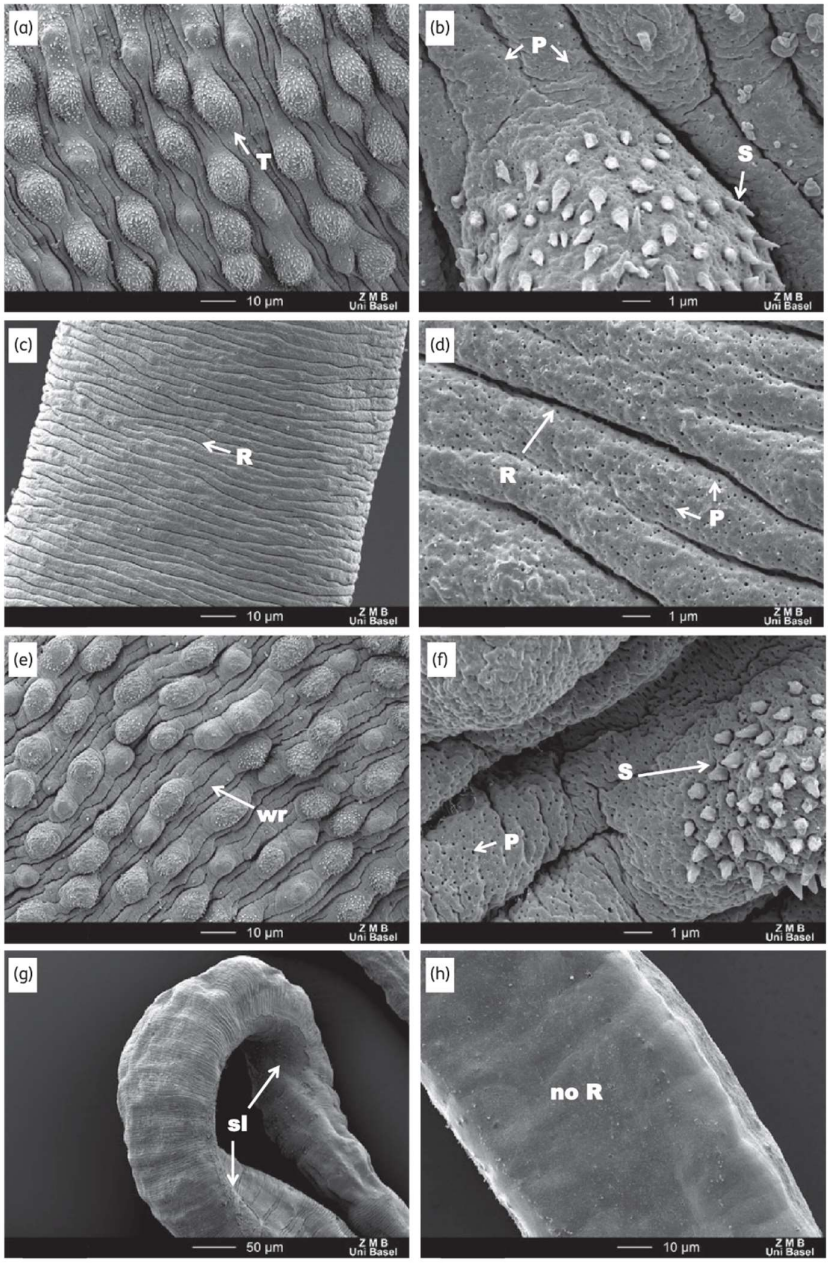

Figure 4. SEM observations of *in vitro* untreated and Ro 13-3978-treated (100 µg/mL for 72 h) worms. (a and b) Male control worms show a healthy tegument: tubercles (T) and spine (S) are intact and the tegument in-between is ridged and taut. Female control worms (c and d) are marked by a ridged (R) texture. Treated male worms (e and f) show a slight puffing and wrinkling (wr) of the between-tubercle tegument and some tubercles have a less rounded shape and fewer spikes. Female treated worms were mostly unaffected. However, two female worms (g and h) exhibit some areas of sloughing (sl) and a loss of ridged texture (no R). High magnification scans (×8000; b, d and f) reveal no differences between the control and treated worms, except that male treated worms appear to have slightly more pores (P; part f).

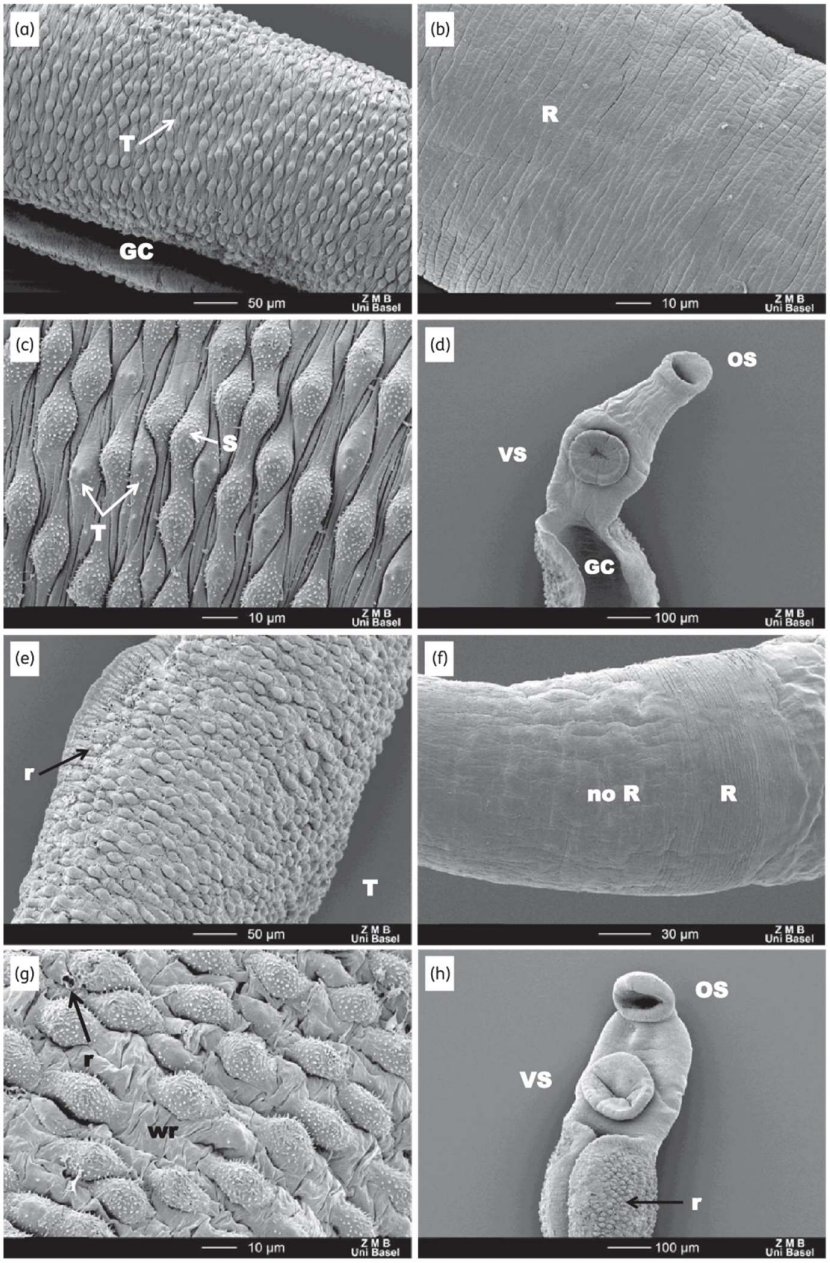

Figure 5. SEM observations of untreated worms and worms extracted from mice treated with 100 mg/kg Ro 13-3978, 24 and 48 h post-treatment. (a–d) Tegument of control worms with intact tubercles (T) and spines (S). OS, VS and GC denote oral sucker, ventral sucker and gynecophoral canal, respectively. (e–h) At 24 h there is mild damage to the male and female worms. For male worms, the tegument between the tubercles exhibits extensive wrinkling (wr) and some tubercles exhibit rupturing (r). The female worms lose features—the ridges (R) found in the control worms start to disappear. (i–l) At 48 h, extensive damage to the tegument is observed: along with wrinkling and rupturing (l), massive blebbing (bl), sloughing (sl) and general disintegration (di) are apparent along the entire length of the worm.

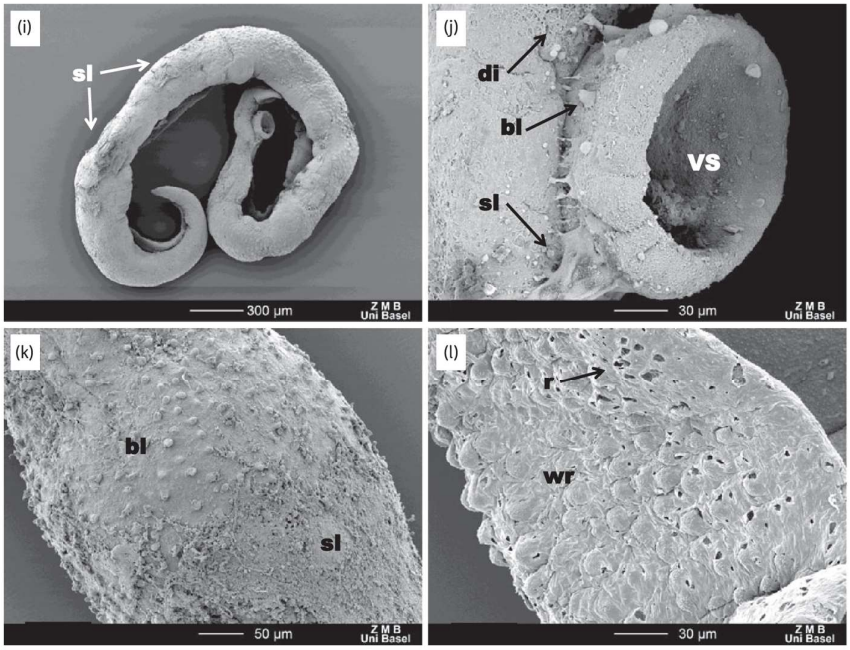

Figure 5. *Continued.*

Hepatic shift

Figure 3 depicts the distribution of worms in the liver and the mesenteric veins. Four hours post-treatment, all worms were present in the mesenteric veins. Eight hours post-treatment, the first worms had shifted to the liver, but the majority of worms still resided in the mesenteric veins and all worms were still alive. Most of the worms had shifted to the liver 28 h post-treatment and all of these showed clearly reduced viability. On the next day (48 h post-treatment), all worms had died and the majority of worms had been expelled.

SEM studies

As reported above, *in vitro*-treated worms showed moderately slowed activity at 72 h without an apparent change to the tegument. SEM examinations of the male worms revealed no obvious changes in tegument, except that it appeared less taut (Figure 4e and f). Most female worms appeared unaffected; however, two female worms from the 100 and 25 µg/mL treatment arms were marked by a few patches of mild to moderate sloughing and a few patches where a loss of ridged texture had occurred (Figure 4g and h). In *S. mansoni*-infected mice treated with a 100 mg/kg oral dose of Ro 13-3978, a progressive effect was observed (Figure 5). At 24 h post-treatment (Figure 5e–h), the worms were slightly more contorted. The tegument was still mostly intact; however, the areas between the tubercles were very wrinkled and small ruptures could be seen on the external tegument at the edges of the gynecophoral canal as well as the edges of the oral sucker. Moreover, the female tegument had started to lose its ridged texture (parallel ridges disappeared). At 48 h post-treatment, severe damage to the worms could be observed throughout all regions. The tegument showed blebbing, sloughing and rupturing all along the body, as well as the head and suckers (Figure 5i–l), and the edges of the gynecophoral canal were deeply furrowed.

Activity of Ro 13-3978 in immunocompromised mice and mice pretreated with 1-aminobenzotriazole

To further elucidate whether active metabolites or immunological mechanisms might help to account for the striking difference between the *in vitro* and *in vivo* activity of Ro 13-3978, further *in vivo* studies were conducted (Table 2). We found that very similar percentage WBR values were observed when single 100 mg/kg oral doses of Ro 13-3978 were administered to *S. mansoni*-infected mice with (100% WBR) and without (95% WBR) pretreatment with 1-aminobenzotriazole as a pan-CYP450 inhibitor, an unsurprising result given that this aryl hydantoin appears to be metabolically inert (see above). The same dose of Ro 13-3978 administered to *S. mansoni*-infected immunocompromised nude (*Foxn1*^{nu}) mice reduced the worm burden by 88%.

Activity of Ro 13-3978 against E. caproni and F. hepatica

We tested whether Ro 13-3978 exhibits blood fluke-specific *in vivo* activity or whether the range of activity would also include the liver fluke *F. hepatica* and the intestinal fluke *E. caproni*. No effect

Table 2. Activity of Ro 13-3978 administered at 100 mg/kg in immunocompromised mice and in mice pretreated with 1-aminobenzotriazole

Experiment	Dosage (mg/kg)	No. of mice investigated	No. of mice cured	Mean number of worms (SD)					Total WBR (%)	*P* value	Female WBR (%)	*P* value
				liver	mesenteric veins	total	males	females				
Nude mice	control	5	0	0.8 (1.1)	18.2 (7.5)	19.4 (7.3)	9.8 (4.1)	9.6 (3.2)	—	0.014	—	0.014
	100	4	0	0	2.3 (1.5)	2.3 (1.5)	0	2.3 (1.5)	88.2		76.1	
Pretreatment with 1-aminobenzotriazole	control	8	0	0.3 (0.5)	35.1 (14.0)	35.4 (13.8)	18.8 (6.3)	16.6 (7.6)	—	0.01	—	0.01
	100	5	5	0	0	0	0	0	100		100	

on the worm burden was observed when rodents infected with these two trematode species were treated with 100 mg/kg oral doses of Ro 13-3978 (Table 3).

Discussion

At least 230 million people worldwide are infected with *Schistosoma* spp.,[2] yet we only have the lone drug praziquantel for treatment and control of this neglected tropical disease. A recent systematic review, which assessed the state of the research and development pipeline of drugs and vaccines for neglected diseases, highlighted a clear deficiency in this field.[19] No drug is currently undergoing clinical testing for schistosomiasis;[19] hence a backup drug will not be available in the next decade.

We restarted investigations on Ro 13-3978, an aryl hydantoin with excellent antischistosomal properties, discovered in the early 1980s by Hoffmann La-Roche.[10] Our experiments confirmed that Ro 13-3978 has high activity against *S. mansoni* in the mouse model, with mature *S. mansoni* being more susceptible to the drug than juvenile flukes. We found that Ro 13-3978 had a single oral ED_{50} of 15 mg/kg in *S. mansoni*-infected mice (adult worms), a value close to the previously reported ED_{50} of 38 mg/kg.[10] In this same schistosome mouse model, praziquantel is considerably less effective against adult *S. mansoni*, with reported ED_{50} values ranging from 172 to 202 mg/kg,[10,20] and it has no significant activity against juvenile stages of the parasite. In contrast, in mice infected with juvenile *S. mansoni*, Ro 13-3978 had a single oral ED_{50} of 140 mg/kg. Interestingly, 7 and 14 day-old worms were not affected by Ro 13-3978, which might be explained by the location of the developing worm in the host (worms migrate via the heart and lungs to the liver, where they establish after ~2.5 weeks).[21]

Considerable unpublished preclinical data on Ro 13-3978 have been generated by Hoffmann La-Roche, ranging from parasitological to toxicological studies. Most importantly, Ro 13-3978 is active against all three major schistosome species—*S. mansoni*, *S. haematobium* and *S. japonicum*.[10] When Ro 13-3978 was administered to three different monkey species (*Cebus* monkeys, baboons and *Erythrocebus* monkeys) infected with *S. mansoni*, high activity was observed.[10] Our studies further document that, in contrast to praziquantel, the activity of Ro 13-3978 is schistosome-specific with no activity observed on the intestinal fluke *E. caproni* and the liver fluke *F. hepatica*. A range of toxicology studies were conducted at Hoffmann La-Roche, which confirmed that the drug is safe when administered at a single dose (unpublished data). For this class of aryl hydantoins, antiandrogenic effects were observed in multiple-dose studies using male castrated rats,[12] which is likely to be of less concern with single-dose treatment regimens. A recent study[9] demonstrated that, in contrast to nilutamide, Ro 13-3978 had no measurable interaction with the androgen receptor at concentrations up to 27 μM (7.8 μg/mL), although it did block dihydrotestosterone-induced cell proliferation in an androgen-dependent cell line.

The mechanism of action of Ro 13-3978 is not known; particularly striking is the difference between its *in vitro* and *in vivo* efficacy. In *in vitro* assays, worms were slightly affected by Ro 13-3978; only moderate impairment to motility was seen and only at very high concentrations. Interestingly, supplementation of the medium with 2% RBC resulted in the death of worms,

Table 3. Activity of Ro 13-3978 administered at 100 mg/kg against *F. hepatica* and *E. caproni*

Experiment	Dosage (mg/kg)	No. of rodents investigated	No. of rodents cured	Mean number of worms (SD)	Total WBR (%)	*P* value
F. hepatica	control	3	0	5.3 (2.3)	—	>0.05
	100	3	0	5.6 (1.5)	0	
E. caproni	control	4	0	12.5 (6.5)	—	>0.05
	100	4	0	14.3 (2.8)	0	

although only at a high concentration of 100 μg/mL. Further, SEM studies showed no membrane damage for most worms—results that, based on *in vitro* data alone, would have dismissed Ro 13-3978 from any further consideration. Yet *in vivo* studies have repeatedly confirmed the high activity of this compound. From the data that we have generated to date, the lack of significant *in vitro* activity of Ro 13-3978 is not explained by active metabolites or by effects on host immunology. In fact, experiments using human and mouse S9 fractions and 1-aminobenzotriazole-treated *S. mansoni*-infected mice indicate that Ro 13-3978 is not metabolized. Our data with the largely T cell-deficient *Foxn1*^{nu} mice suggests that cytotoxic T cell activity is not required for Ro 13-3978 to effectively clear adult *S. mansoni* from the host. On the other hand, the antischistosomal drugs oxamniquine and praziquantel were found to kill fewer adult *S. mansoni* worms in T cell-deprived mice than in immunologically intact controls.[22] However, T cell-independent worm antigens may be released or unveiled following Ro 13-3978 treatment, allowing for a humoral response to either be generated or effectively coat the adult worm *in vivo*. Further studies are in progress to identify the most likely mechanisms that might account for the high *in vivo* efficacy of Ro 13-3978.

The hepatic shift, which characterizes the forced migration of schistosomes to the liver, indicates that the onset of action of Ro 13-3978 starts rather slowly—at around 1 day post-treatment. For comparison, in parallel experiments with praziquantel, by 30 min post-treatment, the majority of worms shift to the liver.[23] Mefloquine, another recently identified antischistosomal agent, showed a hepatic shift 72 h post-treatment.[24]

SEM studies corroborate the slow onset of action of Ro 13-3978. At 24 h post-treatment, all of the worms were still found in the mesenteric veins and the corresponding images show that the worms are still mostly intact, although some damage (occasional ruptures in the tegument) can be seen. At 48 h post-treatment, the damage is clearly visible throughout the worm: the tegument is completely disintegrated, characterized by blebbing, rupturing and sloughing. At this time point, the vast majority of the worms were shifted to the liver and the females expelled. Damage to the tegument is likely not caused by the hepatic shift, as previous non-effective treatments, e.g. the inactive enantiomer of praziquantel (*S*-praziquantel),[23] have also caused a hepatic shift, followed by a return to the mesenteric veins, with the worms completely intact and viable.

In conclusion, we have confirmed that Ro 13-3978 has excellent antischistosomal properties against juvenile and adult *S. mansoni* infections *in vivo*. The aryl hydantoins should be considered candidates for the antischistosomal drug discovery and development pipeline. Structure–activity relationship studies have been launched to identify a derivative with minimal antiandrogenic effects to elucidate active pharmacophores and to further investigate the mechanism of action of this compound class.

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Transparency declarations

None to declare.

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Chapter 4b

Immunohistochemical investigations of treatment with Ro 13-3978, praziquantel, oxamniquine and mefloquine in *Schistosoma mansoni*-infected mice

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EXPERIMENTAL THERAPEUTICS

Immunohistochemical Investigations of Treatment with Ro 13-3978, Praziquantel, Oxamniquine, and Mefloquine in *Schistosoma mansoni*-Infected Mice

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ABSTRACT To date, there is only one drug in use, praziquantel, to treat more than 250 million people afflicted with schistosomiasis, a debilitating parasitic disease. The aryl hydantoin Ro 13-3978 is a promising drug candidate with *in vivo* activity superior to that of praziquantel against both adult and juvenile *Schistosoma mansoni* organisms. Given the drug's contrasting low activity *in vitro* and the timing of its onset of action *in vivo*, it was postulated that immune-assisted parasite clearance could contribute to the drug's *in vivo* activity. We undertook histopathological studies to investigate this hypothesis. Infected mice were treated with an effective dose of Ro 13-3978 (100 mg/kg of body weight) and were dissected before and after the drug's *in vivo* onset of action. The veins and livers were excised, paraffin-embedded, and sectioned, and macrophages (IBA-1), neutrophils (Neutro), B cells (CD45R), and T cells (CD3) were stained by immunohistochemistry. For comparison, samples from infected untreated mice and mice treated with effective doses of praziquantel (400 mg/kg), oxamniquine (200 mg/kg), and mefloquine (200 mg/kg) were examined. At 24 h after treatment with Ro 13-3978, significant macrophage recruitment to the veins was observed, along with a modest increase in circulating B cells, and at 48 h, neutrophils and T cells are also present. Treatment with praziquantel and oxamniquine showed similar patterns of recruitment but with comparatively higher cellular levels, whereas mefloquine treatment resulted in minimal cell recruitment until 3 days posttreatment. Our study sheds light on the immediate immune responses to antischistosomal treatment in mice and provides further insight into immune effector mechanisms of schistosome clearance.

KEYWORDS *Schistosoma mansoni*, immunohistochemistry, praziquantel, mefloquine, oxamniquine, Ro 13-3978

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Schistosomiasis is a chronic parasitic disease which afflicts more than 250 million people worldwide (1, 2). Behind malaria and intestinal helminthiasis, it is the third most impactful parasitic disease, where most of the disease burden is attributable to chronic inflammation and fibrosis of certain visceral organs and the resulting downstream consequences (3, 4). It is caused by trematode flatworms of the *Schistosoma* genus, where *Schistosoma haematobium*, *S. japonicum*, and *S. mansoni* are the main species affecting humans. Mature schistosomes attach to the mesenteric or urogenital vein walls and produce tens to thousands of eggs per day; the granulomas that form around these eggs are what cause the pathology (5).

The crux of the schistosomiasis control strategy is morbidity reduction using preventative chemotherapy with praziquantel, the sole drug currently available (6, 7). While the activity and safety profile of praziquantel are adequate, the drug nonetheless

carries drawbacks, such as inactivity against the juvenile stage of the worm. Importantly, the reliance on a single drug to treat hundreds of millions spurs concerns of resistance (8, 9). Treatment alternatives are therefore urgently needed.

As praziquantel was adopted as the WHO drug of choice, Hoffman-La Roche abandoned its antischistosomal lead, Ro 13-3978. A distant cousin to the obsolete antischistosomal, niridazole, Ro 13-3978 has an intriguing activity profile (10). *In vivo*, it has significantly superior activity to that of praziquantel against both adult and juvenile stage worms, with 50% effective dose (ED_{50}) values of 14.6 and 138.9 mg/kg of body weight, respectively, compared to praziquantel's ED_{50} of 172 to 202 mg/kg for adult worms and no appreciable effect against juvenile-stage worms. However, *in vitro*, it is barely active, with only moderate viability reduction after 72 h of exposure to 100 μg/ml of the drug (11–13).

Further investigations revealed this discrepancy between *in vitro* and *in vivo* activity is likely not due to drug metabolism (12). In addition, hepatic shift experiments were conducted to determine the point at which the worms get shunted to the liver as they lose viability, a rough metric for the *in vivo* onset of action of candidate compounds (14). Coupled with scanning electron microscope (SEM) imaging of *ex vivo* worms, it was shown that 24 h after drug treatment, the worms are still in the mesenteric veins of the intestines and only minor damage to the worm is visible. However, 48 h posttreatment, the worms have been shunted to the hepatic portal vein of the liver and there is extensive damage to the tegument (12). Since the liver is where adult worms mature and the temporary shunting of worms from intestinal veins to the hepatic portal veins of the liver (due to, for example, subcurative drug doses) is not itself lethal to adult schistosomes, we can rule out that the extensive damage is due to a hostile environment in the liver (14, 15).

A plausible hypothesis is that clearance could be immune assisted, as has been observed for many antischistosomal drugs, including praziquantel and oxamniquine (16). In *S. mansoni*-infected B-cell deficient mice, treatment with praziquantel is much less effective (17). Moreover, praziquantel and oxamniquine efficacies were shown to be markedly increased with coadministration of serum from rabbits immunized with worm membrane extract (the administration of serum alone is not effective) (18, 19). In addition, the clearance of schistosomes after treatment with praziquantel, oxamniquine, hycanthone, and some antimonials was markedly impaired in T cell-deficient (nude) mice (17, 20). However, worm clearance with chemotherapy does not always require immune assistance; in the same mouse model, mefloquine, niridazole, or amoscanate efficacy was not affected (20, 21).

In the case of Ro 13-3978, activity is also not compromised in athymic nude mice (12). However, T cell-independent humoral or innate immune components might still be triggered (22). Though a few studies have profiled medium and long-term changes to the host's immune system after treatment of schistosomiasis with praziquantel, there are no studies that demonstrate what immune cell components are recruited at the time of worm clearance (23–25).

The aim of this study was to use immunohistochemistry to look at *in situ* immune responses to treatment with Ro 13-3978 before and after its onset of action in an *S. mansoni* mouse infection model. Praziquantel and oxamniquine, two drugs that are somewhat T cell dependent for efficacy, were studied as comparators. Since Ro 13-3978 efficacy is not lowered in T cell-deficient mice, another drug that also exhibits T cell-independent efficacy (mefloquine) was also included.

RESULTS

The rationale of the study design was to take a snapshot of local cellular immune responses to adult *S. mansoni* after treatment with effective doses of Ro 13-3978 and the comparator drugs. This was done by examining mesenteric vein and liver histological samples of infected and treated mice before the drugs' onsets of action (before hepatic shift, when the worms are still in the mesenteric veins) and after the drugs' onsets of action (after hepatic shift, when the worms have been shunted to the liver)

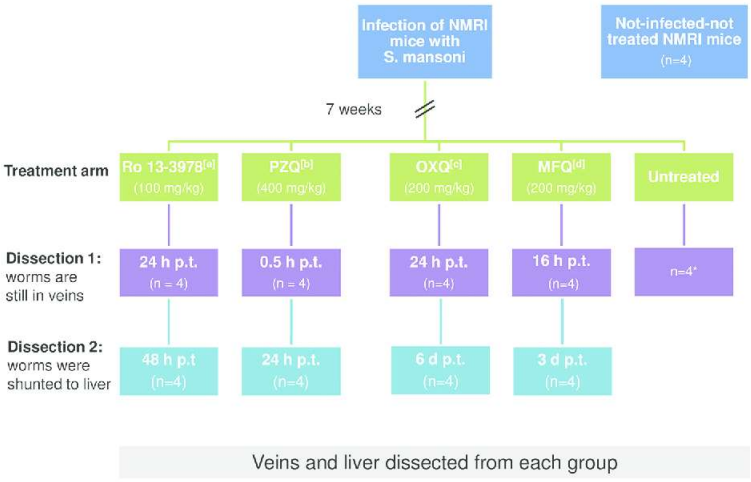

FIG 1 Study flow. Infected mice were allocated to a treatment arm and subdivided into two dissection time points, namely, either before the occurrence of hepatic shift (when worms are still in the veins) or after hepatic shift (when worms are in the liver). Effective doses and time spans of the hepatic shifts were determined in previous publications. [a], reference 12; [b], reference 44; [c], reference 18; [d], reference 45; *, untreated mice have only one dissection time point because without treatment, the worms stay in the veins.

and comparing the histology to that from the vein and liver sections of untreated mice (infected and not infected). The design, as well as drug-specific effective doses and onsets of action, is presented in Fig. 1. All vein and liver sections were examined for each group at each dissection time point. However, changes in immune cell populations of treated mice compared to those from control mice were observable only in tissues where the schistosomes were present, i.e., in veins only at dissection 1 and in the liver only at dissection 2 (with the exception of two samples where a single worm had already been shunted to the liver at dissection 1 [examined in Discussion]). Therefore, below, we present the findings first for untreated mice and then the relevant tissue sections for each time point for the treated mice.

Untreated mice. In mesenteric vein sections of untreated infected mice, no particular immune responses were visibly triggered by *S. mansoni* (Fig. 2). A few macrophages were present in the adipose tissue of the mesenteries (Fig. 2A). Circulating B cells (Fig. 2C) but no circulating neutrophils or T cells were detectable (Fig. 2B and D). In uninfected untreated mice, the immune cell populations were similar to those of infected mice in the veins, with the presence of some resident tissue macrophages and B cells circulating in the blood (see Fig. S1 in the supplemental material).

In the liver, eggs triggered eosinophilia and the recruitment of B cells, T cells, and especially, macrophages, both to the granuloma and to peripheries of the hepatic veins, by which the eggs get shunted to the liver (Fig. 3). Neutrophils (Fig. 3C) were generally absent except in small clusters around granulomas (not shown). However, in contrast to those of infected mice, the liver sections of uninfected mice showed only minimal evenly dispersed populations of B cells, T cells, and macrophages (Kupffer cells) and only a trace amount of neutrophils (see Fig. S2). No accumulations of cells could be observed.

Effect of Ro 13-3978 treatment. Twenty-four hours after treatment with 100 mg/kg Ro 13-3978, there was significant recruitment of macrophages to the tissue areas surrounding the worms in the mesenteric veins, though macrophage attachment to the worm tegument was not observed (Fig. 4B). There was no increase in circulating neutrophils (Fig. 4C); however, the few that were present appeared to attach to the worms' teguments (not shown), which was not seen in untreated mice. In addition,

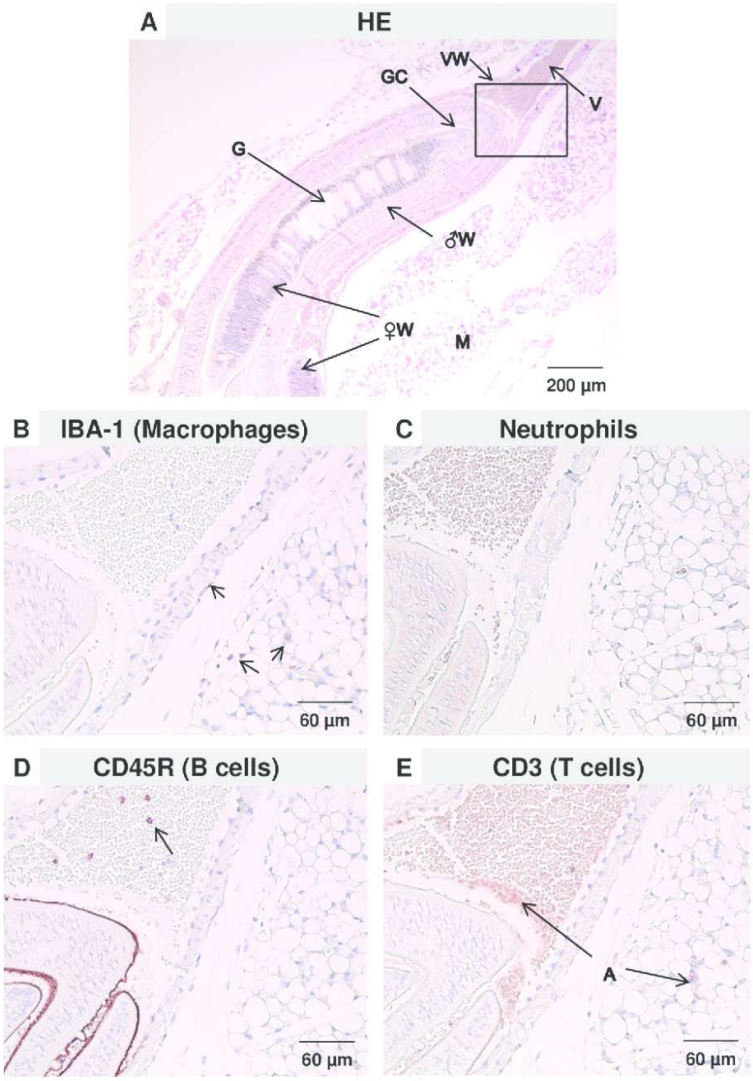

FIG 2 Presence of worms in the veins did not elicit immune cell recruitment in an untreated mouse. (A) Hematoxylin and eosin (HE) staining highlights the structure of the worm pair (the female is surrounded by the male) inside the vein. (B) IBA-1 staining for macrophages showed no recruitment except for a few resident tissue macrophages. (C) No neutrophils were visible. (D) CD45 staining showed some circulating B cells. (E) CD3 staining showed no T cells. Abbreviations: A, artifact; G, worm gut; GC, gynecophoral canal; M, mesenteries; V, vein; VW, vein wall; ♀W, female worm; ♂W, male worm.

some infiltration and attachment of B cells was observed (Fig. 4D), whereas T cells were not appreciably increased (Fig. 4E).

At 48 h posttreatment, all worms had been shunted to the liver. Inside the liver, the immune reaction appeared to follow a pattern similar to those seen in livers of untreated mice, though the numbers for each cell type were increased. The worms had already been surrounded by a dense cluster of immune cells, namely, eosinophils (Fig. 5A), macrophages, (Fig. 5B), neutrophils (Fig. 5C), B cells (Fig. 5D), and T cells (Fig. 5E). Moreover, the attachment of B cells to the worm itself was visible (Fig. 5D).

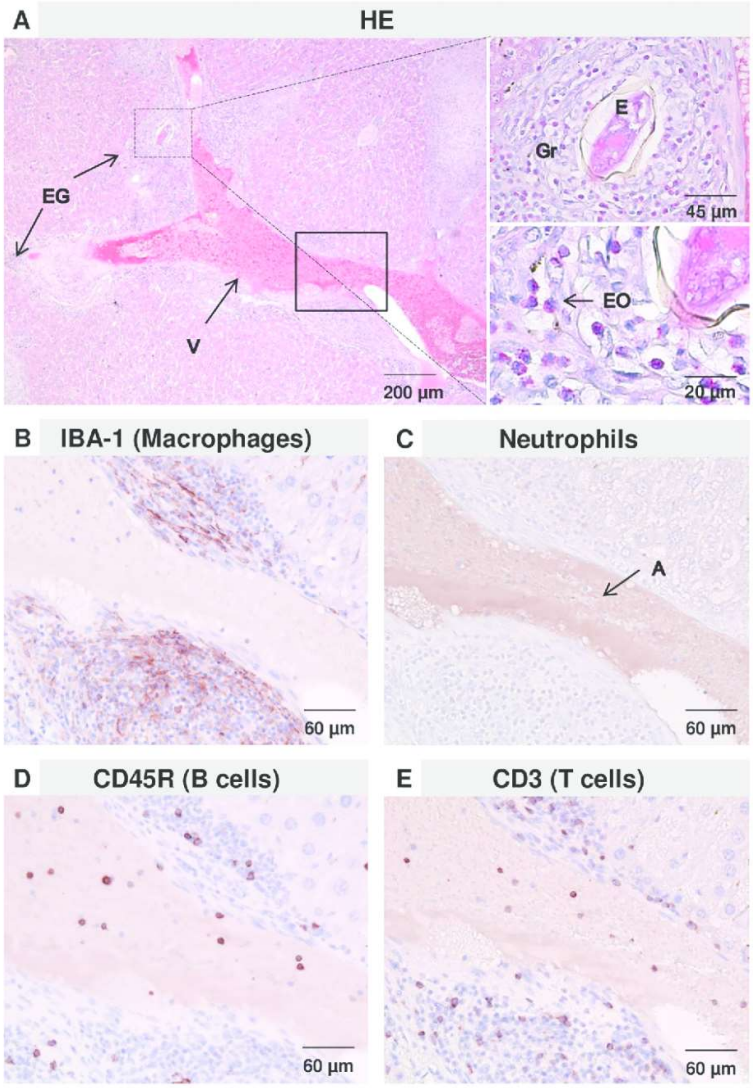

FIG 3 Infected but untreated mice displayed significant inflammation in the liver. (A) Hematoxylin and eosin (HE) staining illustrates the liver, liver vessel, and egg granuloma structures, with strong eosinophilia around the eggs. (B) Macrophages are visible along the vein wall as well as around granulomas. (C) Some circulating neutrophils were present (not show in this section). Circulating B cells (D) were slightly more numerous and recruited to egg granulomas as well, as were T cells (E). Abbreviations: A, artifact; E, egg; Gr, granuloma; EG, egg granuloma; EO, eosinophils.

Comparison to treatment with praziquantel, oxamniquine, or mefloquine. Immune responses to treatment differed between the comparator drugs in both the mesenteric veins (Fig. 6) and the liver (Fig. 7). In the veins, at 0.5 h posttreatment with 400 mg/kg praziquantel, significant macrophage recruitment to the site of the worm was visible (Fig. 6A2). Despite the strong macrophage presence at the vein walls, very few penetrated the vein wall, and no attachment to the worm was detected. This was also observable with a treatment of 200 mg/kg oxamniquine (24 h posttreatment) (Fig. 6B2). For oxamniquine, no circulating or infiltrating neutrophils were noted;

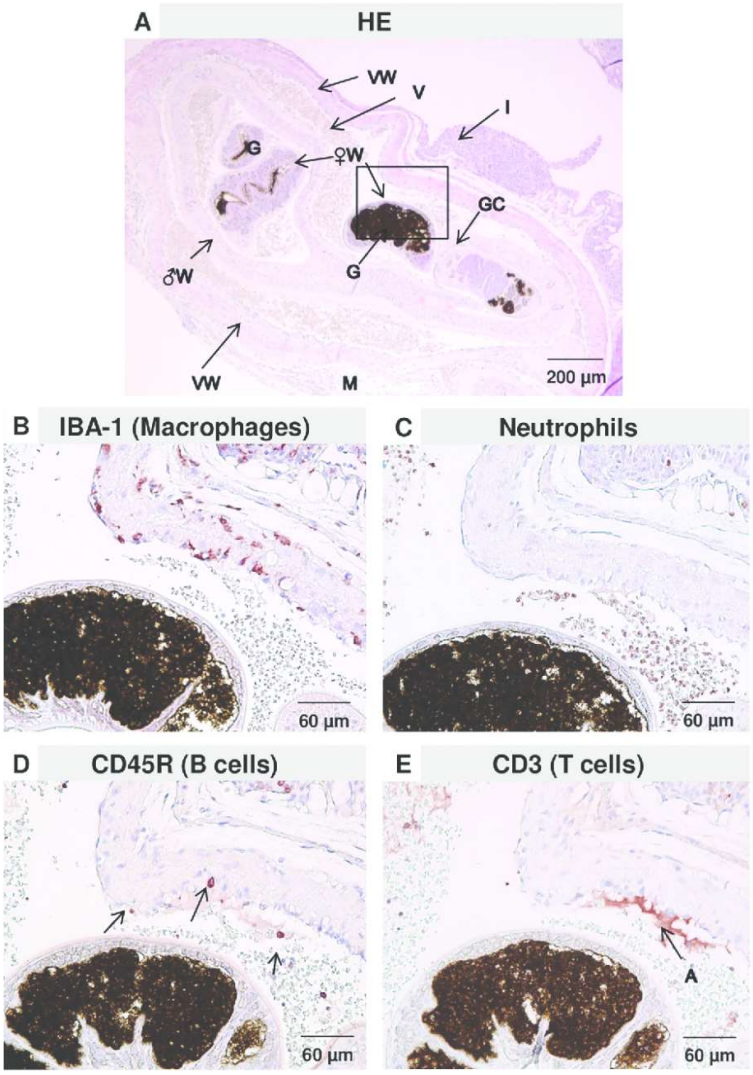

FIG 4 Treatment with Ro 13-3978 caused significant macrophage and some B cell recruitment to the worms in the veins 24 h after treatment. (A) HE staining shows a worm in the vein. (B) Macrophages were recruited to the vein walls surrounding the worm. (C) Circulating neutrophils were not increased but some were attached to the worm tegument (not shown). (D) B cell circulation was slightly increased, with some B cells attached to the worm tegument and the vein wall. (E) T cell staining, even when overstained, did not reveal appreciable T cell recruitment. Abbreviations: A, artifact; G, worm gut; GC, gynecophoral canal; I, host intestinal wall; M, mesenteries; V, vein; VW, vein wall; ♀W, female worm; ♂W, male worm.

however, some B cells and some T cells were visible that infiltrated through the mesenteric adipose tissue (Fig. 6B4 to B5). Treatment with 200 mg/kg mefloquine did not elicit strong immune responses to the worm, except for a modest recruitment of macrophages and B cells (Fig. 6C2 and 6C4, respectively). Staining for neutrophils or T cells showed no infiltration of these cells to the site of infection (Fig. 6C3 and 6C5, respectively).

At the second dissection point, in the liver, multiple immune cell populations were responsive, though at various degrees (Fig. 7). At 24 h after treatment with praziqu-

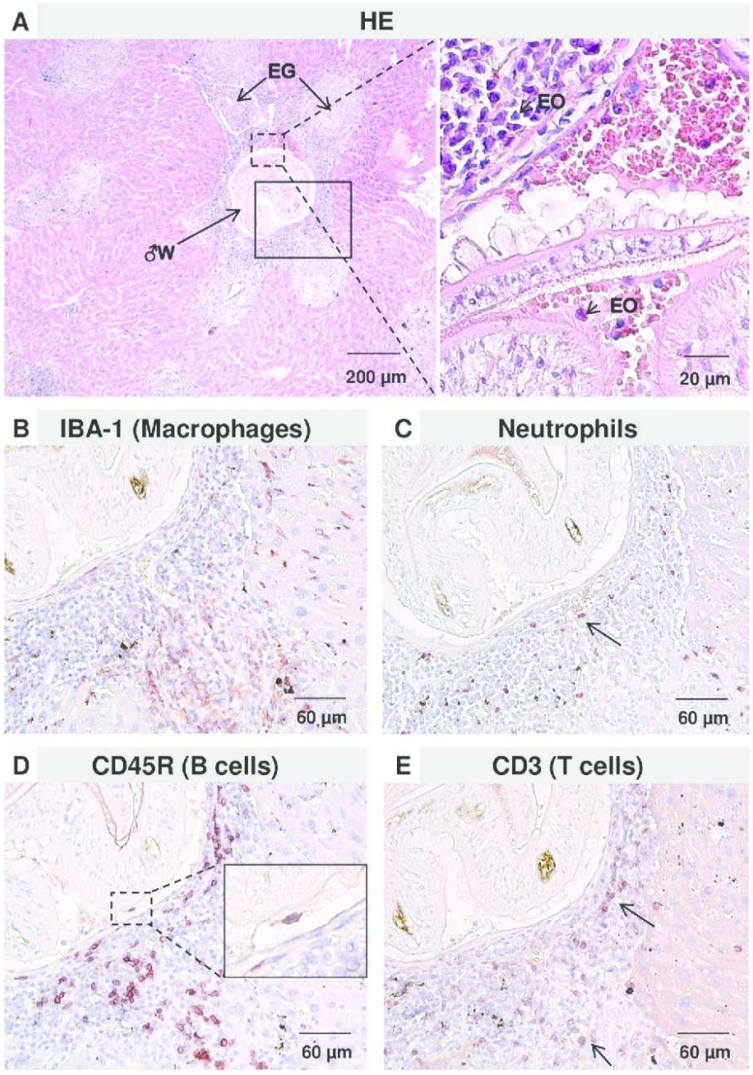

FIG 5 Worms are shifted to the liver 48 h after treatment with Ro 13-3978, where phagocyte and leukocyte levels were elevated. (A) HE staining revealed a dense cluster of immune cells surrounding the worm, including eosinophils. (B) Most macrophages were not attached to the worm, but macrophage levels in the liver section were higher than for the untreated controls (see Fig. S2A in the supplemental material). (C) Slightly elevated levels of neutrophils in the liver tissue were notable, but there was not a large amount surrounding the worm. (D) B cell recruitment to the vessel and attachment to the worm tegument were visible. (E) T cell levels were generally slightly elevated but mostly present in egg granulomas. Abbreviations: EG, egg granuloma; EO, eosinophils; ♂W, male worm.

antel, many macrophages had been recruited to the site of the worm, and the numbers of macrophages in the liver were highly elevated (Fig. 7A2). Neutrophils were not numerous, but some attachment to the worm tegument was visible (Fig. 7A3). B cell and T cell recruitment to the vessel containing the worm were apparent (Fig. 7A4 and A5) as was some attachment of the cells to the worm (not shown).

At the time point of hepatic shift for oxamniquine treatment, 6 d after treatment, macrophages were highly present and were distributed throughout the liver tissue and

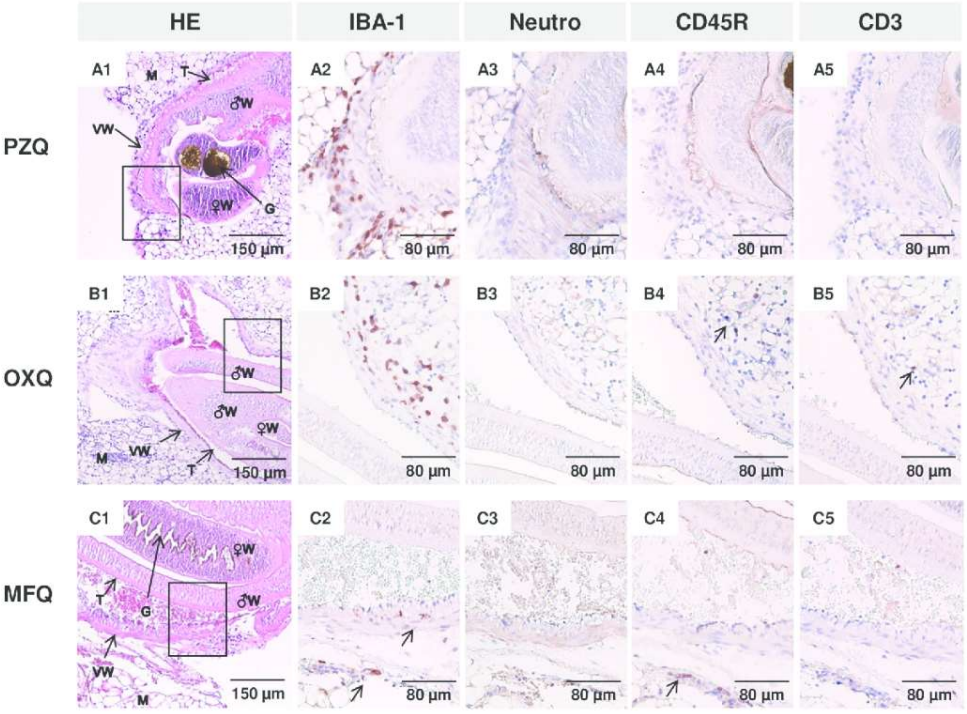

FIG 6 Treatment with praziquantel and oxamniquine resulted in a significant recruitment of macrophages to the sites of the worms, while mefloquine induced a milder response. Worms in veins after treatment with 400 mg/kg praziquantel, 200 mg/kg oxamniquine (B), or 200 mg/kg mefloquine (C). PZQ, praziquantel; OXQ, oxamniquine; MFQ, mefloquine; G, gut; M, mesenteries; VW, vein wall; ♀W, female worm; ♂W, male worm.

the fibrotic tissue surrounding the worm (Fig. 7B2). B and T cell levels were moderately elevated compared to those from infected untreated control mice (Fig. 7B4 and B5). Neutrophils did not appear to be highly present around intact worms; however, worms that were disintegrating were predominantly surrounded with this cell type (Fig. 7B3; see also Fig. S3).

At 3 d posttreatment, all worms treated with 200 mg/kg mefloquine were shunted to the liver. The liver sections demonstrate only modestly elevated numbers of macrophages, neutrophils, B cells, and T cells close to vein vessels containing a shunted worm (Fig. 7C). However, as with the oxamniquine-treated mice, the samples also contained disintegrating worms with larger entourages of each cell type, especially, those with elevated neutrophilic responses (Fig. S4).

DISCUSSION

Long-term posttreatment immunomodulatory effects of antischistosomal drugs have been described, yet very little is understood about what happens as the adult worm is killed and removed, especially, at the local level (16, 24, 26, 27). The potent but slow-onset *in vivo* activity of Ro 13-3978, which stands in contrast to its poor *in vitro* activity, prompted us to investigate potential immunomodulatory effects of this drug by looking at general immune cell populations surrounding the worm using histopathology techniques. To elucidate if such effects were drug specific or a general response to worm death, we also probed the immunomodulatory activities of praziquantel, oxamniquine, and mefloquine. As these are also understudied, our work presents the first immunohistochemical examinations of the short-term local immune response to these drugs and to schistosome clearance in general.

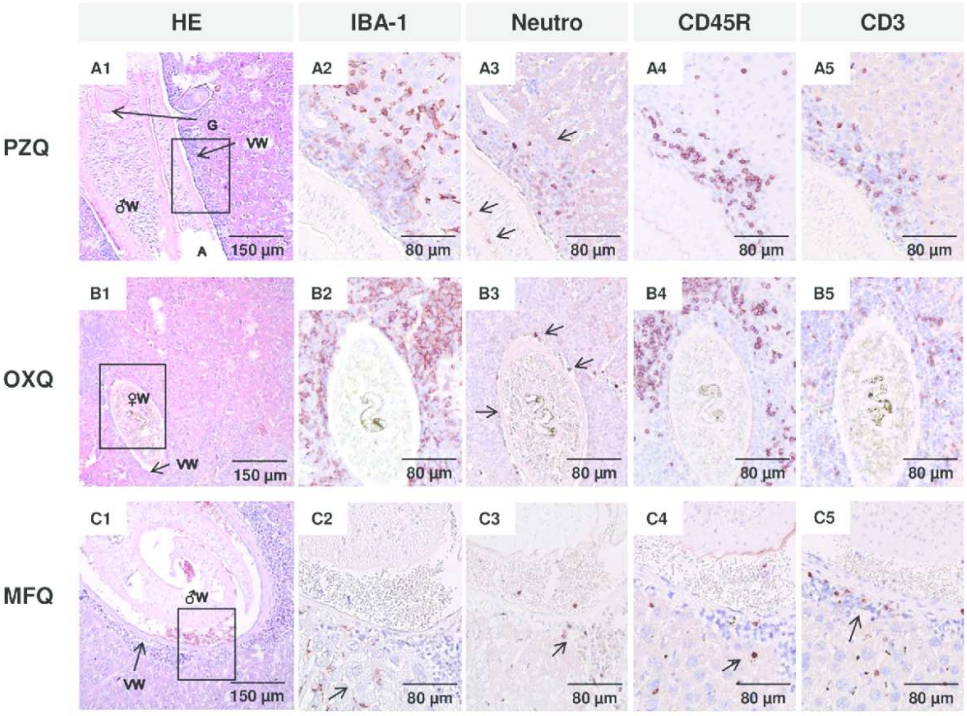

FIG 7 Worms that were shunted to the liver provoked responses to various degrees and cell types, depending on the treatment. Immune responses to worms after being shunted to the liver were strong in praziquantel- and oxamniquine-treated mice but weaker for those treated by mefloquine. High levels of macrophages were observed in the liver tissue and surrounding the worm, as well as significant recruitments of B and T cells, in samples from mice treated with praziquantel (A) or oxamniquine (B). Immune response to mefloquine treatment was more tempered. An elevated presence of B and T cells at the worm site was also observed. PZQ, praziquantel; OXQ, oxamniquine; MFQ, mefloquine; A, artifact; G, gut; VW, vein wall; ♀W, female worm; ♂W, male worm.

We first examined histological vein and liver sections from infected untreated mice to serve as a reference point. In infected untreated mice, little to no immune cell recruitment to the adult worm in the mesenteric veins was observed, which is in stark contrast to the strong immune responses that the eggs elicit in the liver. This is in line with previous hematoxylin and eosin staining of histological examinations of intravascular worms (28), but is now confirmed by immunohistochemistry. Adult *S. mansoni* employs a variety of mechanisms to evade the hostile vascular humoral environment. These include blocking and neutralizing complement components C1q, C2, C3, C8, or C9, releasing decay-accelerating factor (DAF)-like molecules that accelerate the dissociation of C3 convertases, and producing large quantities of antioxidant proteins (superoxide dismutases, glutathione peroxidases, and peroxiredoxins) that protect against oxidative attack from monocytes and leukocytes (29–31). These complex immune evasion mechanisms are likely one reason why it is difficult to acquire immunity against this pathogen (32).

Generally, it was shown that similar cellular responses were induced regardless of treatment but to greatly variable degrees. Comparative levels of each cell type for each dissection time point (and sample type) are summarized in Table 1.

In the veins (dissection 1), treatment with Ro 13-3978 predominantly induced a significant recruitment of macrophages to the site of infection. However, this was also observed after treatment with praziquantel, oxamniquine, or mefloquine at the first dissection time point, before the hepatic shift (at 0.5 h, 24 h, and 16 h posttreatment, respectively). Nonetheless, the quantities differed greatly, with praziquantel- and oxamniquine-treated mice displaying the most acute macrophage response, whereas

TABLE 1 Summary of immune cell proliferation and recruitment to the sites of infection after treatment

Time point[a]	Treatment	Observance of[b]: Macrophages	Neutrophils	B cells	T cells
Dissection 1					
24 h	Ro 13-3978	+++	−	+	−
0.5 h	Praziquantel	++++	−	+	−
24 h	Oxamniquine	++++	−	++	tr
16 h	Mefloquine	+	−	+	−
	Infected Untreated	tr	−	+	−
Dissection 2					
48 h	Ro 13-3978	+++	+	+++	+++
24 h	Praziquantel	++++	+	+++	+++
6 days	Oxamniquine	+++++	+	++++	+++
3 days	Mefloquine	++	+	++	++
	Infected Untreated	++	tr	++	++

[a]Posttreatment time points are for dissection 1, before the hepatic shift, when all worms are still in the veins, and for dissection 2, after the hepatic shift, when all worms have been shunted to the liver.
[b]Valuations refer to cell types surrounding the intact worms (not eggs or decayed worms). −, no cells present; tr, a few trace cells present in some slides; +, some cells recruited; ++, moderate cell recruitment; +++, significant cell recruitment; ++++, strong cell recruitment; +++++, very strong cell recruitment.

Ro 13-3978-treated mice showed a marked but moderate response and mefloquine-treated mice showed only a minor response.

The macrophage recruitment levels for praziquantel before hepatic shift are particularly striking, considering that the samples are from 0.5 h posttreatment with the drug. Praziquantel has been shown to act immediately *in vitro* and in a way that strongly damages the tegument and thus facilitates the attachment of antibodies to the worm tegument as soon as 1 h after treatment (18, 33). Therefore, it might be tempting to conclude that macrophage recruitment is simply proportional to the level of tegumental damage and therefore worm antigen release. Yet by this logic, mefloquine should have provoked a far stronger immune response than was observed in this study (Fig. 5). Mefloquine also acts within an hour *in vitro*, extensively damaging the tegument (34). Moreover, the *ex vivo* SEM studies of adult worms 24 h after treatment with 100 mg/kg Ro 13-3978 show there is a very meek morphological effect, especially, compared to those of *ex vivo* worms taken 24 h after treatment with 200 mg/kg mefloquine (12, 35). Yet, treatment with Ro 13-3978 elicited stronger macrophage recruitment than treatment with mefloquine. In addition, the action of oxamniquine treatment is usually slow, requiring a few days to act on the tegument, as observed from *ex vivo* worms (36). Nonetheless, macrophage recruitment to the worm was higher in mice treated with oxamniquine (Fig. 5) than in those treated with Ro 13-3978 and much higher than in those treated with mefloquine. Thus, other factors may be at play. The drugs could vary in how quickly they affect the above-described immune-evasion mechanisms or they may expose different antigens, which may be differently immunogenic, as is often demonstrated in the area of schistosome vaccinology (37). Finally, it is possible that the drugs themselves possess variable phagocyte stimulatory effects.

Responses from neutrophils, B cells, and T cells in the veins highly varied between treatments. B cell recruitment was notable for Ro 13-397-8 and oxamniquine-treated mice but less so for those treated with praziquantel and mefloquine. In the case of praziquantel, the dissection time point is likely too soon for B cell activation to be observable. Surprisingly, neutrophil recruitment to the worm was rarely observable, even though neutrophils are usually the "first responders" to an infection site. It is difficult to discern if this is due to *S. mansoni*-specific factors or if it is related to the infection model, as neutrophils are known to respond differently in mice than in humans (38). Meanwhile, T cells were rarely present in the veins before the hepatic shift (except in one vein sample from a mouse treated with mefloquine) and rather were recruited to the liver after the hepatic shift.

Immune cell responses after the hepatic shift (in the liver [dissection 2]), are more difficult to compare, as egg granulomas already elicit the recruitment of all cell types to the liver even in untreated infected mice. Nonetheless, it is apparent that while treatment using any of the drugs results in elevated immune cell populations in the liver compared to that in untreated infected mice, praziquantel and oxamniquine prompted the highest recruitment of macrophages. The posthepatic shift time point for oxamniquine, 6 days, is much longer than that for the other drugs, and the large immune cell amounts could rather be an artifact of the time point. Indeed, one of the four liver samples at 24 h posttreatment of oxamniquine (dissection 1) happened to contain a single worm that had already been shunted to the liver; the immune cell recruitment in this sample was more moderate (Fig. S5 in the supplemental material), resembling that in the liver sections from Ro 13-3978-treated mice. However, the liver samples from praziquantel-treated mice were taken at a time point that was earlier than for other treatments (24 h posttreatment compared to 48 and 72 h posttreatment for Ro 13-3978 and mefloquine, respectively), and nonetheless showed much more elevated macrophage levels. Moreover, one of the four dissection 1 time point (0.5 h posttreatment) liver samples from praziquantel-treated mice already contained a shunted worm, around which a strong macrophage presence was also already evident (Fig. S6). This macrophage response after praziquantel treatment, evident in both the vein and liver samples, may be indicative of the direct immunostimulatory effects of praziquantel on macrophages and warrants further investigation.

In the liver samples from mice treated with oxamniquine and mefloquine, it was possible to observe immune responses to disintegrating and intact worms in the same sections (Fig. S3 and S4, respectively). This might be because of the slower onset of action of these drugs, where the shunting of schistosomes to the liver occurs gradually over this period. The disintegrating schistosomes were thus likely already shifted to the liver earlier on, whereas shift of the intact ones might have occurred closer to the dissection 2 time point. There was a distinctly different neutrophil response to a decaying schistosome compared to the response to intact worms (already described above), regardless of treatment, with a dense neutrophil cluster surrounding the decaying parasite. Whether the function of these neutrophils is to clear the extracellular parasite (39), facilitate wound healing (40), or stimulate or dampen adaptive immune responses (41, 42) is not known and may be worth investigating further to elucidate the general mechanisms of parasite clearance and possibly eventual acquired immunity.

In conclusion, we have described and presented the general localized host responses to treatment of a chronic *S. mansoni* infection in the rodent model with a panel of drugs. Taken together, our results suggest that though each drug treatment elicits an immune response, variables beyond the onset of action and quantity of antigens exposed are associated with the type and strength of the immune response after drug treatment. The treatment of *S. mansoni*-infected mice with Ro 13-3978 does lead to phagocytic immune responses, but the effect is milder than what is observed for praziquantel and oxamniquine. Hence, the activity of Ro 13-3978 is likely not due (at least solely) to immune-assisted clearance. Nonetheless, to characterize more precisely the time sequence in which responses are mounted and to compare drug treatments more directly, further *in vivo* studies using quantitative techniques, such as fluorescence-activated cell sorting (FACS), and those using *in vitro* cell activation cultures are required.

MATERIALS AND METHODS

***S. mansoni* mouse model.** Female outbred NMRI mice were obtained and infected with *S. mansoni* cercariae as previously described (43). Briefly, mice (weight, ~20 to 22 g) were purchased from Charles River, Germany, and were allowed to acclimatize to in-house conditions (temperature, ~25°C; humidity, ~70%; 12-hour light and 12-hour dark cycle). Cercariae of *S. mansoni* were obtained from infected *Biomphalaria glabrata* snails by placing them under light for a few hours and collecting the cercarial suspension. Mice were infected subcutaneously with ~100 cercariae and allowed 7 weeks to develop an adult-stage infection.

TABLE 2 Antibodies used for immunohistochemistry

Antibody characteristic	Macrophages	Neutrophils	B cells	T cells
Primary	IBA-1	Neutro	CD45R	CD3
Clone	Polyclonal	NIMP-R14	RA3-6B2	CD3-12
Company and catalog no.	Wako, 019-19741	Abcam, ab2557	Bio-Rad, MCA1258G	Bio-Rad, MCA1477
Host species	Rabbit	Rat	Rat	Rat
Dilution	1:2,000	0.18056	1:2,000	0.18056
Antigen retrieval (pretreatment)	Citrate buffer (10 mM)	None	Borate buffer (20 mM)	Borate buffer (20 mM)
Blocking solution	1.5% goat serum (S-1000; Vector Laboratories)	Dako diluent (S2022; Agilent)	Dako diluent (S2022; Agilent)	Dako diluent (S2022; Agilent)
Secondary	Biotinylated goat anti-rabbit IgG (1:200) (BA-1000; Vector Laboratories)	Histofine (no. 414311F; Biosystems Inc.)	Histofine (no. 414311F; Biosystems Inc.)	Histofine (no. 414311F; Biosystems Inc.)

Treatment and dissection. Seven weeks postinfection, mice ($n = 8$) were allocated to 4 treatment arms receiving the effective doses of either Ro 13-3978 (100 mg/kg; kindly prepared by Jonathan Vennerstrom), praziquantel (400 mg/kg; Sigma-Aldrich), oxamniquine (200 mg/kg; donated by Quentin Bickle), or mefloquine (200 mg/kg; Sigma-Aldrich). All drugs were dissolved in a 70:30 Tween-ethanol (EtOH) mixture dissolved in distilled water (dH_2O [10%]) right before use and were administered orally by gavage. The treatment arms were further subdivided into two dissection time points, namely, dissection 1, closely before the hepatic shift, when the worms are still in the veins, and dissection 2, after all worms are shunted to the liver. The onset of hepatic shift was previously determined and is specific for each drug (denoted and cited in Fig. 1). In addition, 4 mice were infected but not treated and 4 mice were not infected and not treated to serve as controls.

Immediately after euthanizing the mice, the livers and intestinal apparatuses from all groups were extracted. Using a dissecting microscope, sections of the mesenteric veins with or without worms inside were excised.

Histopathological analysis. The livers and vein sections were immediately placed in 50- or 15-ml BD Falcon tubes, respectively, filled with 10% neutral buffered formalin solution (4% formaldehyde; Sigma, Switzerland) for 24 h, after which they were stored at 4°C in 70% EtOH until use. Samples were embedded in paraffin, cut into 5-μm-thin sections with an HM 335 E rotary microtome (Microm International GmbH), retrieved on Superfrost Plus slides (Thermo Scientific, Germany), and allowed to dry overnight. All samples were first cut and stained with hematoxylin (Sigma) and eosin (Biosystems, Switzerland) until at least one worm per sample ($n = 4$ samples) was located.

For the staining, slides were deparaffinized and rehydrated by successive immersions in UltraClear (Biosystems), 100%, 95%, 90%, and 70% EtOH, and ddH_2O. Immunohistochemical staining was undertaken to identify general immune cell populations. The primary antibodies used and their specifics are summarized in Table 2.

The protocols differed slightly depending on the antibody used. Slides were pretreated (except those for neutrophil staining) (Table 2) and internal peroxidases were blocked with 3% H_2O_2 in phosphate-buffered saline (PBS) for 20 min. IBA-1 slides were additionally blocked with 1.5% goat serum in PBS thereafter. Slides were then incubated for 1 h with the primary antibody diluted in antibody diluent (Dako), except for the IBA-1 slides, where the antibody was diluted in PBS with 0.1% Tween. Afterwards, Histofine (Biosystems), or goat anti-rabbit IgG for the IBA-1 slides, was applied for 30 min. IBA-1 slides were additionally incubated with an avidin-biotin-peroxidase conjugate (ABC kit) for 30 min. The final development for all slides was done using the Vector NovaRed (Vector Laboratories) kit according to the manufacturer's protocol. Meyer's hematoxylin (Sigma) was used as a counterstain. The slides were then left to dry, mounted (Eukitt mounting medium; Sigma, Switzerland), and coverslipped (Menzel-Gläser coverslips; Thermo Fisher), and images were taken using a Leica DM5000B microscope.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01142-17.

SUPPLEMENTAL FILE 1, PDF file, 5.2 MB.

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Chapter 4c

Novel organometallic oxamniquine derivatives are active against *Schistosoma mansoni in vitro* and *in vivo*

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Ferrocenyl, Ruthenocenyl, and Benzyl Oxamniquine Derivatives with Cross-Species Activity against *Schistosoma mansoni* and *Schistosoma haematobium*

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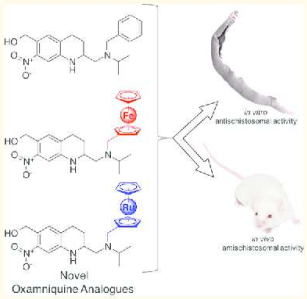
Supporting Information

ABSTRACT: Schistosomiasis is a parasitic disease that affects more than 250 million people annually, mostly children in poor, tropical, rural areas. Only one treatment (praziquantel) is available, putting control efforts at risk should resistance occur. In pursuit of treatment alternatives, we derivatized an old antischistosomal agent, oxamniquine (OXA). Four organometallic derivatives of OXA were synthesized and tested against *Schistosoma mansoni* in vitro and in vivo. Of these, a ferrocenyl derivative, **1**, killed larval and adult worms 24 h postexposure in vitro, in contrast to OXA, which lacks in vitro activity against adult worms. A dose of 200 mg/kg of **1** completely eliminated the worm burden in mice. Subsequently, a ruthenocenyl (**5**) and a benzyl derivative (**6**) of OXA were synthesized to probe the importance of the ferrocenyl group in **1**. Compounds **1**, **5**, and **6** were lethal to both *S. mansoni* and *S. haematobium* adults in vitro. In vivo, at 100 mg/kg, all three compounds revealed *S. mansoni* worm burden reductions of 76 to 93%, commensurate with OXA. Our findings present three compounds with activity against *S. mansoni* in vitro, comparable activity in vivo, and high activity against *S. haematobium* in vitro. These compounds may possess a different binding mode or mode of action compared to OXA and present excellent starting points for further SAR studies.



KEYWORDS: *anthelmintics; bioorganometallic chemistry; medicinal organometallic chemistry; oxamniquine; schistosomiasis*

Schistosomiasis, a neglected tropical disease, has been affecting humans since Ancient Egyptian times.[1] It is caused by skin penetration of aquatic cercarial forms of any of the six *Schistosoma* species: *S. mansoni, S. haematobium, S. japonicum, S. mekongi, S. intercalatum* and *S. guineensis*, with the former three being the most common ones. The acute form of schistosomiasis is manifested as Katayama fever, characterized by symptoms of high fever, coughing, swimmers itch, and malaise.[2] Left untreated, the chronic form of the disease gradually becomes apparent. Inflammatory responses from worm eggs that become lodged in visceral organs result in extensive tissue fibrosis and gradual swelling and decline of organ functions. Patients can also experience hypertension, anemia, blood shunting, and cognitive impairments.[3] Today, schistosomiasis affects more than 250 million people worldwide every year, mostly children and predominantly in impoverished rural areas of the tropics and subtropics.[4,5] The current WHO control strategy is and continues to be morbidity reduction using preventative chemotherapy in the affected populations.[6] The sole drug used is praziquantel, which is safe and effective. However, sporadic reports of treatment failure and the reliance on a single drug render the control strategy vulnerable to drug resistance.[7]

In parallel with praziquantel, another potent antischistosomal drug, oxamniquine (OXA), was introduced in 1972. OXA was shown to eliminate worm burden in animals and patients; however, it is effective only against *S. mansoni* and not against the other species that cause human schistosomiasis. OXA was used as the drug of choice to control schistosomiasis in Brazil until the late 1990s.[8] Unfortunately, resistant parasites were already isolated from patients soon after its introduction, providing the first clear evidence of resistance of a human helminth parasite to this drug.[9] Since then, studies have aimed to find the mode of resistance, targets, and mechanism of action of OXA. Research lead by Pica-Mattoccia, Cioli, and colleagues has demonstrated that OXA is a prodrug, which binds to a 30 kDa endogenous *S. mansoni*-specific sulfotransferase (SmSULT). In the presence of the sulfotransferase's cofactor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), a sulfate

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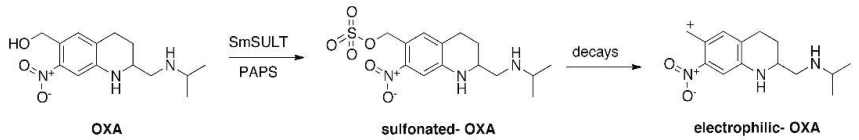
Scheme 1. Proposed Mechanism of Action of Oxamniquine (OXA)[a]



[a]SmSULT is *S. mansoni*-specific sulfotransferase; PAPS is 3′-phosphoadenosine-5′-phosphosulfate.[11]

group is transferred to the hydroxyl group of OXA, which results in an unstable product that decays into an alkylating electrophile with affinity for DNA, proteins, and other macromolecules (Scheme 1).[10]

More recently, Valentim, Cioli, and colleagues were able to pinpoint the genetic locus as well as crystallize OXA bound to the target sulfotransferase along with PAPS, providing not only the genetic basis for resistance and species specificity but also insight into the exact structure−function relationship of OXA.[11] Comparison of the species-specific *S. mansoni* versus *S. haematobium* sulfotransferases revealed the 16 amino acid residues that interact with OXA. Only three amino acid residues differed between the two species, and of these, only one was considered to have an impact on the binding because it is assumed to change the polarity and the size of the active site.[11]

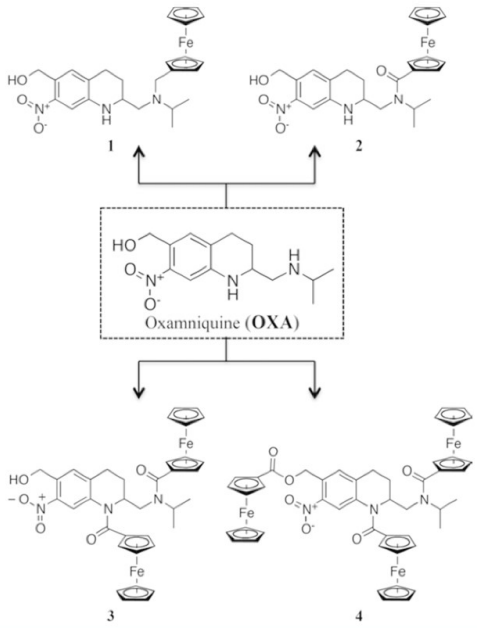
■ RESULTS AND DISCUSSION

With this perspective, our groups have initiated a program to derivatize OXA via the conjugation of organometallic moieties with the aim of overcoming the species-specific activity and thus eventually also resistance. Recently, such a strategy was proven to be very successful in the development of other antiparasitic drug candidates. Metal-based derivatization of organic anthelmintic drug with an organometallic moiety was able to modulate the activity profile of the parent drug.[12−14] The introduction of a ferrocenyl unit into a drug can allow for additional redox properties that can result in compounds with improved toxicity and an improved activity profile.[15−17] With the attachment of a ferrocenyl moiety to an organic drug, the ADMET (adsorption, distribution, metabolism, excretion, and toxicity) properties can be preferentially modulated, resulting in favorable physicochemical properties.

Initially, we designed four derivatives with up to three ferrocenyl units attached to the OXA core (Scheme 2). The exocyclic amine of OXA was *N*-alkylated with a ferrocenyl unit to obtain compound **1**. *N*-acylation instead of alkylation at the same position resulted in compound **2**, and double *N*-acylation of the OXA core afforded compound **3**. We were also eager to probe the importance of the hydroxyl group in **3** and converted it to a ferrocenyl ester (Scheme 2, compound **4**). We sought to elucidate if compounds **1**−**4** could lead to more activity and perhaps represent molecules that could overcome the species-specific action of OXA. The resulting compounds were tested on *S. mansoni* larval and adult stages in vitro as well as in an *S. mansoni*−mouse infection model.

All four ferrocenyl derivatives were prepared using one- or two-step procedures, and the detailed procedures are presented in the Methods and Supporting Information. All newly synthesized compounds (**1**−**4**) were unambiguously characterized by ^{1}H and ^{13}C NMR spectroscopy, IR spectroscopy, ESI mass spectrometry, and elemental analysis. For one of the

Scheme 2. Structures of Oxamniquine (OXA) and Four Ferrocenyl-Oxamniquine Derivatives (1−4)



compounds (**2**), the molecular structure was further confirmed by X-ray crystallography (Figure S1, Tables S1−S4).

With the four ferrocenyl-OXA derivatives (**1**−**4**) in hand, we tested their antischistosomal potency first against *S. mansoni* newly transformed schistosomula (NTS, larval stage) in an in vitro drug sensitivity assay at a drug concentration of 100 μM, where the average worm viability is microscopically assessed and compared to untreated controls. (See Methods for more details.) The results are presented in Table 1. Compound **1** killed all of the worms after 72 h (100% reduction in average worm viability as compared to nontreated worms). Exposure to compound **2** over 72 h resulted in a 76% reduction in worm viability. The other ferrocenyl OXA derivatives (**3** and **4**) showed little effect (less than a 30% reduction in worm viability). OXA showed a moderate effect (67% reduction in viability), which is consistent with previous in vitro studies on NTS.[18] Importantly, the results from an in vitro cytotoxicity assay using L6 rat cells revealed that the compounds are only mildly toxic against mammalian cells (Table 1).

We then evaluated the antischistosomal effect of **1**−**4** on adult worms at a concentration of 100 μM. The viability of the worms over a time period of 72 h in vitro is demonstrated in Figure 1. Although OXA did not have a significant effect on

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Table 1. Evaluation of the in Vitro Antischistosomal Activities of Compounds 1−4 and Oxamniquine (OXA) at a Concentration of 100 μM against Newly Transformed Schistosomula (NTS) to Test Their Activity[a]

compound	% *S. mansoni* NTS viability reduction (72 h)	L6 rat cell 72 h IC_{50} (μM)
1	100	77.6
2	76.2	57.9
3	28.6	100.3
4	19.1	98.3
OXA	66.7	≫90
control		PPT (0.01)

[a]Worms incubated in culture medium and 1% DMSO served as a control. Compounds were then also tested against L6 rat muscle skeletal cells.

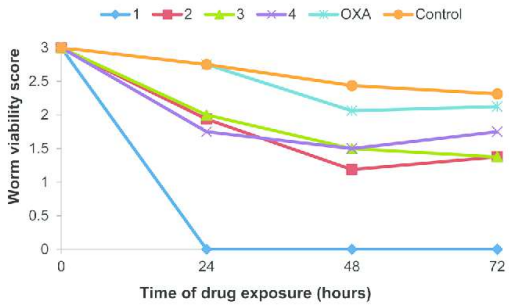


Figure 1. Viability of adult worms at 24, 48, and 72 h when incubated with the ferrocenyl-OXA derivatives (**1**−**4**) at a concentration of 100 μM, OXA, and culture medium with 1% DMSO (control).

worm viability even after 72 h, compounds **2**−**4** had mild effects, reducing the worm viability by 29.6, 27.3, and 36.4%, respectively, after 24 h and by 40.5, 40.5, and 24.3% after 72 h, respectively (as compared to nontreated control worms). Strikingly, compound **1** turned out to be extremely potent in the same assay and killed all of the worms 24 h after drug exposure. A comparison of activities between **1**, derivatives **2**−**4**, and OXA suggests that the attachment of a ferrocenyl unit at the exocyclic amine of OXA significantly improves the in vitro potency of the organic drug not only against *S. mansoni* NTS but also against adult *S. mansoni*.

The inactivity of OXA against adult worms over a 72 h incubation period in vitro is again consistent with previous findings.[18] OXA is active only in vivo or only after 12 days of exposure to a very high concentration of the drug in vitro.[11,19] The fast-acting in vitro activity of compound **1** against both the NTS and adult worms, therefore, differentiates it from OXA with regard to either its potency or its target.

However, to be considered a plausible drug candidate, the compound should demonstrate in vivo activity. Because OXA is itself active only in vivo, all four compounds (**1**−**4**) were tested in the mouse model. OXA was used as a positive control. The worm burden reductions are presented in Table 2. At an oral dose of 200 mg/kg, OXA eliminated all worms from the host, as did compound **1**. The other derivatives demonstrated low activity, with worm burden reductions (WBRs) of 4.3, 25, and 32% for **2**−**4**, respectively. Very importantly, the compounds did not cause any toxicity in mice.

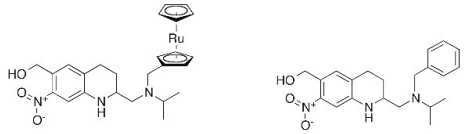
Table 2. Worm Burden and Worm Burden Reductions of *S. mansoni*-Infected Mice Treated with 200 mg/kg OXA and 1−4

		worm burden		worm burden reduction (%)	
compound	no. of mice	female	total	female	total
control batch 1	8	18.2	29.2		
control batch 2	8	13.4	23.0		
OXA[a]	4	0.0	0.0	100[d]	100[d]
1[a]	4	0.0	0.0	100[d]	100[d]
2[b]	3[c]	14.7	22.0		4.3
3[b]	4	11.0	17.3	17.8	25.0
4[a]	2[c]	13.5	20.0	25.9	31.6

[a]Worm burden reduction calculated on the basis of control batch 1. [b]Worm burden reduction calculated on the basis of control batch 2. [c]The initial group size was 4, but mice died prematurely. [d]Significantly different than the control worm burden ($p \leq 0.05$).

Putting the results together, it would appear that the formation of a tertiary amine increases the in vitro activity, whereas none of the amide compounds (**2**−**4**) were active. Because **1** was as active as OXA in the mouse model but much more active than OXA in vitro, we designed two additional analogues of **1** in order to probe the importance of its ferrocenyl unit. In the ruthenocenyl derivative (**5**), the Fe(II) center of **1** was replaced with a Ru(II) core to assess whether the iron-mediated redox properties are responsible for the antischistosomal activity.[15,16] A ruthenocenyl derivative was chosen because the Ru(II) center in ruthenocene exhibits completely different electrochemical behavior than Fe(II).[20−22] An organic analogue of **1**, benzyl derivative **6**, was prepared to probe the relevance of the metallocene unit of **1** and **5** for their activity (Scheme 3).

Scheme 3. Structures of the Ruthenocenyl-OXA Derivative (5) and the Benzyl-OXA Derivative (6)



5 **6**

The in vitro efficacy of **1**, **5**, and **6** was tested against adult worms using a drug dose−response assay in order to directly compare their IC_{50} values over time. As shown in Table 3 and Figure S2, compounds **1**, **5**, and **6** were comparably active, with IC_{50} values of 11.4, 8.7, and 11.1 μM at 72 h, respectively. A comparison of IC_{50} values against adult worms and against L6 rat cells suggests that the compounds show approximately 6−9-fold selectivity toward worms over mammalian cells (Table 3).

When tested in vivo at 100 mg/kg, the activity of compounds **1**, **5**, and **6** differed slightly (Table 4). All three compounds were very active, displaying total WBRs ranging from 76 to 99%, with OXA showing the highest activity (differences between compounds not significant). Again, no toxicity was observed. Hence, iron-mediated redox properties do not appear to drive the activity of **1**.

To determine if the promising activities of compounds **1**, **5**, and **6** are due to the compounds themselves and not by-products, we investigated their stabilities first in [d^6]-DMSO

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Table 3. Comparative IC_{50} Values of Compounds 1, 5, and 6 against *S. mansoni* Adult Worms and against L6 Rat Cell Skeletal Cells

compound	adult worm 24 h IC_{50} (μM)	adult worm 72 h IC_{50} (μM)	L6 rat cell 72 h IC_{50} (μM)	selectivity index[a,b]
1	37.2	11.4	77.6	6.8
5	33.8	8.7	75.7	8.7
6	24.4	11.1	73.8	6.6
OXA	inactive	inactive	≫90	not applicable
control	not applicable	not applicable	PPT (0.03)	not applicable

[a]Calculated on the basis of 72 h values for L6 cells vs adult worms. [b]The selectivity index is like an in vitro measure of the therapeutic window. There is no agreed upon threshold, but previously an SI > 1 for oral anthelmintics has been used.[23,24]

Table 4. Worm Burden and Worm Burden Reductions of *S. mansoni*-Infected Mice Treated with 100 mg/kg OXA, 1, 5, and 6

		worm burden		worm burden reduction (%)	
compound	no. of mice	female	total	female	total
control batch 1	8	11.1	19.9		
control batch 2	8	14.2	30.2		
OXA[a]	4	0.3	0.3	98.2[c]	99.2[c]
1[a]	4	4.5	5.8	68.4	81.0[c]
5[b]	4	1.0	1.3	91.0[c]	93.7[c]
6[b]	4	3.5	4.8	68.5	76.1[c]

[a]Worm burden reduction calculated on the basis of control batch 1. [b]Worm burden reduction calculated on the basis of control batch 2. [c]Significantly different than the control worm burden ($p \leq 0.05$).

using NMR spectroscopy and then in the adult worm culture medium (RPMI buffer, 5% FCS, 1% penicillin/streptomycin mixture) using UPLC-ESI-MS analysis. All three derivatives (**1**, **5**, and **6**) were found to be stable in [d^6]-DMSO for up to 72 h (Supporting Information, Figures S3–S5). In the culture medium, **1** and **6** decayed slightly but remained mostly stable, with 88 and ~100% of the compound remaining after 24 h and 73 and 79% remaining after 72 h (Figure 2).

Compound **5** was less stable, with only 42% remaining at 24 h. A more detailed analysis shows that >76% of **5** is intact over 9 h, after which the decay progresses (Figure S6). However, **1**, **5**, and **6** exert their activity on adult worms 4 h after exposure (Figure S7), long before these compounds start to hydrolyze.

Nonetheless, the gradual decay of **5** prompted a more detailed examination of the derivative breakdown (See the Supporting Information for UV traces in Figure S8–S11). Using UPLC-ESI-MS, we were able to identify that **1** and **5** were hydrolyzed in adult worm culture to OXA and ferrocenylmethanol (**7**) or ruthenocenylmethanol (**8**), respectively (Figures S16 and S17). However, in vitro tests against adult worms show that these breakdown products were not very active. That is, **7** and OXA were not at all active, and **8** showed moderate activity at 100 μM, but with much higher IC_{50} values than for its parent compound **5** (Figure S18). With this result, together with the early onset of action of **1**, **5**, and **6**, it can therefore be inferred that their potent in vitro and in vivo activity arises from their intact forms.

The activity of OXA against only a single species of *Schistosoma* (*S. mansoni*) is limiting in its utility in global treatment control programs. We thus tested if **1**, **5**, and **6** could also be active against other *Schistosoma* spp. and hence overcome the species-specific activity of OXA. An in vitro drug sensitivity assay against adult *S. haematobium* worms was

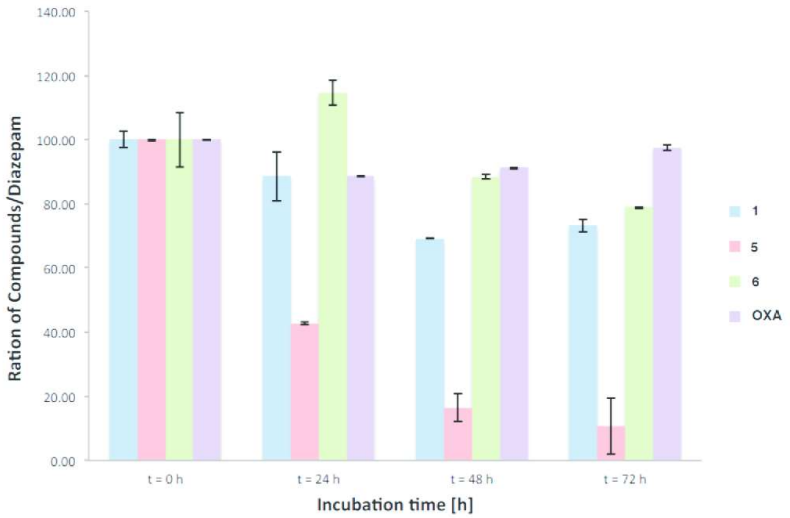

Figure 2. Ratio [%] of **1**, **5**, **6**, and oxamniquine to diazepam (internal standard) at 0, 24, 48, and 72 h in supplemented RPMI medium (5% FCS, 1% penicillin/streptomycin mixture).

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carried out. At 100 μM drug concentration, all three compounds reduced worm viability by 75% after 4 h of exposure and completely killed all worms after 48 h of exposure (Figure S20). OXA had no effect. This is highly comparable to the compounds' activity profile against *S. mansoni* (Figure S8).

This work sought to design organometallic OXA derivatives that could potentially have a different binding mode with respect to *S. mansoni* sulfotransferase or a unique mechanism of action due to the attached organometallic portions. Here we present three OXA derivatives that demonstrate in vivo activity and tolerability in the range of OXA itself and much more potent in vitro activity. Moreover, we present OXA derivatives that, for the first time, extend their activity beyond *S. mansoni* and thus, unlike OXA, are also active against *S. haematobium*, the most prevalent *Schistosoma* species.[9] The observation that altering the groups attached to the secondary amine of OXA nonetheless result in nearly identical in vitro and similar in vivo activity spurs questions of how these groups might be interacting with their target. However, the rather immediate effects in vitro against both *S. mansoni* and *S. haematobium*, in contrast to OXA, could indeed hint at either a different binding mode to the same target as OXA or perhaps another mode of action altogether. Further studies would be warranted to better elucidate the mechanism of action of these derivatives and whether they have the potential to be applied against the full range of human-parasitizing *Schistosoma* spp., including OXA-resistant *S. mansoni*. Nonetheless, these compounds present excellent starting points for further SAR studies with organometallic OXA derivatives.

METHODS

Materials. All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or distilled using standard procedures.[25] All preparations were carried out using standard Schlenk techniques. Thin layer chromatography (TLC) was performed using silica gel 60 F-254 (Merck) plates with the detection of spots achieved by exposure to UV light. Column chromatography was performed using Silica gel 60 (0.040−0.063 mm mesh, Merck). Eluent mixtures are expressed as volume to volume (v/v) ratios. Ruthenocene[26] and chlorocarbonyl ferrocene[27] were synthesized according to literature procedures. Oxamniquine was kindly provided by Dr. Quentin Bickle from the London School of Hygiene and Tropical Medicine. All compounds were dissolved as 10 mM stock solutions in dimethyl sulfoxide (DMSO, purchased from Sigma-Aldrich, Buchs, Switzerland) and stored at −20 °C until use.

Culture medium components were obtained as follows: RPMI 1640 and Medium 199 were purchased from Gibco (Basel, Switzerland), fetal calf serum (FCS) was obtained from Connectorate AG (Dietikon, Switzerland), and L-glutamine and penicillin-streptomycin (10 000 units of penicillin and 10 mg/mL streptomycin) were purchased from Sigma-Aldrich (Buchs, Switzerland).

Instrumentation and Methods. ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents on Bruker AV2-401, AV-400, AV-500, and AV-501 at room temperature. The chemical shifts, δ, are reported in ppm (parts per million). The signals from the residual protons of deuterated solvent have been used as an internal reference.[28,29] The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad). ESI mass spectrometry was performed using a Bruker Esquire 6000 spectrometer. In the assignment of the mass spectra, the most intense peak is listed. Infrared spectra were recorded on PerkinElmer FTIR spectrometer using an ATR platform. Peak intensities are given as broad (b), very strong (vs), strong (s), medium (m), and weak (w). Elemental microanalyses were performed on a LecoCHNS-932 elemental analyzer.

X-ray Crystallography. Crystallographic data were collected at 183(2) K with Mo Kα radiation (λ = 0.7107 Å) that was graphite-monochromated on an Oxford Diffraction CCD Xcalibur system with a ruby detector. A suitable crystal was covered with oil (Infineum V8512, formerly known as Paratone N), placed on a nylon loop that is mounted in a CrystalCap magnetic sample mount (Hampton Research), and immediately transferred to the diffractometer. Program suite CrysAlis^{Pro} was used for data collection, multiscan absorption correction, and data reduction.[30] The structure was solved with direct methods using SIR97[31] and was refined by full-matrix least-squares methods on F^2 with SHELXL-2014.[32] A disorder at atom positions C(17) and C(28) was found in a ratio of 79:21, which leads to the generation of the other enantiomer. Because the space group is P-1 and is therefore centrosymmetric, both enantiomers are equally present in the crystal. CCDC 1528705 contains the supplementary crystallographic data for this article. This data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

NTS Drug Sensitivity Assay. Newly transformed schistosomula (NTS) were prepared as previously described.[24] In short, infected snails were placed under light for 2 to 3 h to induce cercarial shedding, after which the cercarial suspension was collected and cooled on ice for 30 min. Thereafter, the suspension was vigorously pipetted and vortex mixed twice with a 30 min incubation step in between, and then rinsed with HBSS to separate the tails. The resulting NTS were kept in a culture medium overnight (medium 199 supplemented with 5% FCS and 1% penicillin/streptomycin mixture) at 37 °C and 5% CO_2. To set up the assay, the drugs were diluted in a 96-well plate in a total volume of 250 μL culture medium and 100 NTS for a final drug concentration of 100 μM. Wells with a culture medium, 1% DMSO, and 100 NTS served as negative controls. All drugs were tested once in triplicate. The assay was incubated at 37 °C and 5% CO_2 for 72 h, after which the plate was evaluated microscopically using a viability scale from 0 to 3 where 3 denotes viable, motile NTS with no change to tegument and 0 denotes death of the worms.

Adult Drug Sensitivity Assay. Adult *S. mansoni* worms were obtained as described by Keiser et al.[33] All in vitro and in vivo studies were conducted at the Swiss TPH, Basel, and approved by the veterinary authorities of the Canton Basel-Stadt (permit no. 2070) based on Swiss cantonal and national regulations. Female 3-week-old NMRI mice were purchased from Charles River (Sulzfeld, Germany) and allowed to adapt under controlled conditions (temperature ca. 22 °C, humidity ca. 50%, 12 h light and dark cycle, free access to rodent diet and municipal tap water) for 1 week. Mice were infected subcutaneously with approximately 100 *S. mansoni* cercariae (obtained as described above). The infection was allowed to develop for 7 weeks until worms reached the adult stage. The mice were then euthanized, and the adult worms were extracted from the mesenteric veins. For the adult *S. haematobium* worms, LVG hamsters infected with *S. haematobium* cercariae

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were ordered from the Biomedical Research Institute (Rockville, MD, USA). Upon arrival at the Swiss TPH, the hamsters were allowed to acclimatize to the above-described conditions. The infection was allowed to develop for 4.5 months, after which the stools were checked for *S. haematobium* eggs and the hamsters were euthanized and dissected as described above.

All adult worms were kept in RPMI supplemented with 5% FCS and a 1% penicillin/streptomycin mixture at 37 °C and 5% CO_2 until used. For the assay setup, in a 24-well plate, 2 wells per drug were each filled with culture medium and drug stock solution for a final drug concentration of 100 μM and a final well volume of 2 mL. Two additional wells served as negative controls with culture medium and 1% DMSO. Two worm pairs were placed in each well. The worms were incubated at 37 °C and 5% CO_2 and evaluated microscopically at 24, 48, and 72 h using the viability scale described above. The onset of action assay was set up the same as described above but monitored at 0.5, 1, 2, 4, 7, 10, 24, 48, and 72 h. The IC_{50} determination assay was similarly set up, but with drug concentrations of 100, 50, 25, 12.5, and 6.25 μM and monitored at 24, 48, and 72 h.

Cytotoxicity Assay. In a 96-well plate, rat skeletal myoblast L6 cells were seeded at a concentration of 2×10^3 cells/well in 100 μL of RPMI 1640 medium supplemented with 10% FCS and 1.7 μM L-glutamine. The plates were incubated for 24 h at 37 °C and 5% CO_2 to allow a monolayer to form. The drugs were then added in a 3-fold serial drug dilution, starting at 90 μM, with podophyllotoxin (Sigma-Aldrich) serving as a positive control (starting at 100 ng/mL). Along with every drug dilution, tested in duplicate, one cell-free drug dilution served as a baseline. After 70 h of drug incubation, Alamar Blue (Sigma-Aldrich) was added to the assay and incubated for another 2 h for a total of 72 h of incubation time. The plates were then read (536/588 nm Ex/Em) using a SpectraMax M2 plate reader (Molecular Devices) coupled with Softmax software (version 5.4.1). The assay was repeated three times.

In Vivo Studies. Female 3-week-old NMRI mice were purchased, maintained, and infected as described above. Seven weeks postinfection, four mice were assigned to each drug treatment, whereas eight mice were left untreated to serve as controls. Compounds were prepared in a 70:30 Tween/EtOH mixture dissolved in dH_2O (10%). Doses were adjusted for the mouse weight and were administered orally. Three weeks post-treatment, the mice were killed by the CO_2 method and dissected, and the worms were sexed and counted. Mean worm burdens of treated mice were compared to the mean worm burden of untreated animals, and worm burden reductions were calculated.

Statistics. For the NTS and adult worm assays, the % viability reduction was calculated by normalizing average viability scores across replicates to average control viability scores using this formula via Microsoft Office Excel (2010): % viability reduction = 100% − (100%/ $AVGscore_{control}$ × $AVGscore_{treatment}$). IC_{50} values for the adult worm assays were computed using CompuSyn2 (ComboSyn Inc., 2007) by converting viability scores to effect scores for each drug concentration using the same formula as above. The L6 rat skeletal cell Alamar Blue assay IC_{50} values were calculated automatically by the Softmax software (version 5.4.1).

The worm burden (WB) of treated mice was calculated and compared to the worm burden of control mice in order to obtain the worm burden reduction (WBR), calculated as follows: WBR (%) = 100% − (100%/$WB_{control}$ × $WB_{treatment}$). The Kruskal−Wallis and Mann−Whitney U tests were employed to evaluate the significant differences in WBR between treated and control mice.

Stability Assay by ^{1}H NMR Spectroscopy. The stability of **1**, **5**, and **6** was investigated by ^{1}H NMR spectroscopy on a Bruker AV-500 at room temperature. For this purpose, DMSO-d_6 solutions of **1** (3.2 mM), **5** (3.2 mM), and **6** (3.2 mM) were prepared, and ^{1}H NMR spectra were recorded at different time intervals (0, 24, 48, and 72 h).

Stability Assay in Supplemented RPMI Buffer. A recently described procedure by Gasser and co-workers was adapted to assess the stability of **1**, **5**, **6**, and oxamniquine.[34,35] Diazepam was obtained from Sigma-Aldrich and used as the internal standard. For each experiment, fresh stock solutions of **1** (6.4 mM), **5** (6.4 mM), **6** (6.4 mM), oxamniquine (6.4 mM), and diazepam (3.2 mM) were prepared in DMSO and kept protected from light.

To 975 μL of RPMI buffer, 12.5 μL of the respective solution containing the compound to be studied (**1** (6.4 mM), **5** (6.4 mM), **6** (6.4 mM), and oxamniquine (6.4 mM)) and 12.5 μL of diazepam solution were added to a total volume of 1000 μL. The resulting aqueous solutions were incubated depending on the compound to be studied for 0, 3 (only for **5**), 6 (only for **5**), 9 (only for **5**), 24, 48, and 72 h at 37 °C with continuous and gentle shaking (700 rpm) while being protected from light. The progress of the stability was checked at each individual time point by the injection of the respective solution into a UPLC (Acquity Ultra Performance LC, Waters) connected to a mass spectrometer (Bruker Esquire 6000) operated in ESI mode. An Acquity UPLC BEH C18 (2.1 × 50 mm) reverse-phase column was used for the analysis with a flow rate of 0.6 mL min^{-1}. The UV absorption was measured at 254 nm.

For oxamniquine and **6**, the runs were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC grade) and B (distilled water containing 0.02% TFA and 0.05% HCOOC): t = 0−1 min, 1% A; t = 1−1.5 min, 2% A; t = 4.0 min, 100% A; and t = 5 min, 100% A.

For **1** and **5**, the runs were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC grade) and B (distilled water containing 0.02% TFA and 0.05% HCOOC): t = 0−2 min, 2.5% A; t = 6 min, 20% A; t = 11 min, 30% A; t = 12 min, 31% A; t = 14 min, 100% A; and t = 15 min, 100% A.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00054.

Synthesis and compound characterization, NMR spectra of compounds, molecular structure, crystal data, structure refinement, atomic coordinates, equivalent isotropic displacement parameters, bond lengths and angles, anisotropic displacement parameters, in vitro IC_{50}, histograms of compound stability, onset of action of compounds, UV traces, and compound activity (PDF)

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Notes

The authors declare no competing financial interest.

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Chapter 5

Dynamics of schistosomiasis infection and praziquantel treatment in pre-school and school-aged children

Chapter 5a

Efficacy and safety of praziquantel in preschool-aged and school-aged children infected with *Schistosoma mansoni*: a randomized controlled, parallel-group, dose-rangin, phase 2 trial

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Efficacy and safety of praziquantel in preschool-aged and school-aged children infected with *Schistosoma mansoni*: a randomised controlled, parallel-group, dose-ranging, phase 2 trial

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Summary

Background Praziquantel has been the drug of choice for schistosomiasis control for more than 40 years, yet surprisingly, the optimal dose for children younger than 4 years is not known. We aimed to assess the efficacy and safety of escalating praziquantel dosages in preschool-aged children (PSAC).

Methods We did a randomised controlled, parallel-group, single-blind, dose-ranging, phase 2 trial in PSAC (2–5 years) and school-aged children (SAC; aged 6–15 years) as a comparator group in southern Côte d'Ivoire. Children were randomly assigned (1:1:1:1) to 20 mg/kg, 40 mg/kg, or 60 mg/kg praziquantel or placebo. Participants, investigators, and laboratory technicians were masked to group assignment, while the investigator providing treatment was aware of the treatment group. The primary objective was to estimate the nature of the dose–response relation in terms of cure rate using the Kato Katz technique. Dose–response curves were estimated using E_{max} models. Available case analysis was done including all participants with primary endpoint data. This trial is registered with International Standard Randomised Controlled Trial, number ISRCTN15280205.

Findings Between Nov 11, 2014, and Feb 18, 2015, 660 PSAC and 225 SAC were assessed for eligibility; of whom 161 (24%) PSAC and 180 (80%) SAC had a detectable *Schistosoma mansoni* infection. 161 PSAC were randomly allocated of whom 154 received treatment: 42 were assigned to 20 mg/kg praziquantel, of whom 40 received treatment; 38 were assigned to 40 mg/kg praziquantel, of whom 38 received treatment; 41 were assigned to 60 mg/kg praziquantel, of whom 39 received treatment; and 40 were assigned to placebo, of whom 37 received placebo. 180 SAC were randomly allocated of whom 177 received treatment: 49 were assigned to 20 mg/kg praziquantel, of whom 47 received treatment; 46 were assigned to 40 mg/kg praziquantel, of whom 46 received treatment; 42 were assigned to 60 mg/kg praziquantel, of whom 42 received treatment; and 43 were assigned to placebo, of whom 43 received treatment. Follow-up (available-case) data were available for 143 PSAC and 174 SAC. In PSAC, the 20 mg/kg dose resulted in cure in 23 children (62%; 95% CI 44·8–77·5), 40 mg/kg in 26 children (72%; 54·8–85·8), 60 mg/kg in 25 children (71%; 53·7–85·4), and placebo in 13 children (37%; 21·5–55·1). In SAC, the 20 mg/kg dose resulted in cure in 14 children (30%; 95% CI 17·7–45·8), 40 mg/kg in 31 children (69%; 53·4–81·8), 60 mg/kg in 34 children (83%; 67·9–92·8), and placebo in five children (12%; 4·0–25·6). For both age groups, the number of adverse events was similar among the three praziquantel treatment groups, with fewer adverse events observed in the placebo groups. The most common adverse events in PSAC were diarrhoea (11 [9%] of 124) and stomach ache (ten [8%]) and in SAC were diarrhoea (50 [28%] of 177), stomach ache (66 [37%]), and vomiting (26 [15%]) 3 h post treatment. No serious adverse events were reported.

Interpretation Praziquantel shows a flat dose-response and overall lower efficacy in PSAC compared with in SAC. In the absence of treatment alternatives, a single dose of praziquantel of 40 mg/kg, recommended by the WHO for *S mansoni* infections in SAC can be endorsed for PSAC in preventive chemotherapy programmes.

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Introduction

Schistosomiasis is a major public health problem in many parts of the developing world, especially in sub-Saharan Africa. The disease is caused by blood flukes (trematode worms) of the genus *Schistosoma*, with *Schistosoma haematobium*, *Schistosoma japonicum*, and *Schistosoma mansoni* triggering most infections.[1–5] Indeed, more than 200 million people are infected globally, with about half of them suffering from morbid sequelae, including haematuria, dysuria, nutritional deficiencies, anaemia, hepatic granulomas leading to (severe) peri-portal fibrosis and consequent portal hypertension, and delayed physical and cognitive development.[6,7] Praziquantel is the drug of choice for treatment of infections with all *Schistosoma* species in the framework of preventive chemotherapy programmes.[8] While

Research in context

Evidence before this study

We searched PubMed for studies published before Dec 1, 2016, using the search terms "praziquantel", "schistosomiasis", "dose finding", "school-aged children", and "preschool-aged children". Our search identified numerous articles on the use of 40 mg/kg praziquantel in school-aged children, which concluded that this regimen is efficacious and safe. We noted from the year 2000 onwards, there was an increase of studies elucidating the efficacy and safety of crushed praziquantel (40 mg/kg dosage) in preschool-aged children (PSAC). Although this treatment regimen was deemed efficacious and safe, the nature of the dose-related effect has not been studied in both age groups.

Added value of this study

The results of our randomised controlled, parallel-group, single-blind, dose-ranging, phase 2 trial study show increasing cure rates and egg reduction rates (ERRs) for school-aged children (SAC) with escalating dosages of praziquantel, while a dose–response relation could not be observed in PSAC using the diagnostic of choice, the Kato-Katz method. The E_{max} model predicted an ERR of 99% at 65 mg/kg in SAC and an ERR of 95% at 50 mg/kg in PSAC, while the ERR of 99% was out of the observed range. Adverse events were mild and transient and included stomach ache, cough, diarrhoea, and vomiting.

Implications of all the available evidence

Our dose finding study supports the widely used dose 40 mg/kg of praziquantel for schistosomiasis morbidity control in SAC. Based on our results this dose can also be recommended for PSAC. Drug discovery efforts should be strengthened to have safe and effective alternative treatment options for schistosomiasis available in a timely manner.

school-aged children (SAC) are the main target population for treatment, it is becoming increasingly clear that younger children (<6 years) are also affected by schistosomiasis and suffer from morbidity.[9–12] Hence, in 2010, WHO recommended inclusion of preschool-aged children (PSAC) in large scale treatment programmes. In the absence of an appropriate paediatric formulation, which is currently under development, broken or crushed praziquantel tablets have been recommended.[13] Indeed, the efficacy and safety of crushed praziquantel tablets in infants have been assessed in several endemic settings mainly throughout Africa.[11,14–17]

Despite the above-mentioned studies, surprisingly, the effective dose for children younger than 4 years is not known. At the moment, praziquantel is widely used off-label at a standard dose of 40 mg/kg to treat PSAC,[18,19] because this is the recommended dose used for SAC and adults. However, a simple extrapolation of adult praziquantel dosages to children is very uncertain in view of the maturational differences in absorption, metabolism, and elimination.

In more detail, the oral bioavailability of drugs might vary in paediatric and adult populations due to differences in gastric pH and emptying time, intestinal transit time, immaturity of secretion, and the activity of bile and pancreatic fluid. Drug distribution in children and adults differs due to changes in membrane permeability, plasma protein binding, and total body water. Finally, the immaturity of enzyme systems (cytochrome P450), glomerular filtration, renal tubular secretion, and tubular reabsorption in children account for a different excretion of drugs in the paediatric population compared with in adults.[20,21] Despite this, thorough, quality, dose-finding clinical studies using praziquantel have not been done in PSAC to date.

We aimed to determine the nature of the dose–response of praziquantel in PSAC infected with *S mansoni* to determine the dose of praziquantel that shows an efficacy comparable to the standard dose of 40 mg/kg in SAC in an area where *S mansoni* is endemic.[22] Our findings, along with concurrent investigations pertaining to the pharmacokinetics of praziquantel in SAC and PSAC (Kovač and colleagues, under preparation) might be pivotal to further optimise the control of schistosomiasis.

Methods

Study design and participants

We did a randomised controlled, parallel-group, single-blind, dose-ranging, phase 2 trial was done in five villages located in the health district of Azaguié, southern Côte d'Ivoire. A detailed census was done in November, 2014, which generated lists of PSAC and SAC, including their name, age, and sex. PSAC (aged 2–5 years) and SAC (6-15 years) were enrolled. While the age of SAC was assessed based on their birth certificate at school level, the age of PSAC was confirmed by three potential sources: birth certificate, child's health card—where the date of birth is mentioned, and the verbal statement of the mother in case the two previously mentioned documents were unavailable. The village census identified 141, 167, 139, and 290 PSAC (2–5 years) and 231 SAC (6–15 years). All PSAC (n=737) and SAC registered during the census were invited to participate to the baseline survey.

Ethical approval for the study was obtained by the National Ethics Committee of the Ministry of Health in Côte d'Ivoire (CNER, reference number 037/MSLS/CNER-dkn) and the Ethical Committee of Northwestern and Central Switzerland (EKNZ; reference numner 162/2014).

Community meetings were held to explain the purpose, procedures, potential risks, and benefits of the study. Written informed consent was obtained from parents or legal guardians of participants. SAC were invited to give

their assent by writing their name and ticking the following sentence, "I agree to participate in this study" on the assent form. Parents or legal guardians were well informed on the fact that the participation was voluntary hence, they could withdraw their children from the study at any time with no further obligations.

Children were assessed for the presence of an *S mansoni* infection. *S mansoni*-positive children were eligible to participate in this trial. A clinical examination and an oral medical history by active questioning were obtained from all eligible children. Of note, mothers or guardians of the PSAC were asked about the medical history on behalf of their children. Children were excluded and treated with a standard dose of 40 mg/kg praziquantel if they had taken an antimalarial or anthelminthic drug in the past 4 weeks or had any systematic illness—namely, clinical malaria (presence of fever plus positive rapid malaria diagnostic test [ICT Malaria *Plasmodium falciparum* (HRPII), Cape Town, South Africa] according to the national guidelines) or hepatosplenic schistosomiasis. To determine the presence of hepatosplenic schistosomiasis, the extension of the left liver lobe beneath the sternum was measured in centimetres from the mid-sternal line and the extension of the right liver lobe beneath the rib cage was measured in centimetres from the right mid-clavicular line. At the end of the study, 3 months after the inclusion of the first participant, all children enrolled in the study were offered albendazole (400 mg) and praziquantel (40 mg/kg) for the treatment of helminth infections.

Randomisation and masking

PSAC and SAC with a parasitologically confirmed *S mansoni* infection were stratified by light, moderate, or heavy baseline infection intensities and randomly assigned (1:1:1:1) to placebo or 20 mg/kg, 40 mg/kg, or 60 mg/kg praziquantel using computer-generated stratified block randomisation codes provided by an independent statistician (stratified by two infection intensity strata; block size of eight). Children and laboratory technicians undertaking the diagnostics were masked, while the investigator delivering the treatment was aware of the treatment assignments. Masking was maintained throughout the trial until data cut off. Randomisation codes were released after the database was unlocked.

Procedures

During the baseline survey, at school level, children were provided with plastic containers labelled with unique identification numbers (IDs) and asked to deliver a fresh stool and urine sample. For the PSAC, mothers or guardians were given the plastic containers labelled with a unique ID and they were asked to obtain a fresh stool and urine sample of their child. From each participating child, two stool samples over two consecutive days and a single urine sample were collected. Stool and urine samples were transferred to a nearby laboratory in Azaguié town and examined on the day of collection. For the diagnosis of *S mansoni*, stool samples were each subjected to duplicate Kato-Katz thick smears (standard template of 41·7 mg).[23] Eggs of soil-transmitted helminths—ie, *Ascaris lumbricoides*, hookworm, and *Trichuris trichiura*—were also assessed and recorded for each parasite species separately. A subsequent independent quality control of sample results (about 10%) was done. In brief, the result from each slide among the 10% slides is considered correct if the following tolerance margin is not exceeded between the reading of two laboratory technicians: (1) for counts of 100 eggs or less, the difference between technicians' egg counts must not be greater than 10 eggs; and (2) for counts of 100 eggs or more the difference between technicians' egg counts must not be greater than 20 eggs. In case of discrepancy between the results of quality control and the initial reading, all the slides were read once again by the senior technician. Urine samples were subjected to the urine filtration technique for the diagnosis of *S haematobium* using the same quality control process. A commercially available Point-of-Care Circulating Cathodic Antigen (POC-CCA) cassette test (batch number: 34066; Rapid Medical Diagnostics, Pretoria, South Africa) for the diagnosis of *S mansoni* was applied on urine samples from the first day of samples collection. The POC-CCA tests were done and read as described elsewhere.[24] In addition, a finger prick blood sample was taken, and thick and thin blood smears were prepared for the diagnosis of *Plasmodium* species. Blood smears were stained with Giemsa and examined under a microscope using 100 x oil immersion. The *Plasmodium* density was counted against 200 leucocytes, assuming 8000 leucocytes per µL of blood. If less than 10 *Plasmodium* were found, the reading was continued up to 500 leucocytes. All slides were double-checked by a second laboratory technician and only considered negative if no *Plasmodium* were detected in 100 x oil immersion field by the two independent microscopists. In addition, a rapid malaria diagnostic test was used and the haemoglobin value measured using a calibrated HaemoCue device (HaemoCue 301 system, Ängelholm, Sweden) according to the manufacturer's instructions. Weight was measured in kg using the domestic HAMSON bathroom weighing scale (Graduation increments of 0·1 kg) and height was measured using a common builder's measuring tape.

To assess treatment efficacy, another two stool samples and one urine sample were collected between 21 days and 25 days post-treatment for the follow-up and subjected to the same diagnostic approaches applied at the baseline survey.

Procedures

After the baseline screening and clinical examination, eligible participants were treated. Before treatment, each participant received breakfast. The breakfast comprised

of an equally sized piece of buttered baguette for each child. Praziquantel tablets (600 mg Cesol, kindly provided by Merck (Darmstadt, Germany) or placebo tablets (Fagron, Germany) were given according to the calculated dose per kg of bodyweight in half tablet increments (eg, 1·3 tablets calculated=1·5 tablets given or 1·2 tablets calculated=1 tablet given). For PSAC, tablets were crushed using a mortar and pestle and dissolved in a small volume of syrup-flavoured water. SAC and the mothers or guardians of PSAC were interviewed 3 h, 24 h, 48 h, and 72 h after treatment about the occurrence of adverse events and mitigating drugs were provided if necessary. An adverse event was defined as any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal or investigational product, whether or not related to the treatment. All adverse event intensities were judged by the study physician, following guidelines by the European Medicine Agency and were graded as mild, moderate, severe, or intolerable.

Outcomes

Our primary outcome was the cure rate (the percentage of egg-positive children at baseline who became egg-negative after treatment) resulting from different doses of praziquantel based on the Kato-Katz method. The secondary outcomes were egg reduction rate (ERR) and the safety of different doses of praziquantel.

Statistical analysis

Simulations showed that with 36 children enrolled in each of the four treatment study groups (0 mg/kg, 20 mg/kg, 40 mg/kg, and 60 mg/kg), the dose response prediction model should have a median precision (one half length of the 95% CIs) of 10% points, assuming associated cure rates of 2·8%, 50%, 75%,[25] and 90%, respectively. The suggested sample size is also in line with the recommendations from Klingenberg in 2009.[26] To account for losses in the follow-up, the sample size was increased to 40 children in each study group.

Data were double entered into an Excel spreadsheet, transferred into EpiInfo version 3.5.2 (Centers for Disease Control and Prevention; Atlanta, GA, USA) and cross-checked.

Data were analysed with Stata version 13 (Stata Corp; College Station, TX, USA). Participants who had at least one stool sample examined each with duplicate Kato-Katz thick smears at follow-up, were present at treatment day, and not excluded due to a medical condition were included in the final analysis (available case analysis). Imputation of missing data with treatment failure or success was assessed in an intention-to-treat analysis.

Eggs per gram of stool (EPG) were assessed by adding up the egg counts from the duplicate or quadruplicate Kato-Katz thick smears and multiplying this number by a factor of 12 or six, respectively. We classified infection intensity as light (<100 EPG), moderate (100–399 EPG), or heavy (>400 EPG).[27]

Geometric mean egg counts were calculated as:

$$e^{1/n \sum \log(EPG + 1)} - 1$$

And the corresponding ERR 100 as:

$$\frac{100 - (e^{1/n \sum \log(EPGfollow\text{-}up + 1)} - 1)}{(e^{1/n \sum \log(EPGbaseline + 1)} - 1) \times 100}$$

A bootstrap resampling method with 5000 replicates was used to calculate 95% CIs for ERRs.

We used an E_{max} model as a primary model to predict the dose–response curves in terms of cure rates and ERRs. The analysis was done with the DoseFinding package (version 0·9–14) of the statistical software environment R (version 3.3.0). E_{max} model was predicted in two stages. For binary data, first cure rates and their covariances were estimated on logit scale via logistic regression. In the second stage, doses and estimated cure rates were fitted to the non-linear E_{max} model to determine the basal effect (treatment effect at dose 0), the asymptotic maximum effect, and ED_{50} (the dose resulting in 0·5 * E_{max}). We converted estimates on logit scale to probabilities in the cure rate related dose-response figures.

Role of funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Nov 11, 2014, and Feb 18, 2015, 968 children were invited to participate in the study. 660 PSAC and 225 SAC participated in the baseline survey (figure 1). Of these, 161 (24%) PSAC and 180 (80%) SAC had a detectable *S mansoni* infection and were randomly assigned to treatment groups. In the PSAC group, 42 were assigned to 20 mg/kg praziquantel, of whom 40 received treatment; 38 were assigned to 40 mg/kg praziquantel, of whom 38 received treatment; 41 were assigned to 60 mg/kg praziquantel, of whom 39 received treatment; and 40 were assigned to placebo, of whom 37 received placebo. In the SAC group, 49 were assigned to 20 mg/kg praziquantel, of whom 47 received treatment; 46 were assigned to 40 mg/kg praziquantel, of whom 46 received treatment; 42 were assigned to 60 mg/kg praziquantel, of whom 42 treatment; and 43 were assigned to placebo, of whom 43 received treatment.

Follow-up (available-case) data were available for 143 PSAC and 174 SAC (figure 1). Of note, 11 PSAC and six SAC provided only a single stool sample at follow-up.

For all treated children, demographic and parasitological baseline data are shown in table 1. The median age, weight, height, and sex of PSAC and SAC were balanced among the treatment groups. At pre-treatment, the geometric mean EPG of faeces ranged between 24·7 and 39·4 in the PSAC treatment groups, and between 71·7 and 84·1 in the SAC treatment groups (table 2). 129 (80%) of PSAC harboured a light *S mansoni* infection versus 93 (52%) of SAC, while about half (87 [49%]) of the SAC had moderate or heavy infection intensities versus 32 (20%) of PSAC. More than half of the SAC were co-infected with *P falciparum* (112 [62%]), while PSAC harboured fewer *Plasmodium* co-infections (53 [33%]). Co-infections with *T trichiura* were common in the study setting, with 56 (35%) PSAC and 97 (54%) SAC co-infected. By contrast, co-infections with *Ascaris*, hookworm, and *S haematobium* were rare, and ranged between 0% and 11% in the different treatment groups. Haemoglobin values were in the normal range with median values between 10·5 and 11·3 in PSAC and between 11·4 and 12·0 in SAC (table 1).

In PSAC, based on the Kato-Katz technique, both cure rates and ERRs increased only incrementally starting at 20 mg/kg. In more detail, cure rate and ERR (geometric mean) for the 20 mg/kg group were 62·1% (95% CI 44·8–77·5) and 90·7% (82·0–95·7), respectively. In the 40 mg/kg and 60 mg/kg treatment groups, similar cure rates (72·2% [95% CI 54·8–85·8] and 71·4% [53·7–85·4], respectively) were observed (table 2; figure 2). The corresponding ERRs (geometric mean) were 94·8% (95% CI 89·1–98·0) and 95·8% (90·2–98·5), respectively. PSAC in the placebo group, *S mansoni* eggs were not detected in a proportion of 37·1% (95% CI 21·5–55·1) of children with a corresponding ERR of 80·1% (66·3–88·9).

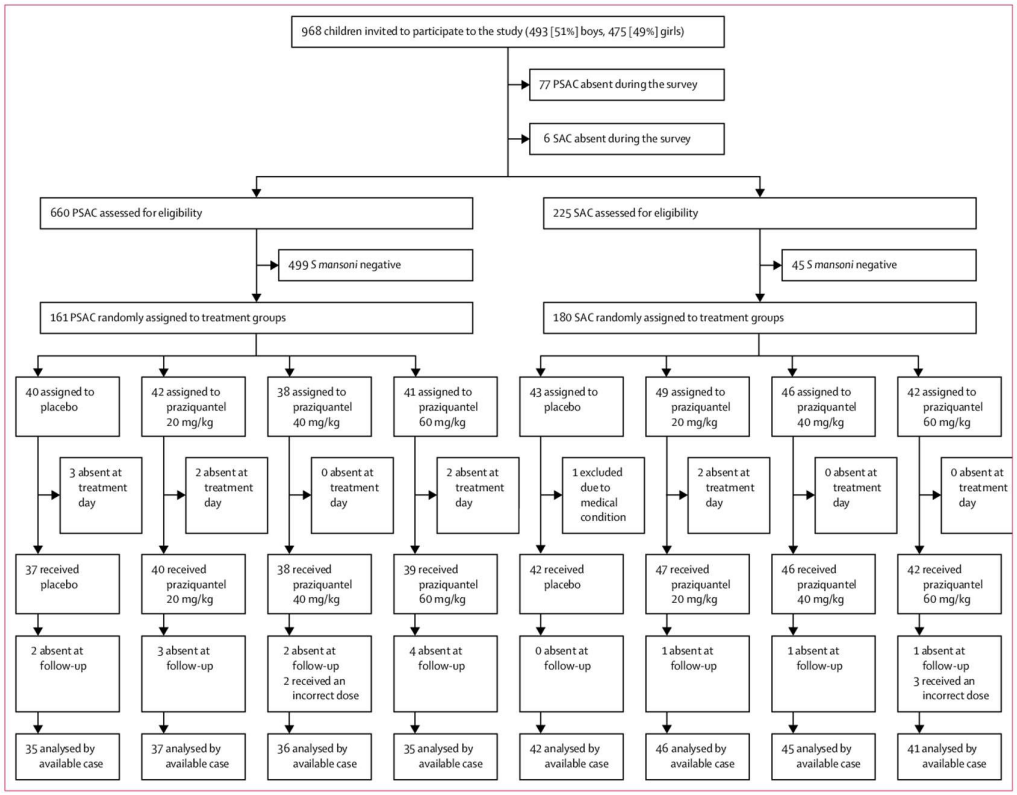

Figure 1: **Trial profile**
PSAC=preschool-aged children. SAC=school-aged children. *S mansoni*=*Schistosomiasis mansoni*.

	Preschool-aged children					School-aged children				
	Placebo (n=40)	Praziquantel 20 mg/kg (n=42)	Praziquantel 40 mg/kg (n=38)	Praziquantel 60 mg/kg (n=41)	Total (n=161)	Placebo (n=43)	Praziquantel 20 mg/kg (n=49)	Praziquantel 40 mg/kg (n=46)	Praziquantel 60 mg/kg (n=42)	Total (n=180)
Age, years	4 (2–5)	4 (2–5)	4 (2–5)	4 (2–5)	4 (2–5)	8 (6–14)	9 (6–12)	9 (6–13)	9 (6–12)	9 (6–13)
Weight, kg	14 (8–18)	14 (8–18)	14 (11–18)	13 (10–18)	14 (9–18)	23 (15–46)	23 (17–40)	23 (15–41)	24 (16–40)	23 (16–43)
Height, cm	100 (83–105)	95 (77–108)	97 (84–110)	95 (81–105)	96 (80–108)	123 (108–164)	128 (106–152)	126 (106–152)	128 (107–147)	127 (106–156)
Haemoglobin (g/dL)	10·5 (10·3–11·2)*	10·9 (10·3–11·6)†	11·3 (10·2–12·0)‡	10·8 (9·9–11·8)§	10·8 (10·1–11·6)¶	11·6 (10·6–12·6)	11·4 (10·8–12·4)	12·0 (11·3–12·5)	11·6 (10·9–12·9)	11·7 (10·9–12·5)
Sex										
Girls	17 (43%)	21 (50%)	22 (58%)	20 (49%)	80 (50%)	19 (44%)	26 (53%)	21 (46%)	25 (60%)	91 (51%)
Boys	23 (58%)	21 (50%)	16 (42%)	21 (51%)	81 (50%)	24 (56%)	23 (47%)	25 (54%)	17 (40%)	89 (49%)
Infection intensity										
Light	30 (75%)	34 (81%)	32 (84%)	33 (81%)	129 (80%)	23 (54%)	26 (53%)	23 (50%)	21 (50%)	93 (52%)
Moderate	8 (20%)	6 (14%)	6 (16%)	7 (17%)	27 (17%)	15 (35%)	16 (33%)	16 (35%)	12 (29%)	59 (33%)
Heavy	2 (5%)	2 (5%)	0	1 (2%)	5 (3%)	5 (12%)	7 (14%)	7 (15%)	9 (21%)	28 (16%)
Co-infections										
Schistosoma haematobium	1 (3%)	0	1 (3%)	3 (7%)	5 (3%)	3 (7%)	3 (6%)	3 (7%)	2 (5%)	11 (6%)
Ascaris lumbricoides	5 (13%)	1 (2%)	0	3 (7%)	9 (6%)	0	3 (6%)	5 (11%)	1 (2%)	9 (5%)
Trichuris trichiura	15 (38%)	15 (36%)	12 (32%)	14 (34%)	56 (35%)	23 (54%)	24 (49%)	27 (59%)	23 (55%)	97 (54%)
Hookworm	0	1 (2%)	1 (3%)	0	2 (1%)	4 (9%)	3 (6%)	5 (11%)	2 (5%)	14 (8%)
Plasmodium falciparum (based on thin or thick smear)	10 (25%)	17 (41%)	13 (34%)	13 (32%)	53 (33%)	24 (56%)	33 (67%)	28 (61%)	27 (64%)	112 (62%)
Plasmodium falciparum (based on RDT)	10 (25%)	14 (33%)	11 (26%)	13 (32%)	48 (30%)	24 (57%)	32 (67%)	27 (57%)	25 (58%)	108 (61%)

Data are median (IQR) or n (%). RDT=rapid diagnostic test. *27 participants. †34 participants. ‡30 participants. §33 participants. ¶124 participants.

Table 1: **Baseline characteristics of treatment groups**

The E_{max} model predicted that in PSAC, an ERR of 99% is out of the observed range, but an ERR of 95% is estimated at 50 mg/kg (figure 3). ERRs based on arithmetic means are shown in table 2.

The observed cure rates in SAC increased with escalating dosages (table 2). The 20 mg/kg dose resulted in cure in 14 (30·4%; 95% CI 17·7–45·8) children, the 40 mg/kg resulted in cure of 31 (68·9%; 53·4–81·8) children, and the 60 mg/kg dose resulted in cure of 34 (82·9%; 67·9–92·8) children based on the Kato-Katz method. Egg reduction rates (geometric mean) were moderate for the 20 mg/kg dose (84·2% [95% CI 70·9–91·5]) and high for the 40 mg/kg and 60 mg/kg treatment groups (98·3% [96·7–99·3]) and 99·1% [97·9–99·8], respectively. In the SAC in the placebo group, we observed a cure rate of 11·9% (95% CI 4·8–25·6) and an ERR of 56·0% (36·9–69·7). ERRs based on arithmetic means are shown in table 2. The E_{max} model predicted an ERR of 99·0% at 65 mg/kg in SAC (figure 3).

See Online for appendix

Cure rates in PSAC were lower based on the POC-CCA cassette test compared with the Kato-Katz technique. Considering "traces" as a positive result revealed a slightly higher cure rate at 40 mg/kg and 60 mg/kg (32·0% [95% CI 14·9–53·5]) and (33·3% [15·6–55·3]) compared with 20 mg/kg (15·4% [4·4–34·9]) and placebo (21·1% [8·3–40·9]). Considering "traces" as a negative result yielded cure rates from 21·7% (95% CI 7·5–43·7; 20 mg/kg) to 52·2% (30·6–73·2; 60 mg/kg). The POC-CCA confirmed the dose–response in SAC (table 2). Considering "traces" as a positive result revealed cure rates ranging from 19·0–55·0% for 20–60 mg/kg praziquantel and a cure rate of 10·8 in placebo treated SAC. Considering "traces" as a negative result yielded cure rates from 12·1% (placebo) to 28·9% (20 mg/kg) to 65·7% (60 mg/kg).

Imputation of missing data with treatment failure or success in the intention-to-treat analysis did not change the observed outcomes (appendix p 3). Although our study was not powered to detect differences in sex, our study did not show an effect of sex on the efficacy of praziquantel (data not shown).

Three children in the 60 mg/kg treatment group were wrongly dosed: one received a dose of 16·8 mg/kg (PSAC), one a dose of 74·5 mg/kg (PSAC), and one a dose of 36·1 mg/kg (SAC). Two PSAC in the 40 mg/kg treatment

group were treated with 60·8 mg/kg and 26·3 mg/kg, respectively (figure 1). Actual doses given in PSAC were 15·2–37·5 mg/kg (20 mg/kg), 24·4–60·8 (40 mg/kg), and 36·1–72·0 mg/kg (60 mg/kg). SAC doses ranged from 13·5–28·7 mg/kg (20 mg/kg dose), 33·1–49·5 mg/kg (40 mg/kg), and 51·4–74·5 (60 mg/kg). The E_{max} model based on actual doses is shown in the appendix (p 4). The E_{max} model based on actual doses also showed a flat dose response for PSAC, whereas the response for SAC showed increasing efficacy at escalating dosages.

In an assessment of the cure rates for each *S mansoni* infection intensity category, we found that cure rates decreased as the infection intensity increased (table 2) in both age groups (PSAC or SAC) and at all praziquantel doses (appendix p 5).

Table 3 shows the number of treated children with adverse events and their dynamics over time. Adverse events data were available for 124 PSAC and 177 SAC. In the PSAC group, 30 children were absent (placebo [n=9], 20 mg/kg [n=5], 40 mg/kg [n=9], and 60 mg/kg [n=7]) following treatment and were not assessed for adverse events. Before treatment, overall 78 (63%) of 124 PSAC and 104 (59%) of 177 SAC reported mild symptoms. The recorded number of complaints was similar among treatment groups, with stomach ache and coughing most frequently reported. At 3 h post treatment, PSAC reported fewer symptoms compared with pre-treatment. In SAC there were slightly more reported episodes compared with pre-treatment at this timepoint. Overall, 29 (23%) of 124 PSAC and 124 (70%) of 177 SAC reported adverse events 3 h post treatment. Most adverse events were mild (29 [91%] of 32 episodes in PSAC and 124 [98%] of 126 episodes in SAC). No serious adverse events were reported. For both age groups, the number of adverse events was similar among the three praziquantel treatment groups, with fewer adverse events observed in the placebo groups. The most common adverse events in PSAC 3 h post treatment were diarrhoea (11 [9%] of 124) and stomach ache (ten [8%] of 124). Adverse events commonly observed in SAC were stomach ache (66 [37%] of 177), diarrhoea (50 [28%] of 177), and headache (27 [15%] of 177; table 3).

24 h post-treatment, 16 episodes (13%) were noted by PSAC and 67 (38%) episodes by SAC. All reported adverse events were mild. No difference in the number of adverse events was noted among the different treatment groups and placebo group. Diarrhoea (six [5%] of 124) and vomiting (four [3%] of 124) were the two most common symptoms in PSAC 24 h post-treatment. Stomach ache (28 [16%] of 177), cough (20 [11%] of 177), and diarrhoea (18 [10%] of 177) were most frequently observed in SAC. Adverse events observed 48 h and 72 h post treatment are presented in the appendix p (6).

Discussion

In the absence of a paediatric formulation of praziquantel, the accurate management of young children with schistosomiasis at point-of-care and at community levels

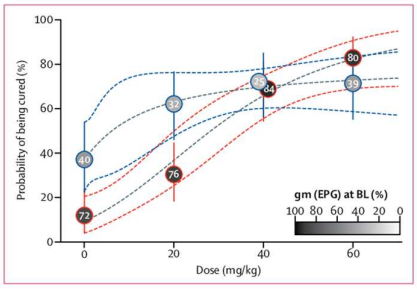

Figure 2: **Cure rates in PSAC (blue lines) and SAC (red lines)**
Circles show observed cure rates with 95% CIs (vertical lines). Numbers and colour code in the circles show geometric mean infection intensities at baseline (BL). Dashed lines represent the estimated dose–response curve and corresponding 95% confidence bands predicted by the E_{max} models. Epg=eggs per gram of stool. PSAC=preschool-aged children. SAC=school-aged children.

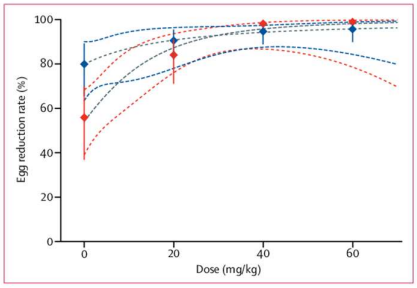

Figure 3: **Egg reduction rates in PSAC (blue lines) and SAC (red lines)**
Diamonds show observed cure rates with 95% CIs (vertical lines). Dashed lines represent the estimated dose response curve and corresponding 95% confidence bands predicted by the E_{max} model. PSAC=preschool-aged children. SAC=school-aged children.

may be challenging. Yet, by focusing treatment in preventive chemotherapy programmes on the school-aged population, children of preschool age are neglected, thus preventing them from benefiting from treatment given to their older peers, and hence creating a potential health inequity. If children as young as age 2 years can be infected (as shown in ours and previous studies),[12,22] but must wait until school age to be treated, they are probably already facing consequences of the chronic infection at a young age, which might carry on into older age. At the moment, praziquantel is widely used off-label at a standard dose of 40 mg/kg to treat PSAC, because this is the dose used for SAC and adults.[25,28–30] However, the effective dose for children younger than 4 years is unknown. Although the efficacy and safety of praziquantel has been intensively assessed in SAC,[28–30] and more recently in PSAC,[31–34] surprisingly, well designed dose–response relation studies are lacking in both age groups.

	Preschool-aged children (PSAC)				School-aged children (SAC)			
	Placebo	Praziquantel 20 mg/kg	Praziquantel 40 mg/kg	Praziquantel 60 mg/kg	Placebo	Praziquantel 20 mg/kg	Praziquantel 40 mg/kg	Praziquantel 60 mg/kg
Kato-Katz								
Infected children before treatment	35	37	36	35	42	46	45	41
Cured children after treatment	13 (37·1%; 21·5–55·1)	23 (62·2%; 44·8–77·5)	26 (72·2%; 54·8–85·8)	25 (71·4%; 53·7–85·4)	5 (11·9%; 4·0–25·6)	14 (30·4%; 17·7–45·8)	31 (68·9%; 53·4–81·8)	34 (82·9%; 67·9–92·8)
Cured children by infection intensity†								
Low	11/27	19/27	23/30	21/26	5/24	11/25	16/17	19/20
Moderate	2/6	4/8	3/6	3/8	0/14	3/16	10/15	9/10
Heavy	0/2	0/2	0/0	1/1	0/4	0/5	5/13	6/11
Geometric mean EPG								
Before treatment	40·0	31·7	24·7	39·4	71·7	76·5	84·1	80·2
After treatment	7·9	2·9	1·3	1·7	31·5	12·1	1·4	0·7
Egg reduction rate	80·1% (66·3–88·9)	90·7% (82·0–95·7)	94·8% (89·1–98·0)	95·8% (90·2–98·5)	56·0% (36·9–69·7)	84·2% (70·9–91·5)	98·3% (96·7–99·3)	99·1% (97·9–99·8)
Arithmetic mean EPG								
Before treatment	112·3	140·1	56·5	87·6	179·6	193·7	220·3	292
After treatment	45·6	26·8	9·0	16·1	132·9	80·2	7·3	29·4
Egg reduction rate	59·4% (21·6–76·3)	80·9% (56·4–88·6)	84·1% (61·9–94·6)	81·6% (57·1–94·6)	26·0% (0–58·5)	58·6% (24·1–75·9)	96·7% (93·2–98·0)	89·9% (61·9–99·5)
POC-CCA* (with "trace" as positive)								
Infected children before treatment	28	26	25	24	37	42	39	40
Cured children after treatment	6 (21·4%; 8·3–40·9)	4 (15·4%; 4·4–34·9)	8 (32·0%; 14·9–53·5)	8 (33·3%; 15·6–55·3)	4 (10·8%; 3·0–25·4)	8 (19·0%; 8·6–34·1)	15 (38·5%; 23·4–55·4)	22 (55·0%; 38·5–70·7)
POC-CCA (with "trace" as negative)								
Infected children before treatment	24	23	21	23	33	38	35	35
Cured children after treatment	4 (16·7%; 4·7–37·4)	5 (21·7%; 7·5–43·7)	7 (33·3%; 14·6–57·0)	12 (52·2%; 30·6–73·2)	4 (12·1%; 3·4–28·2)	11 (28·9%; 15·4–45·9)	16 (45·7%; 28·9–63·3)	23 (65·7%; 47·8–80·9)

Data are n, n (%), n/N, or n (%; 95% CI). EPG=eggs per gram. POC-CCA=Point-of-Care Circulating Cathodic Antigen cassette test. *POC-CCA tests were applied on the first urine sample collected from each participant on the first day of collection. Among PSAC and SAC, respectively (seven *vs* five) in placebo, (11 *vs* four) in 20 mg/kg, (11 *vs* six) in 40 mg/kg, and (11 *vs* one) in 60 mg/kg did not provide urine samples on the first day of urine collection †*Schistosoma mansoni* infection intensity was stratified into low (1–99 EPG), moderate (100–399 EPG), and heavy (≥400 EPG) infection.

Table 2: **Available case analysis of cure and egg reduction rates of 20 mg/kg, 40 mg/kg, and 60 mg/kg praziquantel versus placebo against intestinal schistosomiasis in PSAC and SAC based on Kato-Katz and POC-CCA**

Our randomised controlled trial, assessing the efficacy and safety of 20 mg/kg, 40 mg/kg, and 60 mg/kg praziquantel in PSAC and SAC to uncover the nature of the dose-related effect, aimed to fill this gap. We used baseline and follow-up surveys at a reasonably sensitive diagnostic approach aiming for four Kato-Katz thick smears for the diagnosis of *S mansoni* in all study participants. Additionally, a urine POC-CCA cassette test was used for diagnosis of *S mansoni*.

In SAC, an increasing efficacy was noted with escalating doses of praziquantel. The highest cure rate and ERR (geometric mean) were observed with 60 mg/kg praziquantel. The E_{max} model predicted an ERR of 99·0% at 65 mg/kg in SAC (figure 3). 60 mg/kg showed a slightly better performance than did 40 mg/kg praziquantel in terms of cure rate and ERR; however, the overlapping confidence intervals suggest that both doses perform similarly. Our findings are in line with a recent meta-analysis, which reported a cure rate of 74·6% (95% CI 68·3–80·6) using 40 mg/kg praziquantel to treat *S mansoni* infections in SAC, and a significant relation between the cure rates and dose in the treatment of *S mansoni*.[27] In addition, a multicentre study comparing the efficacy of 40 mg/kg versus 60 mg/kg praziquantel concluded that the higher dose offers no significant efficacy advantage over the standard dose.[28]

We did not identify a no-effect dose range in PSAC. Moderate cure rate and ERR were observed with 20 mg/kg (cure rate of 62·1%; ERR of 90·7%), which slightly increased when 40 mg/kg (cure rate of 72·2%; ERR of 94·8%) or 60 mg/kg (cure rate of 71·4%; ERR of 95·8%) were given. The E_{max} model predicted that in PSAC an ERR of 99% is out of the observed range. If we calculate arithmetic means, then ERRs in PSAC ranged from 80·9–84·1%. Hence, cure rates and ERRs were

	Preschool-aged children (PSAC)					School-aged children (SAC)				
	Placebo (n=27*)	Praziquantel 20 mg/kg (n=35*)	Praziquantel 40 mg/kg (n=29*)	Praziquantel 60 mg/kg (n=33*)	Overall (n=124)	Placebo (n=42)	Praziquantel 20 mg/kg (n=47)	Praziquantel 40 mg/kg (n=46)	Praziquantel 60 mg/kg (n=42)	Overall (n=177)
Before treatment										
Moderate	0	0	0	0	0	0	0	0	0	0
Mild	19 (70%)	21 (60%)	17 (59%)	21 (64%)	78 (63%)	22 (52%)	29 (62%)	28 (61%)	25 (60%)	104 (59%)
None	8 (30%)	14 (40%)	12 (41%)	12 (36%)	46 (37%)	20 (48%)	18 (38%)	18 (39%)	17 (40%)	73 (41%)
Stomach ache	3 (11%)	3 (9%)	8 (28%)	4 (12%)	18 (15%)	12 (29%)	6 (13%)	10 (22%)	8 (19%)	36 (20%)
Cough	9 (33%)	11 (31%)	10 (35%)	6 (18%)	36 (2%9)	10 (24%)	22 (47%)	20 (43%)	14 (33%)	66 (37%)
Diarrhoea	6 (22%)	6 (17%)	4 (14%)	5 (15%)	21 (17%)	4 (10%)	2 (4%)	7 (15%)	0	13 (7%)
Headache	0	2 (6%)	0	1 (3%)	3 (2%)	5 (12%)	5 (11%)	1 (2%)	6 (14%)	17 (10%)
Vomiting	2 (7%)	0	3 (10%)	1 (3%)	6 (5%)	2 (5%)	2 (4%)	1 (2%)	2 (5%)	7 (4%)
Itching	2 (7%)	2 (6%)	2 (7%)	4 (12%)	10 (8%)	1 (2%)	3 (6%)	2 (4%)	1 (2%)	7 (4%)
Fever	3 (11%)	7 (20%)	7 (24%)	11 (33%)	28 (23%)	0	0	1 (2%)	2 (5%)	3 (2%)
3 h post treatment										
Moderate	0	1 (3%)	2 (7%)	0	3 (2%)	1 (2%)	0	0	1 (2%)	2 (1%)
Mild	3 (11%)	7 (20%)	8 (28%)	11 (33%)	29 (23%)	20 (48%)	36 (77%)	36 (78%)	32 (76%)	124 (70%)
None	24 (89%)	27 (77%)	19 (66%)	22 (67%)	92 (74%)	21 (50%)	11 (23%)	10 (22%)	9 (21%)	51 (29%)
Stomach ache	0	1 (3%)	5 (17%)	4 (12%)	10 (8%)	11 (26%)	22 (47%)	17 (37%)	16 (38%)	66 (37%)
Cough	0	2 (6%)	2 (7%)	2 (6%)	6 (5%)	3 (7%)	8 (17%)	4 (9%)	9 (21%)	24 (14%)
Diarrhoea	1 (4%)	5 (14%)	2 (7%)	3 (9%)	11 (9%)	5 (12%)	12 (26%)	17 (37%)	16 (38%)	50 (28%)
Headache	1 (4%)	1 (3%)	1 (3%)	0	3 (2%)	4 (10%)	7 (15%)	5 (11%)	11 (26%)	27 (15%)
Vomiting	0	1 (3%)	1 (3%)	2 (6%)	4 (3%)	2 (5%)	7 (15%)	9 (20%)	8 (19%)	26 (15%)
Itching	0	0	0	0	0	1 (2%)	3 (6%)	2 (4%)	1 (2%)	7 (4%)
Fever	1 (4%)	1 (3%)	0	0	2 (2%)	2 (5%)	2 (4%)	0	1 (2%)	5 (3%)
24 h post treatment										
Moderate	0	0	0	0	0	0	0	0	0	0
Mild	4 (15%)	5 (14%)	1 (3%)	6 (18%)	16 (13%)	16 (38%)	17 (36%)	18 (39%)	16 (38%)	67 (38%)
None	23 (85%)	30 (86%)	28 (97%)	27 (82%)	108 (87%)	26 (62%)	30 (64%)	28 (61%)	26 (62%)	110 (62%)
Stomach ache	0	0	0	0	0	7 (17%)	9 (19%)	4 (9%)	8 (19%)	28 (16%)
Cough	0	0	0	2 (6%)	0	5 (12%)	5 (11%)	5 (11%)	5 (12%)	20 (11%)
Diarrhoea	0	5 (14%)	0	1 (3%)	6 (5%)	5 (12%)	4 (9%)	3 (7%)	6 (14%)	18 (10%)
Headache	1 (4%)	0	1 (3%)	1 (3%)	3 (2%)	2 (5%)	2 (4%)	3 (7%)	4 (10%)	11 (6%)
Vomiting	2 (7%)	1 (3%)	0	1 (3%)	4 (3%)	2 (5%)	2 (4%)	3 (7%)	4 (10%)	11 (6%)
Itching	0	0	0	1 (3%)	0	4 (10%)	3 (6%)	3 (7%)	3 (7%)	13 (7%)
Fever	1 (4%)	0	0	1 (3%)	2 (2%)	1 (2%)	0	0	0	1 (1%)

Data are n (%). *30 kids were absent (placebo [n=9], 20 mg/kg [n=5], 40 mg/kg [n=9], and 60 mg/kg [n=7]) following treatment and were not assessed for adverse events.

Table 3: **Main type of clinical symptoms before treatment and adverse events 3 h and 24 h after praziquantel administration in SAC (n=177) and PSAC (n=124)**

lower at 40 mg/kg and 60 mg/kg praziquantel in PSAC compared with in SAC (regardless of diagnostic tool). Notably, recent WHO Standard Operating Procedures suggested a reference drug efficacy value of 90% or higher based on arithmetic means for treating *S mansoni* infections with praziquantel.[15] All doses investigated in PSAC—namely, 20 mg/kg, 40 mg/kg, and 60 mg/kg—would therefore not fulfil the criteria of clinical efficacy. Enzymatic processes and the immune system have been well documented to be age-dependent and might affect the absorption, distribution, metabolism, and elimination of drugs in young children.[18,20,21,36] Because SAC were treated side-by-side with the same praziquantel dosages, in the same ecological setting (villages were all within a radius of 5 km), using the same diagnostic tests, and administering the same food item, confounding factors can be ruled out. However, crushing the tablets might have altered the bioavailability and pharmacokinetic properties of praziquantel and might therefore play a part in the difference in efficacy noted in PSAC.

The efficacy observed for 40 mg/kg in PSAC corresponds with results reported by the above-mentioned meta-analysis,[27] which calculated a cure rate of 69% and an ERR of 85·6% for this praziquantel dose in PSAC. A recent study in a small cohort in Ugandan

children showed higher cure rates and ERRs with 60 mg/kg praziquantel (ERR 91%, cure rate 82%) versus 40 mg/kg (ERR 82%, cure rate 70%),[34] however two-thirds of the examined children were school-aged and hence no real conclusion on the efficacy of praziquantel on young children could be drawn.

One limitation of our study was that, based on the tablet formulation used in the current study (600 mg praziquantel tablets; Cesol), only half tablets increments (ie, 300 mg) could be given. Using tablets, which could have been divided in four parts (ie, Distocide) could have allowed us to dose slightly more accurately. As proposed recently by Olliaro and colleagues in 2013,[31] we strongly recommend that when working with PSAC, a formulation that can be divided into four parts (to give 150 mg increments) be used, particularly in children weighing less than 10 kg as long as a novel paediatric formulation based on oral dispersible tablets is under development.

Another limitation of our study is a high proportion of PSAC treated with placebo showing no eggs at the follow-up using the Kato-Katz technique. The Kato-Katz method has been well documented to be less sensitive, especially in populations with low infection intensity such as PSAC or in communities having received treatment. In our study, most PSAC had a light infection intensity. 45 PSAC (about 14%) had only one single egg found in all four Kato-Katz thick smears taken together at baseline. In 2015, Siqueira and colleagues[37] assessed parasitological and molecular techniques for the diagnosis and assessment of cure in individuals infected with *S mansoni* harbouring a light infection and concluded that an increased number of Kato-Katz slides or a test with higher sensitivity is required for participants with a very low parasite load situation, such as after therapeutic interventions. Our study therefore included the sensitive POC-CCA cassette test as an alternative diagnostic instrument. As expected, cure rates were significantly lower using the POC-CCA compared with the Kato-Katz, but across all treatment groups, due to the higher sensitivity of this device.[38] However, this finding does not only point to treatment failures of chronic *S mansoni* infections. Indeed, praziquantel is largely refractory against young developing stages of the worms,[14] and hence antigens might be present in the urine of young children due to acute infections contributing to the low cure rates observed with POC-CCA in this study.

The nature of the dose–response relation based on POC-CCA were similar to the one observed with Kato-Katz in SAC. Regardless of whether traces were considered as positive or negative, a dose–response relation was observed in SAC with the highest efficacy observed for SAC at 60 mg/kg (55·0 and 65·7% cure rate, respectively). In PSAC, similar cure rates were observed at 40 mg/kg and 60 mg/kg considering traces as positive (32·0% and 33·3%), which were higher than the ones recorded for 20 mg/kg (cure rate of 15·4%). However, when considering traces as negative, increasing cure rates were observed with increasing dosages in this group (21·7–52·2%). Of note, debate is still ongoing about the correct interpretation to give to POC-CCA "trace" scores in the diagnosis of *S mansoni*,[39] hence the Kato-Katz diagnosis remains the diagnostic of choice at the moment, also for regulators.

We found that praziquantel at the investigated doses, was well tolerated by SAC and PSAC with only mild adverse events observed over time. Almost all adverse events disappeared within the 24 h following praziquantel administration. Our findings are in line with previous studies assessing safety of praziquantel in SAC[28–30] and PSAC.[31–33] Several issues are worth highlighting. First, fewer adverse events were noted in the placebo group compared with the praziquantel groups for both age groups. Second, no statistical difference was observed in the number of adverse events between treatment groups. Praziquantel shows good tolerability even at the highest dosage of 60 mg/kg. Third, although the frequency of adverse events peaked at 3 h post treatment in SAC; in PSAC, fewer clinical symptoms were reported 3 h post treatment compared with symptoms before treatment. However, we note that mothers were acting on behalf of their children in the statement of adverse events which could strongly influence this result.

In conclusion, praziquantel shows lower efficacies in PSAC compared with in SAC with none of the doses achieving a satisfactory ERR based on WHO guidelines[34] and moderate cure rates observed at all doses. However, in the absence of alternative treatments, a single dose of praziquantel (40 mg/kg), as recommended by WHO can be recommended for both *S mansoni* infections in PSAC and SAC in preventive chemotherapy programmes.

Contributors

JTC, JH, and JKe designed the study. JTC, GP, KDS, JKe, and JKo implemented the study. JTC, GP, JH, and JKe analysed and interpreted the data. JTC and JKe wrote the first draft of the report. GP, KDS, and JH revised the report. All authors read and approved the final version of the report.

Declaration of interest

We declare no competing interests.

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Chapter 5b

Characterizing the biochemical response to *Schistosoma mansoni* infection and treatment with praziquantel in preschool and school-aged children

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Characterizing the Biochemical Response to *Schistosoma mansoni* Infection and Treatment with Praziquantel in Preschool and School Aged Children

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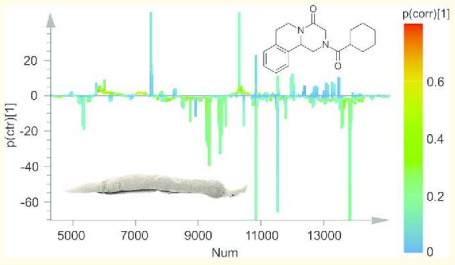
Supporting Information

ABSTRACT: Schistosomiasis is a widespread chronic neglected tropical disease prevalent mostly in children in under-resourced rural areas. Its pathological effects have been clinically characterized, yet the molecular-level effects are understudied. In this study, the biochemical effects of *Schistosoma mansoni* infection and praziquantel treatment were studied in 130 preschool aged and 159 school aged infected children and 11 noninfected children in Azaguié, Côte d'Ivoire. Urine samples were collected prior to receiving 20, 40, or 60 mg/kg of praziquantel or a placebo, as well as 24 h post-treatment, and at the 3-week follow up. Urinary metabolic phenotypes were measured using ^{1}H NMR spectroscopy, and metabolic variation associated with *S. mansoni* infection and praziquantel administration was identified using multivariate statistical techniques. Discriminatory metabolic signatures were detected between heavily infected and noninfected children at baseline as well as according to the dose of praziquantel administered 24 h post treatment. These signatures were primarily associated with the metabolic activity of the gut microbiota, gut health and growth biomarkers and energy and liver metabolism. These analyses provide insights into the metabolic phenotype of schistosomiasis and treatment with praziquantel in two important demographics.



KEYWORDS: *Schistosoma mansoni, metabolic profiling, metabolism, preschool aged, school aged, children, praziquantel*

INTRODUCTION

With just under 190 million people infected, resulting in over 1.8 million Disability Adjusted Life Years (DALYs) lost, schistosomiasis is the third single most important parasitic disease next to malaria and intestinal nematode infections.[1,2] The disease is common throughout the tropics and subtropics and mostly affects people, especially children, living in poor rural areas of low and middle income countries.[3] The infective agent is a blood fluke of the *Schistosoma* genus, where the intestinal form of the disease is caused predominantly by *Schistosoma mansoni*. Adult-stage worms reside in the veins of the mesenteries alongside the intestine, where they lay tens to thousands of eggs per day.[4] The bulk of the pathology is due to eggs that become lodged in proximal organs such as the liver or intestines where they provoke strong Th2-type responses that envelop the eggs in granulomas, which eventually fibrose.[5] This results in both chronic inflammation and degradation of organ tissue, which also leads to portal hypertension and blood shunting, anemia, intestinal polyps and abscesses, blood in stools, and esophageal varices. The severity and frequency of symptoms are thought to be associated with infection intensity.[4] In children, schistosomiasis is associated with malnutrition, and growth and cognitive stunting.[6]

As most of the disease burden is due to disability rather than lethality, the crux of the WHO control strategy is morbidity reduction by preventative chemotherapy in the form of regular mass drug administrations (MDA) to target populations.[3] The drug of choice is praziquantel, a safe, effective and easy to use oral tablet administered at a single dose. For a long time, the main target population was school aged children. However, cumulative evidence has shown that preschool aged children (<6 years) are also significantly impacted,[7,8] leading to a WHO recommendation to treat them on a case by case basis.[9] Aside

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from a handful of efficacy and safety studies, little is known about the effects of praziquantel in this age group, which is pivotal in light of parallel efforts to develop a pediatric praziquantel formulation.[10]

While the macro-level effects of schistosomiasis are well characterized, the disease is poorly understood at the molecular level. Metabolic phenotyping (metabonomics) is a field of "omics" defined as the study of the metabolic responses of multicellular organisms to patho-physiological stimuli.[11] With respect to schistosomiasis, metabonomic analyses in rodent models of infection have revealed disturbances in metabolites related to energy (glycolysis, TCA cycle) and amino acid metabolism, inflammation and collaborative gut microbial-mammalian co-metabolism, in infected versus noninfected rodents.[12−14] In contrast, metabolic phenotyping of urine or stool samples from human schistosomiasis cases are scarce. A study by Ballog et al. investigated the biochemical response of *S. mansoni*-infected school aged children and adults in the Mayuge district of Uganda pre- and post-treatment with praziquantel.[15] Differences were observed in metabolites associated with energy metabolism and gut microbial activity between heavily infected and noninfected adults, whereas differences in children were less certain.

In this present study, we have applied a metabolic phenotyping approach to determine the metabolic variation between *S. mansoni*-infected and noninfected preschool and school aged children from the rural region of Azaguié in Côte d'Ivoire. We studied the metabolic responses of praziquantel treatment in a dose−response manner, with the aim of elucidating whether they differ between school aged and preschool aged children. Urinary metabolite profiles were measured using ^{1}H nuclear magnetic resonance (NMR) spectroscopy and the metabolic variation associated with infection and treatment response was identified using multivariate statistical techniques.

■ METHODS

Study Design and Ethical Considerations

This study was embedded within a pediatric praziquantel dose−response study in the health district of Azaguié in southern Côte d'Ivoire, which is detailed in a publication by Coulibaly et al.[16] Ethical approval for the study was obtained by the National Ethics Committee of the Ministry of Health in Côte d'Ivoire (CNER, reference no. 037/MSLS/CNER-dkn) and the Ethical Committee of Northwestern and Central Switzerland (EKNZ; reference no. 162/2014). In brief, 161 preschool aged children (aged 2−5) from the villages of Makouguié, Odoguié, M'Bromé and Elevi, and 180 school aged children (aged 6−15 years) from Makouguié, infected with *S. mansoni* were eligible for the study. The children were stratified according to infection intensity (light (<100 eggs per gram feces) or moderate and heavy (100 to 400 eggs per gram feces and >400 eggs per gram feces, respectively)), and randomized to either 20, 40, or 60 mg/kg praziquantel or placebo. Complete urine samples were collected from 130 preschool and 159 school aged children at 3 time-points: prior to treatment, 24 h post-treatment and 3 weeks post-treatment. Urine samples were also collected from 11 noninfected children from Makouguié at parallel time-points, as a comparator group. Anthropometric and parasitological data from the sampling population are provided in Table S1. Samples were immediately stored on ice throughout the day and transported to a −80 C° freezer until cold-chain shipment, and then stored at −80 C° until use.

^{1}H NMR Spectroscopy

Urine samples (630 μL) were thawed to room temperature and combined with phosphate buffer (70 μL; 1.5 M KH_2PO_4, 2 mM NaN_3 and 1% TSP in D_2O; Sigma-Aldrich, Switzerland). The samples were vortexed, centrifuged (13 000g for 10 min) and 600 μL of the supernatant was transferred to a 5 mm diameter NMR tube. A pooled urine sample was also created to monitor instrument stability. Spectral profiles were acquired using a 600 MHz Bruker Avance III spectrometer at the Clinical Phenotyping Centre (CPC), Imperial College London. ^{1}H NMR spectra were acquired using a standard one-dimensional (1D) pulse sequence using the first increment of the NOE pulse sequence for water suppression, as previously described.[17] Raw spectra were phased, baseline corrected and calibrated to TSP using Topspin 3.2 (Bruker Biospin) before being digitized and imported into the MATLAB environment (Version R2014a; Mathworks Inc., USA). Redundant peaks derived from water, urea and TSP were removed from the spectra before manual alignment and normalization to the probabilistic quotient using in-house MATLAB scripts developed at Imperial College London.

Data Analysis

The clinical trial metadata and preprocessed spectra were imported into SIMCA (Version 14.1; Umetrics) for multivariate data analysis. Principal components analysis (PCA) was initially performed on all the spectral profiles to identify outliers in the data set, which were subsequently removed (13 samples were removed due to implausibly excessive acetate concentrations, which were suspected to be contaminated due to improper storage, and 2 due to the presence of large amounts of unidentified foreign metabolites). Supervised statistical analyses namely, orthogonal partial least-squares (OPLS) and OPLS-discriminant analysis (OPLS-DA), were used to study the refined data set and in the case of OPLS-DA to analyze intergroup variations, for categories of interest. The predictive ability (Q^2Y) of the OPLS models was calculated using a 7-fold cross-validation approach. The significance of the Q^2Y values was assessed by permutation testing (1000 permutations, with a p value threshold of $p < 0.05$). For all valid models, both an OPLS or OPLS-DA coefficients plot constructed in MATLAB were used to identify peaks that significantly influenced the model. Significant metabolic associations were identified using correlation coefficient (R) cutoff values ($p \leq 0.05$) of 0.115, 0.311, 0.116, 0.174, and 0.152 for the age, infection status, praziquantel all, praziquantel preschool age and praziquantel school-age models, respectively. Metabolites were identified using in-house databases, referencing to previous literature and Statistical Correlation Spectroscopy (STOCSY).

■ RESULTS AND DISCUSSION

Metabolic Differences Associated with *S. mansoni* Infection Status

A PCA model was built on all preintervention profiles to identify metabolic variation of the study population, and thus also to identify potential confounders. From these PCA models, metabolites were found not to vary across village or sex but were observed to vary according to the age of the child (Figure S1). An OPLS model was constructed to identify metabolic variation associated with age (N = 289; 1 predictive and 1

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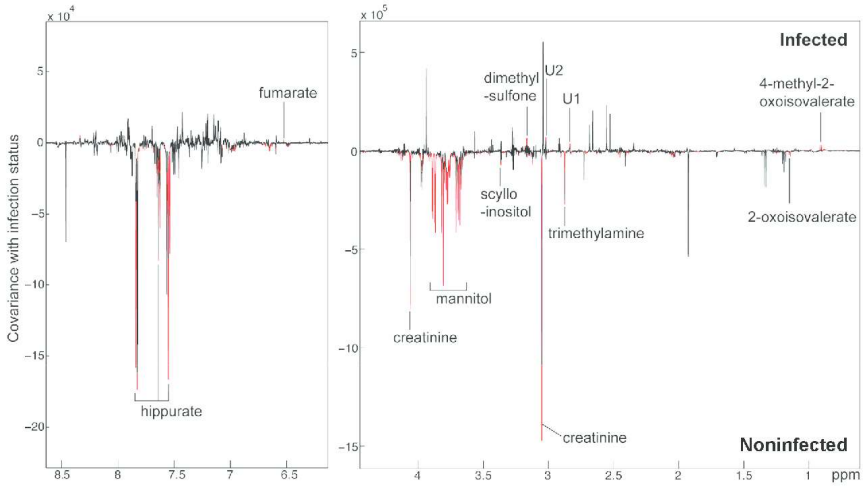

Figure 1. Urinary metabolic profiles of heavily infected (>400 eggs per gram feces) and noninfected children at baseline. Coefficients plot from an OPLS-DA model comparing heavily infected and noninfected children (N = 39, 1 predictive and 1 orthogonal component, Q^2Y = 0.317, p < 0.05). Significant metabolites (correlation coefficient (R) > 0.311) are shown in red and listed in Figure 3. U1, unidentified metabolite 1; U2, unidentified metabolite 2.

orthogonal component; Q^2Y = 0.698; p < 0.01), from which an OPLS coefficients plot was produced to identify significantly contributing metabolites (Figure S2; Figure 3). Creatinine and citrate excretion was positively associated with age, while the excretion of acetate, alanine, creatine, glycine and succinate was negatively associated. This is largely consistent with findings from metabolic studies of aging in children by Gu and colleagues (2009). The excretion of several metabolites associated with the metabolic activity of the gut microbiota were also observed to change in an age-dependent manner, which differed from the Gu et al. study. Hippurate excretion increased with age while 4-hydroxyphenylacetate (4-HPA), dimethylamine (DMA), dimethylglycine (DMG), and trimethylamine (TMA) (metabolites associated with gut microbial choline metabolism) decreased.[18] Reduced excretion of 4-HPA, DMA and TMA was also incidentally observable in mice fed on a protein or zinc deficient diet,[19] which potentially reflects progressive malnourishment with age. This is consistent with lower WAZ scores in the older children of this study (Table S1). Due to the above-described age-related variation, subsequent models were verified to ensure homogeneously distributed age profiles, to prevent potential bias.

An OPLS-DA model built on pretreatment profiles comparing 28 heavily infected (>400 eggs per gram feces) and 11 noninfected children identified discriminatory metabolites between the groups (1 predictive and 1 orthogonal component; Q^2Y = 0.317, p < 0.05) (Figure 1). Heavily infected children were observed to excrete lower amounts of 2-oxoisovalerate, TMA, creatinine, hippurate and mannitol (Figure S3), compared to uninfected children (see Figure 3), while 4-methyl-2-oxovalerate (ketoleucine), fumarate, dimethylsulfone and 2 unknown metabolite peaks (δ 2.85 singlet and δ 3.02 singlet), which were found to correlate strongly by statistical correlation spectroscopy (STOCSY), were excreted in greater amounts. Some of these metabolites are associated with metabolic pathways of the liver, where schistosomiasis causes severe inflammation and fibrosis. For example, 2-oxoisovalerate is a branched chain organic acid and is a metabolite of valine produced in the liver.[20] Aside from a positive association with muscle mass, decreased excretion of creatinine is associated with liver cirrhosis and renal function.[21] Hippurate is a microbial-host cometabolite produced in the liver: gut microbiota metabolize diet polyphenols to benzoic acid, which is subsequently conjugated with glycine in the liver of the host to produce hippurate. Lower excretion of hippurate in the presence of infection might indicate altered hepatic metabolism and/or a disruption to the metabolic activity of the intestinal microbiota, which was also recently found to be perturbed by schistosomiasis.[22−24]

Lower mannitol excretion with infection is of particular interest. Mannitol is present in large concentrations in cassava, a local staple, and is not readily metabolized. The mannitol-lactulose test is frequently employed as a measure of gut permeability where low urinary excretion of mannitol following the intake of a set dose is reflective of malabsorption.[25] Lower mannitol excretion has also been associated with gut inflammation.[26] Conversely, in a recent study of malnourished children in Brazil, where the mannitol-lactulose test was also employed, higher excretion of mannitol was positively associated with healthy growth rate.[27] Low urinary excretion of mannitol in the infected children may therefore be a product of infection-induced gut pathology and its resultant impairment of gut function, which can lead to growth stunting. Consistently, infected children displayed lower TMA excretion, which was also associated with decreased villus height (a metric of intestinal health) in a group of malnourished Zambian children,[28] and higher urinary excretion of ketoleucine, which is directly associated with a lower body-mass index (BMI).[29]

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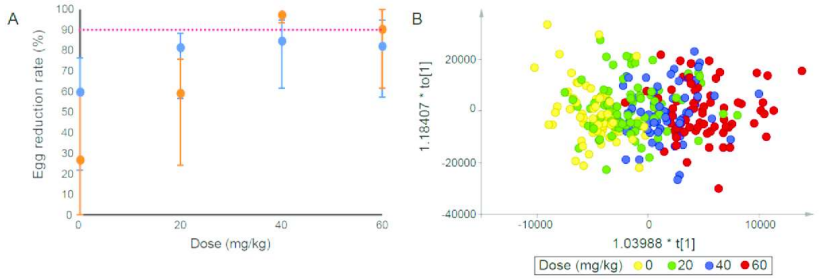

Figure 2. Dose−response to praziquantel. (A) Egg reduction rate based on arithmetic mean for preschool aged children (blue) and school aged children (red), determined from the dose-finding study from which the urine samples are sourced.[16] The red dotted line is the WHO drug efficacy benchmark of a 90% egg reduction rate. (B) OPLS model (N = 285; 1 predictive and 1 orthogonal component; Q^2Y = 0.371, p = 0.01) comparing the dose−response of urinary metabolic profiles 24 h post praziquantel administration for both preschool and school aged children (yellow = 0 mg/kg (placebo), green = 20 mg/kg, blue = 40 mg/kg and red = 60 mg/kg).

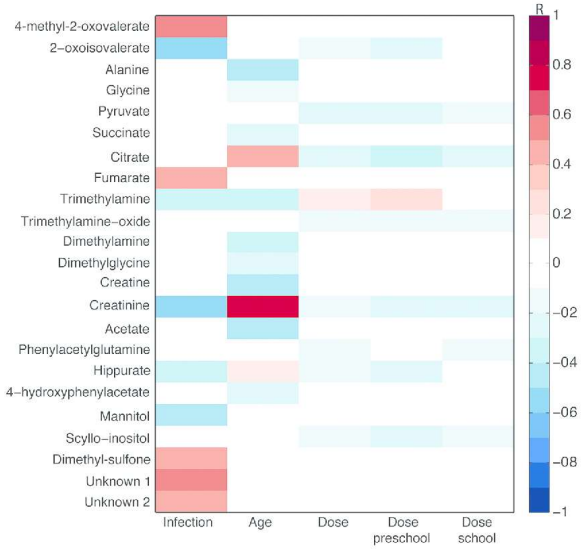

Figure 3. Metabolites associated with age, infection status and praziquantel intake. Dose refers to metabolites associated with increasing praziquantel dose 24 h post-treatment and are shown also separately for preschool (Dose preschool) and school aged children (Dose school). $R(P(\mathrm{cor}))$ values were extracted from the OPLS models constructed for each comparison.

Metabolic Responses to Praziquantel Treatment

In the clinical trial on which this study was based, praziquantel had a slightly lower efficacy in preschool aged children compared to school aged children. While the 40 mg/kg dose met the WHO drug efficacy benchmark for 90% egg reduction rate in the school aged children, none of the doses administered achieved this threshold in the preschool aged children (Figure 2A; Coulibaly et al. 2017[16]). We were thus interested in the immediate metabolic responses to praziquantel treatment and how they differed between the two age groups at 24 h post-treatment. An OPLS model was built to identify significant urinary metabolic alterations associated with increasing praziquantel doses in all study children 24 h post-treatment (Figure 2B; 1 predictive and 1 orthogonal; Q^2Y = 0.371 p < 0.01). The excretion of citrate, creatinine, pyruvate, trimethylamine-oxide (TMAO) and *scyllo*-inositol was negatively correlated with praziquantel dose 24 h after treatment (see Figure 3). A decrease in citrate and pyruvate indicate perturbations to the TCA cycle. The depletion of creatinine could reflect an increase in the glomular filtration rate in response to treatment, which may also explain the increased excretion of *scyllo*-inositol, a sugar alcohol from the coconut palm, which is an abundant part of the children's diet.[30] No traces of praziquantel or praziquantel metabolites were found at

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this time point, which is consistent with its clearance rate ($t_{1/2}$ of 2−4 h).[31]

To identify age-dependent differences in the metabolic response to praziquantel, separate OPLS models were constructed on the urine profiles collected at 24 h for preschool and school aged children, using praziquantel dose as the response variable (Figure 3). Praziquantel intake reduced the excretion of pyruvate, citrate, creatinine, TMAO, and *scyllo*-inositol in both age groups. In the preschool aged children, praziquantel also reduced the excretion of 2-oxoisovalerate and hippurate but increased the excretion of TMA (N = 127; 1 predictive component; Q^2Y = 0.358; p < 0.05). This was not observed with school aged children, who excreted less phenylacetylglutamine (PAG) following praziquantel intake (N = 158; 1 predictive and 1 orthogonal component; Q^2Y = 0.507; p < 0.05). As TMA, hippurate and PAG are gut microbial cometabolites, these differences might indicate that gut microbes are differently affected by praziquantel intake between the two age groups. Indeed, a microbial analysis of 24-h post-treatment stool samples from the same children found that praziquantel intake did affect the abundance of the *Bacilli* and *Erysipelotrichi* classes, the ML615J_28 order and *Actinobacillus* genus.[24] However, the abundances did not vary between age groups. This suggests that although praziquantel did not differentially affect the microbial community structure between the two age groups, it did have a different impact on its functional status.

No praziquantel-associated changes were observed at the 3-week follow up time-point. This indicates that the biochemical perturbations induced by a single dose of praziquantel do not persist. It also suggests that the functional modifications to the gut microbiota induced by praziquantel are transient, consistent with findings from Schneeberger et al.[24]

A limitation of this study is that it is difficult to differentiate whether these biochemical alterations are due to the metabolic impact of praziquantel or due to clearance of the infection or both. The linear dose−response metabolic effect observed in preschool aged children, which stands in contrast to the rather flat dose−response observed in praziquantel efficacy in this age group, suggest that most of these metabolic alterations are due to the metabolic impact of praziquantel. Nonetheless, the differential metabolic responses to praziquantel treatment between preschool and school aged children, especially with regard to microbial cometabolites, may provide first insights with regard to the differing praziquantel efficacy in these two demographics.

■ CONCLUSION

For the first time, we have conducted a metabonomics study embedded in a clinical trial for dose determination of praziquantel in preschool and school aged children infected with *S. mansoni*, in order to extend our understanding of both disease and treatment dynamics. A number of metabolites were shown to differ between noninfected and heavily infected children in pretreatment urine samples. The metabolic alterations observed were indicative of disruptions to metabolism in the liver, functional changes in the gut microbiota and the gut itself and impoverished nutrition status. These observations may help to understand the biochemical mechanisms through which schistosomiasis can contribute to growth and developmental stunting. Moreover, several metabolic perturbations were identified 24 h after praziquantel intake, some of which differed between preschool and school aged children, which then resolved 3 weeks post-treatment. These were primarily related to gut microbial-host metabolic interactions. A combined analysis of these metabolic profiles with gut microbial composition from matching stool samples and matching pharmacokinetic profiles from the study is envisioned to shed light on these interactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00910.

Table S1: Baseline characteristics of sampled population; Figure S1: Metabolite distribution of sampled population at baseline; Figure S2: OPLS model of metabolite distribution according to age; Figure S3: STOCSY for the identification of mannitol (PDF)

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Notes

The authors declare no competing financial interest.

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Chapter 6

Discussion

6 Discussion

Every few years, the global disease burden numbers are published, where schistosomiasis prevalence and disease burden numbers indicate that schistosomiasis remains a significant public health burden ((Murray et al., 2016; Murray et al., 2012; Vos et al., 2016). To that end, the World Health Assembly Resolution WHA 65.1 coupled with the London Declaration make for bold statements to place schistosomiasis on the path to control or elimination, by, amongst other things, increasing treatment coverage (Barry et al., 2013; Webster et al., 2014). Yet ensuring proper coverage means ensuring all relevant populations are targeted and this includes pre-school aged children, for which special adaptations may be required (Stothard et al., 2013). Moreover, the increased distribution of praziquantel, while necessary, increases drug pressure and therefore places the antischistosomal therapeutic options in a nefarious situation. This is not an affordable risk and therefore, treatment alternatives and a modernization to the antischistosomal pipeline are required.

The two main aims of the thesis were to boost the antischistosomal pipeline and to broaden our understanding of schistosomiasis infection and treatment needs of young children. Within the framework of the first aim, we sought to accelerate the drug screening process by investigating automated drug assay alternatives. We reviewed the drug repurposing landscape and leveraged it to screen a medium library of diverse FDA approved compounds. We sought to rescue an old antischistosomal lead, Ro 13-3978, and the now phased out oxamniquine, by profiling the activity and mode of action of the former and testing potent organometallic analogues of the latter. The second aim was carried out by undertaking a praziquantel dose-finding study to inform effective doses for a paediatric formulation. In the framework of this study, we elucidated the metabolic effects of infection and treatment in this group.

Much of the results have been discussed in detail within the manuscripts. Here in this discussion, I would like to extract the lessons learned from these studies in order to outline prospects and challenges for the respective areas covered in this thesis, namely automated drug screening against *S. mansoni,* microfluidics and electrical impedance spectroscopy, drug repurposing and drug rescuing, treatment of pre-school aged children with praziquantel, and the relevance of metabonomic and integrated “omics” for schistosomiasis.

6.1 Automated drug screens against *S. mansoni*

With publications of the *S. mansoni, S. haematobium* and *S. japonicum* genomes and proteomes, combined with enzyme expression systems as well as advances in chemoinformatics, target-based screening is increasingly aiming to integrate into the antischistosomal drug discovery pipeline (Berriman et al., 2009; Neves et al., 2016; Sotillo et al., 2015; Verjovski-Almeida and DeMarco, 2008; Young et al., 2012; Zhou et al., 2009). Indeed, such approaches have helped to identify oxadiazole 2 oxides (though this candidate is no longer undergoing development) as well as a few other chemical entities such as thioredoxin glutathione reductase (TGR) and peroxiredoxin (Prx) inhibitors of *S. mansoni* (Sayed et al., 2008; Simeonov et al., 2008). Though this will continue to be an important developing field, an incessant issue is that *in silico* or enzyme-based target validation does not necessarily correlate to real biological systems, as demonstrated by our FDA screen and analyzed by Neves et al. (Neves et al., 2016; Panic et al., 2015b). Validation with a whole organism screen is always required and therefore *in silico* approaches are rather complementary and informative instead of a replacement (Neves et al., 2016).

Indeed, whole organism screening remains the most important source of new chemical leads for helminths and the drug discovery field in general (Caffrey, 2012; Lang et al., 2006). Yet the drawbacks of the current standard of compound evaluation, manual microscopic evaluation, are undeniably an obstacle to processing large compound libraries and, in addition, can be subjective (Peak and Hoffmann, 2011; Ramirez et al., 2007).

In our pursuit to establish an automated and higher throughput drug sensitivity assay for *S. mansoni* NTS (larval-stage worms), we explored a plethora of options. The easiest and most obvious was to simply add a fluorescent or luminescent dye to the existing assay where the fluorescence or luminescence signals are a proxy for worm viability or cytotoxicity and can be read by a simple plate reader. Indeed, many such assays have been validated before, be it for cell lines or protozoans (Page et al., 1993; Smilkstein et al., 2004; Sykes et al., 2012). The ways in which such dyes function are thoroughly described in Chapter 2a, hence I will not review them again.

Of the 11 markers tested, we were successful in identifying only one, CellTiter-Glo®, that appeared to correspond well to worm viability. Moreover, its use in a small screen allowed for identification of single drug concentration “hits” (with 100% correspondence to hits identified by visual assessment) as well as extrapolation of IC_{50} values for some drugs. Incidentally, Lalli and colleagues also published on the CellTiter-Glo® assay with similar findings, (Lalli et al., 2015). Though this presents a development towards automation, there are clear limitations with this and marker-based assays in general. First, the use of CellTiter-Glo® method incorrectly classifies praziquantel as completely inactive at concentrations that are

known to severely damage larval-stage worms (confirmed by both publications). As we discussed in Chapter 2a, this phenomenon is not unique to this assay, but is observed also in other marker-based assays and in isothermal calorimetry (Manneck et al., 2011; Mansour and Bickle, 2010; Peak et al., 2010). The reasons for this are unknown, however praziquantel is known to disrupt Ca^{2+} homeostasis, which could potentially disrupt mitochondrial ATP production (Chan et al., 2013; Tarasov et al., 2012). As anthelmintics often operate by disrupting ion homeostasis, which could affect ATP homeostasis, these phenotypes could be missed in such screens (Greenberg, 2014; Khanna et al., 2014).

This leads to the second point- though Lalli et al. used this method for a larger 1712 compound screen, the accuracy of the assay has not been properly validated for a large-scale library (Lalli et al., 2015). A z-score for each assay was calculated, but this is only a measure of assay quality based on signal variation (Zhang et al., 1999). Accuracy validation would require a visual microscopic cross-check for a larger compound library, which we performed in our publication on a very small sub-set of compounds. However, we decided not to validate further because of the marker's above-mentioned and two additional limitations, namely its cost and single-point measurement. In other words, consumable costs for CellTiter-Glo® are too high -and for us, not worth investing into making a cheap "in house" version- to justify replacing visual assessment. Moreover, dye-based assays in general are suitable for only one end-point screen; if additional time-points are desired, additional assay set ups are required (Peak and Hoffmann, 2011). I will elaborate on the importance of this latter point later on. In brief, in theory it is possible to use this method for large-scale Yes/No screens, if one decides the possible false negatives and cost of consumables are acceptable, however it would be prudent to conduct additional validation steps.

In recognizing these limitations, we aimed to investigate a label-free method where additional time-points could be measured. In that light, investigations with the novel calScreener™ isothermal calorimeter appeared promising. Initial investigations by Manneck et al. showed good correlation to adult worm viability but required 400 to 1000 NTS per replicate to observe any signal (Manneck et al., 2011). The calScreener™ should have greater sensitivity due to its smaller ampules (300 µl volume vs 3 ml for the TAM 48) and their increased heat capacity (Braissant et al., 2015). Indeed, as we demonstrated, it was more sensitive to picking up adult worm activity for a number of helminths including *S. mansoni.* However, NTS heat flow could still not be sufficiently detected and the small ampule volume proved to be too small for adult *S. mansoni* to survive. From this and previous studies, I would conclude that microcalorimetry is an excellent tool to more specifically characterize compound activity on

adult-stage helminths, but it would take major developments for it to be sensitive enough for larval stage screens (Flores et al., 2015; Manneck et al., 2011; Tritten et al., 2012).

Developments in high-content microscopy drove the development of a label-free high-throughput image-based system screening assay for *S. mansoni* larvae (Paveley et al., 2012; Starkuviene and Pepperkok, 2007). In their initial paper, they described a method in which both motility and morphology of NTS could be scored using image overlay software and a library of NTS drug-treated phenotypes. Recently, they published their screen of 300 000 compounds employing this method, of which 18774 NTS hits were presented (Mansour et al., 2016). Although this definitely presents as the largest screening campaign conducted on *S. mansoni* NTS thus far, improvements to this system are both possible and necessary. First, the original published protocol shows praziquantel as having a moderate effect on NTS morphology and an overall combined morphology/motility effect score in the range of oxamniquine. Yet as mentioned, praziquantel has a rather severe morphological effect on larval schistosomes, whereas oxamniquine only has a minor one, and in manual screens the IC_{50} values for these two compounds are at least an order of magnitude apart (Meister et al., 2014; Panic et al., 2015a). Since both are classified as hits under the protocol, likely many false positives are generated as also indicated in their initial publication. Second, again, both onset of action and IC_{50} values are not yet determinable using this approach. It is arguable that this is simply a pre-screening method and that more refined methods can be applied at the adult worm *in vitro* screening stage. Yet the increasing availability of compounds for repurposing would allow us to compare these two parameters -onset of action and *in vitro* IC_{50} values- with available pharmacokinetic parameters which, when combined, could be more indicative of good *in vivo* activity than simply yes or no screens. In the section that follows, we present a platform that could potentially offset the drawbacks and make the case for how this could be useful in the antischistosomal screening cascade.

6.2 Microfluidics and electrical impedance spectroscopy for *in vitro* drug screening against *S. mansoni* NTS

The field of microfluidics coupled with electrical impedance spectroscopy (EIS) is rapidly expanding into the drug discovery realm (Kang et al., 2008). Microfluidics allows for the handling of biological organisms at single-cell resolution and a tight control of its microenvironment. Consequently, microfluidic devices have been successfully applied to study organisms such as invertebrate and vertebrate embryos, tissue samples or cultured tissue spheroids and even *C. elegans* at various growth stages (Haandbæk et al., 2014). Meanwhile EIS allows for the study of electrical properties of such organisms, which can

reveal information about changes in cell/tissue structure, membrane integrity and even organelles (Bürgel et al., 2015).

In 2014, in order to search for automated compound screening alternatives, we started a collaboration with the Bio Engineering Laboratory (BEL) group at the ETH Zürich on a microfluidic platform for evaluating *S. mansoni* NTS viability using electrical impedance spectroscopy (EIS), at single larvae resolution. A simple EIS microfluidics chip was fabricated to test if NTS viability could be measured using such a device (Fig 1A). The general principle theory is as follows: a microfluidic channel filled with medium and a single NTS is interfaced with two electrodes. When a current is applied an electric field is generated between the electrodes. A passing object (in this case the NTS) creates resistance to the current which is proportional to membrane permeability (i.e. membrane damage) as well as object size, amongst other things. The impedance can be measured and correlated to visually inspected worm viability. Initial results indicate differential impedance signals between live and dead NTS across a range of frequencies. (Fig 1B and C).

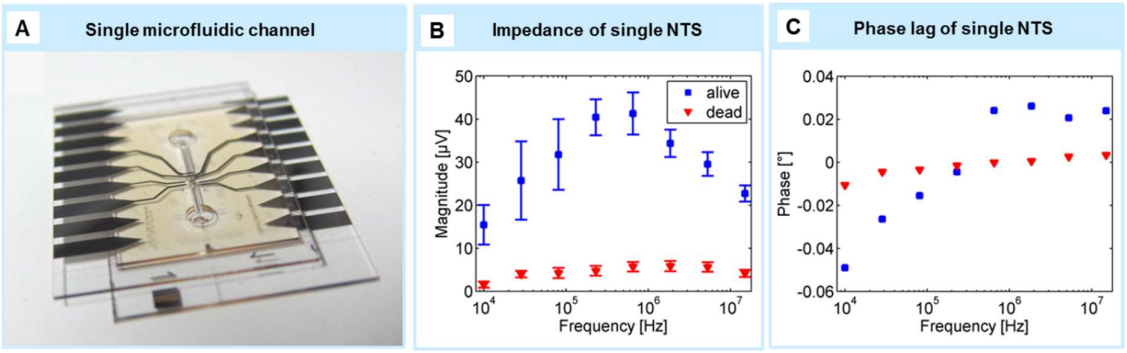

Figure 1 A) Test chip with a single microchannel and platinum electrodes, wherein single NTS can be cultured and measured for B) Impedance of a single NTS (replicates) and C) phase lag over increasing frequencies.

Subsequent tests measured if not only morphological properties but also motility could be measured using this system. This is based on the fact that impedance is altered according to changes in both the area and length of the object, which can correspond to NTS contractions. Our tests show sizable fluctuations in impedance with motile NTS and no significant fluctuations from non-motile NTS (Fig 2A). These fluctuations can be correlated to gradients in motility resulting from exposure to serially diluted drug concentration from which an IC_{50} value can be extracted (Fig 2 B). Because it is possible to culture the NTS within the chip, all of these parameters can be measured at single time-points or continuously.

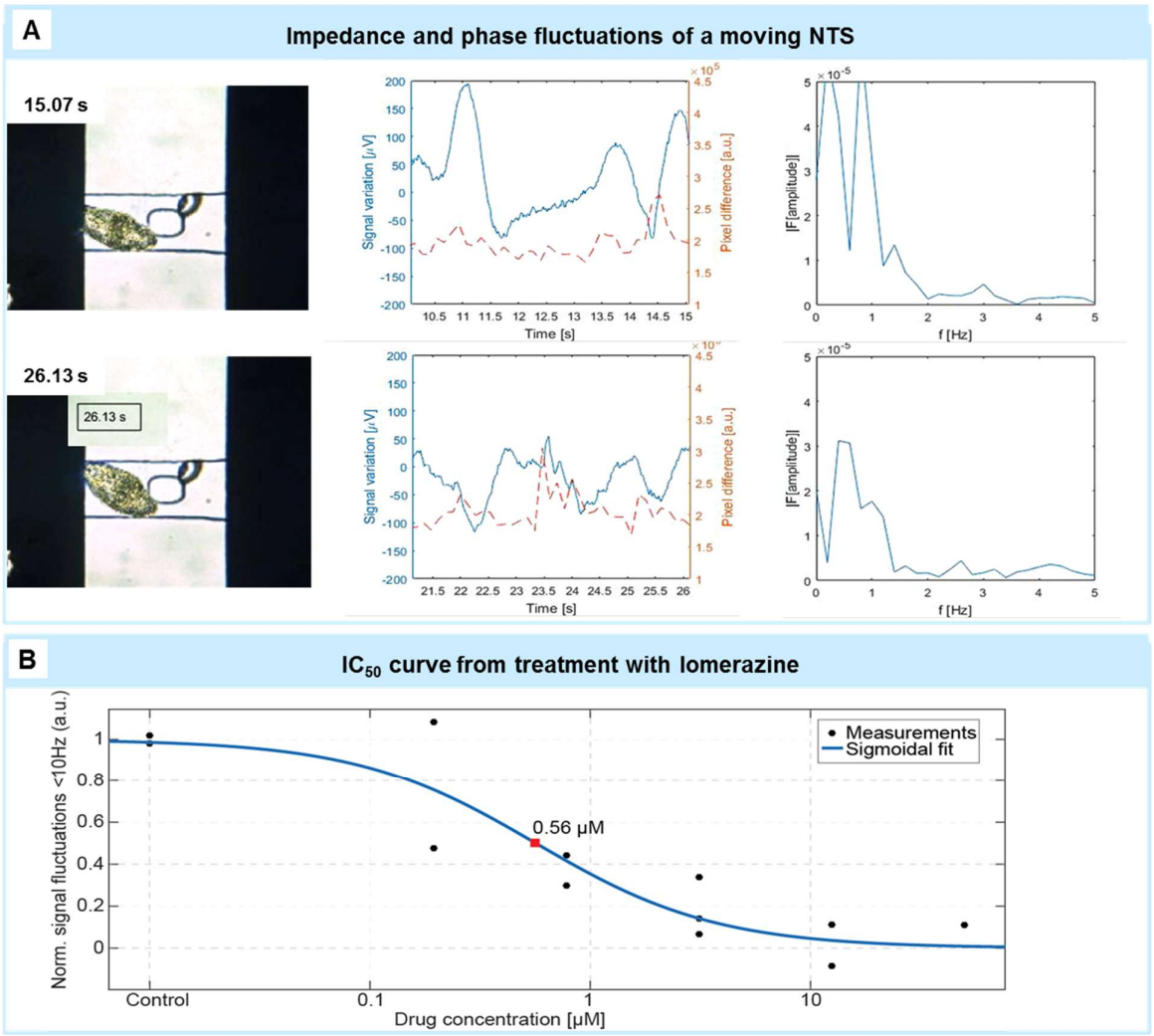

Figure 2 Impedance and phase fluctuations are observable for moving NTS. (A) A recording was taken of a viable NTS pegged between two electrodes; here a snapshot at 15.07 s and 26.13s. B) NTS treated with a dilution series of an *in vitro* active drug, lomerazine, show gradually decreased capabilities in motility, which are measurable using EIS. These were converted to an IC_{50} value (0.56 μM) that was near identical to the value determined by manual microscopy (0.54 μM).

The ability to characterize NTS drug responses at single or over several time points and at single or multiple drug concentrations (using only a few larvae) would present as another and precise automated screening method. However, it also provides richer quality data, which allows us to take this device a step further towards more informative *in vitro* lead selection. The ability to screen a high content of compounds on NTS (as displayed by Paveley et al.) brings into question how to prioritize hits. Often it has been the case that a compound exhibits excellent *in vitro* activity, with a low IC_{50} and a rapid onset of action on all worm stages, yet is inactive or poorly active *in vivo* (Cowan and Keiser, 2015; Guidi et al., 2016;

Ingram-Sieber et al., 2014). Several assays can be employed after activity characterization to help estimate, for example, cytotoxicity (such as the L6 rat skeletal assay), the logP of the compound (such as the PAMPA assay), or compound stability in the face of liver metabolism (such as the microsomal stability assay) (Cowan et al., 2015; Gülden, 1993; Obach, 2001). These are, however, laborious additional steps with extra costs.

Conversely, there is no way to identify a prodrug (a drug where not the parent compound but its metabolites are active) without testing *in vivo* first. This is both ethically and logistically not feasible with a compound library. Though we could in theory accept this as an affordable loss, many drugs are actually prodrugs and neither of the very active *in vivo* compounds discussed in Chapter 4 would have been captured in a typical screening cascade (Filho et al., 2007, 2007; Valentim et al., 2013).

Beyond evaluating drug effects on NTS, we envisioned a means of stream-lining the above described processes by leveraging recent “body on a chip” (BoC) developments (Bhatia and Ingber, 2014; Huh et al., 2010). Human spherical microtissues can be grown in a hanging drop format and their functionality also assessed in a microfluidic EIS chip (Frey et al., 2014). Such tissues have been used, among other things, to assess cardiotoxicity and hepatotoxicity, which might be more accurate and informative than using single enzymes or cell lines (Kunz-Schughart et al., 2004).

In our conceptualized “infected body on a chip” (iBoC), NTS and spheroid cultures could be co-cultured to measure drug efficacy and toxicity simultaneously (Fig 3A). In addition, incubating the drugs with both the liver tissue and the NTS could reveal if *in vivo* activity is expected to be minimal due to drug metabolism, or conversely, if an *in vitro* inactive drug becomes active upon liver metabolism. Many different chip designs can be fabricated for the different drug assays described and integrated on a single platform (Fig 3B).

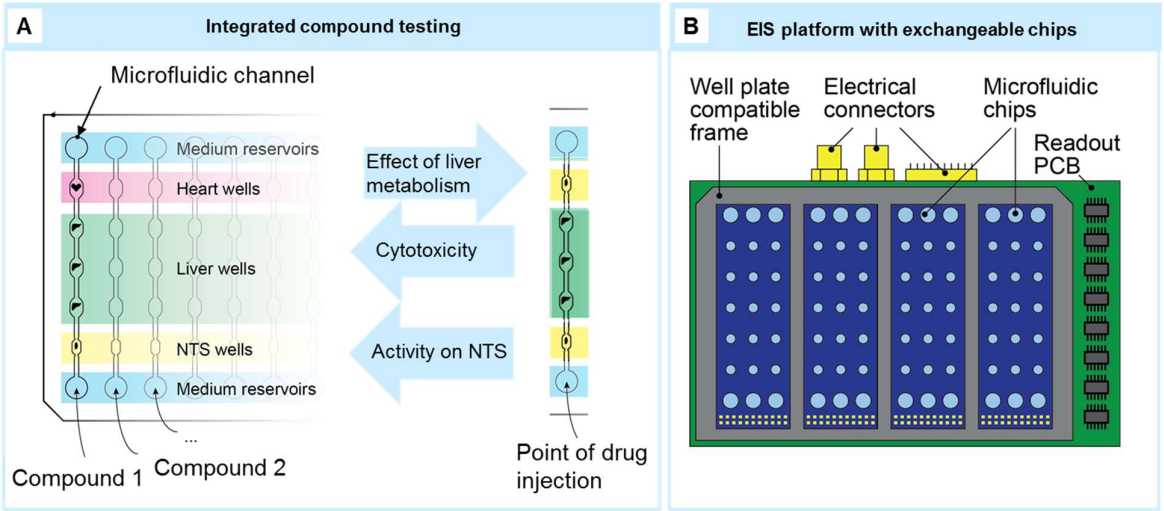

Figure 3 (A) Integrated NTS and spherical tissue in a single channel can test cytotoxicity as well as effects of liver metabolism on drug activity. (B) Different chip designs can be integrated on a single platform for simultaneous and continuous read-outs.

The project requires developments in handling, optimal co-culturing and evaluation parameters and chip design, as well as read-out software for mutli-channel impedance read-outs. These developments are underway, supported by the recent awarding of an SNF Interdisciplinary Grant in collaboration with the group from BEL.

6.3 Drug repurposing challenges and opportunities

There is no doubt that drug repurposing has been an extremely important source of anthelmintics and pre-clinical antischistosomal candidates, as reviewed in Chapter 3a. Since its publication, a number of marketed drugs have been tested on *Schistosoma spp.*, some with more success, such as the chlorambucil anticancer drug showing a 75% WBR in juveniles (Eissa et al., 2017), and some with less success, such as the poor in vivo activity observed for histone/lysine deacetylase inhibitors (Chua et al., 2017).

The repurposing approach is likely to continue as an important source of new leads, especially with increasing avenues for compound sharing, such as the aforementioned NIH initiative for drug repurposing or the Re-search Wisdom Research program (Allarakhia, 2013). Moreover, screens of already marketed drugs bring new information about active pharmacophores which can be shared via open source databases such as DrugBank or Therapeutic Target Database (Knox et al., 2011; Ma'ayan et al., 2007) which can further be explored using cheminformatics approaches and SAR studies (Neves et al., 2016).

All this presents exciting opportunities for antischistosomal drug discovery, yet this does not guarantee a cornucopia. There are some pitfalls to this approach, which are important to examine. First, as we saw with our FDA library screen, many compounds are *a priori* not suitable for oral treatment, as they are either toxic, or poorly absorbed. They may also only be active at a dose that is much different from its original indication. As described in Figure 4 of the introduction, such compounds would then need to be treated as an NCE, which defeats some of the advantages of repurposing (Mucke, 2010). This would then rather be an informational screen about potentially active pharmacophores which is nonetheless informative, even if not truly a repurposing effort.

There may also be a drawback to repurposing drugs with an existing target in humans. In our FDA screen, a very interesting group initially was the angiotensins/ antihypertensives. *In*

vitro, they displayed both low inhibitory concentrations as well as a rapid onset of action. The potent *in vitro* activity was also intriguing due to the indicated target of these compounds, calcium ion or voltage-gated ion channels, which is purportedly also the target of praziquantel, as well as other anthelmintics. It might have indicated an interesting group of compounds to further explore. In addition, their published pharmacokinetic profiles indicated good absorption, which is complementary to their intended target, the blood-dwelling schistosomes. However, *in vivo,* they displayed mild activity at best. A similar finding was presented by Guidi et al. who studied the *in vitro* and *in vivo* efficacy of perhexiline, a drug that was also potent in our FDA screen, but that we decided not to pursue do to its known hepatotoxicity (Guidi et al., 2016). On the one hand, it's possible that clearance is too quick to act on the worms, though we crosschecked the onset of action with the ADME profile. Moreover, praziquantel itself is quickly cleared, but because it is fast acting, the AUC achieved is sufficient to clear the worms (Meister et al., 2014). Many of these drugs are highly protein binding, yet the relationship between protein binding and *in vivo* efficacy is not straightforward (Smith et al., 2010). Hence, since these compounds have a direct target in the host, and in addition, are distributed throughout the circulatory system, they might indeed be binding stronger to their "indicated" target than to the schistosomes. Here, target docking studies would be of interest to assess differential binding affinities.

Finally, even if the repurposed drugs display excellent pre-clinical profiles, caution should be exercised when thinking about their field applications. This was emphasized when the intriguing pre-clinical and clinical profiles of the antimalarials mefloquine and the artemisinins against schistosomiasis were described (Keiser et al., 2010, 2014). It was warned that due to their importance in antimalarial control, these should not be broadly applied for schistosomiasis preventative chemotherapy (Keiser and Utzinger, 2012).

Nonetheless, many of these investigations can be a short-cut to uncovering new pharmacophores that are active against *Schistosoma spp.* Indeed, precisely the mechanism of action and structure-activity relationship studies of the artemisinins inspired a new generation of synthetic peroxides with analogues that are active against *Schistosoma spp.*, (Boissier et al., 2009; Keiser et al., 2012). These can further be fed into open source drug banks and chemoinformatic databases to better inform both *Schistosoma* drug targets as well as promising drug scaffolds for further re-design (Neves et al., 2016), the latter of which lead to successful *in vivo* active organometallic oxamniquine derivatives discussed below.

6.4 Drug rescuing and redesign

In our investigation of the dropped antischistosomal candidate, Ro 13-3978, we characterized the potent activity of this compound against all worm life stages, which was more efficacious than praziquantel *in vivo.* Despite its broad stage activity, Ro 13-3978 was found to be very species-specific, with no activity against the related trematodes, *Echinostoma caproni* and *Fasciola hepatica.* Its rather weak activity *in vitro* was contradictory to its potency *in vivo*, and the lack of significant structural damage *in vitro* was confirmed with our SEM studies comparing worms exposed to high concentration of the drug *in vitro* and *ex-vivo* worms treated with 100 mg/kg. The SEM studies coupled with hepatic shift experiments were also revealing of the onset of action; at 24 h some disruptions to the tegument are visible but by 48 h, the tegument is completely damaged. Dispelling the notion that it is a prodrug (Ro 13-3978 was stable when incubated with liver microsomes), this time frame, along with some visible but vague and unidentifiable cell attachments to tegument (not shown) prompted the theory that activity could somehow be immune-mediated, as was demonstrable for praziquantel and oxamniquine (Doenhoff, 1989).

In considering the size of the adult worm, it was decided that a good first approach to investigating this theory was with immunohistochemical staining of mesenteric vein and liver sections, from *S. mansoni*-infected mice, containing the worm at 24 and 48 h post-treatment (before and after hepatic shift and around the onset of action). In contrast to other techniques, such as fluorescence-activated cell sorting flow cytometry (FACS), this allowed us to illustrate the recruitment of leukocytes to the locus of infection, rather than just adjustments in circulating cells (Dunphy, 2004). We observed a significant increase of macrophages and B cells to the infection site in the veins at 24 h, and recruitment of T cells and some neutrophils at 48 h post-treatment. In comparing this to our sections of praziquantel, oxamniquine and mefloquine-treated mice, we noted that similar responses occur after treatment with these drugs as well, however to a different magnitude. Mefloquine treatment, for example, results in some macrophage recruitment, while oxamniquine and praziquantel induce recruitment of far more macrophages within a similar time-frame. To place it in context, Ro 13-3978 is somewhere in between. As we discussed in Chapter 4b, when these results are compared to published onset of action and SEM studies, immune-cell recruitment is not necessarily correlated to rapid tegument degradation, which elicits questions of drug-immune cell interactions.

To understand these findings further, two lines of investigation are planned. The first is to culture Ro 13-3978 and comparator drugs with phagocytes and lymphocytes to observe if these cells are activated by the drug. This is being undertaken by Dr. Paul Davies from the University of Nebraska, Omaha as well as by in house studies. Furthermore, we would like to classify the sub-populations of the cells we labeled, by for example, employing markers

specifics for dendritic cells vs. macrophages vs. Kupfer cells and to identify if and how the recruited cells are activated. In doing so, we would like to illustrate further the relationship between chemotherapy, the immune system and clearance of *S. mansoni* worms.

As for identifying the target of Ro 13-3978, this may prove to be a bit more challenging. For example, although Ro 13-3978 is a close structural analogue to nilutamide, an androgen receptor antagonist, it has been shown that its antischistosomal activity is not related to androgen receptor binding (Wang et al., 2014). Initially, we had hoped to try and identify the target using affinity chromatography with *S. mansoni* lysed extracts, as previously described (Manneck et al., 2012), however conversations with our chemist, Dr. Jonathan Vennerstrom, revealed that it was difficult to synthesize a linker to Ro 13-3978 that was stable in the pH requirements for affinity chromatography. Moreover, the recently explored click chemistry approach of tracking drug alkylation of proteins was not possible, as an alkyne derivative is not synthesizable for this drug (Ismail et al., 2016). Further possible approaches may include photo-affinity labelling (PAL) or fluorescent tagging, though they might encounter similar chemical synthetic challenges (Schenone et al., 2013; Smith and Collins, 2015). Perhaps with further refinement of chemoinformatic techniques described above, combined with novel gene knock-out technologies, target identification may be more easily attainable (Jurberg and Brindley, 2015; Neves et al., 2016).

Indeed, target identification isn't just a fun game for basic research scientists. The synthesis of the organometallic oxamniquine derivatives we tested were guided in part by the well described interaction of oxamniquine to its sulfotransferase target. This provides an example of how such information can be translated to the possible rescue of an otherwise obsolete drug (Pica-Mattoccia et al., 2006). The ferrocenyl, ruthenocenyl and benzyl oxamniquine derivatives displayed *in vivo* activity against *S. mansoni* on par with oxamniquine, but importantly, far more potent *in vitro* activity. This is interesting for two reasons. First, it may indicate a different mode of binding to the target than oxamniquine, which could be more flexible or independent of co-factors such as PAPS (required for oxamniquine binding) (Valentim et al., 2013). Or it could indicate a different target altogether. Moreover, our histological examinations and previous evidence indicate that oxamniquine also relies on immune assistance and requires about 3 days to start clearing worms from the veins *in vivo* (Botros et al., 1989; Fallon et al., 1992, 1996). The rapid onset of action of organometallic derivatives may be reflective of their *in vivo* kinetics, though further studies would be required to elucidate that. Either way, both possibilities may mean these compounds could also be active in other *Schistosoma spp.* important to humans, namely, *S. haematobium* and *S. japonicum*. Testing with these drugs against *S. haematobium* is planned.

6.5 Dynamics of praziquantel treatment in school-age and pre-school age children

Since the London Declaration of 2012 has set a global aim to place schistosomiasis on the path towards control by 2020, there was an acknowledgment that an increase in treatment coverage is necessary (Barry et al., 2013). Contemporarily, evidence had accumulated that the target population for preventative chemotherapy programs should expand to pre-school aged children, as many were found to harbor infections of varying intensity (Stothard et al., 2013). However, the doses provided to very young children were simply back-calculated from doses intended for adults, which is a compromise at best. Many of these doses would be in the form of half a tablet or tablet which are too crudely adjusted to the children's small weight. Moreover, due to their size, the tablets need to be crushed and swallowed with liquid, producing a very bitter taste (Meyer et al., 2009). The latter point might appear cosmetic and trivial, but it bears significant consequences for treatment adherence and appropriate dosing, as it is difficult for many young children to keep the medication down (Coulibaly et al., 2012). Finally, the metabolisms and immune responses of very young children vary from those of adults, which could greatly influence the pharmacokinetics and efficacy of praziquantel.

In response to this important medical need, the Pediatric Praziquantel Consortium was formed to fabricate pediatric formulations of praziquantel (Pediatric Praziquantel Consortium: http://www.pediatricpraziquantelconsortium.org). However, since it will take another several years untill the new tablet formulation is available, it was our goal is to generate an evidence base for the effective dose and pharmacokinetic profile of praziquantel in very young children, which was the aim of our dose-finding study (Coulibaly et al., under review). To make a robust comparison, school-age children and placebo groups were included. Our study presented several important findings which have been discussed in the paper, though I would like to reiterate and elaborate on a few points. First, the study added to the evidence that pre-school aged children are affected by schistosomiasis. Though many bore light infections, 20% bore moderate to heavy infections. Considering the pathology of schistosomiasis, it is plausible that from a very young age, childhood development is affected by this chronic disease, as is likely the case for other helminthiases (Kvalsvig and Albonico, 2013). It is, however, difficult to ascertain the directionality of this altered development and difficult to design robust longitudinal studies (Müller et al., 2011). There is evidence that helminth infection can promote inflammatory homeostasis by triggering immunoregulatory responses (Mishra et al., 2014). Nonetheless, considering the widespread and cumulative pathology of schistosomiasis, it is likely better to remove the infection as early as possible (Gray et al., 2011).

We presented evidence that the efficacy of praziquantel in pre-school aged children infected with *S. mansoni* is somewhat diminished. This could be in part due to the lower infection intensity observed for pre-schoolers and therefore lower diagnostic precision for this group. However, considering that both Kato-Katz and POC-CCA tests (whether "trace" was interpreted as positive or negative for POC-CCA) showed a similar trend, there might be other reasons. One point, for example, is the oft cited immunomodulatory effects of praziquantel. As we reviewed in our histological examination of drug-treated mice (Chapter 4b), clearance of worms after treatment with praziquantel is greatly aided by host T-cell, B-cell and antibody responses (Brindley and Sher, 1987; Doenhoff, 1989). Yet immune responses in young children are very different from older children, with regards to Th1 versus Th2 response orientation, antibody production as well as cytokine response (Jaspan et al., 2006; Prescott, 2003) and this could very well influence treatment efficacy. This could mean that, as we suggest, the paediatric formulation may require higher doses of praziquantel, or an adjuvant, though the latter complicates clinical trials and administration.

With the aim of elucidating molecular processes behind schistosomiasis and treatment with praziquantel, we undertook a metabonomic investigation of pre-treatment, 24h post-treatment and follow up urine samples from this clinical trial. Our findings revealed metabolic differences in several sub-groups. First, we characterized our population before treatment intervention where pre-schoolers and school-aged children were found to have fairly differential metabolic profiles with respect to muscle mass, energy and gut microbial metabolism, results which were largely in line with previous metabonomic characterizations in children, (Gu et al., 2009). These could have important implications for the differential treatment outcomes between school-aged and pre-school aged children, described above.

Infection status could also be differentiated with a metabolic fingerprint, however only for heavy infections and only before intervention. Metabolites were largely associated with metabolic pathways associated with the liver and, intestinal permeability and gut microbiota. The instability of the metabolites at 3 week follow up could rather reflect the nature of the disease- though the infectious agent is cleared, the pathology persists and therefore those that are not infected might still share similar metabolic signatures as those that are infected. This, unfortunately, rather indicates that a metabolic marker for infection was not retrievable. However, it may be useful for studying progression or regression of general disease pathology, though more longitudinal studies would be warranted.

Finally, several metabolic signatures were correlated to uptake of praziquantel. Considering the immediate antischistosomal effects of praziquantel (only 30 min for hepatic shift) and its supposed immunomodulatory action, this is perhaps not surprising. Our models show the

effect is transitory as, at follow up, no significant differences even between 60 mg/kg and placebo can be seen. Interestingly, metabolic responses of pre-schoolers vs school-aged children to praziquantel treatment were alike with the exception of a few gut microbial metabolites. This suggests a possible role of the gut microbiota in praziquantel treatment success, which was partly reflected by findings from Schneeberger et al. (2018), a study conducted using stool samples from the same trial.

At the moment, these metabolic signatures provide first snap shots into metabolic differences between school age and pre-school age children and the dynamics of schistosomiasis infection and its treatment. In a next step, we would like to integrate the findings from our metabonomic investigations with gut microbiome investigations from stool samples (Schneeberger et al., 2018) and pharmacokinetic profiling from dried blood spots (Kovac et al., 2018) from the same clinical trial, to provide a system-level “omics” analysis of parameters of interest such as the correlations between microbiome and drug uptake and metabolic and microbial predictors of treatment efficacy or failure. This would require a deeper-level analysis, employing further orthogonal multivariate statistics such as Two-Way Orthogonal Partial Least Squares (O2PLS) or penalized regression methods (LASSO and ENET) (Bouhaddani et al., 2016; Pineda et al., 2015). Pharmacometabonomics, the integration of pharmacokinetics and metabonomics, has been successfully employed for personalized medicine purposes such as detecting metabolic correlates to drug uptake and adverse events, which can be more informative than pharmacogenomics, as it takes into account environmental factors (Clayton et al., 2009a; Huang et al., 2015). Moreover, there is mounting evidence that host-microbial interactions are integral to drug metabolism and response, which can be well characterized by integrating microbiome and metabolome profiles (Clayton et al., 2009b; Wilson, 2009). Apart from gaining interesting insights into the dynamics of microbiota, schistosomiasis and praziquantel treatment, it could also inform differential treatment needs between very young and older children with respect to treatment dose, nutritional requirements and innate inter-individual variation. As treatment with praziquantel is often blemished by a few cases of treatment failure, such an investigation would help in illuminating the factors involved and teasing apart host and environmental factors from potential resistance.

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