

**Research on anthelmintic drugs,  
with an emphasis on structure-activity relationship  
and pharmacokinetic studies**

INAUGURALDISSERTATION

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## Table of contents

Acknowledgements.....	III
Summary.....	IV
Table of abbreviations.....	VII

<b>Chapter 1: General introduction.....</b>	<b>1</b>
1.1 Burden and control of helminth infections.....	2
1.2 Schistosomiasis.....	3
1.2.1 Epidemiology and life cycle.....	3
1.2.2 Disease and symptoms.....	4
1.2.3 Diagnosis and treatment.....	5
1.2.4 Recent research for new treatments.....	6
1.2.5 The need for new antischistosomal drugs.....	9
1.3 Trichuriasis.....	10
1.3.1 Epidemiology and life cycle.....	10
1.3.2 Disease and symptoms.....	11
1.3.3 Diagnosis and treatment.....	11
1.3.4 Recent research for new treatments.....	12
1.3.5 Oxantel pamoate, a drug of clinical relevance against trichuriasis.....	13
1.3.6 The need for assessing pharmacokinetics and drug-drug interactions of promising co-administrations against trichuriasis.....	16
1.2 Aim of the dissertation.....	18

<b>Chapter 2: Elucidation of the in vitro and in vivo activities of bridged 1,2,4-trioxolanes, bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides, silyl peroxides, and hydroxylamine derivatives against <i>Schistosoma mansoni</i>.....</b>	<b>25</b>
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<b>Chapter 3: Activities of <i>N,N'</i>-diarylurea MMV665852 analogs against <i>Schistosoma mansoni</i>.....</b>	<b>33</b>
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<b>Chapter 4: Repurposing of anticancer drugs: in vitro and in vivo activities against <i>Schistosoma mansoni</i>.....</b>	<b>41</b>
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<b>Chapter 5: Pharmacokinetic drug-drug interaction study of co-administrations oxantel pamoate plus albendazole, and albendazole plus mebendazole - a preclinical safety evaluation for human trichuriasis treatments.....</b>	<b>51</b>
<b>Chapter 6: General discussion and conclusion.....</b>	<b>59</b>
6.1 Antischistosomal drug discovery.....	60
6.1.1 Synopsis of three drug sets tested for antischistosomal activity.....	60
6.1.2 Relevance physico-chemical properties and “drugability”.....	64
6.1.3 Importance of public-private and academic partners.....	69
6.1.4 Continuation of the projects.....	71
6.2 <i>Trichuris</i> drug research.....	72
6.2.1 Main findings and interpretation of the safety assessment of the two co-administrations albendazole plus oxantel pamoate, and albendazole plus mebendazole.....	72
6.2.2 Gastrointestinal metabolic interactions and the determination of the compartment responsible for drug activity against <i>T. muris</i> .....	75
6.2.3 Next steps in trichuriasis drug research.....	79
6.3 Conclusion.....	80
Appendix: Curriculum vitae.....	85

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## Summary

More than one third of the world's population is infected with at least one helminth, among which schistosomiasis and trichuriasis are highly prevalent. The control of schistosomiasis relies heavily on the treatment of people living in endemic areas with praziquantel. Praziquantel is currently the only available antischistosomal drug and 28 million patients are treated every year, which leads to a high drug pressure on schistosomes. The low efficacy on the juvenile stage of *Schistosoma* and the risk of development of drug resistance urges the development of an alternative treatment. The current chemotherapy of trichuriasis, using two benzimidazoles albendazole and mebendazole, results in unsatisfactory treatment outcomes. The co-administration of the two drugs cures more trichuriasis patients than the drugs administered separately. Also the co-administration of albendazole with the rediscovered trichuricidal drug oxantel pamoate is a promising treatment. In order to apply these co-administrations on the large scale, safety has to be assured.

The objectives of this thesis were to advance the development of antischistosomal drugs by conducting structure-activity relationship studies and by defining new pharmacophores for lead optimization. Moreover, we aimed to determine the safety of two co-administrations (albendazole plus oxantel pamoate, and albendazole plus mebendazole) by studying their preclinical in vitro and in vivo drug-drug interactions.

Three compound sets were investigated for antischistosomal activity by testing them first against the larval and adult stage of *S. mansoni*, followed by a cytotoxicity determination to finally evaluate the in vivo efficacy in the chronic *S. mansoni* mouse model.

The first set tested consisted of five different synthetic peroxide classes: bridged 1,2,4-trioxolanes, bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides, silyl peroxides, and hydroxylamine derivatives. The trioxolanes, tetraoxanes, and the tricyclic monoperoxides showed high in vitro activities in the low micromolar to nanomolar range. None of the compounds revealed significantly high drug efficacy in vivo. The highest efficacy was achieved by the trioxolane class displaying a worm burden reduction (WBR) of 44%. Since low solubility of the peroxides was noted and assumed to be a major limiting factor for drug efficacy, the peroxides were next packed into a cyclodextrin complex. However, the WBRs were unchanged. Further lead optimization of peroxides should aim to synthesize compounds with higher aqueous solubility.

In a further project, we assessed the antischistosomal activities of MMV665852 analogs to identify new pharmacophores. *N,N'*-diarylureas, *N*-phenyl benzamides, and *N*-aryl

phenylcarbamates revealed high and fast in vitro activity at concentrations in the low micromolar to the nanomolar range within few hours of drug exposure. For high activity, the presence of a conjugated ring system on both sides of the structures was essential. The highest WBRs were observed with a *N*-phenyl benzamide (66%) and a *N,N'*-diaryurea (43%). For this drug set, we investigated the “drugability” of the compounds using in silico and in vitro tools, testing physico-chemical parameters, solubility, permeability through the intestinal wall, and metabolic stability. Solubility appeared to be the main reason for low “drugability” of the compounds. Also this set of molecules demonstrated the necessity of the compound to have drug-like features. Therefore, it is recommended to apply a “structure-bioavailability-activity”-based drug design and screen flow.

Several marketed cancer drugs contain the *N,N'*-diaryurea or *N*-phenyl benzamide structures. Additionally, cancer drugs are believed to target drug action present in schistosomes and in humans. For these reasons, a cancer drug library of 114 compounds was tested for antischistosomal activity. Eleven drugs demonstrated in vitro activity below 10  $\mu$ M, of which two demonstrated in vivo activity (trametinib: 64% WBR, vandetanib: 48% WBR). Future studies might involve multiple administrations of the drugs to simulate their long half-lives in humans, which are short in the mouse, or the assessment of the drugs' influence on schistosome development, with which cancer drugs were suggested to interfere.

The potential for drug-drug interactions of albendazole plus mebendazole and albendazole plus oxantel pamoate was assessed using two approaches: first, using an in vitro metabolic assay, assessing the inhibition of Cytochrome P450 (CYP), and second, comparing pharmacokinetic parameters after applying co-administrations or monotherapy to rats.

The in vitro CYP inhibition assay showed only interaction against CYP1A2. In more detail, this interaction was presented by a 2.6-fold decreased  $IC_{50}$  value, when the enzyme was simultaneously exposed to albendazole and oxantel pamoate compared to separate exposures.

For analysis of the plasma concentrations from the in vivo study, a High Pressure Liquid Chromatography (HPLC) method with UV detection was developed for oxantel pamoate, albendazole sulfoxide and sulfone (the two major metabolites of albendazole), and mebendazole and validated according to FDA guidelines. No interaction between albendazole and oxantel pamoate was observed in vivo, presumably due to low bioavailability of oxantel pamoate. However, a moderate interaction was observed in the disposition of mebendazole. Mebendazole co-administered with albendazole resulted in a significantly increased area under the plasma curve (AUC) (3.5-fold increase) and a maximal plasma concentration ( $C_{max}$ )

(2.8-fold increase). However, the observed interaction might not apply to humans, since the plasma levels observed in humans, after the standard treatment dosages are ten to thirty times lower than the levels we observed in rats. It is further not known what role the species differences of the quantitative biotransformation of benzimidazole plays. Therefore, further safety studies on co-administration albendazole plus mebendazole in healthy humans might be needed to confirm the safety of the regimen. A safety study of the co-administration albendazole plus oxantel pamoate may only be considered, if tests for absorption and bioavailability of oxantel pamoate indicate exposure of the body to oxantel pamoate.

In conclusion, the projects of antischistosomal drug discovery identified new active pharmacophores and provided evidence for the hypothesized antischistosomal activity of cancer drugs. The pharmacokinetic safety evaluation detected a potential interaction when albendazole and mebendazole are co-administered.



## Table of abbreviations

ABZ	Albendazole
ABZSO <sub>2</sub>	Albendazole sulfone
ABZSO	Albendazole sulfoxide
ADME	Absorption, distribution, metabolism, elimination
AUC	Area under the curve
BCS	Biopharmaceutics Classification System
C <sub>max</sub>	Maximal concentration
CYP	Cytochrome P450
ED <sub>50</sub>	Dose of 50% efficacy
GI	Gastrointestinal
HPLC-UV	High pressure liquid chromatography with UV detection
HsPBGS	Human prothobilinogen synthase
IC <sub>50</sub>	Concentration of 50% inhibition
LD <sub>50</sub>	Dose lethal to 50% of test animals
LLOQ	Lower limit of quantification
LogP	Octanol-water partition coefficient
MBZ	Mebendazole
MIC	Minimal inhibitory concentration
MMV	Medicines for Malaria Venture
NTD	Neglected tropical disease
OxP	Oxantel pamoate
PAMPA	Parallel artificial membrane permeation assay
PPP	Private-public partnership
SI	Selectivity index
t <sub>1/2</sub>	Half-life
t <sub>max</sub>	Time at maximal concentration
WBR	Worm burden reduction
WHO	World Health Organization



# **Chapter 1**

## **General introduction**

## 1.1 Burden and control of helminth infections

More than one third of the world's population (2.5 billion of 6.7 billion in 2008) is infected with at least one helminth. People living in poor conditions in subtropical and tropical areas are most affected by helminthiases [1]. The mortality rates of many neglected tropical diseases are not high. However, the diseases are characterized by lengthy periods of suffering and often by a lifetime of disablement [2].

The World Health Organization (WHO) recommends an approach to overcome the global impact of neglected tropical diseases through five interventions: 1) innovative and intensified disease management; 2) preventive chemotherapy, vector ecology and management; 3) veterinary and public-health services; 4) provision of safe water, sanitation and hygiene [3].

In 2010, preventive chemotherapy reached about 35% of those who needed treatment, at least once during a 12-month period. This is more than 700 million people, and the current approach aims to accelerate preventive chemotherapy programs and to widen their range [4]. The implementation of mass drug administration urges the scientific and global health community to better understand the pharmacology of the commonly used drugs, to better monitor and understand drug resistance, and to conduct drug discovery to have alternative treatments if resistance occurs [5].

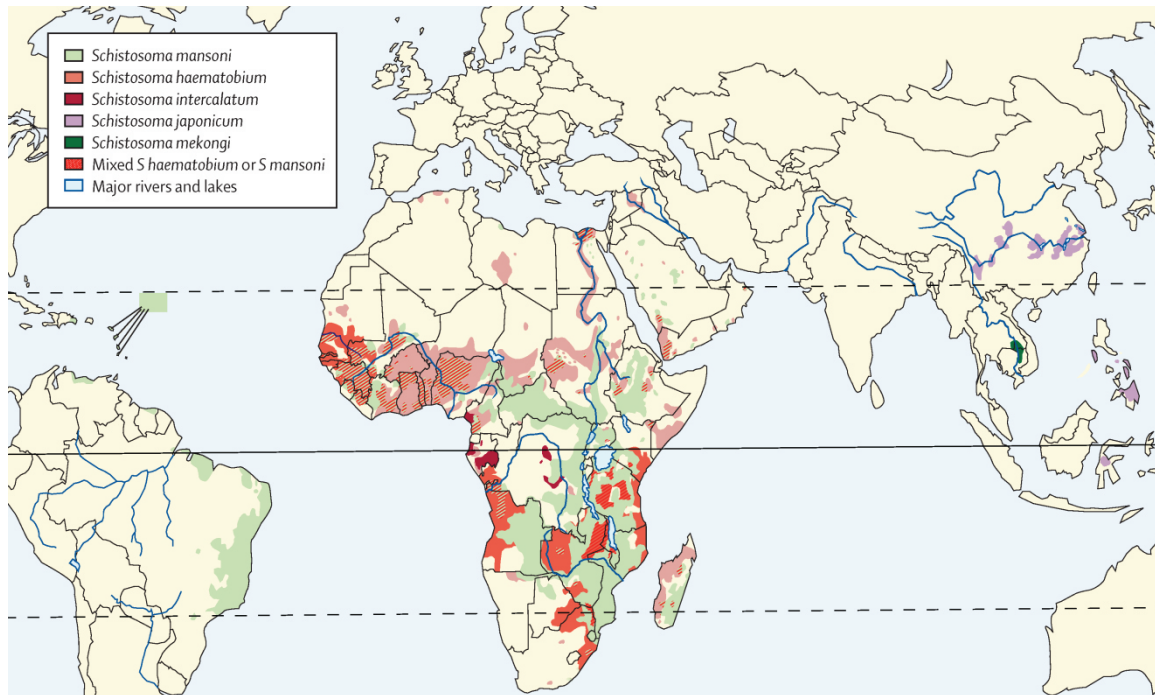
Schistosomiasis and trichuriasis are two helminth infections belonging to the neglected tropical diseases. Approximately 207 million people suffer from an infection with *Schistosoma spp.*, a blood-dwelling trematode. Trichuriasis is an intestinal nematode infection, caused by the soil-transmitted helminth *Trichuris trichiura*, and affects approximately 604 million people worldwide [1].

In spite of their global health significance, research and development of novel drugs for schistosomiasis and trichuriasis has been limited. For instance, there is only one antischistosomal drug on the market, which is a perilous situation in case of emergence of drug resistance. Moreover, a better knowledge about pharmacokinetics of promising drug co-administrations against trichuriasis is desired. In general, both diseases are in need for increased research on better treatment options.

## 1.2 Schistosomiasis

### 1.2.1 Epidemiology and life cycle

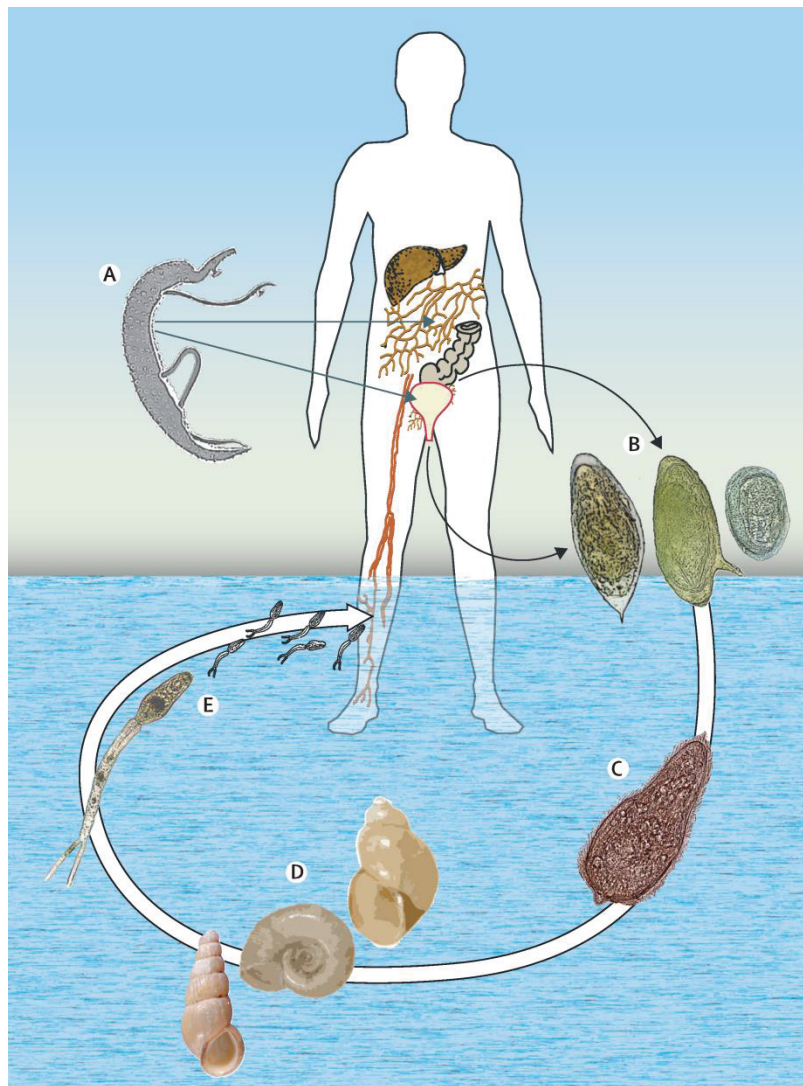
Schistosomiasis is caused by flukes (trematodes) of the genus *Schistosoma*. The main species pathogenic for humans are *S. mansoni* (prevalent in Africa, Middle East, and the Americas), *S. japonicum* (East Asia), and *S. haematobium* (Africa and Middle East). Less common are *S. mekongi* (Mekon River basin), *S. intercalatum* and *S. guineensis* (west and central Africa) (Figure 1) [6].



**Figure 1: Global prevalence of infections with *Schistosoma spp.* infecting humans [6]**

People become infected via cutaneous penetration of exposed skin in schistosome-infected waters (Figure 2). In the human body, schistosomes mature from the larval stage (schistosomula) in the blood stream to the juvenile stage in the lung, and finally to the adult stage in the mesenteric and portal veins (hepatic schistosomiasis), or the veins of the bladder (urinary schistosomiasis, exclusively caused by *S. haematobium*). The maturation to the adult stage takes approximately 5-7 weeks [7]. Adult schistosomes have a long live span of 3-10 years on average, and up to 40 years [6], during which male and female worms in copula produce hundreds of eggs per day [8]. The eggs migrate throughout the body and cause inflammation - the actual cause of morbidity. Eggs that reach the gastrointestinal (GI) tract are excreted via the feces and hatch after contact with freshwater to miracidia. This aquatic form locates snails using

chemotaxis and infects them. Within the snails, miracidia undergo several rounds of asexual proliferation to develop into cercariae. After 6 weeks, the cercariae are shed into the water and locate humans (the final host), which they infect percutaneously [7].



**Figure 2: Life cycle of *S. mansoni*, *S. haematobium*, and *S. japonicum***

Paired adult worms (A) produce eggs (B), which are excreted into water and transform into miracidia (C). In the snail host (D), miracidia multiply to cercariae (E), which penetrate people standing in infectious waters [7].

### 1.2.2 Disease and symptoms

There are two manifestations of schistosomiasis: acute and chronic. Acute symptoms can be caused by the percutaneous penetration of cercariae, which results in urticaria, also known as “swimmer’s itch”. The migrating schistosomula lead to a hypersensitivity reaction few weeks after infection, also called Katayama fever, which can manifest as cough, head ache, and fever [7]. The chronic stage is responsible for most of the disease burden, which occurs once the

worms have matured to adult fluke pairs and are releasing eggs. Proteolytic enzymes released during egg migration provoke inflammatory reactions in the surrounding tissues. The symptoms of urinary and intestinal schistosomiasis are non-specific, such as anemia, malnutrition, bloody urine or stool, and impaired childhood development. Moreover, urinary schistosomiasis is a risk factor for bladder cancer [6].

### 1.2.3 Diagnosis and treatment

Schistosomiasis can be diagnosed by microscopic or molecular detection of viable eggs in the stool, or urine. These techniques are however limited by low sensitivity and heterogeneous distribution of the eggs in the excretes [6]. Of the microscopic methods, FLOTAC showed higher sensitivity in stool samples of *S. mansoni* patients than the most widely used Kato-Katz fecal thick smear method [9]. The serological detection of antibodies against schistosome infection is useful for diagnosing schistosomiasis in travelers after their return, but less useful for diagnosing people living in endemic areas, since antibodies remain in the blood circulation after elimination of the infection [6]. Diagnosis using polymerase chain reaction (PCR) to multiply schistosome-specific DNA sections, is very sensitive and selective, but is generally less applicable in endemic areas due to lack of specialized facilities and personnel [10]. A rapid diagnostic test detects circulating cathodic or anodic antigens (CCA) in urine of *S. mansoni* patients. The test showed the sensitivity of triple Kato-Katz diagnosis, without cross-reactivity to *S. haematobium* [11]. A test based on circulating anodic antigens (CAA) is under development.

Chronic schistosomiasis of all species can be treated with praziquantel. For *S. haematobium* and *S. mansoni*, the standard treatment used in preventive chemotherapy programs is a single oral dose of 40 mg/kg body weight; for *S. japonicum* and *S. mekongi* it is 60 mg/kg. Praziquantel is, however, inefficacious against premature schistosomes, and therefore not adequate for treatment of acute infections [6]. Hence, a second dose of praziquantel may be recommended after the parasites have fully developed. The cure rate of praziquantel is 60 to 90%, and is often overestimated due to the low sensitivity of diagnostic tools [12].

Every year, hundreds of millions of tablets are distributed to people at risk of infection. This is possible through private-public partnerships (PPPs). For example, in 2004, MedPharm donated 14 million praziquantel tablets, and Merck KGaA pledged 200 million tablets in the years 2008 to 2017 [13]. In 2012, 28 million people were treated with praziquantel [14].

No vaccine against schistosomiasis is available to date. However, a few candidates showed 50 to 70% worm burden reduction in animal models, and some progressed to early phase clinical trials. Efficacy in humans has yet to be proven. Additionally, decoding the genomes of schistosomes in the past years, may allow the generation of recombinant proteins, e.g. of surface or intestinal proteins, that will facilitate and thus probably accelerate the development of a vaccine [15].

#### 1.2.4 Recent research for new treatments

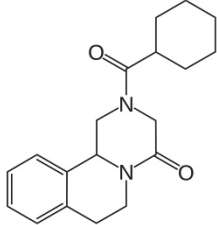
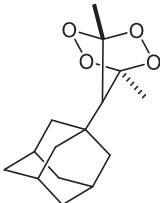
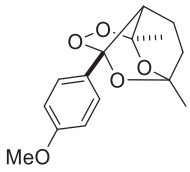
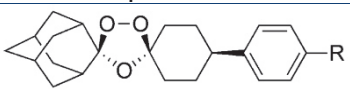
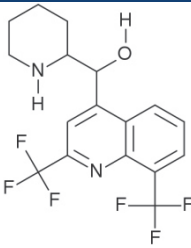
Since praziquantel is the only treatment available against schistosomiasis, and emergence of drug resistance cannot be excluded, research for new antischistosomal is needed [12].

During the last two decades, derivatives of the antimalarial artemisinin (product of *Artemisia annua*) have been studied on schistosomes intensively. Since most of this work was conducted at Swiss TPH, findings on these drugs are presented in greater detail. Several **artemisinin derivatives** demonstrated antischistosomal activity, such as artemether [16], the fully synthetic ozonides (or trioxolanes) [17],[18], trioxaquinines [19], dioxolanes [20], bridged tetraoxanes and tricyclic monoperoxides [21]. Among these, OZ418 demonstrated high and species-independent drug efficacy in the *S. mansoni* and the *S. haematobium* mouse model, displaying worm burden reductions (WBRs) of 80% and 86%, respectively [18]. Current lead compounds at the Swiss TPH are presented in Table 1. The most recent structure-activity relationship studies of the peroxides demonstrated high in vitro activities of a bridged tetraoxane after a 72-hour exposure (IC<sub>50</sub> of 0.1 µM against larval stage, and IC<sub>50</sub> of 0.3 µM against adult stage) and a WBR of 75%. The in vitro activities of a tricyclic monoperoxide were slightly lower (IC<sub>50</sub> of 14.4 µM against larval stage, and IC<sub>50</sub> of 11.8 µM against adult stage), however, showed higher in vivo activity with 83% WBR [21].

Both the antimalarial and the antischistosomal mechanism of action of artemisinin and the peroxidic derivatives is most probably due to an interference of the drugs' peroxide with heme polymerization, leading to hemozoin accumulation, which is toxic to the parasites [22].



**Table 1: In vitro and in vivo activities of molecules with promising antischistosomal activity studied at the Swiss TPH, compared with praziquantel**

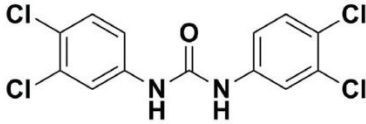
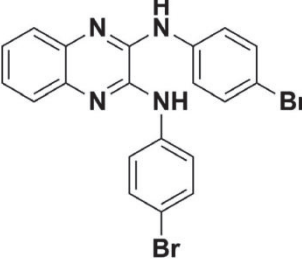
Molecular structure	IC <sub>50</sub> larval <i>S. manoni</i> [μM]	IC <sub>50</sub> adult <i>S. mansoni</i> [μM]	SI	WBR [%]	Ref.
Current drug of choice: praziquantel					
 <p>2-(Cyclohexylcarbonyl)-2,3,6,7-tetrahydro-4H-pyrazino[2,1-a]isoquinolin-4-one</p>	2.2 <sup>1</sup>	0.1 <sup>1</sup>	>960 <sup>1</sup>	>90% <sup>2</sup>	[21] <sup>1</sup> [31] <sup>2</sup>
Artemisinin-inspired synthetic peroxides					
 <p>Bridged 1,2,4,5-tetraoxane</p>	0.1	0.3	5.7	75.4	[21]
 <p>Tricyclic monoperoxide</p>	14.4	11.8	4.9	83	
 <p>Ozonide OZ418 (R = OCH<sub>2</sub>COOH)</p>	Not done	>200 <sup>3</sup>	Not done	80% <sup>4</sup>	[32] <sup>3</sup> [18] <sup>4</sup>
Mefloquine					
 <p>Quinoline</p>	6.6 <sup>5</sup>	12.7 <sup>5</sup>	0.3 <sup>5,6</sup>	73 <sup>5</sup>	[23] <sup>5</sup> [33] <sup>6</sup>

IC<sub>50</sub>: Concentration of 50% drug effect

SI: Selectivity index (IC<sub>50</sub> cytotoxicity divided by IC<sub>50</sub> of adult schistosomes)

WBR: Worm burden reduction

**Table 1: continued**

Molecular structure	IC <sub>50</sub> larval <i>S. manoni</i> [μM]	IC <sub>50</sub> adult <i>S. mansoni</i> [μM]	SI	WBR [%]	Ref.
MMV Box					
 <i>N,N'</i> -diaryllurea (MMV665852)	4.7	0.8	40	53	[26]
 2,3-dianilinoquinoxaline	>33.3	Not done	7.1	41	

IC<sub>50</sub>: Concentration of 50% drug effect

SI: Selectivity index (IC<sub>50</sub> cytotoxicity divided by IC<sub>50</sub> of adult schistosomes)

WBR: Worm burden reduction

**Mefloquine** is another antimalarial drug found to possess antischistosomal activity. It is an interesting candidate due to its seemingly species-independent and developmental stage-independent activity. Briefly in more detail, mefloquine revealed an IC<sub>50</sub> of 6.6 μM against *S. mansoni* larvae in vitro. The adult stage of *S. mansoni* and *S. japonicum* was less susceptible, with IC<sub>50</sub> values of 12.7 μM and 16.4 μM, respectively. Artificially infected mice that were treated with 200 mg/kg mefloquine per body weight showed a decreased *S. mansoni* worm burden of 73%, or *S. haematobium* burden of 81%. *S. japonicum* had an EC<sub>50</sub> of 53 mg/kg on the juvenile stage [23]. The few clinical trials showed inconsistent results, thus, more studies are needed to describe the extent of efficacy in schistosomiasis patients. It might be worth highlighting that mefloquine treatment raises concern about development of resistance against *Plasmodium falciparum*, which is co-endemic in areas where schistosomiasis persists [24].

The fact that *Schistosoma* shares mechanisms of action with *P. falciparum* lead to the screen of a compound library assembled by the Medicines for Malaria Venture (MMV), the so called **MMV Box**. The library consists of 400 drug-like compounds with attested in vitro activity against *P. falciparum* [25]. Indeed, the screen identified several lead candidates for which lead optimization would be worth perusing (Table 1). MMV665852 was most promising due to its activity and chemical attractiveness. In vitro activity was high with an IC<sub>50</sub> value of

4.7  $\mu\text{M}$  against the larval and 0.8  $\mu\text{M}$  against the adult stage of *S. mansoni*. However, the WBR was only moderate with 53% [26].

Other great efforts have been made in antischistosomal drug discovery over the last decades, screening large compound libraries of new [27],[28] or marketed drugs [29]. Furthermore, a wide assortment of natural products with antischistosomal activity has been identified [30].

### **1.2.5 The need for new antischistosomal drugs**

Since its appearance on the market in 1975, after almost half a century of praziquantel use, and millions of people being treated annually [14], no other drug has been marketed for antischistosomal treatment [34],[28]. Indeed, praziquantel eliminated the urgent need for antischistosomal drug research, and replaced the then widely used antischistosomal chemotherapies. Praziquantel had clearly advantages over oxamniquine, which was only efficacious against *S. mansoni* [35], and for which drug-resistance had been reported [36], and also antimony-tartrate, which was given intravenously [35], or metrifonate, which is less widely applicable than praziquantel, since it is only active against *S. haematobium* [35].

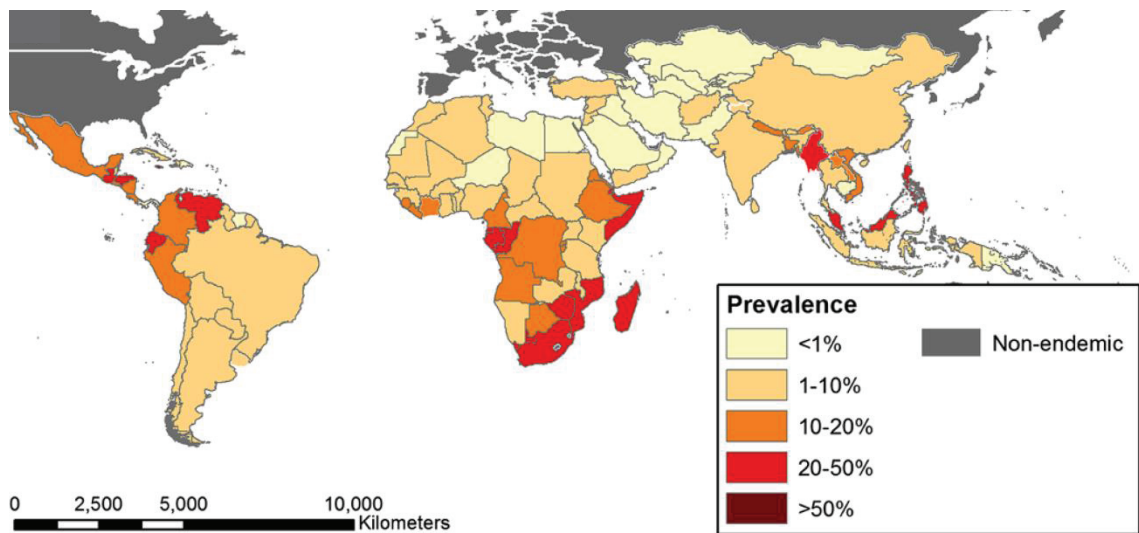
There is a general discussion about whether new drugs against schistosomiasis are needed. Some stakeholders are not satisfied with only one drug on the market, mainly due to anticipation of drug resistance [5],[12], but also due to its moderate cure rate and inactivity against juvenile schistosomes [23]. Others, however, do not expect drug resistance, due to 1) the long life cycle of schistosomes; 2) refugia, where susceptible strains do not experience pressure of mass drug administrations; 3) biological inferiority of schistosomes with experimental praziquantel-resistance compared to praziquantel-susceptible worms; and 4) the dependence on the host's immune system in praziquantel's mode of drug action [37].

Undoubtedly, the various efforts to discover new antischistomials, as described above, demonstrate the general desire to find alternatives to praziquantel. Since no other product has been marketed yet [38], research efforts should be continued.

## 1.3 Trichuriasis

### 1.3.1 Epidemiology and life cycle

*Trichuris trichiura* causing trichuriasis or human whipworm infection, infects an estimated 600 to 700 million people and is one of the most prevalent helminth infections [1],[39] next to the other soil-transmitted helminths *Ascaris lumbricoides* (1 billion infected) and hookworm infections (600 million infected) [39]. *T. trichiura* prevails in tropical and subtropical areas all over the globe (Figure 3). Approximately 50% of all trichuriasis cases are found equally in sub-Saharan Africa, East Asia and the Pacific Islands; 40% are found in Latin America and the Caribbean, South Asia, or India. China, and middle, east and north Africa host the least *T. trichiura* number of infections [39].



**Figure 3: Global prevalence of trichuriasis [40]**

Infection occurs via ingestion of *T. trichiura* eggs. The larvae hatch and settle preferably in the cecum - in stronger infections also in the colon and rectum - where they develop into adult worms within 12 weeks (Figure 4) [39]. The thin anterior part of the parasite is anchored to approximately one third of its length in the epithelial cells of the intestinal lumen. In this “syncytial tunnel”, the cuticle of the worm is in contact with the cytosol of the ruptured epithelial cells. The posterior part, however, is located in the lumen of the GI tract [41]. Adult female worms lay eggs, which are released into the environment via fecal excretion by the human [39].

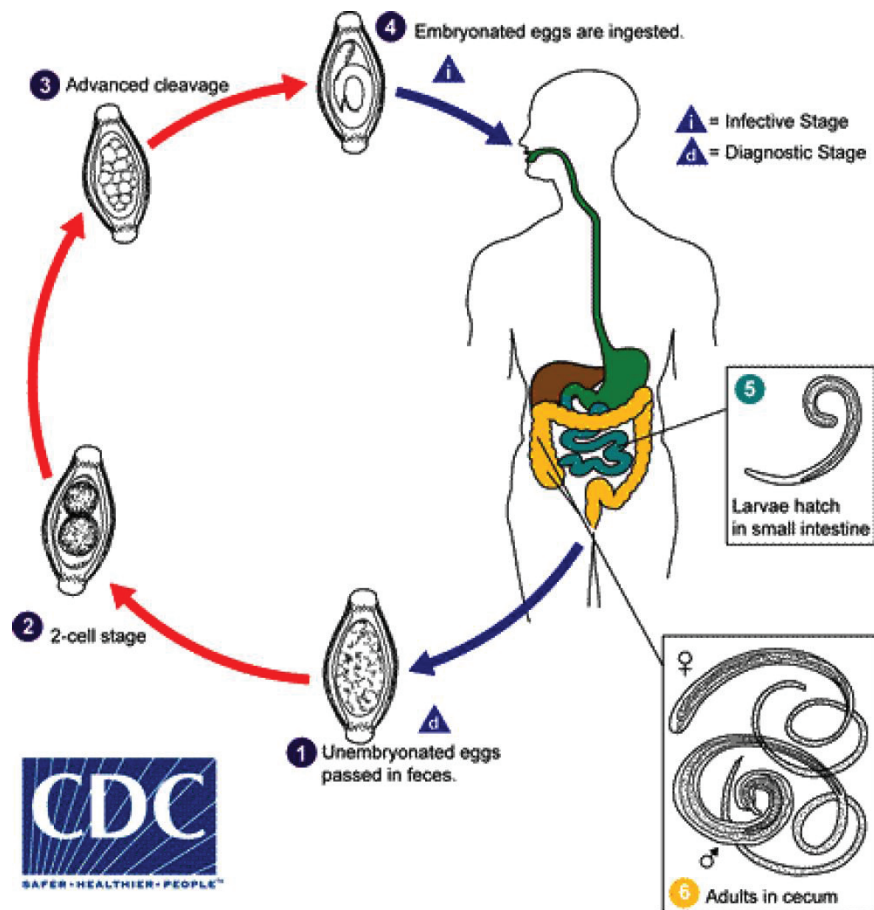


Figure 4: Life cycle of *T. trichiura* [42]

### 1.3.2 Disease and symptoms

Light infections persist unrecognized. At site of parasite attachment, inflammation occurs and can lead to colitis. An array of symptoms can result from colitis, such as chronic abdominal pain, diarrhea, anemia, impaired growth, finger clubbing, and in more serious cases chronic dysentery and rectal prolapse. Most intense infections occur in children aged 5-15 years. Whether the decline of intensity and frequency of infections in adulthood is due to changes in exposure, acquired immunity, or both, remains controversial [39].

### 1.3.3 Diagnosis and treatment

The presence and intensity of *T. trichiura* infection can be examined with light microscopy by determining the number of eggs per gram feces, using the Kato-Katz fecal thick smear, the McMaster method, or FLOTAC [43]. Light infections can be detected by concentration of the eggs with formalin ethyl acetate sedimentation [39].

Trichuriasis, like the other soil-transmitted helminth infections, is treated with albendazole or mebendazole in the frame of preventive chemotherapy programs [44]. Around 30% of children at risk for acquiring soil-transmitted helminth infections are reached in these programs, using single-dose albendazole or mebendazole [45]. However, clinical trials conducted between 1960 and 2007 showed unsatisfying treatment outcomes. Albendazole cured on average merely 28% of infected people, and mebendazole 36% [46].

No vaccine is available against trichuriasis yet. One candidate showed promising result, expelling all worms from mice, 35 days after application [15].

#### 1.3.4 Recent research for new treatments

Despite the urgent need for better chemotherapies against trichuriasis [47], only few potential drugs have been investigated in recent years.

The antiprotozoal drug **nitazoxanide** emerged and disappeared again as a drug candidate against trichuriasis. In vitro activities against the larval ( $IC_{50} < 1 \mu\text{g/ml}$ ) and adult stage ( $IC_{50} = 12.9 \mu\text{g/ml}$ ) of *T. muris* were promising, and the in vivo WBR was moderate (56% after 100 mg/kg p.o.) [48]. Unfortunately, the efficacy could not be translated to humans [49].

Similarly, **monepantel** was an interesting candidate due to its broad-spectrum activities against intestinal parasites [50], and its availability on the veterinary drug market. However, lack of in vitro and in vivo activity in *T. muris* infection models prevented further investigations [51].

**Emodepside** is a semi-synthetic product from the fungus *Mycelia sterilia*, living in the leaves of *Camellia japonica*. Emodepside demonstrated efficacy against trichuriasis in naturally infected livestock and domestic animals [52], as well as experimental *Trichuris* infections [53]. Bayer Animal Health has released a combination of emodepside with toltrazuril (Procox®) to rid dogs from *Trichuris vulpis*, other nematodes, and coccidian [54]. Procox® is highly efficacious in dogs (100%) [55]. In 2014, the Drugs for Neglected Diseases Initiative (DNDi) signed an agreement with Bayer Health Care to develop emodepside for treatment of onchocerciasis patients [56]. Once preclinical and Phase I testing has been completed the trichuricidal properties of emodepside should be tested in phase 2 trials.

Several **benzimidazole anthelmintics for veterinary use** were active in clinical trials, such as oxibendazole, fenbendazole, flubendazole, and thiabendazole. The various dose regimens used in the different clinical trials showed inconsistent cure rates between 17 and 100%. Further interesting candidates, which come from the veterinary field are **doramectin**,

**milbemycin oxime**, and **moxidectin**. Indeed, drug repurposing reveals a way to accelerate and at the same time lower the costs of anthelmintic drug discovery and development [29].

The effect of **trans-cinnamaldehyde**, a component of cinnamon (*Cinnamomum verum*), on *Trichuris suis* was discovered this year. In vitro tests showed a more than 50% mortality rate of larvae (L1), when exposed to 30  $\mu$ M for 2 hours. Trans-cinnamaldehyde orally applied to pigs did not reduce the worm burden. The authors suspect the compound to be well absorbed into the blood stream, thus lowering the concentration in the intestine where the parasite resides. Therefore, altering the oral formulation might improve in vivo efficacy [57].

**Papain**, a cysteine protein kinase from the papaya fruit (*Carica papaya*), demonstrated high efficacy in the *T. suis* pig infection model. A single dose of 450  $\mu$ mol resulted in a WBR of 98%, which was higher than the treatment with 400 mg (1.5 mmol) albendazole, which resulted in a WBR below 39%. The investigators want to proceed with the development of a formulation with the prospects of applying papain in humans [58].

Another promising strategy for developing better treatments is the use of **combination of existing drugs** [5]. This was demonstrated with combinations of albendazole plus mebendazole, where the cure rate was 54%, compared to single albendazole (15%) and mebendazole (20%) [59]. Another combination, albendazole plus ivermectin, showed superior efficacy in multiple studies [60],[61],[62].

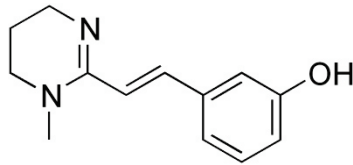
Last but not least, **oxantel pamoate** demonstrated efficacy and safety in trichuriasis patients, and is described in more detail in the section below.

### 1.3.5 Oxantel pamoate, a drug of clinical relevance against trichuriasis

Oxantel pamoate is being studied as co-administration with albendazole, aiming to provide a broad-spectrum antinematodal therapy. In trichuriasis patients, oxantel pamoate plus albendazole resulted in higher cure and egg reduction rates (69% and 99%, respectively), than albendazole plus ivermectin (28% and 95%, respectively), or albendazole plus mebendazole (8% and 52%, respectively). Moreover, oxantel pamoate plus albendazole co-administration did not lead to increased side effects [63].

The profile of oxantel pamoate is summarized in Table 2. Oxantel pamoate is best known in combination with pyrantel pamoate and praziquantel as a broad-spectrum deworming medication for cats and dogs [64]. In dogs, the egg reduction rate is 98%, after the

**Table 2: Profile of oxantel pamoate**

<b>Chemical structure</b>		
<b>Chemical group</b>	Tetrahydropyrimidine	
<b>Year of entry into market</b>	1966	[35]
<b>Clinical relevance</b>	In combination with pyrantel presents effect against multiple intestinal helminths: <i>Ascaris</i> , <i>Enterobius</i> , hookworms, <i>Trichuris</i>	[75]
<b>Countries in which oxantel pamoate-pyrantel pamoate are registered</b>	Columbia	[68]
	Peru	[68]
	Philippines	[68]
<b>Other anthelmintic tetrahydropyrimidine derivatives</b>	Pyrantel pamoate: <i>Ancylostoma</i> , <i>Ascaris</i> , <i>Enterobius</i> , <i>Necator</i> , <i>Trichinella</i> , <i>Trichostrongylus</i> ; horse, pig, and ruminant nematodes; horse cestodes	[75]
	Morantel: Ruminant nematodes	[75]
<b>Pharmacokinetic parameters in humans</b>	Absorption: Low T <sub>max</sub> : 2-4 h post-administration Elimination: 7% urinary Total elimination: 12 h post-administration	[69]
<b>Pharmacokinetic in dogs</b>	Absorption: Negligible	[66]
<b>Veterinary usage</b>	In combination with pyrantel pamoate and praziquantel as broad-spectrum deworming drug for cats and dogs	[64]
<b>In vitro activities against helminths</b>	<i>T. muris</i> L1 IC <sub>50</sub> : 0.08 μM	[76]
	<i>T. muris</i> L4 IC <sub>50</sub> : 3.9 μM	[77]
	<i>A. ceylanicum</i> L3 and adult IC <sub>50</sub> : >150 μM	[77]
	<i>N. americanus</i> L3 IC <sub>50</sub> : >150 μM <i>N. americanus</i> adult IC <sub>50</sub> : 19.5 μM	[77]
<b>In vivo activities against helminths</b>	<i>T. muris</i> in mice ED <sub>50</sub> : 4.7 mg/kg	[77]
	<i>T. suis</i> in swine: 4-5 mg/kg expelled >99% of <i>T. suis</i>	[67]
<b>Mechanism of action</b>	Agonist of the N-subtype cholinergic receptor	[71]
<b>Mode of action</b>	Interference with acetylcholin-neurotransmission paralyzes the worm, leading to expulsion by the host's bowel movement	[35]
<b>Non-anthelmintic bioactivities</b>	Reduces bacterial biofilm of <i>Porphyromonas gingivalis</i> by inhibiting the fumarate reductase (IC <sub>50</sub> =2.2 μM).	[72]
	Reduces the heme biosynthesis by stabilizing the HsPBGS hexamer quaternary structure (IC <sub>50</sub> =18 μM).	[74]
<b>Analytical methods published</b>	Oxantel pamoate and pyrantel pamoate from tablets using HPLC-UV	[70]



prescribed dose (Dolpac®: 20 mg/kg oxantel, 5 mg/kg pyrantel, 5 mg/kg praziquantel) [65]. The absorption of oxantel after oral administration to dogs is negligible [66].

Oxantel pamoate also demonstrated high efficacy in the *T. suis* swine model. After a dose of 4 to 5 mg/kg more than 99% of *T. suis* were eliminated [67].

An oxantel pamoate product also exists for human use, however, only in Columbia, Peru, and the Philippines [68], although, it is currently not produced in these countries. The product is combined with pyrantel pamoate and prescribed against *Trichuris trichiura*, *Enterobius vermicularis*, *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, *Trichostrongylus orientalis*, and *Trichostrongylus colubriformis*. The absorption through the intestinal wall is low. The maximal plasma concentration ( $C_{max}$ ) is reached approximately 2-4 hours after oral application, and about 7% of the dose is found in the urine. Most of the drug is excreted from the body 12 hours after application [69].

Beside the information of the package, no data on the pharmacokinetics of oxantel have been published so far (date of information search: 05.11.2015). This has the consequence, that almost no quantitative analytical methods are available. Only one method using high pressure liquid chromatography (HPLC) and UV detection has been published in 1988, describing the quantification of oxantel and pyrantel in oxantel-pyrantel tablets [70].

On the molecular level, oxantel interferes with acetylcholine receptors [35]. More precisely, oxantel is an N-subtype cholinergic agonist. Levamisole and pyrantel on the other hand are agonists of the L-subtype cholinergic receptor. Therefore, the combination of oxantel and pyrantel were proposed to have therapeutic advantages, covering N- and L-subtypes, and thus increasing the spectrum of action and reducing the potential for development of drug resistance [71].

Other bioactivities were found for oxantel pamoate. Oxantel disrupts biofilm development of the oral bacterium *Porphyromonas gingivalis* with a minimal inhibitory concentration (MIC) of 125  $\mu$ M. The target enzyme is fumarate reductase, which has an important role in the fermentation of amino acids. Fumarate reductase is inhibited at an  $IC_{50}$  of 2.2  $\mu$ M [72]. The growth of other anaerobic bacteria, such as *Helicobacter pylori* and *Campylobacter jejuni*, are also inhibited, but to a lesser extent (MIC >750  $\mu$ M) [73].

Oxantel was found to be an inhibitor of the human porphobilinogen synthase (HsPBGS) with low activity ( $IC_{50}$ =18  $\mu$ M). HsPBGS is an essential enzyme involved in heme biosynthesis. Stabilization of the lower active hexamer structure of HsPBGS, opposed to the higher active octamer, reduces HsPBGS activity, resulting in reduced heme biosynthesis. This can implicate toxicities such as lead poisoning [74].

### **1.3.6 The need for assessing pharmacokinetics and drug-drug interactions of promising co-administrations against trichuriasis**

Co-administered therapies could be a solution for higher efficacy and a wider range of efficacy against intestinal nematodes [5]. Most promising partner therapies are albendazole with ivermectin [60],[62], mebendazole [59], or oxantel pamoate [63]. So far, safety studies regarding drug-drug interactions and pharmacokinetic parameters have only been conducted for albendazole plus ivermectin [78],[79]. Therefore, assessing drug-drug interactions and pharmacokinetic parameters for albendazole plus mebendazole and albendazole plus oxantel pamoate is desired.

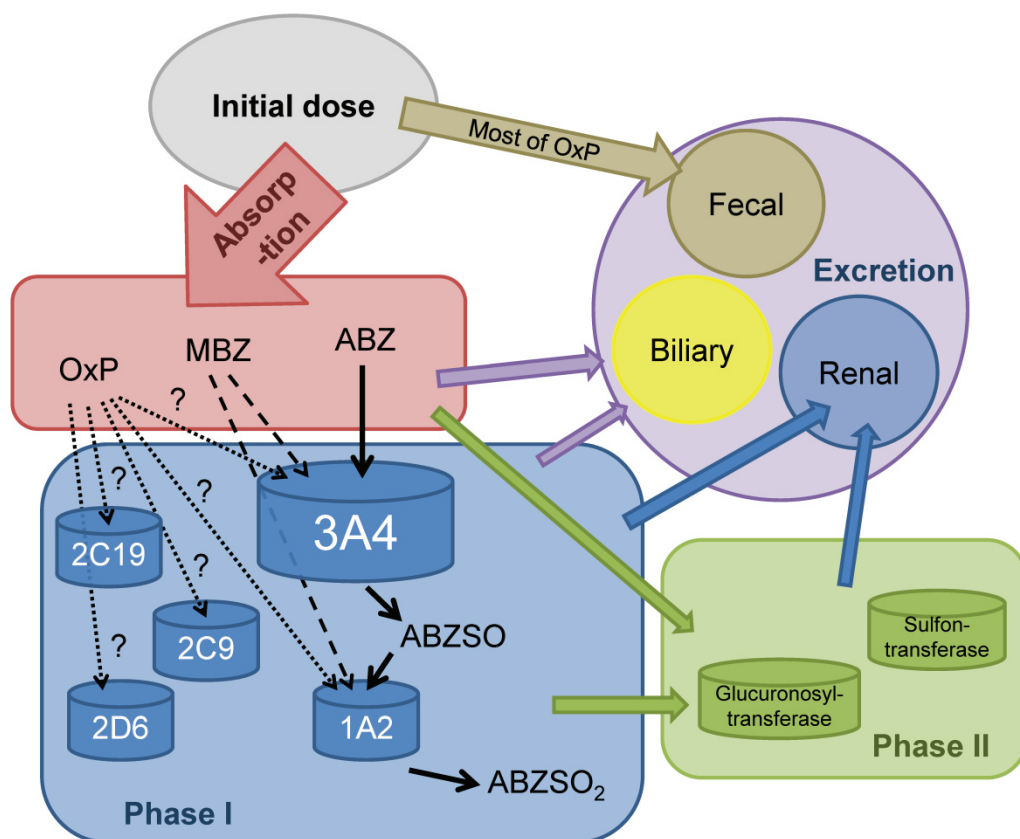
Drug-drug interactions can occur, when one drug alters the other drug's rate of elimination, leading to reduced efficacy or emergence of side effects [80]. After absorption through the intestinal wall, xenobiotics typically undergo a phase I metabolism, during which molecules are oxidized, reduced, or hydrolyzed to more hydrophilic molecules. Hydrophilicity is favorable for quick renal excretion. The main family responsible for phase I metabolism is Cytochrome P450 (CYP) [80]. Around half of the marketed drugs are converted by CYPs, mainly by CYP3A4 [81]. After phase I metabolism, or directly after absorption, molecules undergo typically phase II metabolism. Phase II metabolism involves conjugations of large hydrophilic molecules, such as glucuronides and sulfates, to further facilitate renal excretion [80].

Albendazole's metabolism has been studied intensively. After oral administration, albendazole is quickly metabolized to the active sulfoxide metabolite and the inactive sulfone metabolite by the flavin-containing monooxygenase and CYPs. CYP4A3, and to a smaller extent CYP1A2, are involved in albendazole sulfoxide formation. CYP1A2, however, is strongly involved in the biotransformation of albendazole sulfoxide to albendazole sulfone [82]. In plasma and urine, mainly the metabolites are found, whereas albendazole can only be found in trace amounts [83]. Mebendazole is also excessively metabolized by the first-pass effect, however into inactive molecules [83]. Reduction by the CYP-independent carbonyl transferase was suggested to be the main metabolic pathway for mebendazole [84]. The absorption of oxantel pamoate is low and only 7% is excreted via the kidney [69]. Nothing is known about potential metabolic pathway of oxantel (date of information search: 06.11.2015).

In vitro assays are widely used to predict drug-drug interactions [85]. For instance, CYP subspecies can be tested for drug inhibition to reveal potential metabolic interactions between drugs [86]. Figure 5 shows possible pathway of in vitro CYP-inhibition incorporated into the basic pharmacokinetic processes: absorption, metabolism, excretion (distribution is not

depicted). In the scheme, oxantel pamoate, mebendazole, and albendazole are depicted and it is hinted how their pathways could interfere.

Besides in vitro predictions, in vivo studies on pharmacokinetics are essential to form a more accurate picture of the processes in humans, and steer decisions for further drug development steps [85].



**Figure 5: Simplified pathway of drugs in the body and possible CYP drug-drug interactions of ABZ, MBZ, and OxP**

The absorbed portion of albendazole (ABZ) is metabolized by phase I reactions. CYP3A4 and 1A2 are involved (based on literature, as depicted by black arrows). Mebendazole (MBZ) is not known for metabolism by CYP, but CYP-interfere in the presence of ABZ or ABZSO cannot be excluded (improbable interaction, as depicted by dashed arrows). Little oxantel pamoate (OxP) is absorbed into the body. Interactions with CYP are not known (depicted by dotted arrows).

## 1.4 Aims of the dissertation

As mentioned above, new antischistosomal drugs are needed on the market as an alternative to praziquantel. Furthermore, potential drug-drug interactions of promising co-administrations should be assessed. Therefore, the aim of this dissertation was to identify novel antischistosomal drugs and to elucidate the pharmacokinetics of co-administered treatments against trichuriasis. Following objectives were conducted to achieve the overall project aim:

- Progress the optimization of artemisinin-inspired peroxides in antischistosomal drug discovery
- Determine the potential of the hit compound MMV665852 of the MMV Box as a new antischistosomal class
- Explore the antischistosomal potency of a library of approved cancer drugs, of which several present a promising molecular structure or mechanism of action
- Determine in vitro CYP drug-drug interactions of oxantel pamoate and benzimidazole combinations
- Develop and validate an HPLC/UV-Vis method for simultaneous detection of albendazole's metabolites (albendazole sulfoxide and sulfone), mebendazole, and oxantel in plasma
- Compare promising co-administrations against trichuriasis (albendazole plus oxantel pamoate; albendazole plus mebendazole) to single drug treatments in the rodent model, using the developed HPLC/UV-Vis method, for pharmacokinetic profiling of the co-administrations

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## **Chapter 2**

**Elucidation of the in vitro and in vivo activities of bridged 1,2,4-trioxolanes, bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides, silyl peroxides, and hydroxylamine derivatives against *Schistosoma mansoni***



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## Elucidation of the in vitro and in vivo activities of bridged 1,2,4-trioxolanes, bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides, silyl peroxides, and hydroxylamine derivatives against *Schistosoma mansoni*



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### ABSTRACT

Praziquantel is currently the only drug available to treat schistosomiasis. Since drug resistance would be a major barrier for the increasing global attempts to eliminate schistosomiasis as a public health problem, efforts should go hand in hand with the discovery of novel treatment options. Synthetic peroxides might offer a good direction since their antischistosomal activity has been demonstrated in the laboratory. We studied 19 bridged 1,2,4,5-tetraoxanes, 2 tricyclic monoperoxides, 11 bridged 1,2,4-trioxolanes, 12 silyl peroxides, and 4 hydroxylamine derivatives against newly transformed schistosomula (NTS) and adult *Schistosoma mansoni* in vitro. Schistosomicidal compounds were tested for cytotoxicity followed by in vivo studies of the most promising compounds. Tricyclic monoperoxides, trioxolanes, and tetraoxanes revealed the highest in vitro activity against NTS (IC<sub>50</sub>s 0.4–20.2 μM) and adult schistosomes (IC<sub>50</sub>s 1.8–22.8 μM). Tetraoxanes showed higher cytotoxicity than antischistosomal activity. Selected trioxolane and tricyclic monoperoxides were tested in mice harboring an adult *S. mansoni* infection. The highest activity was observed for two trioxolanes, which showed moderate worm burden reductions (WBR) of 44.3% and 42.9% ( $p > 0.05$ ). Complexation of the compounds with β-cyclodextrin with the aim to improve solubility and gastrointestinal absorption did not increase in vivo antischistosomal efficacy. The high in vitro antischistosomal activity of trioxolanes and tricyclic monoperoxides is a promising basis for future investigations, with the focus on improving in vivo efficacy.

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### 1. Introduction

Schistosomiasis is a neglected tropical disease, caused principally by three human *Schistosoma* species, *S. mansoni*, *S. haematobium*, and *S. japonicum*. Chemotherapy using praziquantel is the mainstay of control. Praziquantel is a broad-spectrum anthelmintic agent and the treatment of choice against schistosomiasis since its discovery in the 1970s. Every year millions of people are treated with praziquantel in the frame of mass drug administration (preventive chemotherapy) programs. For example, in 2012, 27.5 million people in 21 countries were treated with praziquantel. In 2018, the World Health Organization aims to treat as many as 235 million people. With increasing drug pressure, the risk for

praziquantel resistance and tolerance is rising.<sup>1</sup> Hence, there is a need for new antischistosomal drugs.<sup>2,3</sup>

In the past years, various semisynthetic and synthetic peroxide classes have been studied for their antischistosomal properties in vitro, in vivo as well as clinical trials, including the artemisinins,<sup>4</sup> ozonides (or trioxolanes),<sup>5,6</sup> trioxaquinones,<sup>7</sup> and dioxolanes.<sup>8</sup> It has been hypothesized that the peroxide moiety interferes with heme polymerization, which is responsible for both the antischistosomal and antimalarial activity.<sup>9,10</sup>

We recently studied the antischistosomal activity of synthetic peroxides (bridged 1,2,4,5-tetraoxanes, aliphatic peroxidated dicarbonyl compounds and their heteroanalogs, tricyclic monoperoxides) and identified two promising classes, bridged 1,2,4,5-tetraoxanes and tricyclic monoperoxides, which revealed IC<sub>50</sub>s of 0.3 and 11.8 μM against adult *S. mansoni* in vitro and WBRs of 75.4% and 82.8% in the *S. mansoni* mouse model.<sup>11</sup>

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In the present work, we synthesized a new set of bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides as well as bridged 1,2,4-trioxolanes, silyl peroxides, and hydroxylamine derivatives. The latter three substance classes were tested for the first time for their antischistosomal activity. Compounds were first tested against the larval and adult forms of *S. mansoni*. Compounds showing a promising antischistosomal activity and a selectivity index >1 in vitro were subsequently tested in vivo. Selected compounds were additionally packed into  $\beta$ -cyclodextrin with the aim to improve bioavailability.

## 2. Material and methods

### 2.1. Drugs and media

We studied 19 bridged 1,2,4,5-tetraoxanes, 11 bridged 1,2,4-trioxolanes, 12 silyl peroxides, 2 tricyclic monoperoxides, and 4 hydroxylamine derivatives (Table 1). For comparison 2 hit compounds of the previous study<sup>11</sup> are presented. The 50 compounds were prepared based upon methods described in literature.<sup>12–20</sup>

For in vitro evaluations, compounds were prepared as 10 mg/ml stock solutions in dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Buchs, Switzerland).

Medium 199 and RPMI 1640 were purchased from Life Technologies (Carlsbad, CA, USA), heat inactivated fetal calf serum (FCS), penicillin, and streptomycin from Lubioscience (Lucerne, Switzerland), and L-glutamine from Sigma–Aldrich.  $\beta$ -cyclodextrin for drug complexation was purchased from Acros Organics (Geel, Belgium). For oral suspensions of in vivo testing, compounds not packed in  $\beta$ -cyclodextrin were suspended in Tween 80 (Fluka, Buchs, Switzerland), ethanol, and H<sub>2</sub>O (7:3:90), whereas drugs packed in  $\beta$ -cyclodextrin were suspended in polyethylene glycol 300 (Sigma–Aldrich) and H<sub>2</sub>O (60:40).

### 2.2. Mice and parasite

In vivo studies were approved by the veterinary authorities of Canton Basel-Stadt (license No. 2070), based on Swiss cantonal and national regulations.

Three week old female NMRI mice ( $n = 64$ ) were purchased from Charles River (Sulzfeld, Germany), kept at 22 °C, 50% humidity, with an artificial 12-h day/night cycle, and free access to rodent diet and water. Four-week-old mice were infected by subcutaneous injection with 100 *S. mansoni* cercariae (Liberian strain), harvested from *S. mansoni*-infected *Biomphalaria glabrata* snails.

### 2.3. In vitro drug assay on newly transformed schistosomula (NTS)

*S. mansoni* cercariae were mechanically transformed to NTS, and stored in Medium 199 supplemented with 5% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C, with 5% CO<sub>2</sub>, as described previously.<sup>21</sup>

For the drug assay, NTS were added (100/well) to 12.5  $\mu$ g/ml compound dilutions in supplemented Medium 199, which were prepared in flat-bottom 96-well plates (BD Falcon). Compounds that killed the NTS after a 72-h incubation period in at least one well were tested at lower concentrations (0.4, 0.8, 1.6, 3.1, 6.3, and 12.5  $\mu$ g/ml) for IC<sub>50</sub> determination. NTS exposed to the highest concentration of DMSO (0.13%) served as control. Assays were performed in triplicate, and repeated once. Drug activity was evaluated microscopically (Carl Zeiss, Germany; 80–200 $\times$  magnification) 72 h post-incubation, using scoring from 3 (normal activity and morphology) to 0 (no motility, impaired morphology, and granularity).<sup>22</sup>

### 2.4. In vitro drug assay on adult *S. mansoni*

Adult schistosomes were harvested by dissection from mesenteric and hepatic portal veins of infected mice, seven to nine weeks post-infection. Schistosomes were stored in RPMI 1640 medium supplemented with 5% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, at 37 °C with 5% CO<sub>2</sub>.<sup>6</sup>

For drug activity assessment, adult schistosomes (three of both sexes) were put into 25.0  $\mu$ g/ml compound dilutions in supplemented RPMI medium using 24-well flat-bottom plates (BD Falcon). Schistosomes incubated in the highest concentration of DMSO in culture medium (0.25%) served as control. Compounds that killed the worms 72 h post-incubation were subsequently tested at lower concentrations (0.3, 0.9, 2.8, 8.3, and 25.0  $\mu$ g/ml) for IC<sub>50</sub> determination, and scored via microscopic readout in the same manner as described above for the NTS. Assays were performed in duplicate, and repeated once.<sup>22</sup>

### 2.5. L6 cytotoxicity drug assay

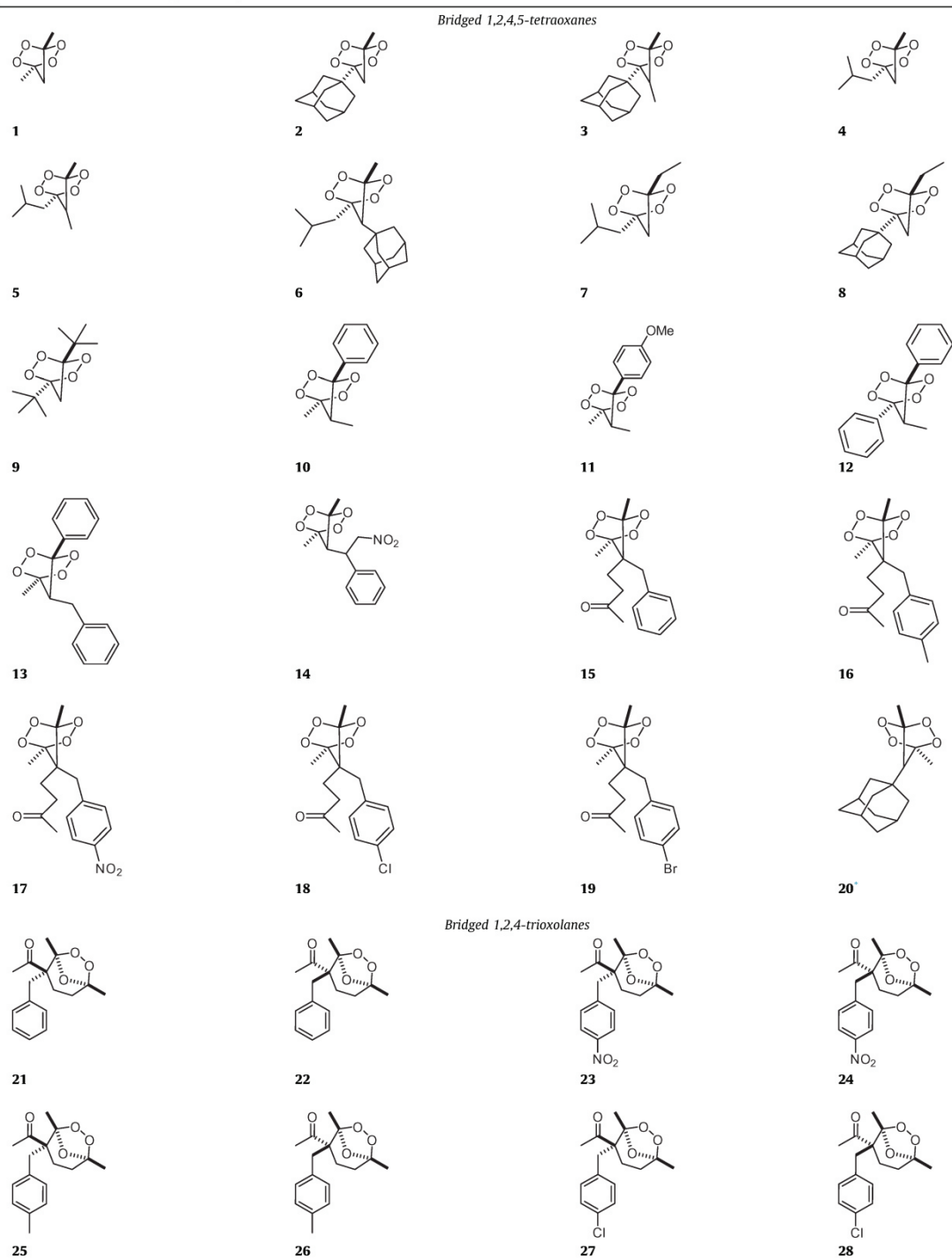
Rat skeletal myoblast L6 cells (ATCC, Manassas, VA USA) were seeded ( $2 \times 10^3$ /well) into 96-well flat-bottom plates (BD Falcon). After a 24-h adherence time, cells were incubated with a 3-fold serial dilution starting at 90  $\mu$ g/ml. After 70 h, resazurin (Sigma–Aldrich) was added to the wells, and after another 2 h, the fluorescence was read using an excitation wavelength of 536 nm and an emission wavelength of 588 nm (SpectraMax, Molecular Devices; Softmax, version 5.4.1). Cells incubated with a 3-fold serial dilution of podophyllotoxin (Sigma–Aldrich) starting at 100 ng/ml served as positive control. IC<sub>50</sub> determination was performed in duplicate, and repeated twice.<sup>23</sup>

### 2.6. Complexation of drugs with $\beta$ -cyclodextrin

Compound solutions in acetonitrile (2 ml) were mixed into a solution of  $\beta$ -cyclodextrin in H<sub>2</sub>O and acetonitrile (70:30; 30 ml), with a molar ratio of 1:1  $\beta$ -cyclodextrin to drug. The heterogeneous mixture was stirred at 20–25 °C for 24 h, and the solvent was subsequently removed with a water jet vacuum pump (Vitalab, Grossostheim, Germany). Analytical data are shown as [Supplementary data \(Supplementary file\)](#).

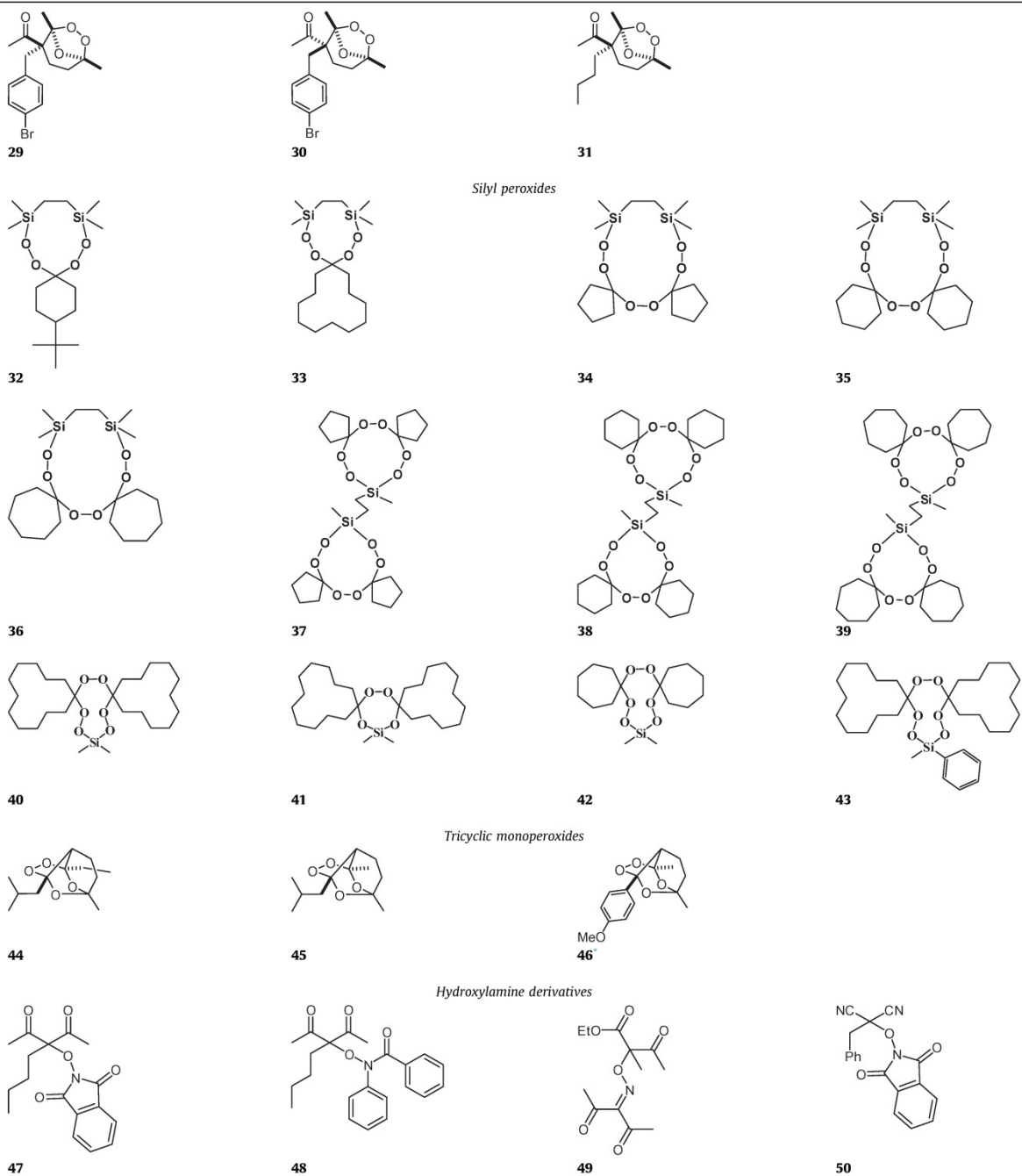
### 2.7. Analytical instrumentation and methods

NMR spectra of compounds were recorded on a Bruker AW-300 (300.13 MHz for <sup>1</sup>H, 75.48 MHz for <sup>13</sup>C) and Bruker Avance 400 (400.1 MHz for <sup>1</sup>H, 100.6 MHz for <sup>13</sup>C) in CDCl<sub>3</sub> and DMSO-*d*<sub>6</sub>. Thin layer chromatography (TLC) analysis was carried out on standard silica gel chromatography plates. Melting point determinations were carried out on a Kofler hot-stage apparatus. Chromatography was performed using silica gel (63–200 mesh and 5–40  $\mu$ m). Elemental analysis on carbon, hydrogen, and nitrogen was carried out using a 2400 Perkin–Elmer CHN analyzer. Determination of purity of all compounds was executed by elemental (combustion) analysis. For all peroxides, deviation from the theoretical values for C, H, and N content was less than 0.4%. High-resolution mass spectra (HRMS) were measured by using electrospray ionization (ESI). The measurements were performed in positive-ion mode (interface capillary voltage 4500 V); the spectra were acquired in the *m/z* range of 50–3000; the external/internal calibration was done with Electrospray Calibrant Solution. Solutions in MeCN were injected with a syringe (flow rate 3 ml/min). Nitrogen was applied as a dry gas; the interface temperature was set at 180 °C. These

**Table 1**  
Chemical structures of investigated compounds

(continued on next page)

Table 1 (continued)



\* Lead compound of previous study (K. Ingram et al., 2012).

data confirmed >95% purity of all compounds. Structures of all compounds were confirmed using  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. Analytical results of the unbound compounds as well as the  $\beta$ -cyclodextrin-compound complexes are shown in the [Supplementary file](#).

## 2.8. In vivo studies in *S. mansoni*-infected mice

Compound suspensions were orally applied to *S. mansoni*-infected mice (groups of  $n = 4$ ) 49 days post-infection, at a single dose of 400 mg/kg. Untreated infected mice ( $n = 8$ ) served as

control. Mice were euthanized and dissected 16–21 days post-treatment to count the worms in the portal and mesenteric veins, and the liver.<sup>5</sup>

## 2.9. Statistics

Scores of the antischistosomal in vitro drug assays were set in relation to the control values. For in vitro activities, IC<sub>50</sub> values were calculated using CompuSyn software (ComboSyn Inc., New York, NY, USA; version 3.0.1, 2007). The *R*-value represents the linear correlation coefficient, which reflects the conformity or goodness of the experimental data.<sup>24</sup> IC<sub>50</sub> and *r*<sup>2</sup>-values of cytotoxicity determination were calculated by Softmax. IC<sub>50</sub>s of both antischistosomal activities and cytotoxicity were converted to molarity. Selectivity indices were calculated by dividing the IC<sub>50</sub> of the mammalian cell line by the IC<sub>50</sub> of the antischistosomal activity against adult schistosomes. For in vivo drug efficacy assessment, WBRs were calculated by comparing worm counts of treated mouse groups to the control group. The Kruskal–Wallis test (StatsDirect Ltd., Altrincham, UK; StatsDirect, version 2.7.2.) was applied for significance determination (*p* = 0.05).

## 3. Results

### 3.1. In vitro activity against NTS

Of the 48 compounds tested, 24 killed all NTS in at least one well after 72 h at 12.5 µg/ml. Of these, compounds **6** and **21** revealed

very high (IC<sub>50</sub> <1 µM) antischistosomal activities with IC<sub>50</sub> values of 0.9 and 0.4 µM, respectively. Twenty compounds showed high (IC<sub>50</sub>s 1–10 µM) activities (9 tetraoxanes, 7 trioxolanes, 2 tricyclic monoperoxides, and 2 silyl peroxides), and 2 compounds were characterized by moderate (IC<sub>50</sub> >10 µM) antischistosomal activity.

In comparison, in our previous study the most active tetraoxane **20** showed an IC<sub>50</sub> at 0.1 µM, the most active tricyclic monoperoxide **46** at 14.4 µM, and the gold standard praziquantel at 2.2 µM (Table 2).<sup>11</sup>

### 3.2. In vitro activity against adult *S. mansoni*

All 48 compounds were tested on adult *S. mansoni*. Twenty-six compounds killed the worms following incubation at 25.0 µg/ml for 72 h. Of these, 16 compounds (7 tetraoxanes, 7 trioxolanes, and 2 tricyclic monoperoxides) revealed high (IC<sub>50</sub> 1–10 µM) antischistosomal activity. Ten compounds showed moderate (IC<sub>50</sub> >10 µM) activity (6 tetraoxanes, 4 trioxolanes) (Table 2). IC<sub>50</sub>s of the hit compounds of our previous study were 0.3 µM for tetraoxane **20** and 11.8 µM for tricyclic monoperoxide **46**, as well as 0.1 µM for the standard drug praziquantel (Table 2).<sup>11</sup>

### 3.3. Selectivity of adult *S. mansoni*-active drugs

Compounds exhibiting IC<sub>50</sub>s ≤10 µM against adult schistosomes were deemed as potent schistosomicidal and therefore tested on a mammalian cell line to determine the compound toxicity and thereof their selectivity (Table 2). Eight compounds

**Table 2**  
IC<sub>50</sub>s of investigated compounds against NTS, adult *S. mansoni*, and L6 cells and the resulting selectivity indices

	Compound	NTS		Adult		L6-cells		SI
		IC <sub>50</sub> (µM)	<i>r</i> -value	IC <sub>50</sub> (µM)	<i>r</i> -value	IC <sub>50</sub> (µM)	<i>r</i> -value	
	Praziquantel <sup>†</sup>	2.2	0.90	0.1	1.9	>96	–	>960
	Tetraoxane 20 <sup>*</sup>	0.1	0.90	0.3	1.0	1.7	–	5.7
	Tricyclic monoperoxide 46 <sup>*</sup>	14.4	0.80	11.8	0.90	8.2	–	4.9
	Tetraoxanes							
	1	20.2	0.98	ND	–	ND	–	–
	4	3.7	0.95	7.4	0.94	4.4	1.00	0.6
	7	4.7	0.86	20.9	0.96	ND	–	–
	9	ND	–	12.1	0.97	ND	–	–
	2	1.3	0.84	2.0	0.90	<0.4	–	<0.2
	8	1.8	0.93	2.0	0.90	<0.4	–	<0.2
	6	0.9	0.96	1.8	0.96	1.0	1.00	0.5
	15	1.6	0.90	10.9	0.96	ND	–	–
	17	ND	–	23.6	0.90	ND	–	–
	16	5.2	0.98	9.8	0.96	2.5	1.00	0.3
	18	3.5	0.90	15.4	0.97	ND	–	–
	19	1.3	0.88	8.4	0.96	2.5	1.00	0.4
	3	4.0	0.92	3.9	0.96	<0.4	–	<0.1
	5	ND	–	20.8	0.99	ND	–	–
	Trioxolanes							
	21	0.4	0.79	1.8	0.89	5.4	0.99	2.9
	22	5.6	1.0	10.3	0.95	ND	–	–
	23	5.7	0.92	7.0	0.92	22.7	0.97	1.7
	24	7.7	0.99	22.8	0.89	ND	–	–
	25	ND	–	10.0	0.97	ND	–	–
	26	12.2	0.98	7.4	0.95	15.3	0.98	7.4
	31	ND	–	12.2	0.89	ND	–	–
	27	2.8	0.81	4.2	0.96	2.7	1.00	1.3
	28	3.2	0.92	11.2	0.95	ND	–	–
	29	6.2	0.98	6.6	0.98	7.3	0.97	1.1
	30	2.2	0.92	4.2	1.00	8.1	0.99	1.6
	Tricyclic monoperoxides							
	44	2.7	0.92	4.4	0.96	24.4	1.00	5.7
	45	2.0	0.88	2.0	0.89	4.9	0.99	3.0
	Silyl peroxides							
	32	4.0	0.94	ND	–	ND	–	–
	33	7.2	0.88	ND	–	ND	–	–

ND: not done, since compounds did not cause death at highest concentration tested.

SI: selectivity index (cytotoxicity IC<sub>50</sub> divided by adult schistosome IC<sub>50</sub>).

<sup>\*</sup> Data reproduced from.<sup>11</sup>



**Table 3**Worm burden reductions of investigated compounds in mice harboring an adult *S. mansoni* infection after a single oral dose of 400 mg/kg

Compound	Number of mice tested	Average worm burden (SD)	Worm burden reduction (%)
Control <sup>1</sup>	8	34.1 (10.3)	—
Control <sup>2</sup>	8	23.6 (11.7)	—
20 <sup>*</sup>	6	6.7 (2.5)	75.4
46 <sup>*</sup>	4	5.3 (5.0)	82.8
30 <sup>1</sup>	3	19.0 (4.6)	44.3
27 <sup>1</sup>	4	19.5 (12.4)	42.9
44 <sup>1</sup>	3	25.0 (7.0)	26.7
26 <sup>1</sup>	4	30.8 (8.7)	9.9
21 <sup>1</sup>	4	32.0 (3.3)	6.2
45 <sup>1</sup>	4	37.0 (12.5)	0.0
CD-44 <sup>2</sup>	3	16.3 (16.6)	30.9
CD-27 <sup>2</sup>	4	22.5 (3.3)	4.8
CD-26 <sup>2</sup>	4	24.5 (6.6)	0.0
CD-30 <sup>2</sup>	3	33.0 (6.7)	0.0
CD-20 <sup>2</sup>	4	33.3 (12.3)	0.0
CD-46 <sup>2</sup>	3	35.7 (18.8)	0.0

CD: complexation with  $\beta$ -cyclodextrin.

SD: standard deviation.

Superscripts 1, 2 refer to the respective control groups.

\* Data reproduced from.<sup>11</sup>

indicated selective toxicity towards the parasite (SI >1), namely compounds **21**, **23**, **26**, **27**, **29**, **30**, **44**, and **45**, all representatives of the tricyclic monoperoxide or the trioxolane class. Tetraoxanes were excluded from in vivo studies due to unselective toxicity. For comparison, the tetraoxanes of the previous study showed SIs  $\leq 5.7$ .<sup>11</sup>

### 3.4. In vivo drug efficacy against adult *S. mansoni*

Four trioxolanes (**21**, **26**, **27**, **30**) and 2 tricyclic monoperoxides (**44**, **45**) progressed into in vivo studies based on antischistosomal activity and selectivity. Compound **29** was not considered for in vivo testing because it showed high structural similarity to compound **27**, which had a more promising antischistosomal profile. Furthermore, compound **23** was excluded because of its higher IC<sub>50</sub> and lower selectivity compared to the other compounds chosen for in vivo studies.

Compounds **30** and **27** showed slight, but not significant ( $p > 0.05$ ), worm burden reductions (WBR) of 44.3% and 42.9%, respectively. Compounds **26**, **44**, **21**, and **45** showed low WBRs of 0.0–26.7%. Compounds **26**, **27**, **30**, and **44** were prepared as  $\beta$ -cyclodextrin complexes with the aim to improve solubility and gastrointestinal wall permeation.<sup>25</sup> For comparison, two lead molecules (**20**, **46**) from our previous study<sup>11</sup> were also packaged. Compounds CD-**20** and CD-**46** showed no WBRs. Compounds CD-**26**, CD-**27**, CD-**30**, and CD-**44** of the present study showed low WBRs between 0.0–30.9% ( $p > 0.05$ ). All in vivo results are presented in Table 3.

## 4. Discussion

Schistosomiasis is a debilitating disease, affecting hundreds of millions of people living in poor, rural areas of the subtropics and tropics. Chemotherapy is the mainstay of control, yet there is no alternative to praziquantel, the gold standard, and no drug is in the clinical pipeline.<sup>26</sup> This is a perilous situation if praziquantel tolerance or resistance should arise.

Given the promising findings obtained with bridged 1,2,4,5-tetraoxanes and tricyclic monoperoxides earlier,<sup>11</sup> in the present study, we tested a new series of peroxidic compounds, including

bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides, bridged 1,2,4-trioxolanes, silyl peroxides, and hydroxylamine derivatives. We tested 48 compounds (depicted in Table 1) in vitro on two stages of *S. mansoni*, the larval (NTS) and the adult, and assessed their cytotoxicity using a mammalian cell line. Subsequently, potent and selective compounds were tested in the *S. mansoni* mouse model.

Of the 48 compounds tested, 24 compounds killed NTS at 25.0  $\mu\text{g/ml}$  (around 100  $\mu\text{M}$ ) of which 22 revealed high activity (IC<sub>50</sub>  $\leq 10 \mu\text{M}$ ). Twenty-six compounds killed adult *S. mansoni* at 25.0  $\mu\text{g/ml}$ . Sixteen of these were characterized by high activity (IC<sub>50</sub>  $\leq 10 \mu\text{M}$ ). Fourteen compounds showed high activity (IC<sub>50</sub>  $\leq 10 \mu\text{M}$ ) toward both stages, with NTS being slightly more affected than adult *S. mansoni*. The trend of higher sensitivity of NTS against synthetic peroxides was already observed previously.<sup>11</sup>

Of the 19 tetraoxanes tested, 7 showed IC<sub>50</sub>  $\leq 10 \mu\text{M}$  against both NTS and adult schistosomes. The 4 adamantyl-containing tetraoxanes were the most potent, with IC<sub>50</sub> values down to 2.0  $\mu\text{M}$  on adult flukes. Replacing the adamantyl moiety with small alkyl substituents lowered or annihilated the activity. Placing aryls at the side position lead to loss of activity as well. For instance, the adamantyl-containing tetraoxane **3** had an IC<sub>50</sub> of 3.9  $\mu\text{M}$ , whereas replacing the analog's adamantyl substituent with an aryl (compound **10**) or an isobutyl (compound **5**) showed no, or moderate (IC<sub>50</sub> 20.8  $\mu\text{M}$ ) activity, respectively. Therefore, this set of molecules agrees upon the supporting but not essential nature of adamantyl, which was noted previously.<sup>11</sup> Due to unselective activity however, no tetraoxane was tested in vivo. The toxicity observed with this set of tetraoxanes is in contrast to our previous findings, where the tested tetraoxanes revealed selectivity (SI  $\leq 5.7$ ).

The 2 tricyclic monoperoxides with simple alkyl substituents showed selective antischistosomal activity in vitro, but in mice they reduced the *S. mansoni* worm burden inefficaciously. The reason for the differing in vivo activity between these two and the previously tested tricyclic monoperoxide derivative remains to be elucidated.

Of the 11 trioxolanes tested, 5 revealed IC<sub>50</sub> values  $\leq 10 \mu\text{M}$  against larval and adult schistosomes, which all showed selective schistosomal toxicity. Some trioxolanes were diastereomers (**21**, **22**; **23**, **24**; **25**, **26**; **27**, **28**; **29**, **30**), but no consistent

configuration-dependent activity was noted. Also the role of the electron-withdrawing residue (e.g., halogen or nitro) could not be determined. Two trioxolanes (**30** and **27**) were tested in vivo, and resulted in the highest WBRs of this study with 44.3% and 42.9%, respectively, but without significance ( $p > 0.05$ ).

Hydroxylamine derivatives were inactive against both NTS and adult *S. mansoni* in vitro. Also the newly synthesized silyl peroxides showed poor to no activity in vitro. Only 2 out of 12 silyl peroxides (**32** and **33**) revealed activity against NTS with  $IC_{50}$  values  $\leq 10 \mu\text{M}$ . Poor solubility of these compounds was observed.

Selected compounds were retested in vivo after their complexation with  $\beta$ -cyclodextrin, since cyclodextrins are known to improve compound solubility and absorption by biological barriers, such as mucosae or skin.<sup>25</sup> Nevertheless, observed WBRs of cyclodextrin-drug complexes were lower than free drugs. Likewise, two lead compounds from our previous work resulted in low WBRs. In general, cyclodextrins can enhance, but also hamper (e.g., with excess cyclodextrin) drug delivery through biological membranes, hence optimization of the complexation procedure is usually needed.<sup>27</sup>

In conclusion, trioxolanes revealed the most potent in vitro schistosomicidal activity and selectivity of all peroxidic drugs investigated in this study, with moderate in vivo worm burden reductions. Tetraoxanes and tricyclic monoperoxides, the lead candidates of the previous study, showed high in vitro antischistosomal activity, but failed demonstrating selectivity, or in vivo efficacy, respectively. Further modifications on the compounds are necessary to improve in vivo efficacy.

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#### Supplementary data

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## **Chapter 3**

**Activities of *N,N'*-diarylurea MMV665852 analogs  
against *Schistosoma mansoni***

# Activities of *N,N'*-Diarylurea MMV665852 Analogs against *Schistosoma mansoni*

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**There is an unmet need to discover and develop novel antischistosomal drugs. As exemplified by MMV665852, *N,N'*-diarylureas have recently emerged as a promising antischistosomal chemotype. In this study, we evaluated the structure-activity relationships of 46 commercially available analogs of MMV665852 on newly transformed schistosomula (NTS) and adult *Schistosoma mansoni* worms *in vitro*. Active compounds were evaluated with a cytotoxicity assay, *in silico* calculations, metabolic stability studies, and an *in vivo* assay with mice harboring adult *S. mansoni* worms. Of the 46 compounds tested at 33.3  $\mu$ M, 13 and 14 compounds killed NTS and adult worms, respectively, within 72 h. Nine compounds had 90% inhibitory concentrations ( $IC_{90}$ s) of  $\leq 10$   $\mu$ M against adult worms, with selectivity indexes of  $\geq 2.8$ . Their physicochemical properties and permeation through an artificial membrane indicated good to moderate intestinal absorption. Their metabolic stabilities ranged from low to high. Despite satisfactory *in vitro* results and *in silico* predictions, only one compound resulted in a statistically significant worm burden reduction (66%) after administration of a single oral dose of 400 mg/kg of body weight to *S. mansoni*-infected mice. Worm burden reductions of 0 to 43% were observed for the remaining eight compounds tested. In conclusion, several analogs of the *N,N'*-diarylurea MMV665852 had high efficacy against *S. mansoni in vitro* and favorable physicochemical properties for permeation through the intestinal wall. To counteract the low efficacy observed in the mouse model, further investigations should focus on identifying compounds with improved solubility and pharmacokinetic properties.**

Schistosomiasis is a parasitic disease caused by blood-dwelling flukes of the genus *Schistosoma*. An estimated 230 million people in 76 countries are infected, and 779 million people live at risk of infection (1). The clinically most relevant species are *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. Preventive chemotherapy is the strategy of choice to control schistosomiasis, and in 2012, approximately 27.5 million people were treated (2). Treatment of schistosomiasis relies on a single drug: praziquantel. Despite praziquantel's single-dose efficacy, drug safety, and relatively low cost, new antischistosomal with differentiated modes of action need to be developed to address emergent drug resistance (3). Indeed, drug pressure on the worms increases continuously, and cases of praziquantel resistance in *S. mansoni* have already been reported (4). Additionally, a clear disadvantage of praziquantel is its lack of activity against the early developing schistosome stage, also highlighting the need to develop novel antischistosomal drugs with multistage activity (5).

We recently screened the Medicines for Malaria Venture (MMV) Malaria Box (6) of 400 commercially available malaria-active compounds for antischistosomal activity (7). This library was initially assayed *in vitro* against schistosomula, and then active compounds were tested against adult worms; from this screen, selected compounds progressed to *in vivo* evaluation. MMV665852 was the most promising lead identified in that work. This *N,N'*-diarylurea inhibited worm viability *in vitro* by 50% at 0.8  $\mu$ M (50% inhibitory concentration [ $IC_{50}$ ]), and it reduced worm burden in *S. mansoni*-infected mice to 53% (7).

*N,N'*-Diarylureas display a broad spectrum of biological activities and have been investigated for their potential use for tuberculosis (8), malaria (9), HIV (10), immunology (11), and, most extensively, oncology (12).

The aim of the present work was to conduct an initial structure-activity relationship (SAR) study of the *N,N'*-diarylurea MMV665852 against *S. mansoni*. A search of commercially available compound libraries for structures similar to that of MMV665852, using a Tanimoto-Rogers similarity coefficient of 0.85 as the cutoff, identified 46 compounds. These were tested against the larval and adult stages of *S. mansoni*. Hits progressed into a cell-based toxicity assay. In addition, physicochemical properties important for oral bioavailability according to Lipinski's "rule of five" (13) were calculated, intestinal wall permeation was assessed using a parallel artificial membrane permeation assay (PAMPA) (14), and metabolic stability was determined before the compounds were tested *in vivo*.

## MATERIALS AND METHODS

**Drugs and culture media.** Identification of molecules chemically similar to MMV665852 was performed in the eMolecules database, using a Tanimoto-Rogers similarity coefficient of 0.85 as the cutoff, followed by a visual inspection and selection. The 46 identified compounds (Fig. 1) and

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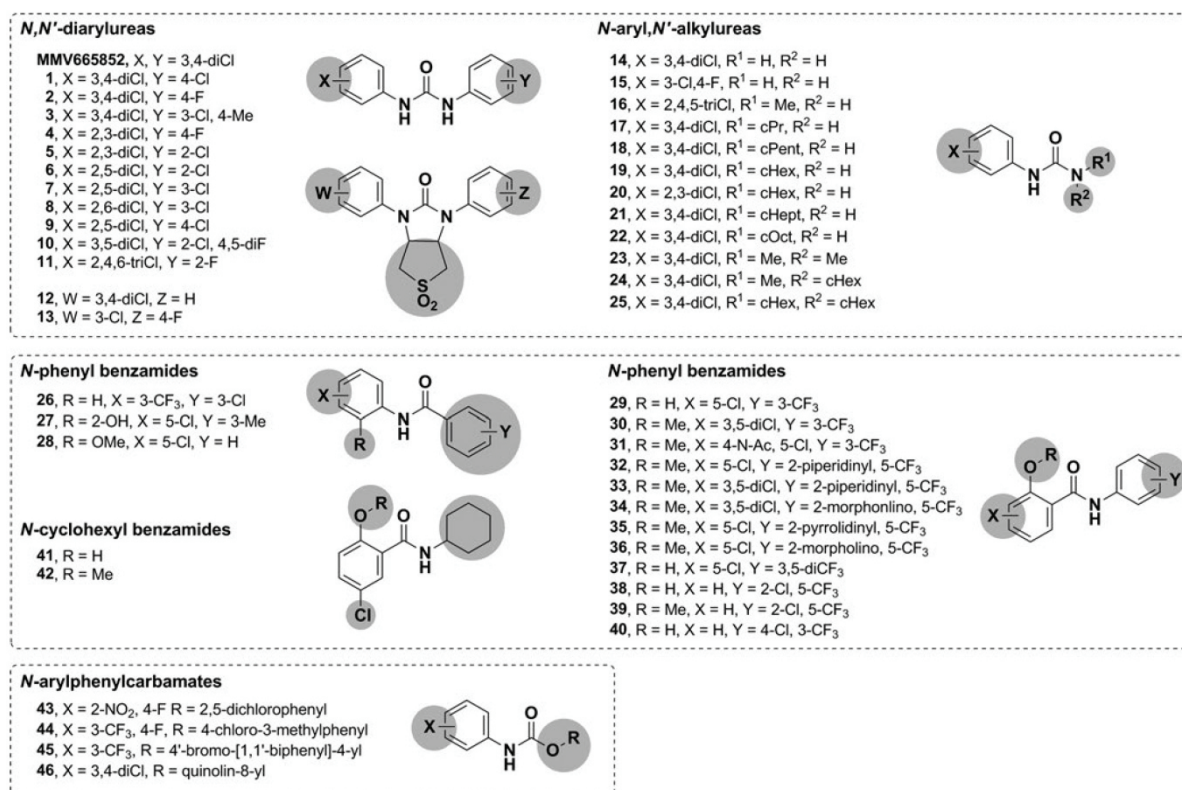


FIG 1 Compound structures.

diuron were purchased from Specs, Sigma-Aldrich, or MolPort. For *in vitro* tests, compounds were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). For *in vivo* tests, compound 37 (15) was synthesized according to the methods of Chen et al. (16) and Hwang et al. (17). The culture media were prepared from medium 199 or RPMI 1640 (Life Technologies) with L-glutamine (Sigma-Aldrich), heat-inactivated fetal calf serum (FCS), penicillin, and streptomycin, which were purchased from LuBioScience.

**Mice and parasites.** *In vivo* experiments were approved by the veterinary authorities of Canton Basel-Stadt, Switzerland (license no. 2070). Female outbred NMRI mice ( $n = 52$ ) were purchased from Charles River (Sulzfeld, Germany), kept at 22°C and 50% humidity with an artificial 12-h–12-h day-night cycle, and provided with water and rodent diet *ad libitum*. The 4-week-old mice were subcutaneously infected with 100 *S. mansoni* (Liberian strain) cercariae, which were collected from *S. mansoni*-infected *Biomphalaria glabrata* snails, as previously described (18).

**Newly transformed schistosomula (NTS) drug assay.** Cercariae were collected from *S. mansoni*-infected *B. glabrata*, mechanically transformed to schistosomula (19), and stored for 12 to 24 h at 37°C and 5% CO<sub>2</sub> in medium 199 supplemented with 5% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin before use.

For the NTS drug assay, 100 NTS per well were incubated in a 96-well plate (BD Falcon) with 33.3 µM compound diluted in supplemented medium 199. Wells containing NTS exposed to drug-free DMSO at a volume equivalent to the highest drug concentration in the assay (0.3%) served as controls. The assay was performed twice in triplicate. The efficacy of the drugs was judged by scoring their overall viability, using phenotypic reference points such as motility, morphology, and granularity. The following scoring scale was used: 3 = normal motility and morphol-

ogy, with no granularity; 2 = movements slowed down, first morphological changes, and signs of granularity were visible; 1 = highly reduced motility and/or altered morphology, with granularity; and 0 = complete immotility, altered morphology, and granularity. Parasites were judged via microscopic readout (magnification, ×80 to ×120; Carl Zeiss, Germany) 24, 48, and 72 h after incubation. Compounds that killed the NTS after 72 h were subsequently tested three times in triplicate, in 2-fold serial dilutions from 33.3 to 0.26 µM, for IC<sub>50</sub> and IC<sub>90</sub> determinations.

**Adult *S. mansoni* drug assay.** Adult *S. mansoni* were collected from mice by dissection at 49 to 70 days postinfection and were maintained in RPMI 1640 culture medium supplemented with 5% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO<sub>2</sub>. Compounds were diluted to 33.3 µM in supplemented RPMI medium in 24-well plates (BD Falcon), to which three worms of each sex were added per well. Worms incubated with drug-free DMSO (0.3%) served as a control. The viability of *S. mansoni* adult was assessed via microscopic readout (in the same manner as described for NTS) at 1, 24, 48, and 72 h postincubation. For compounds that killed the worms after 72 h, IC<sub>50</sub> and IC<sub>90</sub> values were assessed using 3-fold serial dilutions from 33.3 to 0.41 µM in duplicate, and this was repeated once (20). Fast-acting compounds (100% immobilization after 1 h) were monitored 1, 2, 4, 7, 10, 24, 48, and 72 h after incubation. Slow-acting compounds (100% immobilization in 1 to 72 h) were evaluated 4, 24, 48, and 72 h after incubation.

**Rat skeletal myoblast L6 cytotoxicity.** Rat skeletal myoblast L6 cells (ATCC) were seeded at  $2 \times 10^3$  cells/well in 96-well plates (BD Falcon) in RPMI 1640 medium supplemented with 10% FCS and 1.7 µM L-glutamine. Following a 24-h adhesion period at 37°C and 5% CO<sub>2</sub>, cells were incubated with 3-fold serial drug dilutions, starting at 90 µM, for IC<sub>50</sub> determinations. As a positive control, the IC<sub>50</sub> of pod-

phyllo toxin (Sigma-Aldrich) was measured in every experiment, using 3-fold serial dilutions starting at 100 ng/ml. Along with every drug dilution, which was tested in duplicate, one cell-free drug dilution served as baseline. After 70 h of drug incubation, resazurin (Sigma-Aldrich) was added to the assay and incubated for another 2 h. The plates were then read using a SpectraMax M2 plate reader (Molecular Devices) and Softmax software (version 5.4.1), with an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The assay was repeated at least twice (21).

**Calculation of physicochemical properties.** The numbers of hydrogen bond acceptors (HBA) and donors (HBD) were calculated using ChemBioDraw Ultra software by CambridgeSoft. The *in silico* prediction tool ALOGPS 2.1 was used to determine calculated log P (clogP) values of the compounds (22; <http://www.vcclab.org>). Drug-likeness was assessed according to Lipinski's "rule of five" for desirable physicochemical properties, i.e., molecular weight (MW) of  $\leq 500$  g/mol, logP value of  $\leq 5$ , HBD number of  $\leq 10$ , and HBA number of  $\leq 5$  (13).

**Kinetic aqueous solubility.** Compound kinetic aqueous solubility measurements were performed at two pH values (6.0 and 7.4) in triplicate. Compound stock solutions (10 mM) were diluted to 0.1 mM in an aqueous universal buffer solution (Pion). Benzthiazide (Sigma-Aldrich) served as a positive control. After 15 h, 300  $\mu$ l of solution was filtered (96-well, 0.2- $\mu$ m-filter plates; Corning) using vacuum filtration (Whatman Ltd.) to remove precipitates. Equal amounts of filtrate and *n*-propanol (Sigma-Aldrich) were mixed and transferred to 96-well plates for UV detection of the compound spectra (250 to 500 nm) (SpectraMax 190; Molecular Devices). The amount of compound dissolved was calculated by comparing the sample spectra with UV spectra obtained for references and blanks with  $\mu$ SOL Explorer solubility analyzer software (Pion, version 3.4.0.5).

**PAMPA and quantification using LC-MS.** Effective permeation (log  $P_e$ ) was determined in a 96-well format using a parallel artificial membrane permeation assay (PAMPA) (23). The PAMPA sandwich (P/N 110 163), system solution, GIT-0 lipid solution, acceptor sink buffer, and Gut-Box apparatus were purchased from Pion. For each compound, measurements were performed at two pH values (pH 6.0 and pH 7.4) in quadruplicate. Compounds were diluted with system solution to 10  $\mu$ M. Samples (150  $\mu$ l) were withdrawn for reference, and a further 200  $\mu$ l was transferred to donor plate wells of the PAMPA sandwich. The filter membranes at the bottom of acceptor plates were infused with 5  $\mu$ l of GIT-0 lipid solution, and 200  $\mu$ l of acceptor sink buffer was added to the acceptor wells. The sandwich was assembled and placed in the GutBox apparatus. After 16 h, the sandwich was disassembled, and samples (150  $\mu$ l) from donor and acceptor wells, as well as the reference wells, were quantified by liquid chromatography-mass spectrometry (LC-MS).

LC-MS measurements were performed using an 1100/1200 series high-pressure liquid chromatography (HPLC) system coupled to a model 6410 triple-quadrupole mass detector (Agilent Technologies) equipped for electrospray ionization. The system was controlled with Agilent Mass Hunter workstation data acquisition software (version B.01.04). The column used was an Atlantis XBridges amide column (2.1  $\times$  50 mm) with a 3.5- $\mu$ m particle size (Waters). Two mobile phases were prepared to create an organic solvent gradient, i.e., mobile phase A (H<sub>2</sub>O containing 0.1% [vol/vol] formic acid) and mobile phase B (acetonitrile containing 0.1% [vol/vol] formic acid), running at 0.6 ml/min. MS parameters, such as fragmenter voltage, collision energy, and polarity, were optimized individually for each analyte, and the molecular ion was followed for each compound, using the multiple-reaction monitoring mode.

Log  $P_e$  values were calculated from the determined concentrations by using PAMPA Explorer software (version 3.5; Pion) (14).

**Mouse liver microsomes stability assay.** To assess metabolic stability, each compound (1  $\mu$ M) was incubated with liver microsomes (Xenotech) at 37°C and a 0.4-mg/ml protein concentration. The metabolic reaction was initiated by the addition of an NADPH-regenerating system and quenched at various time points over a 60-min incubation period by the

addition of acetonitrile containing diazepam as an internal standard. Control samples (containing no NADPH) were included (and quenched at 2, 30, and 60 min) to monitor for potential degradation in the absence of the cofactor. The samples were analyzed by ultraperformance LC-MS (UPLC-MS) (Waters/Micromass Xevo G2 QTOF instrument) under positive electrospray ionization, and MS spectral data were acquired in the mass range of 80 to 1,200 Da. The microsomes-predicted hepatic extraction ratios ( $E_H$ ), obtained based on the relative rates of test compound degradation *in vitro*, were used to classify compounds as low ( $<0.3$ ), intermediate (0.3 to 0.7), high (0.7 to 0.95), and very-high ( $>0.95$ ) extraction compounds.

***In vivo* studies.** Compounds ( $n = 9$ ) were dissolved in DMSO (5% of the final volume) and diluted with 1% (mass/vol) hydroxypropyl methylcellulose (HPMC) (Sigma-Aldrich) in distilled water to a final concentration of 60 mg/ml. On postinfection day 49, *S. mansoni*-infected mice ( $n = 4$ /group) were treated with single oral doses of 400 mg/kg of body weight. The control group of mice ( $n = 8$ ) remained untreated. At 17 to 21 days posttreatment, the mice were dissected, and the worms residing in the mesenteric veins and the liver were counted and sexed (18).

**Statistics.** *In vitro* activities of the compounds against NTS and adult *S. mansoni* were calculated from the mean viability values in relation to the control value, as follows: drug effect =  $1 - (\text{score}_{\text{drug}}/\text{score}_{\text{control}})$ . From the obtained drug effects, IC<sub>50</sub> and IC<sub>90</sub> values were calculated using CompuSyn software (version 3.0.1; ComboSyn). The linear correlation coefficient ( $r$  value) reflects the conformity of the experimental data (24). IC<sub>50</sub>s against the mammalian cell line and  $R^2$  values (goodness of fit) were generated by the software Softmax. The selectivity index (SI) was calculated by dividing the L6 cytotoxicity IC<sub>50</sub> by the adult *S. mansoni* IC<sub>50</sub>. To assess the *in vivo* efficacy, the mean values for living worms of each treatment group were compared to that of the control group, and worm burden reductions (WBR) were calculated as follows: WBR (%) =  $100\% - 100\% \times (\text{WB}_{\text{treated mice}}/\text{WB}_{\text{control mice}})$ . The statistical significance of the *in vivo* treatments was determined using the Kruskal-Wallis test (at a significance level of 0.05 [ $P$  value]) (StatsDirect, version 2.7.2; StatsDirect Ltd.).

## RESULTS

***In vitro* activity against NTS.** Of the 46 compounds tested at 33.3  $\mu$ M for activity against NTS, 13 killed NTS within 72 h (Table 1). Of these compounds, two (compounds 10 and 37) killed NTS even at the lowest concentration tested (0.26  $\mu$ M). Six compounds (compounds 1, 29, 38, 40, 45, and 46) were characterized by IC<sub>50</sub>s of  $\leq 0.5$   $\mu$ M and IC<sub>90</sub>s of  $\leq 1.4$   $\mu$ M, and five (compounds 2, 25, 32, 41, and 44) had IC<sub>50</sub>s ranging from 1.0 to 9.2  $\mu$ M.

***In vitro* activity against adult *S. mansoni*.** Fourteen of 46 compounds tested at 33.3  $\mu$ M for activity against adult *S. mansoni* killed the worms when incubated for 72 h (Table 1). Of these, eight compounds (compounds 1, 2, 10, 29, 37, 38, 40, and 45) resulted in death of adult *S. mansoni* within 1 h and had IC<sub>50</sub>s of  $\leq 0.8$   $\mu$ M and IC<sub>90</sub> values of  $\leq 2.4$   $\mu$ M after a 72-h incubation period. Figure 2 presents the IC<sub>50</sub>s of these derivatives between 1 and 24 h postincubation. The compounds acted quickly against adult *S. mansoni*: IC<sub>50</sub>s measured after 4 h were similar to those measured after 72 h. The IC<sub>50</sub> of *N*-phenyl benzamide 29 reached the nanomolar range within 1 h. Compounds 3, 4, 22, 25, 26, and 46 were schistosomicidal at 1 to 72 h postincubation, with calculated IC<sub>50</sub>s of 1.8 to 7.4  $\mu$ M.

**Antischistosomal selectivity.** Compounds ( $n = 14$ ) that demonstrated IC<sub>50</sub>s of  $\leq 10$   $\mu$ M against adult worms after 72 h were tested for cytotoxicity by use of L6 rat skeletal muscle cells to select compounds for *in vivo* testing. The antischistosomal selectivities of these compounds appeared to be rather low, with selectivity indexes ranging from 0.8 to 11.6 (Table 1).

TABLE 1 *In vitro* activities of compounds active against NTS and adult *S. mansoni*<sup>a</sup>

Compound	NTS			Adult <i>S. mansoni</i> worms			L6 cells		SI
	IC <sub>50</sub>	IC <sub>90</sub>	<i>r</i>	IC <sub>50</sub>	IC <sub>90</sub>	<i>r</i>	IC <sub>50</sub>	R <sup>2</sup>	
MMV665852 <sup>b</sup>	4.7		1.0	0.8	2.2	1.0	1.5	0.99	1.9
1	0.07	0.3	0.76	0.4	1.4	0.92	3.2	1.00	7.3
2	1.3	2.8	0.90	0.7	2.2	0.94	3.7	0.99	5.4
3	ND			3.6	9.6	0.90	3.3	0.97	0.9
4	ND			7.0	14.7	0.92	5.2	0.98	0.8
10	<0.26			0.2	0.8	0.92	1.4	1.00	7.2
22	ND			6.0	13.9	0.93	5.2	0.99	0.9
25	4.3	8.5	0.97	6.7	13.2	0.91	12.4	0.93	1.9
26	ND			7.4	17.1	0.92	10.4	0.97	1.4
29	0.03	0.2	0.76	0.1	0.5	0.80	1.4	1.00	11.6
32	2.6	501	0.83	ND			ND		
37	<0.26			0.3	1.1	0.89	0.9	1.00	2.8
38	0.2	0.7	0.87	0.6	2.4	0.92	4.0	0.99	4.9
40	0.07	0.3	0.76	0.6	1.8	0.92	2.3	1.00	4.0
41	9.2	30.2	0.87	ND			ND		
44	1.0	3.1	0.97	ND			ND		
45	0.5	1.4	0.93	0.7	2.3	0.93	2.1	1.00	2.9
46	0.2	0.8	0.93	1.8	4.9	0.95	2.1	0.98	1.2

<sup>a</sup> Data are presented for compounds showing activity against NTS and/or adult worms (lethal at 33.3 μM after 72 h of incubation). Worms were incubated in 3-fold serial dilutions of compounds, starting at 33.3 μM, for 72 h, and IC<sub>50</sub> and IC<sub>90</sub> values were calculated. *r*, linear correlation coefficient (values of ≥0.85 are acceptable); R<sup>2</sup>, goodness of fit; SI, selectivity index (IC<sub>50</sub> against L6 cells divided by IC<sub>50</sub> against adult *S. mansoni*); ND, not done (due to lack of activity at 33.3 μM).

<sup>b</sup> See reference 7.

**Physicochemical properties, solubility, and passive intestinal permeability.** For compounds (*n* = 9) with IC<sub>90</sub> values of ≤10 μM within 24 h against both NTS and adult *S. mansoni*, and with selectivity indexes of ≥1, physicochemical properties, solubilities, and PAMPA permeability values were determined (Table 2). MWs and numbers of HBA and HBD of all nine lead candidates were in the acceptable ranges for drug-likeness. Most compounds showed desirable clogP values of ≤5. The aqueous solubilities were generally low (<0.3 μM). The passive permeability of most compounds was predicted to be “good” (−log *P<sub>e</sub>* < 5.7). However, *N,N'*-diarylurea 1 showed a decreased permeability potential at pH 7.4, with a −log *P<sub>e</sub>* value of 6.37 ± 0.35, and arylphenylcarbamate 45 showed moderate to poor permeability potentials at pH 6.0 and pH 7.4, with −log *P<sub>e</sub>* values of 6.23 ± 0.40 and 6.41 ± 0.35, respectively.

**Metabolic phase I stability.** Metabolic stability with mouse liver microsomes was assessed for the nine lead compounds (Table 3). Compound 45 showed the least *in vitro* degradation, with an *E<sub>H</sub>* value of 0.22. Compounds 2, 1, and 40 revealed intermediate degradation, while compounds 10, 37, 29, 38, and 46 revealed high levels of degradation. The metabolic stability for compound 46 could not be determined (due to low solubility).

***In vivo* studies.** All nine compounds progressed to *in vivo* studies (Table 4), where they were tested in mice harboring adult *S. mansoni*. *N*-Phenyl benzamide 38 was the most active compound, with a worm burden reduction (WBR) of 66% (*P* < 0.05). For comparison, praziquantel at 400 mg/kg is characterized by a WBR of 94% (25). Other compounds resulted in statistically insignificant (*P* > 0.05) WBRs, as follows: compound 2, 43%; compound 10, 36%; compound 40, 36%; compound 46, 15%; and

compound 29, 12%. *N,N'*-Diarylurea 1 and aryl *N*-phenylcarbamate 45 did not reduce worm burdens. Toxicity was observed for *N*-phenyl benzamide 37; two mice died, at 1 h and 3 h post-treatment, and one mouse had to be euthanized after 2 h. The majority of compounds did not dissolve completely in the 1% HPMC formulation.

## DISCUSSION

Schistosomiasis is a neglected tropical disease, and research for new antischistosomal drugs is sparse. There is no antischistosomal drug in the developmental pipeline, which is potentially perilous should praziquantel resistance arise. By screening MMV's Open Access Malaria Box, we identified *N,N'*-diarylureas as a new, orally active chemotype against *Schistosoma mansoni* (7).

In this follow-up study, we selected 46 commercially available compounds with chemical similarity to the lead *N,N'*-diarylurea, MMV665852, by using the Tanimoto-Rogers similarity algorithm with a similarity coefficient cutoff of 0.85; these compounds encompassed 13 *N,N'*-diarylureas, 12 *N*-aryl,*N'*-alkylureas, 17 *N*-phenyl benzamides, and 4 aryl *N*-phenylcarbamates. All compounds were first tested *in vitro*, on larval (NTS) and adult schistosomes. Active compounds (IC<sub>50</sub> ≤ 10 μM) were tested for cytotoxicity, and the most selective and active were assessed for drug-likeness (numbers of HBA and HBD, clogP value, solubility, and gastrointestinal permeation [estimated by PAMPA]), followed by microsomal stability determination and oral application to mice harboring chronic *S. mansoni* infections.

Nine compounds had IC<sub>90</sub> values of ≤10 μM against both *S. mansoni* stages after 24 h of incubation, of which 8 were characterized as fast acting. Of these, 7 compounds had higher antischistosomal activity than the lead compound MMV665852 against adult *S. mansoni* and NTS. We observed that fast-acting compounds were also the most potent; compounds that killed adult worms at 33.3 μM within 1 h had IC<sub>50</sub>s of ≤0.8 μM and IC<sub>90</sub> values of ≤2.4 μM (determined after 72 h of incubation), whereas slow-acting compounds (worms were killed within 1 to 72 h at 33.3 μM) had IC<sub>50</sub>s of 1.8 to 7.4 μM. Fast drug action is obviously important for compounds with fast plasma clearance in order to

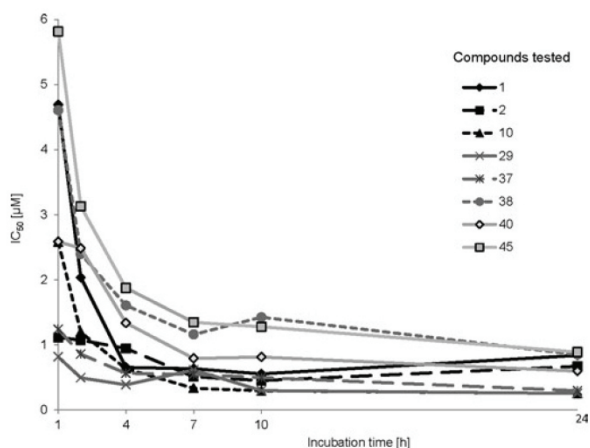


FIG 2 IC<sub>50</sub>s of 8 fast-acting compounds against adult *S. mansoni* observed between 1 and 24 h postincubation.

TABLE 2 Physicochemical properties, solubilities, and intestinal wall permeability potentials of lead candidates<sup>a</sup>

Compound	MW (g/mol)	clogP	No. of HBA	No. of HBD	No. of rule-of-5 violations	Exptl solubility (μM)	PAMPA result (-log P <sub>e</sub> ) (cm/s)
1	315.58	4.71 ± 0.46	3	2	0	<0.3 (pH 6.0) <0.3 (pH 7.4)	5.65 ± 0.64 (pH 6.0) 6.37 ± 0.35 (pH 7.4)
2	299.13	4.28 ± 0.37	3	2	0	<0.3 (pH 6.0) <0.3 (pH 7.4)	4.98 ± 0.18 (pH 6.0) 4.83 ± 0.20 (pH 7.4)
10	351.56	4.89 ± 0.38	3	2	0	<0.3 (pH 6.0) <0.3 (pH 7.4)	5.70 ± 0.25 (pH 6.0) 5.65 ± 0.32 (pH 7.4)
29	315.67	4.40 ± 0.32	3	2	0	<0.3 (pH 6.0) <0.3 (pH 7.4)	5.17 ± 0.28 (pH 6.0) 5.13 ± 0.22 (pH 7.4)
37	383.67	5.34 ± 0.53	3	2	0-1	<0.3 (pH 6.0) <0.3 (pH 7.4)	4.93 ± 0.02 (pH 6.0) 4.67 ± 0.07 (pH 7.4)
38	315.67	4.38 ± 0.27	3	2	0	<0.3 (pH 6.0) <0.3 (pH 7.4)	4.82 ± 0.06 (pH 6.0) 4.62 ± 0.04 (pH 7.4)
40	315.67	4.43 ± 0.35	3	2	0	<0.3 (pH 6.0) <0.3 (pH 7.4)	5.18 ± 0.30 (pH 6.0) 5.27 ± 0.43 (pH 7.4)
45	436.22	6.23 ± 0.54	3	1	1	<0.3 (pH 6.0) <0.3 (pH 7.4)	6.23 ± 0.40 (pH 6.0) 6.41 ± 0.35 (pH 7.4)
46	332.28	4.09 ± 0.34	4	1	0	<0.3 (pH 6.0) <0.3 (pH 7.4)	4.46 ± 0.17 (pH 6.0) 4.53 ± 0.26 (pH 7.4)

<sup>a</sup> MW, molecular weight; HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; clogP, calculated partition coefficient for octanol-water; PAMPA, parallel artificial membrane permeation assay. Permeation values are interpreted as follows: poor, values of >6.3; moderate, values of 6.3 to 5.7; and good, values of <5.7.

impair schistosomes sufficiently before the drug is removed from the body.

High *in vitro* activity against both stages was observed for compounds of three chemical classes: *N,N'*-diarylureas 1, 2, and 10; *N*-phenyl benzamides 29, 37, 38, and 40; and aryl *N*-phenylcarbamates 45 and 46. Ureas with a nonaromatic ring on one side were less active, and those with a hydrogen and/or alkane residue were not active at all. The preferred positions of the halogen groups in the active *N,N'*-diarylureas seemed to be *meta* and *para* rather than *ortho*. *N,N'*-Diarylurea 1 (triclocarban) is an antibacterial agent used in consumer soaps and deodorants. It is mainly active against Gram-positive bacteria but also has slight activity against Gram-negative bacteria and fungi (26). Diuron (com-

pound 23), an herbicidal *N*-aryl,*N'*-alkylurea used in agriculture, was inactive on both parasite stages.

*N*-Phenyl benzamides with 2-hydroxy and 4-chloro substituents at the benzoyl substructure (salicylanilides) and a trifluoromethyl at the *N*-phenyl substructure (e.g., compounds 29 and 37) demonstrated excellent antischistosomal activity. Methylation of the *N*-phenyl benzamide phenol functional group lowered the antischistosomal activity (e.g., compound 38 > compound 39, and compound 41 > compound 42), indicating the importance of the salicylanilide. It is interesting that the active salicylanilides 29, 37, 38, and 40 closely resemble niclosamide, an old nitro-substituted salicylanilide teniacide thought to function by uncoupling oxidative phosphorylation. Due to the low antischistosomal selectivity of these salicylanilides, consistent with previous investigations (15) of this compound class, salicylanilides will be challeng-

TABLE 3 Metabolic stability *in vitro* and calculated *in vivo* hepatic extraction for lead candidates<sup>d</sup>

Compound	<i>t</i> <sub>1/2</sub> (min) <sup>a</sup>	CL <sub>int</sub> (μl/min/mg protein) <sup>b</sup>	<i>E</i> <sub>H</sub> <sup>c</sup>
1	33	52	0.53
2	45	39	0.45
10	8	222	0.83
29	8	223	0.83
37	16	111	0.70
38	6	294	0.86
40	23	77	0.62
45	129	13	0.22
46	UD		

<sup>a</sup> *t*<sub>1/2</sub> (degradation half-life) = ln 2/*k*.

<sup>b</sup> CL<sub>int</sub> = CL<sub>int, in vitro</sub> × liver mass (g)/body weight (kg) × microsomal protein mass (mg)/liver mass (g).

<sup>c</sup> *E*<sub>H</sub> = predicted *in vivo* hepatic extraction ratio, determined as follows: *E*<sub>H</sub> = CL<sub>blood</sub>/Q = CL<sub>int</sub>/(Q + CL<sub>int</sub>); CL<sub>blood</sub> = Q × CL<sub>int</sub>/(Q + CL<sub>int</sub>). *E*<sub>H</sub> values were interpreted as follows: low, values of <0.3; intermediate, values of 0.3 to 0.7; high, values of 0.7 to 0.95; very high, values of >0.95.

<sup>d</sup> The following scaling factors were applied to mouse liver microsome calculations: liver mass = 54.9 g liver/kg body weight; microsomal protein mass = 47 mg/g liver mass; and hepatic blood flow (Q) = 120 ml/min/kg body weight (32). UD, unable to be determined.

TABLE 4 Worm burden and worm burden reductions of lead candidates in mice harboring a chronic *S. mansoni* infection<sup>c</sup>

Compound	No. of mice investigated	Avg (SD) no. of worms	WBR (%)
MMV665852 <sup>a</sup>			53
Praziquantel <sup>b</sup>			94
Control <sup>1</sup>	8	26.9 (20.3)	
Control <sup>2</sup>	8	34.2 (10.3)	
38 <sup>1</sup>	4	9.3 (3.3)	66*
2 <sup>1,2</sup>	3	18.0 (4.2)	43
10 <sup>1</sup>	4	17.3 (12.4)	36
40 <sup>1,2</sup>	4	19.5 (8.7)	36
46 <sup>2</sup>	4	29.0 (10.5)	15
29 <sup>1</sup>	4	23.8 (7.0)	12
45 <sup>2</sup>	4	35.5 (3.5)	0
1 <sup>1</sup>	4	29.5 (15.3)	0
37 <sup>2</sup>	4	21.8 (11.3)	36

<sup>a</sup> See reference 7.

<sup>b</sup> See reference 25.

<sup>c</sup> SD, standard deviation; WBR, worm burden reduction. \*, *P* < 0.05. Values in superscript refer to the corresponding control group.



ing starting points for further optimization. Since only four carbamate isosteres were assessed in this compound set, no conclusions about their SAR can be drawn. However, they may represent an alternative to ureas.

As we noted above for the salicylanilides, we observed relatively low antischistosomal selectivities for the tested compounds by using cytotoxicity data generated from a single mammalian cell line (L6). Indeed, there was a strong correlation ( $r = 0.819$ ;  $P < 0.01$ ) between *S. mansoni* adult  $IC_{50}$ s and L6 cell  $IC_{50}$ s. Interestingly, we previously observed (7) that MMV665852 was an order of magnitude less cytotoxic against the MRC-5 cell line. Thus, obtaining a true picture of cytotoxicity for these compounds will likely require data from multiple mammalian cell lines. Although physicochemical property calculations as well as PAMPA experiments suggested a high potential for intestinal absorption for most of the lead compounds, their measured aqueous solubilities were low. In the microsomal (phase I) stability assay, half of the tested compounds were characterized by good to intermediate stability. Some parameters that were not investigated were efflux effects or protein binding, which can also lower drug plasma levels.

Disappointingly, despite the promising *in vitro* antischistosomal activity of 14 compounds, only *N*-phenyl benzamide (salicylanilide) 38 had a significant WBR (66%) after a single oral dose of 400 mg/kg, which is slightly higher than the WBR of 53% achieved with the same dose of MMV665852 (7). For salicylanilide 38 and its analogs, inactive metabolites due to phase II glucuronidation (11) of the phenol functional group may account for the *in vitro/in vivo* discrepancy of this compound class. Moreover, trilocarban was described to be quickly biotransformed to *N*-glucuronides, leading to fast renal excretion (27, 28). On the other hand, the most likely reason for the *in vitro/in vivo* discrepancy of *N,N'*-diaryureas 1, 2, and 10 is their low aqueous solubility, a liability previously noted (9) in a SAR investigation of antimalarial *N,N'*-diaryureas. As no significant differences were observed between the  $IC_{50}$ s of the *N,N'*-diaryurea MMV665852 in media supplemented with differing amounts of serum (7), protein binding (29) is unlikely to account for the low *in vivo* antischistosomal activity of these *N,N'*-diaryureas. However, structural optimization of *N,N'*-diaryureas can improve their bioavailability, increase drug solubility and plasma peak areas, and decrease drug clearance (30, 31).

In conclusion, we identified several new *N,N'*-diaryureas, aryl *N*-phenylcarbamate, and *N*-phenyl benzamide derivatives of MMV66582 as having high activities against NTS and adult *S. mansoni* worms in *in vitro* experiments. One derivative, *N*-phenyl benzamide 38, had a significant WBR (66%) after a single oral dose, which is a promising result for further investigations. Achieving overall high *in vivo* efficacy and antischistosomal selectivity for these compound classes will likely require identification of more hydrophilic derivatives.

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## **Chapter 4**

**Repurposing of anticancer drugs:  
in vitro and in vivo activities against *Schistosoma mansoni***

RESEARCH

Open Access



# Repurposing of anticancer drugs: *in vitro* and *in vivo* activities against *Schistosoma mansoni*

Noemi Cowan<sup>1,2</sup> and Jennifer Keiser<sup>1,2\*</sup>

## Abstract

**Background:** Drug discovery for the neglected tropical disease schistosomiasis has a high priority. Anticancer drugs, especially protein kinase inhibitors, might serve as a starting point for drug discovery owing to the importance of protein kinases in helminth growth and development. Furthermore, the *Schistosoma mansoni* genome encodes several genes for targets of drugs marketed for human use, including several anticancer drugs.

**Methods:** In this study, we screened the approved oncology drug set of the National Cancer Institute's Developmental Therapeutic Program for antischistosomal activity. Drugs were tested *in vitro* against the larval and adult stage of *S. mansoni*. IC<sub>50</sub> values and albumin binding were determined for active compounds. Lead compounds were tested in the chronic *S. mansoni* mouse model.

**Results:** Eleven of the 114 compounds tested revealed IC<sub>50</sub> values ≤ 10 μM against both *S. mansoni* stages. Five of these lost activity against adult *S. mansoni* in the presence of serum albumin. Of 6 compounds studied *in vivo*, the highest activity was observed from two kinase inhibitors trametinib, and vandetanib, which reduced worm burden by 63.6 and 48.1 % respectively, after a single oral dose of 400 mg/kg body weight.

**Conclusion:** Our study has confirmed that oncology drugs possess antischistosomal activity. There is space for further investigation, including elucidation of the mechanisms of action of schistosome-active cancer drugs, application of different treatment courses, and structure-activity relationship studies for improving drug potency.

**Keywords:** Schistosomiasis, Drug repurposing, Drug screening, Protein kinase inhibitor

## Background

Schistosomiasis is a neglected tropical disease caused by the blood-dwelling fluke of the genus *Schistosoma*. The clinically relevant species are *S. mansoni*, *S. haematobium*, and *S. japonicum*. Approximately 779 million people live at risk of infection, and 230 million are infected [1], causing an estimated 3.3 million disability-adjusted life years (DALYs) [2]. Praziquantel is the sole treatment against all three species. The lack of drugs in the discovery pipeline highly encourages efforts to identify an alternative treatment of schistosomiasis, in anticipation of praziquantel resistance [3, 4]. Drug repurposing is an efficient tool to find new drugs against helminthiases, reducing time and costs of drug research and development [5].

In recent years, imatinib (Gleevec®), a kinase inhibitor used to treat chronic myeloid leukemia, gained attention in

the field of antischistosomal drug research due to its dose- and time-dependent effect on *S. mansoni in vitro* [6]. It has been observed that imatinib causes degenerative changes in the gonads and gastrodermis of schistosomes [7]. Furthermore, protein kinase inhibitors interfere with essential developmental steps in the biology of schistosomes [6, 8].

We have recently identified *N,N'*-diarylureas as a new chemical class potent against *S. mansoni* [9]. A subsequent structure-activity relationship (SAR) study revealed *N,N'*-diarylureas and *N*-phenyl benzamides as the relevant pharmacophores for antischistosomal drug activity [10]. These pharmacophores are also present in some marketed anticancer drugs, such as sorafenib and ponatinib.

The Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) (USA) offers drug repositories free of charge to endorse preclinical research (<https://dtp.nci.nih.gov/repositories.html>); among which is a set of the US Food and Drug Administration (FDA)-approved anticancer drugs.

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There are 54 drugs for human use on the market which exert their pharmacological effect on 26 drug targets which are also encoded by the genome of *S. mansoni* [11]. Fourteen of those drugs are part of DTP's oncology drug set.

The aim of this study was to evaluate DTP's oncology drug set of 114 FDA-approved drugs for antischistosomal activity. We tested the drugs first on the larval stage of *S. mansoni* (schistosomula), followed by screening of larvae-active compounds against adult worms. IC<sub>50</sub> values were then determined against adult *S. mansoni*, and the influence of protein binding on drug activity was assessed using physiological amounts of serum albumin. The most active compounds were subsequently tested in *S. mansoni*-infected mice.

## Methods

### Drugs and media

The cancer drug library used for schistosome *in vitro* assays was gratefully received in June 2014 from the DTP/NCI as 10 mM stock solutions (20 µl) in dimethyl sulfoxide (DMSO) in 96-well plates. Hit compounds were ordered as solid compounds from DTP, and dissolved in DMSO to 10 mM stock solutions. Bosutinib was not available from DTP and was therefore purchased from Sigma-Aldrich. For *in vivo* studies, solid afatinib, bosutinib, ponatinib, trametinib, and vandetanib were purchased from AkScientific. Sunitinib was purchased from VWR as a 100 mM solution in DMSO.

Medium 199, and RPMI 1640 were purchased from Life Technologies. Heat-inactivated fetal calf serum (FCS), penicillin, and streptomycin were purchased from LuBioScience.

### Mouse infection and maintenance

Rodent experiments were authorized by the Canton Basel-Stadt, Switzerland (license no. 2070).

Female NMRI mice, 3-weeks of age, were purchased from Charles Rivers, Germany. After a 1-week adaptation period, mice were infected with cercariae collected from *S. mansoni*-infected intermediate host snails (*Biomphalaria glabrata*), by subcutaneous injection with 100 cercariae [12]. Mice received rodent food and water *ad libitum* and were maintained with a 12-h light/dark cycle, at 22 °C and 50 % humidity.

### Larval schistosome drug assay

*S. mansoni* cercariae were collected from *S. mansoni*-infected *B. glabrata*, and mechanically transformed to newly transformed schistosomula (NTS) [13]. After a resting period of 12–24 h (37 °C, 5 % CO<sub>2</sub>), drugs were tested for NTS activity at a concentration of 33.3 µM in Medium 199 supplemented with 5 % FCS, 200 U/ml penicillin, and 200 µg/ml streptomycin, and prepared in 96-well flat-bottom plates with 100 NTS per well. NTS incubated with

the equivalent volume of drug-free DMSO (0.3 %) served as control. NTS were evaluated 24, 48, and 72 h after incubation via microscopic read out (80–120× magnification; Zeiss; Germany), using a scoring scale from 3 (normal viability, morphology, and granularity) to 0 (no motility, changed morphology, and granularity). Drugs with an activity of ≥ 50 % after 24 h, and/or 90 % after 72 h, and a drug effect on adult schistosomes of ≥ 80 % after 24 h and/or 90 % after 72 h at 33.3 µM, were tested at six different concentrations ranging from 0.14 to 33.3 µM using a 3-fold dilution series for IC<sub>50</sub> determination. All assays were performed in duplicate and repeated once [10].

### Adult schistosome drug assay

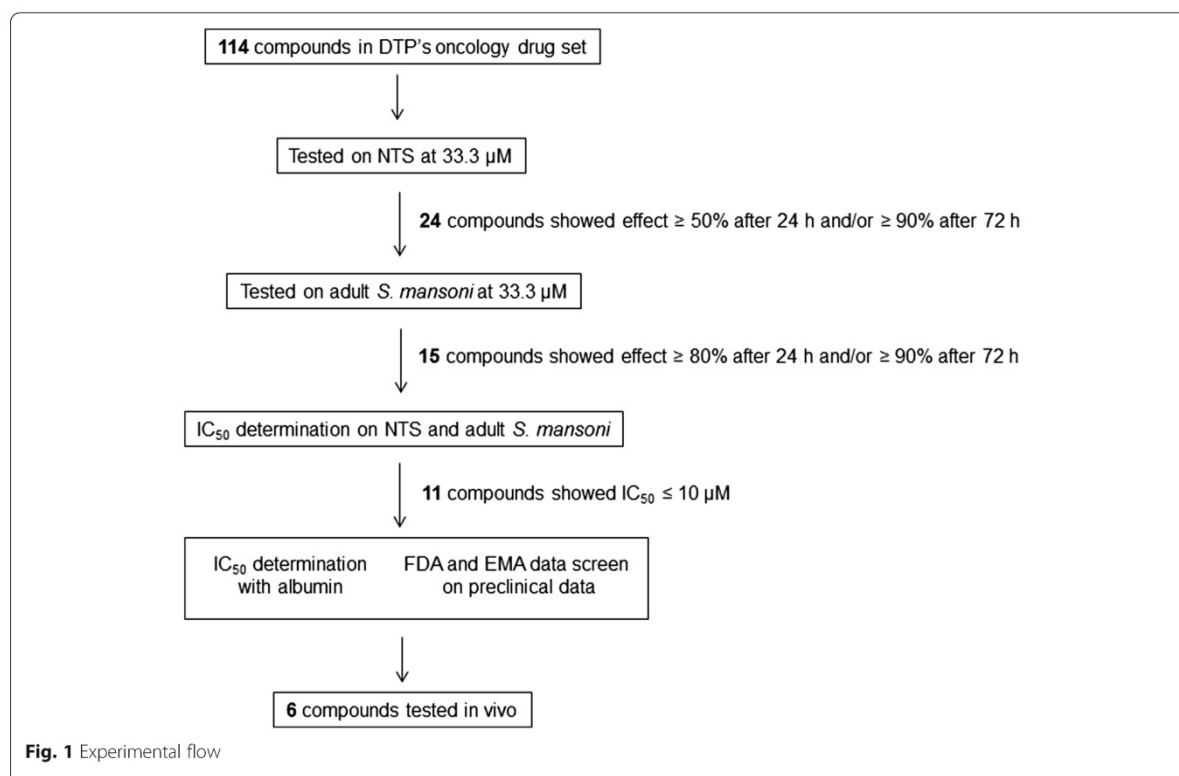
Adult schistosomes were collected from mice with a chronic *S. mansoni* infection (7-week-old) by dissection of the mesenteric veins. Drugs were tested at 33.3 µM in RPMI 1640 culture medium supplemented with 5 % FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin, and prepared in 24-well flat-bottom plates. Three flukes of both sexes were put into the wells, incubated at 37 °C, and 5 % CO<sub>2</sub>, and scored (in the same manner as described for NTS) after 1, 24, 48, and 72 h. Drugs revealing activity against NTS, and adult schistosomes (as explained above), were assessed for their IC<sub>50</sub>, using 3-fold serial dilutions resulting in five different concentrations ranging from 0.41 to 33.3 µM, and scored 4, 24, 48, and 72 h post incubation. IC<sub>50</sub> determinations were performed in duplicate, and repeated once [12]. For compounds exhibiting an IC<sub>50</sub> < 33.3 µM, IC<sub>50s</sub> were determined using culture medium supplemented with 45 g/l bovine serum albumin (AlbuMax® II Lipid-Rich BSA, Gibco): the physiological albumin concentration in humans [14].

### Preclinical and clinical data from FDA and EMA

FDA and European Medicines Agency (EMA) data sheets were used to retrieve drug information such as the maximal plasma concentration (C<sub>max</sub>), plasma half-life (t<sub>1/2</sub>), non-clinical toxicology (lethal single oral dose LD<sub>50</sub>), indication, mechanism of action, and dosage.

### *In vivo* adult schistosome drug assay

For oral application, the drugs were dissolved in 7 % Tween 80 and 3 % ethanol in water (v/v/v), with the exception of sunitinib, which was used as obtained. Groups of 4 mice harboring a chronic *S. mansoni* infection were treated with a single oral dose of 400 mg/kg body weight, or 200 mg/kg for afatinib due to its low LD<sub>50</sub> (382–763 mg/kg in mice) [15]. A control group of 8 mice was left untreated. Three weeks post treatment, the mice were euthanized, and schistosomes residing in the mesenteric veins and the liver were counted and sexed.



**Table 1** IC<sub>50</sub> values of anticancer drugs against larval (NTS) and adult stages of *S. mansoni*

Compound	NTS: IC <sub>50</sub> value [μM]	Adult <i>S. mansoni</i> : IC <sub>50</sub> value [μM]	Adult <i>S. mansoni</i> : IC <sub>50</sub> value in the presence of albumin [μM]
Regorafenib	6.9	1.0	>> 33.3
Ponatinib	0.2	1.1	20.2
Sorafenib	4.1	1.1	>> 33.3
Bosutinib	0.8	1.5	14.8
Afatinib	0.8	1.8	9.9
Sunitinib	1.0	1.8	10.6
Crizotinib	0.3	2.3	18.8
Trametinib	4.6	4.1	21.0
Tamoxifen citrate	2.0	5.7	>> 33.3
Gefitinib	7.8	8.3	>> 33.3
Cabozantinib	19.3	9.0	>> 33.3
Celecoxib	41.9	9.4	>> 33.3
Vandetanib	0.9	9.5	>> 33.3
Idarubicin HCl	12.7	16.2	>> 33.3
Mechlorethamine HCl	>> 33.3	84.8	Not done

## Statistics

Drug effects on schistosomes were determined with the scores of parasites exposed to drug, and the score of the controls. For IC<sub>50</sub> and r value (linear correlation coefficient) determination, the dose-response was calculated with CompuSyn (version 3.0.1; ComboSyn), as described previously [10]. *In vivo* worm burden reductions (WBR) were calculated with the number of worms found in treated mouse groups compared to the control group [10]. *P*-values were calculated using the Kruskal-Wallis test (Stats direct statistical software version 2.8.0).

## Results

### *In vitro* studies

#### *In vitro* activities against NTS

DTP's oncology drug set was first tested at 33.3 μM against NTS (Fig. 1). Twenty-four drugs showed an effect ≥ 50 % after 24 h, and/or ≥ 90 % after 72 h. The most active drugs were crizotinib, ponatinib, and tamoxifen citrate, killing NTS in less than 1 h. Afatinib, idarubicin hydrochloride, regorafenib, sorafenib, and temsirolimus were lethal to NTS within 24 h, everolimus and sirolimus within 48 h, and bosutinib, daunorubicin, and vandetanib within 72 h (Table 1).

***In vitro* activities against adult *S. mansoni***

The 24 NTS-active compounds were subsequently tested on adult *S. mansoni* at 33.3  $\mu\text{M}$ . Tamoxifen citrate was the most active compound, killing all adult worms within 1 h. Afatinib, ponatinib, and sorafenib caused death of all adults within 24 h, whereas adult worms incubated with bosutinib and idarubicin hydrochloride were dead after 72 h. In total, 15 compounds were classified as active against adult *S. mansoni*, characterized by an effect  $\geq 80\%$  after 24 h, and/or  $\geq 90\%$  after 72 h (Table 1).

**Determination of  $\text{IC}_{50}$  values**

These 15 compounds were investigated further by assessing their  $\text{IC}_{50}$  values against adult worms and NTS.

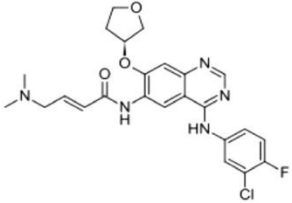
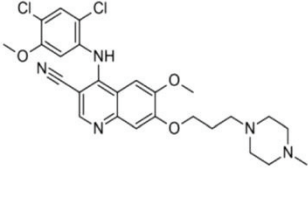
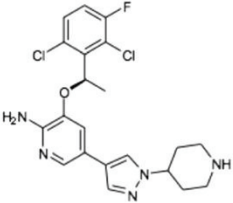
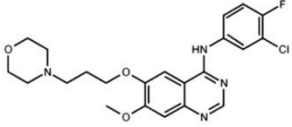
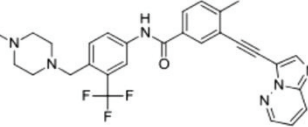
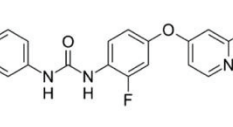
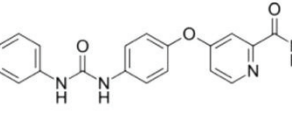
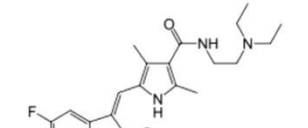
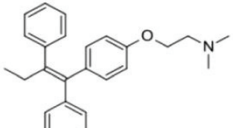
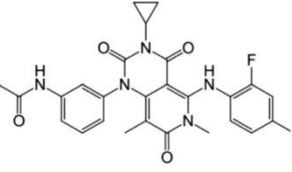
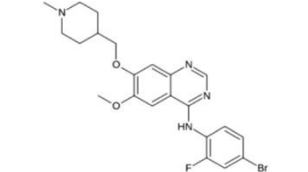
Eleven compounds (structures depicted in Table 2) revealed high activities ( $\text{IC}_{50} \leq 10\ \mu\text{M}$ ) against both stages after 72 h.

However,  $\text{IC}_{50}$  values increased in the presence of albumin. In medium supplemented with 45 g/L BSA, only 6 compounds showed activity ( $\text{IC}_{50}$  of 9–21  $\mu\text{M}$  against adult *S. mansoni*), namely afatinib, bosutinib, crizotinib, ponatinib, sunitinib, and trametinib. For the remaining compounds tested, no  $\text{IC}_{50}$  could be calculated due to lack of activity.

**Preclinical and clinical data**

Data on pharmacokinetic parameters, toxicity, and other information of interest accessible from the FDA and the EMA (summarized in Table 3) were consulted for the 11

**Table 2** Chemical structures of lead compounds

		
afatinib	bosutinib	crizotinib
		
gefitinib	ponatinib	regorafenib
		
sorafenib	sunitinib	tamoxifen
		
trametinib	vandetanib	

**Table 3** FDA and EMA drug description of anticancer drugs of lead compounds

Drug	C <sub>max</sub> : Single oral dose (or otherwise as indicated)	t <sub>1/2</sub> : Single oral dose (or otherwise as indicated)	LD <sub>50</sub> : Single oral dose toxicity	Indication	Mechanism of action	Dosage	Reference (health agency)
Afatinib (GIOTRIF <sup>®</sup> )	NA	37 h (after repeated dosing given to patients)	NA	Metastatic non-small cell lung cancer	Irreversible inhibitor of tyrosine kinase autophosphorylation	40 mg/day	FDA
	397 nmol/l (1×8 mg/kg given to rats)	4.5 h (1×8 mg/kg given to rats)	382–763 mg/kg (mice)				EMA
Bosutinib (BOSULIF <sup>®</sup> )	0.2 µg/ml (500 mg given to patients on 15 consecutive days)	22 h (patients; dose not indicated)	NA	Chronic, accelerated, or blast phase Ph + chronic myelogenous leukemia	Tyrosine kinase inhibitor	500 mg/day	FDA
	NA	2.5–5.4 h (mice and rats; dose not indicated)	>2000 mg/kg (mice and rats)				EMA
Crizotinib (XALKOR <sup>®</sup> )	100–135 ng/ml (250 mg given to patients)	42 h (250 mg given to patients)	>500 mg/kg (rats)	Metastatic non-small cell lung cancer	Tyrosine kinase inhibitor	2×250 mg/day	FDA
	NA	5.8–13 h (rats; dose not indicated)	NA				EMA
Gefitinib (IRESSA <sup>®</sup> )	NA	48 h (healthy volunteers; dose not indicated)	NA	Non-small cell lung cancer	Multiple tyrosine kinase inhibitor	250 mg/day	FDA
	1 µg/ml (after 20 mg/kg given to rats); 0.1 µg/ml (after 250 mg/kg given to healthy volunteers)	10 h (rats; dose not indicated); 30 h (after 250 mg/kg given to healthy volunteers)	Around 2000 mg/kg (rats); >1000 mg/kg (dogs)				EMA
Ponatinib (INCLUSIG <sup>®</sup> )	6 h (patients; dose not indicated)	24 h (patients; dose not indicated)	>2000 mg/kg (mice)	Chronic myeloid leukemia	Tyrosine kinase inhibitor	45 mg/day	FDA
	4 h (patients; dose not indicated)	22 h (patients; dose not indicated)	NA				EMA
Regorafenib (STIVAGRA <sup>®</sup> )	12.5 µg/ml (after 160 mg given to patients)	24 h (after 160 mg given to patients)	NA	Metastatic colon cancer	Multiple protein kinase inhibitor	160 mg/day for first 21 days of a 28-day cycle	FDA
	3.95 mg/l (multiple treatment: 160 mg/day for 3 weeks given to patients)	2 h (multiple treatment: 160 mg/day for 3 weeks given to patients)	>250 mg/kg (mice and rats)				EMA
Sorafenib (INEXAVAR <sup>®</sup> )	NA	25–48 h	NA	Liver, kidney, thyroid cancer	Multiple protein kinase inhibitor	2×400 mg/day	FDA
	0.55 mg/l (after 400 mg given to patients)	22.3 h (after 400 mg given to patients)	>1460 mg/kg (mice and rats)				EMA
Sunitinib (SUTENT <sup>®</sup> )	NA	40–60 h (parent drug in healthy volunteers; 80–110 h (active metabolite in healthy volunteers (dose not indicated)	NA	Gastrointestinal stromal tumor, renal cell carcinoma, well-differentiated pancreatic neuroendocrine tumors	Inhibitor of multiple receptor tyrosine kinases	50 mg/d for the first 28 days of a 42-day cycle	FDA
	NA	NA	>500 mg/kg (mice and rats)				EMA
Trametinib (MEKINIST <sup>®</sup> )	NA	Estimated: 3.9–4.8 days (patients; dose not indicated)	NA	Kinase inhibitor	Kinase inhibitor	2 mg/day	FDA
	222 ng/ml (steady state after 2 mg/day given to healthy volunteers)	5.3 days (healthy volunteers; dose not indicated)	NA				EMA
Tamoxifen citrate (NOLVADEX <sup>®</sup> )	40 ng/ml (after 20 mg given to rats)	5–6 days (after 20 mg given to rats)	NA	Breast cancer	Nonsteroidal antiestrogen	NA	FDA
	NA	NA	NA				EMA
Vandetanib (CAPRELSA <sup>®</sup> )	NA	NA	NA	Medullary thyroid cancer	Multiple tyrosine kinase inhibitor	300 mg/day	FDA
	NA	19 days (after 300 mg given to healthy volunteers)	NA				EMA

NA Not available on the data sheets of the according health agency



compounds characterized by an  $IC_{50} \leq 10 \mu M$  against both stages in order to select good *in vivo* candidates. With regards to potential drug exposure time, vandetanib has a very long half-life (19 days in humans), which we considered an advantageous feature for killing parasites that reside in the bloodstream.

#### Activity in *S. mansoni*-infected mice

Afatinib, bosutinib, ponatinib, sunitinib, trametinib, and vandetanib were chosen for *in vivo* studies based on their *in vitro* activity against schistosomes, and review of the literature. Of note, since crizotinib was not affordable, it was not tested *in vivo*. Drugs were orally applied to mice in a single dose of 400 mg/kg body weight; except for afatinib, which was administered at a single dose of 200 mg/kg, given its lower  $LD_{50}$ . Trametinib, and vandetanib had the highest WBRs of 63.6 %, and 48.1 % respectively ( $p$ -value > 0.05). The remaining compounds were only marginally, or not at all efficacious, with WBRs between 0–27.5 % (Table 4).

#### Discussion

New drugs are needed to treat the neglected tropical disease schistosomiasis. In the present work, we applied a repurposing strategy using a set of FDA-approved anticancer drugs. This library was chosen given proposed overlaps in mechanism of action, active pharmacophores, and matches of human drug targets found in the genome of *S. mansoni* [6, 11, 16].

Because repurposing builds upon previous research and development efforts, new antischistosomal drugs could quickly advance into clinical testing, greatly diminishing the huge costs of drug development [5]. However, it is worth reflecting on the selected library. Anticancer agents are often characterized by the occurrence of numerous and severe adverse events. Since anthelmintic chemotherapy consists typically of a single dose [17], the adverse events occurring during the intensive multiple-dose regimens of

cancer chemotherapy [18], would probably not occur. This encourages studying the anthelmintic properties of anti-cancer drugs further and in more detail. However, the health-risk benefits of repurposing cancer drugs should be evaluated on a case-by-case basis.

We identified 11 cancer drugs in this work with high *in vitro* activity against adult and larval *S. mansoni*. It is worth highlighting that 10 of these drugs are protein kinase inhibitors, which have been suggested as potentially interesting antischistosomal drug discovery candidates, since protein kinase inhibitors can interfere with signaling pathways in schistosome development [6]. The exact mechanism(s) of action of these drugs on schistosomes remain yet to be elucidated, although apoptosis might be involved, due to the fact that many protein kinase inhibitors induce apoptosis [19].

Six of these compounds maintained their antischistosomal activity when exposed to serum albumin - the predominant plasma protein in humans [14] - while the antischistosomal activity of 5 lead candidates was strongly negatively influenced by serum albumin. The loss of *in vitro* antischistosomal activity of imatinib (a protein kinase inhibitor, which did not progress further in our screens) in the presence of alpha-1-acid glycoprotein, serum albumin, and especially with a combination of both, has been described recently [20]. The influence of alpha-1-acid glycoprotein on the *in vitro* activity of our lead compounds was not studied in the present work since binding to this protein might play a more crucial role in rodents than in humans, as described below.

*In vivo* drug efficacy determination, based on our *in vitro* findings (taking into account the loss of activity in the presence of albumin), as well as a literature review on preclinical and clinical data of these drugs (Table 3), revealed two kinase inhibitors: trametinib, and vandetanib, with moderate WBRs of 63.6 and 48.1 % respectively. Vandetanib's efficacy was somewhat surprising, since the addition of albumin to the *in vitro*  $IC_{50}$  determination led to inactivity of the drug. Protein binding to serum albumin, and alpha-1-acid glycoprotein (90 %) was also highlighted by the manufacturer [21]. However, trametinib and vandetanib have a high bioavailability of 100 % [22], or > 90 % [23] in rodents. Additionally, both drugs have long half-lives: 3 days in rats and mice for trametinib [24]; and 28 h in mice for vandetanib [25]. In humans, both trametinib and vandetanib also have exceptionally long half-lives: 4.2 days (3.9 – 4.5 days) [26], and 19 days [27] respectively. The high bioavailabilities combined with the long half-lives might therefore outweigh the negative influence of protein binding on the antischistosomal activity, and explain the efficacy against *S. mansoni* in the mouse model. There might even be a possibility for higher efficacy of vandetanib in humans, since the alpha-1-acid glycoprotein homeostasis is species-dependent. While this serum protein in humans

**Table 4** *In vivo* worm burden reductions after a single oral dose of 200 mg/kg (afatinib) or 400 mg/kg body weight (remaining drugs) to mice harboring a chronic *S. mansoni* infection

Drug	Number of mice treated	Average worm count (SD)	WBR [%]
Control <sup>1</sup>	8	20.4 (12.4)	-
Control <sup>2</sup>	8	23.0 (18.4)	-
Trametinib <sup>1,2</sup>	5	8.1 (4.1)	63.6
Vandetanib <sup>1,2</sup>	5	11.3 (8.4)	48.1
Afatinib <sup>1</sup>	4	14.8 (11.1)	27.5
Ponatinib <sup>1</sup>	3	16.6 (12.9)	18.6
Sunitinib <sup>1</sup>	4	22.5 (8.5)	2.2
Bosutinib <sup>1</sup>	4	25.3 (4.0)	0

*P* value of all WBRs was > 0.05; Values in superscript refer to the corresponding control group  
SD standard deviation

increases 2–5-fold upon inflammatory processes, the increase in mice is 30–40-fold, which is a crucial difference when alpha1-acid glycoprotein-sensitive drugs are being evaluated [16].

Interestingly, none of the 14 drugs, for which the genes of the corresponding human drug targets also exists in *S. mansoni* [11], revealed noteworthy antischistosomal activity ( $IC_{50} > 33.3 \mu\text{M}$ ) (data not shown). Only temsirolimus, and sirolimus killed NTS within 24 or 48 h respectively; but neither of the two reduced the viability of adult *S. mansoni* considerably. However, we would like to highlight that our drug activity assessments are based on alterations on the parasite phenotype. We did not determine the effect on schistosome development, such as the reproductive organs, or egg production and expulsion, which might be affected by the 14 drugs.

When comparing our *in vitro* results with those of other research groups, differences in drug activity are notable. Under our screening conditions, at 33.3  $\mu\text{M}$ , and 72 h drug exposure, imatinib showed <70 % activity against schistosomula, and 76 % against adult schistosomes, while all worms were still moving. In contrast, Beckmann and Grevelding (2010) described the activity of imatinib (72 h postincubation) to be fatal for 30 % of all adult worms after incubation at 10  $\mu\text{M}$ , or 63 % after incubation at 50  $\mu\text{M}$  [6]. According to Katz *et al* (2013), 6 % of worms died after incubation with imatinib (25  $\mu\text{M}$  for 24 h), followed by 48 h in drug-free culture medium [28]. The reason for the different survival rate is not clear, but might originate from differences in drug susceptibilities of different *S. mansoni* strains (Puerto Rican; Luiz Evangelista versus Liberian).

## Conclusion

In summary, the oncology drug set revealed several *in vitro*-active drugs against *S. mansoni*; of which two (trametinib, and vandetanib) were also moderately active *in vivo*. There is room to further investigate trametinib's and vandetanib's potential as antischistosomal drugs, including elucidation of mechanisms of action, application of different treatment courses, and structure-activity relationship studies.

### Competing interests

Both authors declare that they have no competing interests.

### Authors' contributions

NC and JK designed the studies. NC carried out the experiments and wrote the first draft of the manuscript. JK revised the manuscript. Both authors read and approved the final version of the manuscript.

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## **Chapter 5**

**Pharmacokinetic drug-drug interaction study of  
co-administrations oxantel pamoate plus albendazole,  
and albendazole plus mebendazole - a preclinical safety evaluation  
for human trichuriasis treatments**



# In Vitro and In Vivo Drug Interaction Study of Two Lead Combinations, Oxantel Pamoate plus Albendazole and Albendazole plus Mebendazole, for the Treatment of Soil-Transmitted Helminthiasis

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The current treatments against *Trichuris trichiura*, albendazole and mebendazole, are only poorly efficacious. Therefore, combination chemotherapy was recommended for treating soil-transmitted helminthiasis. Albendazole-mebendazole and albendazole-oxantel pamoate have shown promising results in clinical trials. However, *in vitro* and *in vivo* drug interaction studies should be performed before their simultaneous treatment can be recommended. Inhibition of human recombinant cytochromes P450 (CYPs) CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 was tested by exposure to albendazole, albendazole sulfoxide, mebendazole, and oxantel pamoate, as well as albendazole-mebendazole, albendazole sulfoxide-mebendazole, albendazole-oxantel pamoate, and albendazole sulfoxide-oxantel pamoate. A high-pressure liquid chromatography (HPLC)-UV/visible spectroscopy method was developed and validated for simultaneous quantification of albendazole sulfoxide, albendazole sulfone, mebendazole, and oxantel pamoate in plasma. Albendazole, mebendazole, oxantel pamoate, albendazole-mebendazole, and albendazole-oxantel pamoate were orally applied to rats (100 mg/kg) and pharmacokinetic parameters calculated. CYP1A2 showed a 2.6-fold increased inhibition by albendazole-oxantel pamoate (50% inhibitory concentration [IC<sub>50</sub>] = 3.1 μM) and a 3.9-fold increased inhibition by albendazole sulfoxide-mebendazole (IC<sub>50</sub> = 3.8 μM) compared to the single drugs. In rats, mebendazole's area under the concentration-time curve (AUC) and maximal plasma concentration (C<sub>max</sub>) were augmented 3.5- and 2.8-fold, respectively ( $P = 0.02$  for both) when coadministered with albendazole compared to mebendazole alone. Albendazole sulfone was slightly affected by albendazole-mebendazole, displaying a 1.3-fold-elevated AUC compared to albendazole alone. Oxantel pamoate could not be quantified, translating to a bioavailability below 0.025% in rats. Elevated plasma levels of albendazole sulfoxide, albendazole sulfone, and mebendazole in coadministrations are probably not mediated by CYP-based drug-drug interaction. Even though this study indicates that it is safe to coadminister albendazole-oxantel pamoate and albendazole-mebendazole, human pharmacokinetic studies are recommended.

An estimated 465 million people are infected with the soil-transmitted helminth (STH) *Trichuris trichiura*, also known as human whipworm (1). Treatment programs (preventive chemotherapy) using the benzimidazoles albendazole and mebendazole are implemented to deworm patients infected with STHs. However, both drugs show low efficacy in single-dose treatment regimens (2, 3, 36).

To make a step toward better treatment options for trichuriasis (4), combination therapies have received increased attention in the recent past. In more detail, in a recent study in Uganda, albendazole coadministered with mebendazole cured 54.2% of infections and reduced egg excretion by 94.3%, as opposed to low cure rates of single treatments with albendazole (15.4% cure rate; 54.9% egg reduction rate) and mebendazole (20.4% cure rate; 66.7% egg reduction rate) (3). In addition, the combination of ivermectin plus albendazole has been studied in different clinical trials, and higher cure and egg reduction rates were observed for the combination than for the single treatments (2, 5, 6). Oxantel pamoate plus albendazole showed an even higher efficacy (68.5% cure rate; 99.2% egg reduction rate) than ivermectin plus albendazole (27.5% cure rate; 51.6% egg reduction rate) (7). When coadministering multiple drugs, drug-drug interactions should be ruled out. While the combination of albendazole plus ivermectin has been carefully evaluated in preclinical and clinical studies (8, 9) albendazole combined with mebendazole or oxantel pamoate

has not yet been assessed for pharmacokinetic drug-drug interactions.

Xenobiotics are typically neutralized by biotransformation during phase I (oxidation, reduction, and hydrolysis) and phase II (conjugation) metabolism to inactive hydrophilic molecules for renal excretion. Phase I metabolism is driven mainly by the superfamily of cytochrome P450 (CYP) (10). The CYP isozymes convert around half of the drugs on the market (11). If coadministered drugs are both substrates of the same CYP isozyme, inductive or inhibitory effects on this isozyme can mutually decrease or increase the drug plasma levels, respectively. Indisputably important for drug efficacy and safety is the maintenance of optimal drug levels (10).

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Both *in vitro* and *in vivo* studies are recommended to study drug-drug interactions. *In vitro* studies may employ hepatocytes, microsomes manufactured from livers (12), or recombinant CYPs (13). In general, drugs that inhibit metabolic enzymes with 50% inhibitory concentrations ( $IC_{50}$ s) of  $>10 \mu\text{M}$  are considered to be less likely to cause inhibitory drug-drug interactions. Drugs characterized by  $IC_{50}$ s of  $<1 \mu\text{M}$  are considered potent inhibitors and likely cause interactions. For drugs with  $IC_{50}$ s between 1 and  $10 \mu\text{M}$ , other factors, such as CYP isoform inhibition, the stage of the drug discovery process, therapy area, and expected plasma concentrations, should be considered (14). For conducting *in vivo* drug-drug interaction studies, the Food and Drug Administration (FDA) and European Medicines Agency (EMA) endorse a “stress-the-system” approach, e.g., using drug quantities at the dosing limit. A safety range of 80 to 125% of the area under the concentration-time curve (AUC) of the single drugs is accepted. In case plasma levels change due to drug-drug interactions, adjustment of the dosage might be considered (12).

Since albendazole and mebendazole were launched over 30 years ago, their metabolisms have been elucidated in detail. Briefly, when orally taken, albendazole is quickly metabolized to the active sulfoxide metabolite and the inactive sulfone metabolite by the microsomal flavin-containing monooxygenase (FMO) and CYP450 isozymes. CYP4A3 and, to a lesser extent, CYP1A2 are involved in albendazole sulfoxide formation (15). In plasma and urine, mainly the metabolites are present, whereas albendazole can only be found in trace amounts (16). Mebendazole also undergoes excessive first-pass effect, resulting in inactive metabolites (16). The enzymes responsible for mebendazole metabolism have not been identified (17). However, *in vitro* tests have suggested involvement of CYPs (17) and the carbonyl transferase in the biotransformation of mebendazole (18). Finally, the absorption of oxantel pamoate is only 6 to 8% (19). So far, no information about the metabolic pathway of oxantel pamoate is available.

The aim of the present study was to assess potential drug-drug interactions of the two drug combinations albendazole-oxantel pamoate and albendazole-mebendazole. We assessed CYP interactions using validated fluorescence assays and quantified drug levels in rats after the oral application of single-drug treatments or combined treatments. For the purpose of the latter, a high-pressure liquid chromatography (HPLC)-UV method was developed to quantify albendazole sulfoxide, albendazole sulfone, mebendazole, and oxantel pamoate.

## MATERIALS AND METHODS

**Chemicals and solvents.** Albendazole, albendazole sulfoxide, mebendazole, 4-azabenzimidazole, diclofenac sodium, ketoconazole, omeprazole, propranolol hydrochloride, and quinidine were purchased from Sigma-Aldrich (Switzerland). Oxantel pamoate was obtained from Megafine, India, and albendazole sulfone from WITAG (Germany). Dimethyl sulfoxide (DMSO) (Sigma-Aldrich), acetonitrile (Biosolve BV, Netherlands), and methanol (Sigma-Aldrich) were of HPLC grade. Ammonium formate and formic acid were purchased from Sigma-Aldrich.

**CYP450 metabolic drug-drug interaction studies: fluorogenic human recombinant CYP450 assays.** The Vivid CYP450 kits were purchased from Life Technologies, Canada. The assays were performed according to the manufacturer’s recommendations (20). The incubation times were chosen according to the CYP and CYP substrate combination, as described earlier (21). For CYP2D6 and its substrate Vivid 2D6 cyan, the fluorescence was recorded every minute between 0 to 60 min after the

start of the assay. The assay conditions are presented in Table S1 in the supplemental material.

Stock solutions of 10 mM drug in DMSO were prepared in volumetric flasks. Working dilutions of the test compounds were prepared in the Vivid reaction buffer at 2.5-fold-higher concentrations (0.0, 0.34, 1.0, 3.1, 9.3, 27.8, 83.3, and 250  $\mu\text{M}$ ) than the final assay concentrations (0.0, 0.14, 4.1, 1.2, 3.7, 11.1, 33.3, and 100  $\mu\text{M}$ ). For drugs of the combination assays (albendazole-oxantel pamoate, albendazole sulfoxide-oxantel pamoate, and albendazole-mebendazole), the compounds were mixed together in the working solutions. Due to low solubility, the highest concentration of the combination of albendazole sulfoxide-mebendazole was 125  $\mu\text{M}$  in the 3-fold dilution series, resulting in the highest concentration of 50  $\mu\text{M}$  for the two drugs in the assay.

Working dilutions of control inhibitors were prepared either in the same manner as for the test compounds (propranolol, omeprazole, and diclofenac) or at lower concentrations (ketoconazole and quinidine). The latter compounds were prepared at 2.5-fold-higher concentrations (0.0, 0.03, 0.1, 0.3, 0.9, 2.8, 8.3, 25.0  $\mu\text{M}$ ) in reaction buffer than the final assay concentrations (0.0, 0.014, 0.41, 0.12, 0.37, 1.1, 3.3, 10.0  $\mu\text{M}$ ).

For the dilutions of single drugs and the DMSO control, the amount of DMSO was adjusted to the levels of the combination assays. The largest amount of DMSO in the assay was 2%.

Drug working dilutions were placed into Costar 96-well black polystyrene plates (Corning, USA). Baculosomes, which contain the human recombinant CYPs, were mixed with the Vivid regeneration system and Vivid reaction buffer as recommended by the manufacturer, added to the drugs, and left for a 10-min preincubation period. During the preincubation, the background fluorescence was measured (SpectraMax M2 [Molecular Devices]; Softmax version 5.4.1). Finally, the fluorogenic Vivid CYP substrates and  $\text{NADP}^+$  were added to start the enzymatic reaction. After the reaction time, the fluorescence was recorded. The background fluorescence of the CYP assays was subtracted from the assay fluorescence. The CYP inhibition was determined by comparing the fluorescence of test compound assays with that of the DMSO control of the equivalent DMSO amount. The assays were performed in duplicate and repeated once.

**Plasma-level determination of drugs using HPLC-UV/visible spectroscopy.** (i) **Preparation of calibration lines and quality controls.** Stock solutions of 10 mg/ml of albendazole sulfoxide, albendazole sulfone, and oxantel pamoate and 3.3 mg/ml of mebendazole were prepared in DMSO using volumetric flasks. Working solutions were prepared from stock solutions diluted 2-fold in 10% acetonitrile in ammonium formate buffer (25 mM, pH 4.0). The working solutions were used to spike blank plasma (Sprague-Dawley rats; Dunn Labortechnik, Germany) to obtain samples for calibration lines and quality controls for the method validation. The spiked plasma samples had a final volume of 100  $\mu\text{l}$  and contained less than 3% of organic solvent.

(ii) **Plasma sample processing.** Plasma samples (100  $\mu\text{l}$ ) were precipitated using ice-cold methanol containing 10  $\mu\text{g/ml}$  4-azabenzimidazole as an internal standard (300  $\mu\text{l}$ ). After vortex mixing for 30 s, the samples were centrifuged at  $16,000 \times g$  for 10 min. The supernatant was transferred to a new tube and dried with a SpeedVac SPD 111V concentrator (Thermo Fisher Scientific, Germany). The pellet was resuspended with 10% acetonitrile in ammonium formate buffer (25 mM, pH 4.0) and analyzed.

(iii) **Instrumentation.** For the HPLC-UV analysis, an Agilent series 1100 HPLC system (Agilent Technologies, Inc.) coupled to a binary pump (flow rate of 1 ml/min), a microvacuum degasser, an autosampler (10°C), a column heater (25°C), and a UV/visible detector (300 nm) was used. Sample volumes of 50  $\mu\text{l}$  were injected and separated using a reversed-phase Kinetex XB  $C_{18}$  column (4.5 by 150 mm, 2.6  $\mu\text{m}$ ; Phenomenex, Switzerland). An organic gradient was used for analyte elution, using ammonium formate buffer (25 mM, pH 4.0) and acetonitrile.

(iv) **Method validation.** Method validation was conducted according to FDA specifications (22). In addition to the calibration lines, four quality controls (QCs) were prepared from the working solutions: high, inter-

mediate, and low concentrations in the dynamic range and the lower limit of quantification (LLOQ). The concentrations used were 9.6, 2.4, 0.60, and 0.40  $\mu\text{g}/\text{ml}$  for albendazole sulfoxide, albendazole sulfone, and oxantel pamoate and 4.8, 1.2, 0.30, and 0.20  $\mu\text{g}/\text{ml}$  for mebendazole in blank plasma.

(a) **Accuracy and precision.** Two sets of QC samples were prepared and quantified on two different days. The accuracy was calculated as the percentage of measured concentrations with respect to the theoretical value. For the evaluation of the method precision, the coefficient of variation was determined as the percentage of the standard deviation with respect to the mean concentration. Accuracy and precision for both intraday ( $n = 6$ ) and interday ( $n = 2 \times 6$ ) were determined.

(b) **Selectivity.** Plasma samples from four different rodent species (Sprague-Dawley rats and NSA mice from DUNN Labortechnik and Wistar rats and NMRI mice from Charles River, Germany) were spiked to LLOQ samples and processed as described above. LLOQ samples ( $n = 6$ ) were compared to zero samples (blank plasma samples processed with internal standard [IS];  $n = 6$ ).

(c) **Recovery and matrix effect.** For recovery determination, the absolute peak areas of samples spiked to QCs before and after protein extraction were compared. For matrix effect determination, QC samples prepared in protein extract were compared to QC samples prepared in 10% acetonitrile in ammonium formate buffer.

(d) **Dilution.** To enable dilution of samples that exceed the dynamic range, the dilution effect was assessed. Plasma was spiked with a concentration 10-fold higher than the upper limit of quantification (ULOQ): 96  $\mu\text{g}/\text{ml}$  for albendazole sulfoxide, albendazole sulfone, and oxantel pamoate and 48  $\mu\text{g}/\text{ml}$  for mebendazole. The samples ( $n = 4$ ) were then diluted 10-fold with plasma and processed as described above.

(e) **Stability.** QC samples ( $n = 4$ ) were left at room temperature for 24 h and then quantified. The accuracy and precision were determined as described above.

**Pharmacokinetic study.** The pharmacokinetic study using rats was authorized by the cantonal veterinary office of Basel-Stadt, Switzerland (license no. 2070). Sixteen rats were purchased from Charles River, Germany, and catheters were implanted into the jugular vein. The rats were kept at 50% humidity and 22°C, with an artificial 12-hour day/night cycle and with access to rodent food *ad libitum*.

Drugs were prepared as 90-mg/ml suspensions in 7% Tween, 3% ethanol, and water. Four groups of four rats each were treated with 100 mg/kg of the following compounds in monotherapy or combination therapy: single albendazole, single mebendazole, albendazole-mebendazole, and albendazole-oxantel pamoate. Blood samples from four animals per treatment group were withdrawn at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 33, and 24 h posttreatment. The samples were collected in heparin lithium tubes and centrifuged to obtain cell-free plasma. The plasma was stored in aliquots of 100  $\mu\text{l}$  at  $-20^\circ\text{C}$  until usage.

**Statistical and pharmacokinetic analyses.**  $\text{IC}_{50}$ s of CYP inhibitions and their corresponding  $r$  values (correlation coefficients) were calculated from mean inhibition values using CompuSyn software (ComboSyn Inc., USA) (23). Pharmacokinetic parameters obtained from the *in vivo* studies were determined with noncompartmental analysis using PK Solver 2.0 (24). Calculated parameters were the area under the concentration-time curve from 0 to 24 h ( $\text{AUC}_{0-24}$ ) determined by applying the linear trapezoidal method, the maximal plasma concentration ( $C_{\text{max}}$ ), the time at  $C_{\text{max}}$  ( $T_{\text{max}}$ ), and the half-life ( $t_{1/2}$ ). The Kruskal-Wallis test (StatsDirect, version 2.8.0; StatsDirect Ltd., United Kingdom) was applied (at a significance level of  $P < 0.05$ ) to determine the significance of changes of pharmacokinetic parameters.

## RESULTS

**Influence of drug combinations on recombinant CYP450 metabolism.** Single drugs and the active metabolite of albendazole (albendazole, albendazole sulfoxide, mebendazole, and oxantel pamoate) and drug combinations (albendazole-mebendazole, al-

TABLE 1 CYP450 isozyme  $\text{IC}_{50}$ s of single drugs and drug combinations

CYP	Drug	$\text{IC}_{50}$ ( $\mu\text{M}$ )	$r^a$
1A2	Albendazole	8.0	0.99
	Albendazole sulfoxide	14.9	0.99
	Mebendazole	20.4	0.96
	Oxantel pamoate	>100	0.34
	Albendazole-mebendazole	9.4	0.97
	Albendazole sulfoxide-mebendazole	3.8	0.99
	Albendazole-oxantel pamoate	3.1	0.99
	Albendazole sulfoxide-oxantel pamoate	13.1	1.00
	Propranolol	0.54	0.99
2C9	Albendazole	>100	NA
	Albendazole sulfoxide	>100	NA
	Mebendazole	30.3	0.93
	Oxantel pamoate	7.8	0.99
	Albendazole-mebendazole	41.9	0.81
	Albendazole sulfoxide-mebendazole	97.5	0.86
	Albendazole-oxantel pamoate	19.3	0.81
	Albendazole sulfoxide-oxantel pamoate	18.0	0.96
	Diclofenac	4.10	0.95
2C19	Albendazole	>100	NA
	Albendazole sulfoxide	>100	NA
	Mebendazole	>100	NA
	Oxantel pamoate	>100	NA
	Albendazole-mebendazole	>100	NA
	Albendazole sulfoxide-mebendazole	>50	NA
	Albendazole-oxantel pamoate	>100	NA
	Albendazole sulfoxide-oxantel pamoate	>100	NA
	Omeprazole	2.14	0.98
2D6	Albendazole	>100	NA
	Albendazole sulfoxide	>100	NA
	Mebendazole	>100	NA
	Oxantel pamoate	1.7	0.99
	Albendazole-mebendazole	>100	NA
	Albendazole sulfoxide-mebendazole	53.8	0.89
	Albendazole-oxantel pamoate	10.2	0.88
	Albendazole sulfoxide-oxantel pamoate	6.2	0.97
	Quinidine	0.09	0.87
3A4	Albendazole	>100	NA
	Albendazole sulfoxide	>100	NA
	Mebendazole	>100	NA
	Oxantel pamoate	>100	NA
	Albendazole-mebendazole	>100	NA
	Albendazole sulfoxide-mebendazole	29.1	0.93
	Albendazole-oxantel pamoate	>100	NA
	Albendazole sulfoxide-oxantel pamoate	>100	NA
	Ketoconazole	0.10	0.89

<sup>a</sup>  $r$ , correlation coefficient, where  $\geq 0.85$  is acceptable. NA, not applicable.

bendazole sulfoxide-mebendazole, albendazole-oxantel pamoate, and albendazole sulfoxide-oxantel pamoate) were tested for CYP1A2, -2C9, -2C19, -2D6, and -3A4 inhibition. For each enzyme, a standard inhibitor was used as positive control. Findings obtained for the standards were comparable to previously determined  $\text{IC}_{50}$  values. All findings are summarized in Table 1.

(i) **CYP1A2.** Moderate inhibition of CYP1A2 was observed when it was incubated with the single drugs albendazole ( $\text{IC}_{50} = 8.0 \mu\text{M}$ ), albendazole sulfoxide ( $\text{IC}_{50} = 14.9 \mu\text{M}$ ), and mebendazole ( $\text{IC}_{50} = 20.4 \mu\text{M}$ ). Oxantel pamoate did not inhibit CYP1A2



TABLE 2 Intraday and interday accuracy and precision

Analyte	Theoretical concn ( $\mu\text{g/ml}$ )	Intraday <sup>a</sup>		Interday <sup>b</sup>	
		Calculated concn ( $\mu\text{g/ml}$ )	Accuracy $\pm$ CV (%)	Calculated concn ( $\mu\text{g/ml}$ )	Accuracy $\pm$ CV (%)
Albendazole sulfoxide	0.4	0.4	99.0 $\pm$ 1.8	0.4	101.4 $\pm$ 4.6
	0.6	0.6	107.7 $\pm$ 1.4	0.7	108.7 $\pm$ 1.7
	2.4	2.4	101.1 $\pm$ 1.3	2.6	106.4 $\pm$ 5.3
	9.6	9.3	96.9 $\pm$ 2.5	9.7	101.4 $\pm$ 4.9
Albendazole sulfone	0.4	0.4	96.6 $\pm$ 0.7	0.4	95.1 $\pm$ 5.2
	0.6	0.6	106.8 $\pm$ 0.7	0.6	103.0 $\pm$ 5.3
	2.4	2.4	101.3 $\pm$ 1.6	2.4	99.9 $\pm$ 2.4
	9.6	9.4	97.9 $\pm$ 2.5	9.3	96.7 $\pm$ 2.3
Mebendazole	0.2	0.2	101.8 $\pm$ 1.5	0.2	93.3 $\pm$ 9.5
	0.3	0.3	108.4 $\pm$ 1.1	0.3	99.0 $\pm$ 9.7
	1.2	1.2	99.4 $\pm$ 1.6	1.2	96.0 $\pm$ 4.0
	4.8	4.7	97.9 $\pm$ 3.4	4.6	94.8 $\pm$ 4.3
Oxantel pamoate	0.4	0.4	99.5 $\pm$ 1.3	0.4	98.8 $\pm$ 3.6
	0.6	0.6	102.6 $\pm$ 2.0	0.6	103.2 $\pm$ 1.7
	2.4	2.3	97.7 $\pm$ 1.2	2.5	102.7 $\pm$ 5.2
	9.6	9.4	98.0 $\pm$ 2.5	9.9	102.7 $\pm$ 5.1

<sup>a</sup> Mean values for  $n = 6$  samples.

<sup>b</sup> Mean values for  $n = 12$  samples in two independent experiments.

( $\text{IC}_{50} > 100 \mu\text{M}$ ). Albendazole-mebendazole and albendazole sulfoxide-oxantel pamoate had  $\text{IC}_{50}$ s of 9.4  $\mu\text{M}$  and 13.1  $\mu\text{M}$ , respectively. The combination of albendazole plus oxantel pamoate showed a 2-6-fold-increased CYP inhibition, with an  $\text{IC}_{50}$  of 3.1  $\mu\text{M}$ , compared to single albendazole. The strongest interaction was observed with the combination of albendazole sulfoxide plus mebendazole, with a 3.9-fold-higher  $\text{IC}_{50}$  (3.8  $\mu\text{M}$ ) than albendazole sulfoxide (14.9  $\mu\text{M}$ ), the combination partner with the lower  $\text{IC}_{50}$ .

(ii) **CYP2C9.** CYP2C9 was moderately inhibited by mebendazole ( $\text{IC}_{50} = 30.3 \mu\text{M}$ ) and oxantel pamoate ( $\text{IC}_{50} = 7.8 \mu\text{M}$ ) as well as by three of the drug combinations tested ( $\text{IC}_{50} = 41.9 \mu\text{M}$  for albendazole-mebendazole,  $\text{IC}_{50} = 19.3 \mu\text{M}$  for albendazole-oxantel pamoate, and  $\text{IC}_{50} = 18.0 \mu\text{M}$  for albendazole sulfoxide-oxantel pamoate). None of the combinations showed a greater inhibitory effect than the single drugs.

(iii) **CYP2C19.** No inhibition of CYP2C19 was observed at the concentration ranges of the drugs and drug combinations tested.

(iv) **CYP2D6.** The Vivid 2D6 cyan substrate was converted by CPY2D6 in linear relationship ( $r^2 > 0.995$ ) to reaction time between 1 and 40 min. A reaction endpoint of 20 min was chosen for the assay. CYP2D6 was inhibited by oxantel pamoate with an  $\text{IC}_{50}$  of 1.7  $\mu\text{M}$ . For the two oxantel pamoate combinations, 3.6-fold-lower (albendazole sulfoxide-oxantel pamoate;  $\text{IC}_{50} = 6.2 \mu\text{M}$ ) and 6-fold-lower (albendazole-oxantel pamoate;  $\text{IC}_{50} = 10.2 \mu\text{M}$ ) inhibitions were observed. Moreover, a weak inhibition was observed for albendazole sulfoxide-mebendazole ( $\text{IC}_{50} = 53.8 \mu\text{M}$ ), whereas the drugs separately had  $\text{IC}_{50}$ s of  $> 100 \mu\text{M}$ .

(v) **CYP3A4.** An inhibition of CYP3A4 was observed only with the combination of albendazole sulfoxide-mebendazole ( $\text{IC}_{50}$  of 29.1  $\mu\text{M}$ ).

**HPLC-UV/visible spectroscopy method validation.** All four analytes showed linear ( $r^2 > 0.999$ ) concentration-dependent absorbance at the concentration calibration lines (0.4 to 9.6  $\mu\text{g/ml}$

for oxantel pamoate, albendazole sulfoxide, and albendazole sulfone and 0.2 to 4.8  $\mu\text{g/ml}$  for mebendazole). The method was selective in all four rodent plasma samples tested, showing at least a 5-fold-higher peak at the LLOQ than the background absorbance of plasma zero samples. The method determined the three quality controls and LLOQs accurately and precisely (Table 2). The recovery and matrix effects of oxantel pamoate, albendazole sulfoxide, albendazole sulfone, and mebendazole were 89 to 103.2%, all in a non-concentration-dependent manner (Table 3). All analytes were stable in plasma at room temperature for 24 h, showing accuracy and precision for all concentrations assessed. Dilution effects were not apparent: a 10-fold dilution with plasma of a sample with a concentration 10-fold higher than the ULOQ resulted in an accurate and precise quantification of all analytes.

**Effect of coadministration on plasma levels.** Pharmacokinetic parameters of albendazole sulfoxide, albendazole sulfone, and mebendazole for all treatments (albendazole, mebendazole, albendazole-mebendazole, and albendazole-oxantel pamoate) are summarized in Table 4. Oxantel pamoate could not be quantified (LLOQ = 0.4  $\mu\text{g/ml}$ ) in any sample.

Albendazole sulfoxide showed comparable plasma exposure (AUC and  $C_{\text{max}}$ ) in all three albendazole-containing treatments after oral application of 100 mg/kg of each drug (Fig. 1). The half-life of albendazole sulfoxide was increased 1.8-fold in the combination of albendazole plus mebendazole compared to albendazole alone. The time of maximal plasma concentration of albendazole sulfoxide after coadministration with mebendazole was slightly delayed (6.7 h versus 5.0 h) compared to that for albendazole alone. The coadministration of albendazole plus oxantel pamoate resulted in the same pharmacokinetic parameters for as the single albendazole treatment.

Albendazole sulfone showed similar pharmacokinetic parameters following administration of albendazole-mebendazole as albendazole alone (Fig. 2). However, slight differences

TABLE 3 Relative recovery and matrix effect

Analyte	Theoretical concn (µg/ml)	Recovery (%) <sup>a</sup>		Matrix effect (%) <sup>a</sup>	
		Mean ± CV	Overall mean ± CV	Mean ± CV	Overall mean ± CV
Albendazole sulfoxide	9.6	94.2 ± 0.6	92.9 ± 2.2	97.9 ± 1.1	92.4 ± 10.2
	2.4	94.1 ± 1.2		98.5 ± 1.7	
	0.6	90.5 ± 1.7		80.8 ± 5.6	
Albendazole sulfone	9.6	93.1 ± 0.6	93.6 ± 0.8	99.1 ± 1.0	103.2 ± 3.5
	2.4	93.3 ± 1.5		105.2 ± 1.6	
	0.6	94.5 ± 1.7		105.2 ± 4.3	
Mebendazole	4.8	92.0 ± 0.9	90.5 ± 3.7	100.0 ± 2.3	94.8 ± 8.8
	1.2	92.9 ± 0.8		99.8 ± 2.3	
	0.3	86.7 ± 2.4		84.6 ± 5.6	
Oxantel pamoate	9.6	89.9 ± 0.6	89.8 ± 2.2	93.9 ± 1.0	89.1 ± 8.9
	2.4	91.6 ± 1.3		93.9 ± 2.0	
	0.6	87.7 ± 1.9		79.4 ± 6.2	

<sup>a</sup> n = 6 samples.

were observed for albendazole sulfone when albendazole was coadministered with oxantel pamoate compared to albendazole alone, with a 1.6-fold-lower  $C_{max}$  and an earlier  $T_{max}$  (8.0 h versus 13.0 h), which were, however, statistically insignificant ( $P > 0.05$ ).

Mebendazole exposure was elevated when coadministered with albendazole compared to single mebendazole treatment (AUC 3.5-fold higher and  $C_{max}$  2.8-fold higher), with a statistical significance of  $P = 0.02$ .  $t_{1/2}$  and  $T_{max}$  were equal in both treatments (Fig. 3).

## DISCUSSION

Combination chemotherapy is widely used in many therapeutic areas (25) and has been increasingly explored for the treatment of STH infections. In particular, combinations of albendazole plus ivermectin, albendazole plus mebendazole, and albendazole plus oxantel pamoate have been investigated in randomized controlled trials in the past years (2, 3, 6). A key characteristic for anthelmintic treatment is the almost compulsory usage of single-dose regimens (26), since the treatments are used at large scale in preventive chemotherapy programs. Therefore, a combined delivery of drugs to the population is warranted. However, before two drugs can be recommended for simultaneous treatment, *in vitro* and *in vivo* studies are necessary to rule out potential drug interactions. To our knowledge, to date only the coadministration of

albendazole plus ivermectin has been thoroughly studied (8, 9). The aim of the present work was therefore to assess the combined therapies of albendazole plus oxantel pamoate and albendazole plus mebendazole for potential drug-drug interactions.

The anthelmintics studied were not potent inhibitors of recombinant CYP450 enzymes. In more detail, only albendazole and albendazole sulfoxide moderately inhibited CYP1A2 ( $IC_{50}$ s of  $>8 \mu M$ ). In addition, oxantel pamoate was a slight inhibitor for CYP2D6 and CYP2C9, with  $IC_{50}$ s of 1.7 and 7.8  $\mu M$ , respectively. Our finding of the moderate CYP1A2 inhibition by albendazole is in contrast to earlier studies that showed no inhibition of this enzyme (27, 28). Importantly, both albendazole and albendazole sulfoxide not only can inhibit but also can induce CYPs, which *in vitro* may be masked by their inhibitory effect (28).

The drug combinations tested in this study did not show a significant interplay with the major CYP enzymes. Two combinations had an effect on CYP1A2: albendazole-oxantel pamoate showed a 2.6-fold-higher inhibitory effect ( $IC_{50} = 3.1 \mu M$ ) and albendazole sulfoxide-mebendazole showed a 3.9-fold-higher inhibitory effect ( $IC_{50} = 3.8 \mu M$ ) than the respective partner drugs alone. Another interaction was observed with mebendazole in combination with albendazole sulfoxide, the only treatment that inhibited CYP3A4 ( $IC_{50} = 29.1 \mu M$ ). However, plasma levels of mebendazole and albendazole sulfoxide in humans are approxi-

TABLE 4 Pharmacokinetic parameters of albendazole sulfoxide, albendazole sulfone, and mebendazole after oral, single, and/or combination application of albendazole, mebendazole, and oxantel pamoate

Parameter <sup>a</sup>	Mean value for:							
	Albendazole sulfoxide after treatment with:			Albendazole sulfone after treatment with:			Mebendazole after treatment with:	
	Albendazole	Albendazole-mebendazole	Albendazole-oxantel pamoate	Albendazole	Albendazole-mebendazole	Albendazole-oxantel pamoate	Mebendazole	Albendazole-mebendazole
$t_{1/2}$ , h (range)	1.7 (1.1–2.7)	3.0 (1.0–6.4)	1.9 (1.0–2.6)	1.5 (1.2–1.8)	1.6 (1.1–2.1)	1.9 (1.1–2.6)	10.1 (8.5–12.6)	8.8 (3.0–13.5)
$T_{max}$ , h (range)	5.0 (4.0–6.0)	6.7 (6.0–8.0)	5.0 (4.0–6.0)	13.0 (8.0–24.0)	14.0 (6.0–24.0)	8.0 (8.0–10.0)	4.5 (2.0–6.0)	4.7 (2.0–8)
$C_{max}$ , µg/ml (SD)	5.6 (1.5)	4.7 (2.0)	5.2 (0.7)	1.4 (0.3)	1.5 (0.6)	1.2 (0.1)	0.4 (0.3)	1.0 (0.2)
$AUC_{0-24}$ , µg/ml · h (SD)	63.8 (30.9)	67.9 (35.4)	59.7 (9.2)	24.0 (15.6)	31.9 (20.2)	15.0 (1.0)	5.9 (3.5)	20.0 (9.2)

<sup>a</sup>  $t_{1/2}$ , half-life;  $T_{max}$ , time to maximal plasma concentration;  $C_{max}$ , maximal plasma concentration;  $AUC_{0-24}$ , area under the concentration-time curve; SD, standard deviation.

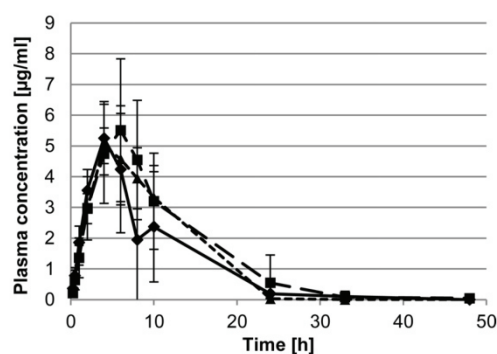


FIG 1 Plasma levels of albendazole sulfoxide after administration of albendazole (◆) as single treatment or combined with mebendazole (■) or oxantel pamoate (▲).

mately 600 times lower than the concentrations we used *in vitro*: after the administration of 1.5 g mebendazole (three times the dose used in preventive chemotherapy programs), its concentration in the plasma was reported to be below 42 µg/liter (12 nM), and after the standard dosage of albendazole (400 mg), the plasma level of albendazole sulfoxide is 0.16 mg/liter (45 nM) (16). Therefore, the CYP1A2 and -3A4-inhibitory interaction of albendazole sulfoxide plus mebendazole observed *in vitro* is most likely of no clinical relevance.

In our *in vivo* study, coadministration of albendazole and mebendazole to rats had an impact on the plasma levels of mebendazole. Mebendazole's  $AUC_{0-24}$  and  $C_{max}$  were significantly elevated (3.5-fold and 2.8-fold, respectively) compared to those with single mebendazole treatment. Since there is no evidence about CYP metabolism of mebendazole, other metabolic interactions might be involved, for instance, at the level of the flavin-containing monooxygenase, which is also strongly involved in albendazole biotransformation (15, 29). In addition, it was demonstrated that mebendazole induced the hepatic monooxygenase (30), which might trigger an interaction when coadministered with albendazole. Moreover, efflux mechanisms might be involved. Albendazole sulfoxide was suggested to be eliminated via efflux from the bloodstream into the intestine in rats and sheep

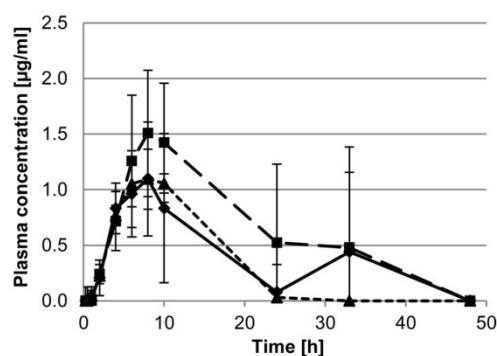


FIG 2 Plasma levels of albendazole sulfone after administration of albendazole (◆) as single treatment or combined with mebendazole (■) or oxantel pamoate (▲).

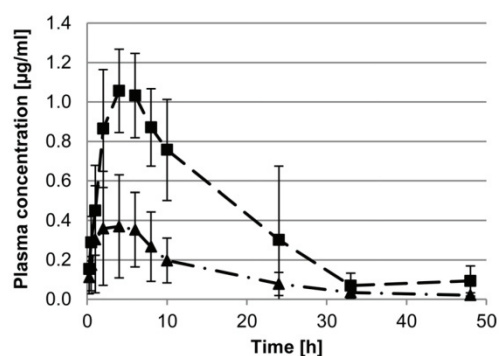


FIG 3 Plasma levels of mebendazole (▲) after administration of mebendazole as single treatment or combined with albendazole (■).

(31), probably by an ATP-binding cassette (ABC) drug efflux transporter, the breast cancer resistance protein (BCRP), as *in vitro* studies have indicated. Albendazole, on the other hand, seems not to be transported by BCRP or by p-glycoprotein, another prominent ABC efflux transporter (32). Mebendazole is also not a substrate of p-glycoprotein (33), and whether there is interaction with BCRP is unknown. However, as mentioned above, the plasma levels of mebendazole and albendazole sulfoxide in humans are relatively low; hence, whether the interactions observed in this study in rats are of clinical relevance is not known. Importantly, the combination of albendazole plus mebendazole was well tolerated in humans (7).

The bioavailability of oxantel pamoate in humans is known to be low; only around 6 to 8% is excreted via the kidneys (19). Surprisingly, we could not quantify any oxantel pamoate in our samples, meaning plasma levels in rats were <0.4 µg/ml, the LLOQ of the analytical method. Roughly calculated, that would correspond to a bioavailability of <0.025%, if in theory the entire dose applied (100 mg/kg) would be absorbed and not metabolized (blood volume calculated according to reference 34). Species differences in the bioavailability of drugs are common (35) and might explain the lower bioavailability observed in our study in rats.

In conclusion, based on *in vitro* and *in vivo* studies, drug-drug interactions are unlikely to occur for the two combinations albendazole-oxantel pamoate and albendazole-mebendazole. However, pharmacokinetic studies in humans might be useful to provide further information about the safety profiles of these combinations.

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## **Chapter 6**

### **General discussion and conclusion**

## 6.1 Antischistosomal drug discovery

With praziquantel as the only drug available against schistosomiasis and the high drug pressure on *Schistosoma*, development of drug resistance is anticipated; thus, research for new antischistosomal drugs is needed [1],[2]. This pressing situation promoted the evaluation of three compound sets for antischistosomal activity: **Set 1**: Lead optimization of synthetic peroxides; **Set 2**: Pharmacophore characterization of MMV665852 (*N,N'*-diarylurea) analogs; and **Set 3**: Exploration of a cancer drug library.

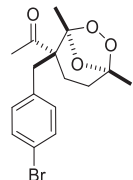
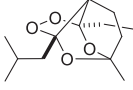
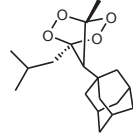
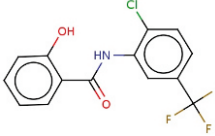
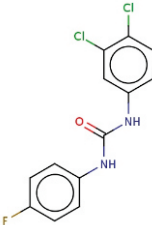
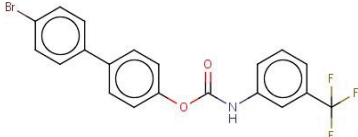
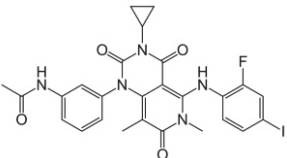
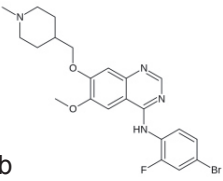
### 6.1.1 Synopsis of three drug sets tested for antischistosomal activity

The investigation of the three drug sets for antischistosomal activity resulted in the following findings:

- Set 1: Strengthened evidence that artemisinin-inspired synthetic peroxides (bridged tetraoxanes, tricyclic monoperoxides, and bridged trioxolanes, also known as ozonides) have excellent antischistosomal activity.
- Set 2: Identification of *N,N'*-diarylurea, *N*-phenyl benzamide, and *N*-aryl phenylcarbamate derivatives as potent novel chemical classes with in vitro and in vivo antischistosomal activity.
- Set 3: Discovery of marketed cancer drugs with in vitro and in vivo activity. The study findings supported the hypothesis of cancer drugs to be potential antischistosomal drugs through their inhibition of protein kinases.

The antischistosomal activities of hit structures of the three sets are summarized in Table 3. When screening compounds of Sets 1 and 2, the most striking observation was the discrepancy between in vitro and in vivo activities. Both sets were potent, demonstrating in vitro activities below 1  $\mu$ M. Set 2 was even fast acting, reaching  $IC_{50}$  values below 1  $\mu$ M after 1 hour of incubation. However, in the mouse model, the worm burden reductions (WBRs) for hits in Sets 1 and 2 were not higher than 44% and 66%, respectively.

**Table 3: Features of the hit compounds of all three sets tested**

Compound	IC <sub>50</sub> larval <i>S. mansoni</i> [μM]	IC <sub>50</sub> adult <i>S. mansoni</i> [μM]	SI	WBR [%]	Other characteristics
<b>Set 1: Peroxides</b>					
 Trioxolane (cpd 30)	2.2	4.2	1.6	44	Low solubility
 Tricyclic monoperoxide (cpd 44)	2.7	4.4	5.7	27	
 Tetraoxane (cpd 6)	0.9	1.8	0.5	not done	
<b>Set 2: MMV665852 analogs</b>					
 N-phenyl benzamide (cpd 38)	0.2	0.6	4.9	66	Fast acting; low solubility; moderate to good predicted pharmacokinetics
 N,N'-diarylurea (cpd 2)	1.3	0.7	5.4	43	
 N-aryl phenylcarbamate (cpd 45)	0.5	0.7	2.9	0	
<b>Set 3: cancer drugs</b>					
 Trametinib	4.6	4.1	<0.1	64	Slow activity; high bioavailability; long half-life
 Vandetanib	0.9	9.5	0.8	48	

IC<sub>50</sub>: Concentration of 50% drug effect; SI: Selectivity index; WBR: Worm burden reduction

Set 3 exhibited a different pattern of activity. In vitro, the cancer drugs were less potent than the previous two sets, displaying higher IC<sub>50</sub> values and a longer time until activity was observed (24-72 hours). Yet, the in vivo results were in the same range as Sets 1 and 2, with WBRs of 63% and 48% for the two hit cancer drugs (trametinib and vandetanib). In the screening of Set 3, we considered plasma protein binding as a potential activity-reducing factor. Indeed, repeating the adult *S. mansoni* in vitro assay with the physiological concentration of serum albumin demonstrated a loss of activity for 8 out of 14 molecules. Testing the influence of plasma protein binding on antischistosomal activity might be worth to be included in the screen flow, or solely used as additional compound characterization of promising candidates, for instance, when attempting to discriminate two compounds otherwise very similar.

Regarding cytotoxicity, Set 1 contained several molecules (mainly tetraoxanes) displaying toxicity in the nanomolar to low micromolar range, whereas Set 2 remained at low micromolar values. Also Set 3 revealed significant cytotoxic values ranging between <0.37 μM (lowest value tested) and 32 μM (data not published). Clearly, these results are not surprising considering the anti-proliferative nature of cancer drugs. When looking at their relative cytotoxicity, all three sets laid in the same range with selectivity indexes (i.e. IC<sub>50</sub>cytotoxicity/IC<sub>50</sub>antischistosomal activity) between 1 and 10. This demonstrates that also marketed drugs, although compelled by safety regulations, show in vitro toxicity. Thus, in vitro toxicity should be interpreted in the context of other available information.

Important to mention is the fact that Sets 1 and 2 showed (microscopically observed) solubility issues, which might have led to the moderate efficacies. For Set 2, we investigated potential reasons for the low in vivo efficacy by testing the following parameters: Number of violations of the rule of 5 (described below), solubility, and permeability through the intestinal wall. The tests indicated low solubility (<3 μM for all compounds tested) to be the reason for low absorption, since only dissolved molecules can cross the intestinal wall, and would therefore lead to low drug exposure of the schistosomes. The other parameters predicted moderate to good bioavailability for the majority of compounds.

As mentioned above, the in vivo efficacy in all sets was moderate to low. In Sets 1 and 2, we attempted to improve the compounds' in vivo efficacies by increasing intestinal absorption via two different methods. In vivo candidates of Set 1 were packed into a complex using β-cyclodextrin, a commonly used method to enhance drug solubility [3]. Unfortunately, no improvement in drug efficacy could be observed. In the case of Set 2, an oily formulation (olive oil, polyethylene glycol, water) was used for the in vivo lead compounds. The



compounds did dissolve better in the oil-based formulation, however, drug efficacy was not increased (data not published). Indeed, the addition of hydrochloric acid to simulate digestive juices led to compound precipitation in the formulation. This might be the reason for insufficient freely available drug for absorption.

Regarding future lead optimization of Set 1, I would add hydrophilic side chains to the three lead scaffolds, the trioxolanes and tricyclic monoperoxides, as well as to the more toxic tetraoxanes. I would not yet declare tetraoxanes as too unselective, since structure optimization may lead to higher selectivity. Cytotoxicity is merely a model estimating toxicity in humans and should be evaluated in context with other factors, such as in vitro activities and existing information about drug safety (e.g. LD<sub>50</sub> values of marketed drugs). In future lead optimization studies, I would incorporate a screen for “drugability”. The goal would be to find a balanced drug profile of antischistosomal activity, selectivity, and bioavailability. In the same manner, future lead optimization of Set 2 should consist of more hydrophilic derivatives of the three potent classes: *N,N'*-diarylhureas, *N*-phenyl benzamides, and *N*-aryl phenylcarbamates.

In contrast to Sets 1 and 2, two marketed drugs of Set 3, despite their moderate in vitro schistosomicidal activity, revealed WBRs similar to top candidates in Sets 1 and 2. Both hit drugs of Set 3 (trametinib and vandetanib) have an exceptionally long half-life in humans (4.2 days and 19 days, respectively), while the half-life in mice is shorter (3 days, and 28 hours, respectively) [4]. As these drugs exert their activity rather slowly (72 hours), using multiple dosing in the mouse to prolong the drug exposure of the worms might simulate the in-human situation more accurately, and result in higher efficacy.

Furthermore, since trametinib and vandetanib target protein kinases, a class involved in important biological mechanisms in helminths (e.g. growth and gonad maturation) [5], the effect of the drugs on schistosome maturation should be assessed as well. Last, we have seen strain differences of in vitro results earlier. Once, when we screened the MMV box (Liberian strain) and compared the results with the MMV box screen at the London School of Hygiene and Tropical Medicine (Puerto Rican strain) [6]; and at another occasion, when we compared the in vitro activity of imatinib (an antischistosomal cancer drug) with earlier published results (Luiz Evangelista strain) [4]. Therefore, it is recommendable to test any new chemotype on various *Schistosoma* strains and species to assess the spectrum of antischistosomal activity.

### 6.1.2 Relevance of physico-chemical properties and “drugability”

During my work, I applied several assays for pharmacokinetic compound profiling to either explain observed in vitro/in vivo discrepancies, or as part of the screening cascade. Indeed, since the introduction of combinatorial chemistry and high throughput screens, a trend toward lower solubility and less permeability of clinical candidates evolved [7]. Dr. Christopher Lipinski investigated the physico-chemical properties of orally administered drugs and created the famous rule of 5. The rule describes drug-likeness according to physico-chemical properties as follows: the molecular weight should be below 500 g/mol, the octanol-water partition coefficient (LogP) should not be greater than 5, and the number of hydrogen bond donors and acceptors should not exceed 10 or 5, respectively. All numbers are multiples of five, which gave the rule’s name. The numbers are a rule of thumb, and thus, many orally available molecules violate the rule of 5 [8]. Similarly to the rule of 5, molecules have been classified according to their solubility and permeability into four types using the Biopharmaceutics Classification System (BCS) [9]. BCS 1 represents molecules with high solubility and high permeability, BCS 2 low solubility and high permeability, BCS 3 low permeability and high solubility, and BCS 4 low solubility and permeability. The classification serves for instance as guidance for bioavailability and bioequivalence studies, as well as for dose formulations and improvement of drug efficacy [10]. The BCS is illustrated in Figure 6, including in vitro drug activity, and exemplified with the three drug sets tested.

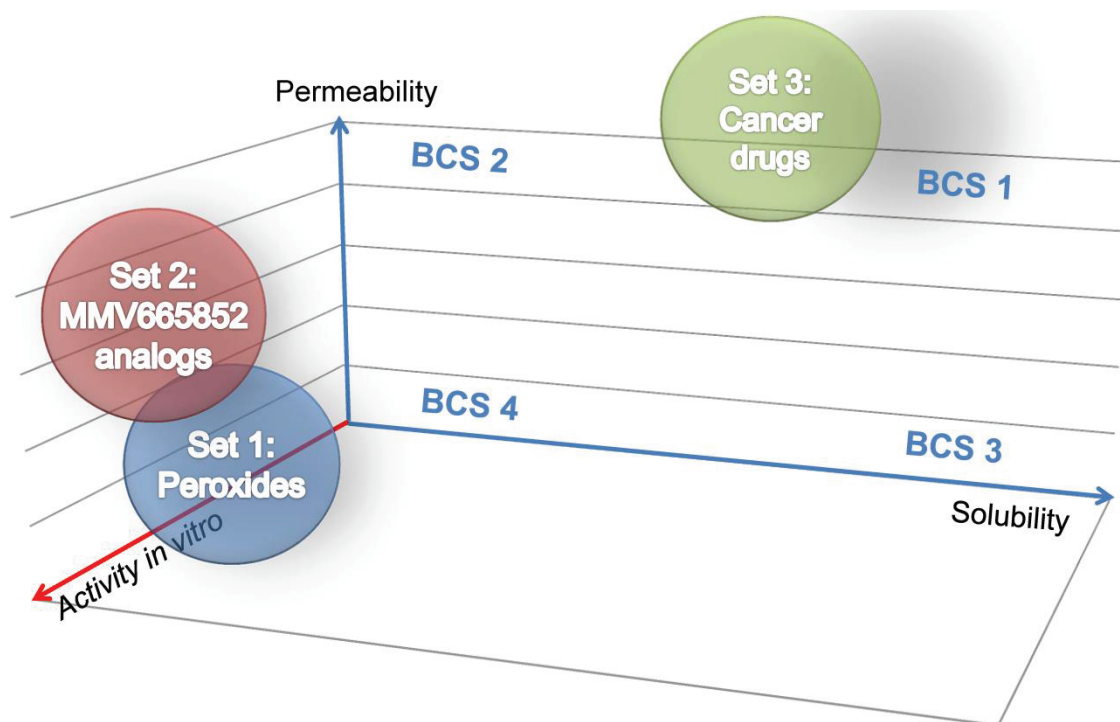
BCS 1 substances are without doubt the preferred molecules in drug discovery. However, as mentioned above, modern drug discovery screens using target-based drug design, without screening first for “drugability”, inherently select for more lipophilic molecules. This trend was examined using clinical candidates at Merck and at Pfizer between the years 1960 and 2000. Merck screened compounds first for bioavailability before proceeding to target-activity studies. Pfizer on the other hand screened bioavailability after the target-activity studies. The evolution of clinical candidates at Merck showed unchanging compound lipophilicity (LogP). In contrast, the clinical candidates at Pfizer were increasingly lipophilic, meaning lower solubility of the compounds [11].

Indeed, also anthelmintic drug discovery might be prone to select for lipophilic molecules, since drug uptake predominantly happens via passive transport into the worms [12].

In general, screening and designing molecules for optimal antischistosomal activity and selectivity is not enough to predict in vivo efficacy. Hence, it is advisable to incorporate

physico-chemical calculations and pharmacokinetic predicting tools in the existing screen flow.

When applying ADME (absorption, distribution, metabolism, elimination) assays before target activity determination, it might be worth to use multiple screening models for the determination of “drugability” to prevent “false-positive” and “false-negative” results in the drug discovery process [14].



**Figure 6: The position of the three drug sets tested according the Biopharmaceutics Classification System (BCS), and in vitro antischistosomal activity.**

Set 1 showed low solubility, but high antischistosomal activity. Their permeability through the intestinal wall is unknown. Set 2 showed low, but high and fast activity. The predicted permeability was moderate to good. Set 3 was less active, with a slow onset of action. Bioavailability for the hit compounds is 90-100%.

Some *in silico* and *in vitro* predictive assays were performed during the present research on antischistosomal drugs. They are described below, and summarized and evaluated for further usage in Table 4.

**Physico-chemical properties.** Of utmost importance is compound solubility. Compounds should be synthesized with consideration for an optimal solubility index, and tested for aqueous solubility. We tested the *in vitro* solubility by dissolving the compound in an aqueous phase, removing the precipitated fraction after 15 hours, and analyzing the amount of compound in solution [15]. Another method to predict solubility is the determination of LogP, which LogP should be below 5, and can be determined *in vitro* [8], or *in silico* [16]. In our study, we assessed rule of 5 with a freely downloadable *in silico* tool [15],[17].

**Permeability and metabolic pathway assays.** Cell-based assays are widely used to simulate the passage of small molecules across the intestinal wall (caco-2 cells) [18], or to assess their hepatic metabolism (hepatocytes) [19]. Newer, non-cell based tools were developed for easier handling and leave more freedom in choosing assay parameters (e.g. pH, or testing of cytotoxic substances). We performed for instance the parallel artificial membrane permeation assay (PAMPA), which simulates penetration through the small intestinal wall by using a donor well (containing drug), acceptor well (initially containing no drug), and a membrane (can be coated with lipids to simulate barriers, such as the intestinal wall, or the blood-brain barrier) separating the two wells from each other. After incubation, the drug concentration in the acceptor well represents the passive drug absorption [20]. Assays using an artificial lipid membrane demonstrated correlation with caco-2 results and can therefore serve as a valid alternative to cell-based assays [14]. Likewise, non-cell based kits for determining metabolic stability are widely used. Microsomes, for instance, are extracted fractions of hepatocytes containing the endoplasmatic reticulum, which expresses CYP metabolic enzymes [21]. A microsomal stability assay was established with a master student in the frame of her master thesis. The assay was able to determine the clearance of *in vivo* candidates. Furthermore, for the determination of CYP inhibition, recombinant isomers of CYP can be purchased [22]. Recombinant CYP isozymes most involved in drug metabolism (CYP1A2, 2C9, 2C19, 2D6, and 3A4), were used in our work for estimation of drug-drug interactions of promising co-administrations as treatment of trichuriasis.

In our study of Set 2, it became apparent, that prediction of bioavailability is only one piece of the puzzle and does not guarantee antischistosomal activity since the rule of 5 was

**Table 4:** Current assays at Swiss TPH used in the framework of this thesis and evaluation of assays used for antischistosomal drug research – experience and recommendations



Lead discovery		Lead optimization		In vivo studies	
<b>Current Assays</b>		<ul style="list-style-type: none"> <li>- SAR studies for characterization of pharmacophore/lead compounds</li> <li>- Estimation of drug selectivity using a L6 rat myoblast cytotoxicity assay</li> <li>- Influence of blood components on activity</li> </ul>		<ul style="list-style-type: none"> <li>- In vivo studies (proof of concept on infected mice)</li> <li>- In depth toxicity studies</li> <li>- Dose-dependent efficacy (e.g. EC<sub>50</sub> determination)</li> </ul>	
<b>Tested Assays</b>					
<b>Assay</b>	<b>Rule of 5</b>	<b>Assay</b>	<b>Aqueous solubility</b>	<b>Assay</b>	<b>β-cyclodextrin complexation</b>
Purpose	In silico bioavailability estimation.	Purpose	Solubility determination. Screen for BCS 1 molecules.	Purpose	Improvement of water-solubility to improve absorption.
Advantage	No costs. Simple procedure. Medium to high throughput.	Advantage	Simple procedure. Medium to high throughput. Spectrometric readout.	Advantage	Established method in drug development.
Disadvantage	Active transport systems are neglected.	Disadvantage	Partly specialized equipment needed. Costly.	Disadvantage	-
Remarks	Can be applied prior to molecule synthesis.	Remarks	-	Remarks	Highly lipophilic molecules can still get stuck in the intestinal wall.
Suggestion for maintenance of assay tested	Recommended for molecule design in SAR studies.	Suggestion for maintenance of assay tested	Appropriate solubility is essential for PK and PD. Sharing this tool in the units working on drug discovery might be a cost-effective solution.	Suggestion for maintenance of assay	Specialized partners need to prepare the complexes. Hit compounds might benefit from complexation.
		<b>Assay</b>	<b>PAMPA</b>	<b>Assay</b>	<b>Oil-based formulation</b>
		Purpose	Prediction of passive transport across the intestinal wall for estimation of drug absorption. Screen for BCS 1 molecules.	Purpose	Increase the amount of dissolved compound in the formulation
		Advantage	Medium to high throughput. Assay conditions are flexible. Correlation to caco-2 cell assay.	Advantage	Easy procedure.
		Disadvantage	LC/MS readout. Specialized equipment needed; thus relatively high costs.	Disadvantage	-
		Remarks	Does not predict active transport.	Remarks	Influence of fatty food drug on absorption is usually unknown. Gastric juices might lead to precipitation after all.
		Suggestion for maintenance of assay	Sharing this tool in the units working on drug discovery might be a cost-effective solution.	Suggestion for maintenance of assay	Can be applied in special cases, e.g. if positive food interactions are implied.

**Table 4:** continued

Assay	<b>Serum albumin binding</b>
Purpose	Assess the influence of serum proteins on antischistosomal activity
Advantage	Conventional microscopic readout
Disadvantage	-
Remarks	-
Suggestion for maintenance of assay	Serum albumin could be added in the culture medium in adult <i>S.mansoni</i> assays, or as an extra screening filter for in vivo candidates.

Assay	<b>Microsomal stability</b>
Purpose	Assess the metabolic phase I stability
Advantage	Validated kits are available
Disadvantage	Low throughput. LC or LC/MS readout.
Remarks	-
Suggestion for maintenance of assay	Can be used for small sets, such as in vivo candidates or lead compound determination in SAR studies.

Assay	<b>CYP450 drug-drug interaction</b>
Purpose	Test for enzyme inhibition or induction
Advantage	Validated kits are available. Spectrometric readout.
Disadvantage	Slightly costly. Reagents are described to be light-and freeze-thaw sensitive.
Remarks	-
Suggestion for maintenance of assay	Recommended for novel co-administered combinations, or drug candidates which need a more detailed drug profiling.

Assays to consider using

Assay	<b>Snap-shot PK</b>
Purpose	Picture plasma levels in animal models
Advantage	Close to true picture in vivo
Disadvantage	Very low throughput
Remarks	-
Suggestion for maintenance of assay	Recommended for compounds of special interest.

true in most cases and the PAMPA assay predicted moderate to good passive absorption. The low solubility of the compounds was most probably the restriction for drug efficacy.

In conclusion, I recommend including physico-chemical calculations in the drug screen. Online tools are available and involve therefore no costs. Moreover, the metabolic stability assay using hepatic microsomes was established in our laboratory and will help to prevent in vivo studies with compounds displaying too fast clearance. The solubility assay and the PAMPA assay are useful tools to evaluate compound solubility or absorbance, respectively. In case the behavior of compounds requires more evidence, assessment of the plasma profile after oral application of the compounds (snap-shot pharmacokinetic profiling) might be useful.

I strongly endorse the establishment of these or equivalent techniques in the screen flow. Including these screening steps will achieve higher drugability of in vivo candidates, and should steer toward higher antischistosomal drug efficacy. From an ethical point of view, the elimination of “undrugable” compounds before in vivo tests is in agreement with the 3R rule (reduce, refine, replace), which promotes the reduction of laboratory animal usage.

### **6.1.3 Importance of public-private and academic partners**

Public-private partnerships have a pivotal role in the control of neglected tropical diseases (NTDs). In 2009, half a billion drug doses were donated for treatment of NTDs in over 80 endemic countries. This was the result of over 320 million partnerships with non-governmental development organizations [23]. Over the next few years, approximately 2 to 3 billion dollars will be needed to realize the ambitious aim of controlling and eliminating NTDs [24]. The increased funding opportunities through national governments and philanthropic institutions (e.g. Rockefeller and Gates Foundations) allowed higher budgets for research and development for new drugs, which are urgently needed [25].

Indeed, our work also clearly profited from valuable existing public-private and academic partnerships. For instance, the Medicines for Malaria Venture (MMV) donated the MMV box for our precursor study of Set 2, and selected compounds for the structure-activity relationship study of Set 2. MMV also provided us with valuable pharmacokinetic and toxicity information about the compounds. In this respect, the support by Dr. Thomas Spangenberg was of great value. Also the cancer drug library of 114 compounds was donated by a non-profit organization, the Developmental Therapeutics Program (DTP) of the US

National Institute of Health (NIH). This project could only be realized by the donation of the drugs, since the purchase of 114 compounds would have been beyond our budget.

Furthermore, the evaluation of Sets 1 and 2 was done in tight collaboration with academic partners. For instance, all peroxides of Set 1 were synthesized by Dr. Alexander Terent'ev and colleagues of the N.D. Zelinsky Institute of Organic Chemistry, Russia. For Set 2, we received support from Dr. Jonathan Vennerstrom of the Department of Pharmaceutical Sciences of the University of Nebraska Medical Center, USA, who confirmed uncertainties about chemical questions. In addition, for Set 2, we performed an array of pharmacokinetic-relevant measurements in collaboration with Dr. Beat Ernst and Philipp Dätwyler at the Department of Pharmaceutical Sciences of the University of Basel.

In general, partners can support research by providing ideas, expertise, and critical input, which are all pivotal for high quality research.



#### 6.1.4 Continuation of the projects

I think that all three projects of antischistosomal drug discovery should be continued because they exhibited good to excellent in vitro activities, as well as in vivo efficacy. Concluding from the discussion above, I would proceed as follows:

##### Future short-term steps

- Include solubility as a feature for drug design
- Use in silico tools for ADME predictions in the drug screen
- Use serum albumin in adult *S. mansoni* assay to assess potential loss of activity of in vivo candidates
- Test hit compounds for antischistosomal activity in other schistosome species (*S. haematobium*, *S. japonicum*) or other *S. mansoni* strains
- Assess the effect of multiple oral doses for trametinib and vandetanib upon experimental *S.mansoni*-infection

##### Future long-term steps

- Incorporate bioavailability as a feature in the screening cascade. Academic or private partners might provide support in this endeavor
- Use snap-shot plasma profiling for compounds of special interest

## 6.2 Trichuriasis drug research

Due to poor treatment outcomes of trichuriasis patients with the drugs of choice albendazole (cure rate: 28%) and mebendazole (cure rate: 36%), a more efficacious treatment is needed [26]. Two co-administration regimens showed superior drug efficacy in trichuriasis patients: albendazole plus oxantel pamoate (cure rate: 69%) [27] and albendazole plus mebendazole (cure rate: 54%) [28]. Both co-administrations could be quickly approved for use, since all three compounds are already safely used in humans worldwide, however, not in these combinations [29],[30].

In general, a better understanding of the relationship between pharmacokinetic parameters and efficacy of anthelmintics against gastrointestinal nematodes should be developed to maximize treatment outcome [31].

I conducted preclinical safety studies of the above-mentioned co-administrations, and carried out preliminary experiments to determine the compartment responsible for trichuricidal efficacy (findings to be published).

### 6.2.1 Main findings and interpretation of the safety assessment of the two co-administrations albendazole plus oxantel pamoate, and albendazole plus mebendazole

The CYP inhibition study assessed the potential for the co-administered drugs to use the same metabolic pathway *in vitro*. Interference of the mutual metabolism can lead to adverse events in the organism treated [21]. Secondly, the co-administrations were assessed in the rodent model to observe potential interactions.

**In vitro:** Moderate CYP interaction was observed in CYP1A2, with the combination albendazole plus oxantel pamoate (2.6-fold compared to separate inhibitions).

**In vivo:** Co-administration of albendazole plus mebendazole led to a significantly increased area under the curve of mebendazole (3.5-fold;  $p=0.02$ ), compared to single mebendazole treatment.

The *in vitro* CYP inhibition assays showed solely interaction at CYP1A2, which was slightly more inhibited (2.6-fold increase;  $IC_{50}$  of 3.1  $\mu$ M) when albendazole and oxantel pamoate

were combined as opposed to the separate inhibitions. Since the absorption of oxantel is low [32],[33], drug-drug interactions of hepatic enzymes are unlikely to occur.

When tested in vivo, the albendazole plus oxantel pamoate co-administration showed no signs of drug-drug interaction; the pharmacokinetic parameters (area under the curve [AUC], maximal concentration [ $C_{max}$ ], time at maximal concentration [ $t_{max}$ ], half-life [ $t_{1/2}$ ]) were almost identical to the parameters of albendazole as single treatment. Oxantel pamoate could not be quantified in any sample, meaning that the plasma levels were below 0.4  $\mu\text{g/ml}$ , which was the lower limit of quantification (LLOQ) of the analytical method. Roughly calculated, that would correspond to a bioavailability below 0.025%, provided that the entire dose applied (100 mg/kg body weight of the rat) would be absorbed and not metabolized (blood volume calculated according to H. B. Lee and M. D. Blaufox [34]). Therefore, the absence of drug interactions is not surprising.

Despite declarations about low bioavailability observed in humans [33] and dogs [32],[35], no precise numbers of the bioavailability of oxantel pamoate are available. To confirm this common knowledge, I would endorse a small study in humans to generate sound evidence about the bioavailability of oxantel pamoate. Also, determining the in vitro absorption of oxantel might be of interest for estimation of drug exposure of the liver. Absorption could be predicted for instance with the above-mentioned PAMPA assay. If oxantel pamoate will be absorbed in vitro and detected in humans, the need for pharmacokinetic drug-drug interaction studies with the co-administration of albendazole plus oxantel pamoate should be evaluated.

Albendazole co-administered with mebendazole on the other hand showed slight interaction, expressed in elevated mebendazole levels. Drug interaction can be defined as a change of the AUC by 25% [36]. Therefore, the statistically significant 3.5-fold increase of mebendazole's AUC upon co-administration ( $p=0.02$ ), can be regarded as a result of drug-drug interaction. Also  $C_{max}$  was significantly higher (2.8-fold;  $p=0.02$ ) than after single treatment. Other pharmacokinetic parameters ( $t_{max}$  and  $t_{1/2}$ ) remained unchanged.

Since the CYP inhibition study did not show any interaction between albendazole and mebendazole, the reason for the higher AUC might be a result of non-CYP enzymes, such as inhibition of the carbonyl transferase, and/or the flavin-containing monooxygenase. The first hypothesis was suggested as the main pathway for mebendazole biotransformation [37]; however, albendazole is not known for biotransformation via the carbonyl transferase [38]. The latter is a major pathway for albendazole, but has not yet been characterized for mebendazole metabolism [39]. Another reason for drug-drug interaction might be the

inhibition of efflux mechanisms from the blood stream into the intestine. In more detail, in rats, the efflux protein p-glycoprotein was suggested to transport albendazole sulfoxide across the intestinal wall into the intestine [40]. If mebendazole and albendazole sulfoxide compete for the same efflux mechanism, plasma level of one or both drugs could potentially increase upon co-administration.

Generally, CYP interactions with the isozyme 3A4, which is the main metabolic pathway for albendazole [38], seem unlikely. A study in 16 healthy volunteers assessed interactions of albendazole or mebendazole with ritonavir, a highly potent CYP3A inhibitor. Ritonavir (200 mg/kg) did not affect the disposition of albendazole (400 mg) or mebendazole (1000 mg) upon a single exposure to the co-administrations. The investigators suggested co-administrations with other CYP3A inhibitors most likely to be safe as well [41].

Undoubtedly, the results of our *in vivo* studies in rats are only an indication about the situation in humans. A diverging factor is for instance the difference in plasma concentrations. In our study, the AUC of a single albendazole treatment with 100 mg/kg resulted in an AUC of 63.8  $\mu\text{g/ml}\cdot\text{h}$  in rats, whereas in humans, a 400 mg dose resulted in 5.4  $\mu\text{g/ml}\cdot\text{h}$  [41]. Similarly, mebendazole applied to rats at 100 mg/kg led to an AUC of 5.9  $\mu\text{g/ml}\cdot\text{h}$ , whereas in humans, a 1000 mg dose lead to an AUC of 0.2  $\mu\text{g/ml}\cdot\text{h}$  [41]. The drug exposure in humans, using standard doses, was clearly lower than in our experimental interaction studies. Thus, interactions that occurred in rats might not translate to humans.

Furthermore, species differences in benzimidazole metabolism should be considered [42]. For example, unequal ratios of ABZSO's (-) and (+) enantiomers were observed: in humans the ratio is 20:80, whereas in rat it is 59:41, respectively [38], which might change the pattern of drug interactions.

These pharmacokinetic studies were a first step to assess drug safety. Without doubt, *in vitro* and *in vivo* experiments do not replace clinical studies. One can argue, that co-administration of well-known and proven-to-be-safe drugs are most likely safe when applied together. Moreover, co-administration therapies are not dangerous per se; many people in the industrialized world are subject of polypharmacy. Hospitalized people older than 65 years old, and diagnosed with hypertension, for instance, take in average 4.4 different drugs a day [43]. Nevertheless, since we do not know the precise toxic threshold of the drugs, it is difficult to estimate the impact of elevated plasma levels on toxicity and adverse events. Furthermore, we do not know how pathologies of helminth infections influence the pharmacokinetics of the drugs, whether they lead to or exacerbate drug interactions.

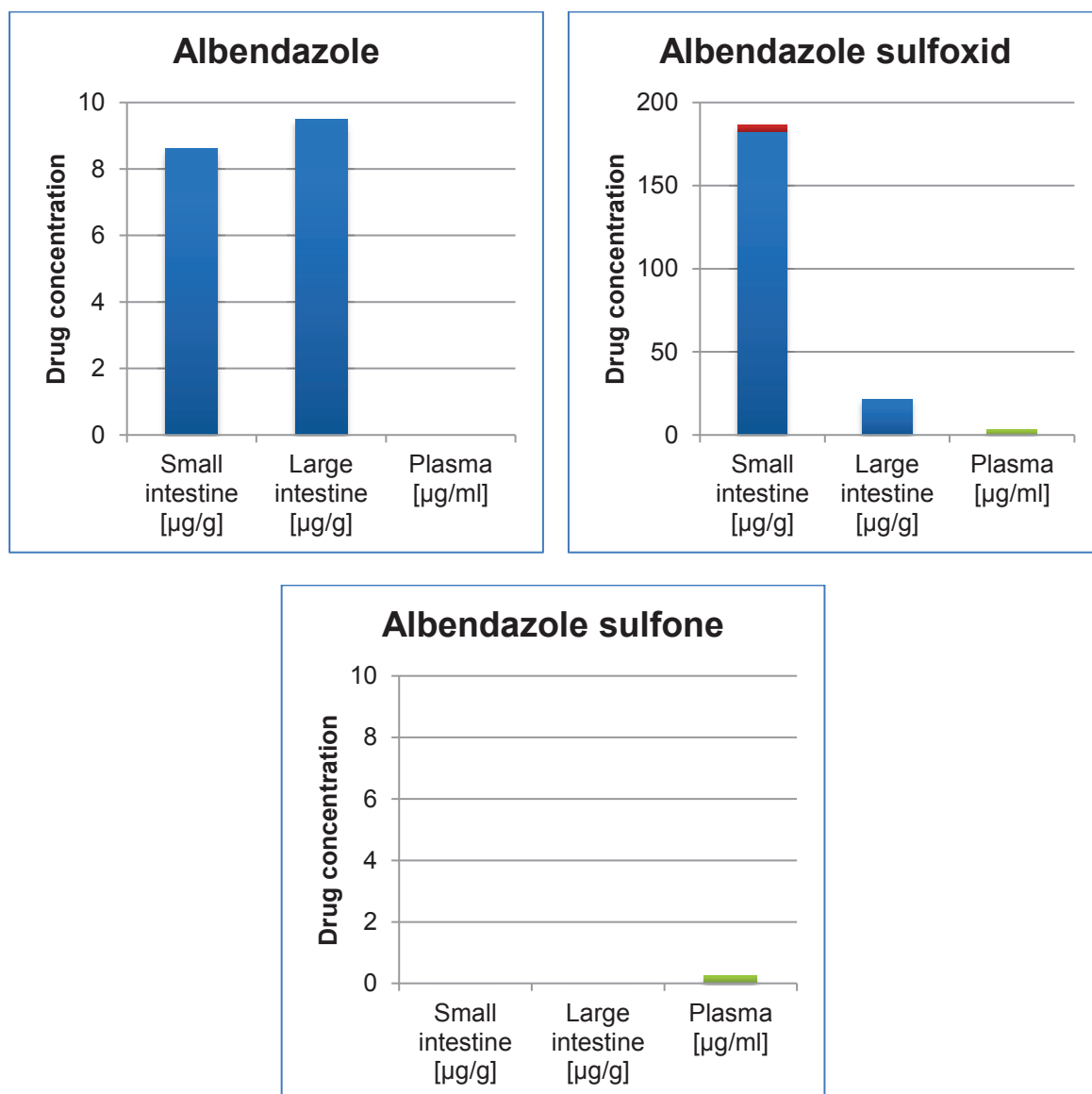
With respect to safety uncertainties still remaining and species difference in benzimidazole biotransformation, I would conduct a study with healthy volunteers and patients in endemic areas. Elucidation of the pharmacokinetics of albendazole plus mebendazole co-administration in humans might ultimately rule out drug-drug interactions.

### **6.2.2 Gastrointestinal metabolic interactions and the determination of the compartment responsible for drug activity against *T. muris***

After reviewing safety considerations, efficacy considerations of aforementioned drug co-administrations will be discussed. The co-administrations showed improved efficacy as opposed to their single treatments. Is the efficacy of albendazole and mebendazole co-administration improved due to metabolic drug-drug interaction in the gastrointestinal (GI) tract? Also in the GI tract, albendazole is exposed to biotransformation into its sulfoxide metabolite [44]. Intestinal nematodes take up drugs from exposure in the intestinal lumen [13]. Since albendazole seems to be more relevant for drug efficacy than albendazole sulfoxide [12], a change in albendazole biotransformation might impact the treatment outcome. Mebendazole has no active metabolite [42]. Therefore, inhibition of mebendazole metabolism would lead to a higher drug exposure of the intestinal worms, hence, probably resulting in higher efficacy. To date, no study has tested the inhibition or induction of albendazole and mebendazole biotransformation in the GI tract (date of information search: 18.11.2015). Analyzing the GI tract content and mucosa for oxantel pamoate, mebendazole, albendazole, albendazole sulfoxide, and albendazole sulfone might provide information about intestinal metabolism and interactions.

Currently, the compartment responsible for drug activity against *Trichuris* has not been identified. The answer is not trivial, since *Trichuris*' anterior is embedded to one third in the "syncytial tunnel", and two thirds of the posterior end is freely laying in the lumen of the GI tract [45]. Studies with *T. suis* suggested parasitic drug uptake via the bloodstream, since the concentration of drug found in the worms correlated best with blood concentrations of oxibendazole, fenbendazole's major metabolite, and less with concentrations observed in the GI tract [46]. On the other hand, drug diffusion through the cuticle (nematodes) or the tegument (trematodes) is the predominant way of drug entry, rather than oral ingestion [12]. Identification of the compartment responsible for drug activity is crucial for drug research against trichuriasis, and should therefore be further investigated.

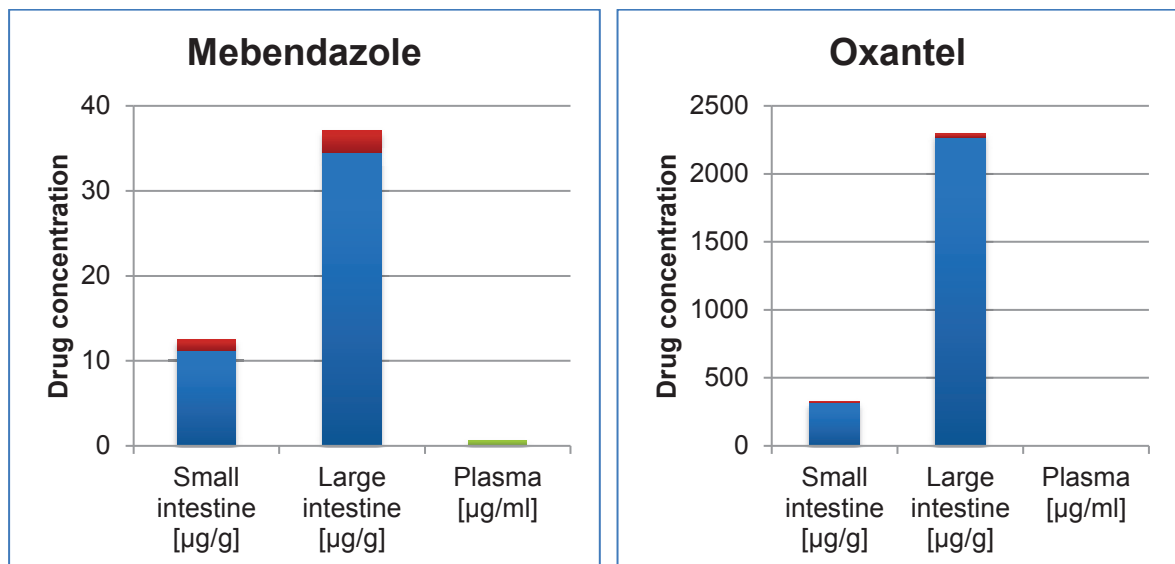
Currently, a project addressing the identification of the compartment responsible for trichuricidal efficacy is ongoing. Preliminary studies demonstrated intensive metabolism of albendazole in the GI tract to albendazole sulfoxide, without further metabolism to the sulfone metabolite (Figure 7). Only little drug quantities could be found in the mucosa.



**Figure 7: Distribution of albendazole, albendazole sulfoxide, and albendazole sulfone.**

Albendazole was applied orally to one mouse at 100 mg/kg body weight. The dissection time point was 4 h post-treatment. **Red**: intestinal mucosa; **blue**: intestinal content; **green**: plasma

Mebendazole and oxantel were predominantly quantified in the GI tract content with low concentrations in the mucosa (Figure 8). The relatively low concentrations of mebendazole (only 4-times higher than the strongly metabolized albendazole) indicate metabolism in the GI tract. In contrast, oxantel seems to experience less biotransformation than the other drugs analyzed, concluded from the high levels in the GIT content.



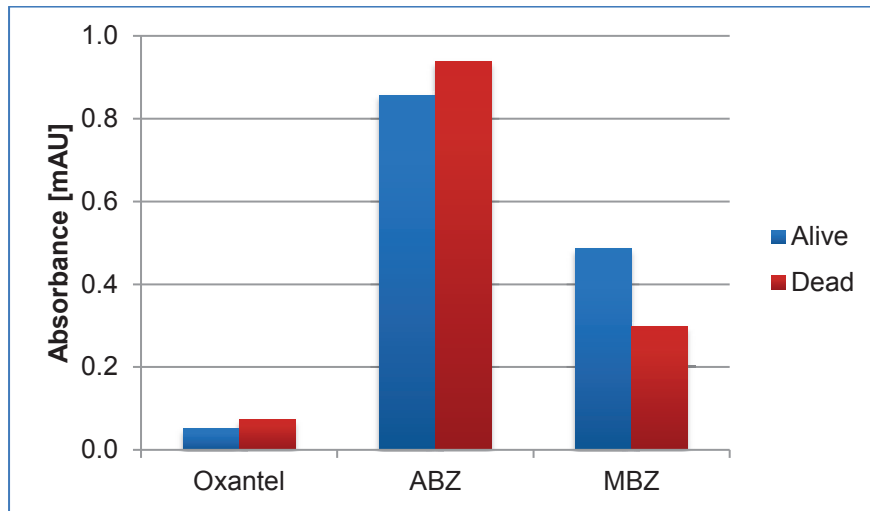
**Figure 8: Distribution mebendazole and oxantel.**

Mebendazole and oxantel pamoate were applied orally to mice (each drug n=1) at 100 mg/kg body weight. The dissection time point was 4 h post-treatment.

**Red:** intestinal mucosa; **blue:** intestinal content; **green:** plasma

Further tests should monitor drug distribution at different time points to estimate the full exposure of the parasite to the active anthelmintics. Of further interest will be the relationship between the drug concentrations observed within the worms and in the different compartments. Correlation of concentrations might then identify the compartment responsible for drug efficacy and serve as a marker for efficacy.

For instance, in a project with bachelor students, we analyzed the in vitro drug uptake into *T. muris* (Figure 9): albendazole was found in highest quantity, followed by mebendazole, and oxantel demonstrated least entry. Oral ingestion seemed not to be the major route of uptake for any of the drugs, since dead and alive worms incubated in drug-containing medium displayed comparable amounts of drug in their tissue. Analyzing the worms concomitantly to the drug distribution might shed light onto the question about which compartment correlates with the drug amounts observed in *Trichuris*.



**Figure 9: Uptake of oxantel, albendazole (ABZ), and mebendazole (MBZ) into *T. muris* in vitro.**

Alive or dead adult *T. muris* (two measurements, each of n=30 worms) were incubated in 10  $\mu$ M drug for 24 hours. After thorough rinsing, the worms were ground and drugs were extracted with methanol, followed by HPLC-UV analysis.



### 6.2.3 Next steps in trichuriasis drug research

In vitro and in vivo data, as well as hints from clinical trials about potential interactions of albendazole and mebendazole, form a mosaic of the actual pharmacokinetics in humans upon co-administration. I would recommend obtaining a clear picture of the actual profile. Also more studies pertaining “host-parasite pharmacokinetics” are needed. The studies would encompass the following objective:

- Confirmation of the superior efficacy of albendazole plus mebendazole co-administration compared to other treatments.
- Study of albendazole plus mebendazole co-administration kinetic profiles in an endemic area. The standard doses of 400 mg albendazole and 500 mg mebendazole could be applied. The influence of infections and infection intensity would be of interest. In this respect, patients with intestinal schistosomiasis should be included in the study.
- Testing albendazole plus mebendazole in different infection models might serve as preliminary study, and justify aforementioned studies in patients.
- Examination of oxantel pamoate’s bioavailability in humans. The standard dose of 20 mg/kg could be applied, but also a higher dose, e.g. 40 mg/kg to “test the limits”. Goal would be to confirm the general assumption of oxantel’s negligible bioavailability with numbers.
- Determination of the in vitro intestinal absorption of oxantel pamoate to estimate the drug exposure of the liver.
- Evaluation of the need for a pharmacokinetic drug-drug interaction study of albendazole plus oxantel pamoate co-administration in humans.
- Assessment of metabolism and drug-drug interaction in the GI tract. Increased exposure to the active drug, due to drug-drug interactions, might explain changes in drug efficacy.
- Determination of the compartment responsible for drug effect using the *T. muris* mouse model. Does drug uptake into the worm happen via blood ingestion or via uptake through the cuticle from the GI tract? This information is important for drug efficacy and pharmacokinetic studies.

### 6.3 Conclusion

The aim of the thesis was to advance anthelmintic drug research, with emphasis on structure-activity relationship and pharmacokinetic studies. The following conclusions can be drawn from the results obtained.

The two structure-activity relationship studies on *S. mansoni* showed high in vitro activities. The IC<sub>50</sub> values were in the nanomolar range, which is hardly seen in antischistosomal drug discovery, close to praziquantel values. However, none of the compounds expressed high efficacy in the *S. mansoni* mouse model. ADME predictions suggested low solubility to be the main issue, possibly leading to low absorption. Hence, synthetic peroxides and the MMV665852 analogs might reveal higher activities in vivo, if the design of the structures aims for higher aqueous solubility. Therefore, a higher in vivo-hit rate might be achieved by using a “structure-bioavailability-activity”-based drug design and screen flow. Testing marketed cancer drugs provided first experimental data on the general hypothesis that the mechanism of action of cancer drugs might translate to antischistosomal activity. The results showed indeed activity of protein kinase inhibitors on *S. mansoni*. However, the in vivo worm burden reductions were very low to moderate. Further studies might involve multiple dosing of trametinib and vandetanib in mice to simulate the long half-lives observed in humans, as well as testing their effect on schistosome development.

The in vitro CYP inhibition studies showed slight interaction between albendazole and oxantel pamoate. This interaction was not observed in vivo, probably because oxantel is hardly absorbed. A significant interaction was observed in vivo for mebendazole, when mebendazole was co-administered with albendazole. Since there is not enough evidence for the safety of albendazole plus mebendazole co-administration, clinical trials in endemic areas should assess pharmacokinetic parameters of this co-administration regimen in case it will be used in larger scale. Additionally, the intestinal absorption of oxantel pamoate is of interest, and is advised to be tested in vitro. To sum up, my studies present a first insight in co-administrations that might soon be applied for preventive chemotherapy in endemic areas.

Ongoing projects assessing the distribution of trichuricidal drugs should be continued to determine the compartment in the host that is responsible for drug efficacy. This knowledge is crucial for drug development against trichuriasis.

In conclusion, my projects advanced helminth drug research and development by identifying new chemotypes with promising activity against *S. mansoni*, which enriched the meager collection of molecules available for antischistosomal drug discovery. Moreover, the

pharmacokinetic evaluation demonstrated the need for safety confirmation of the albendazole plus mebendazole co-administration in humans. This is an important step in the process of developing a better treatment against trichuriasis.

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# Appendix

## Curriculum vitae

## Noemi Cowan

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Date of birth	04.10.1986
Place of birth	Basel, Switzerland
Nationality	Swiss
Maiden name	Hiroshige

### Academic education

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Oct 2012 - Sept 2016	<b>PhD studies, helminth drug discovery and development unit,</b> Swiss Tropical and Public Health Institute (SwissTPH), Basel Early drug discovery and pharmacokinetic studies for helminth infections <ul style="list-style-type: none"><li>• In vitro and in vivo activity evaluation of small molecules against the parasitic fluke <i>Schistosoma mansoni</i></li><li>• Development and validation of an HPLC-UV/vis method for quantification of anthelmintic drugs in biological fluids, according to FDA guidelines</li><li>• Pharmacokinetic studies of co-administered drugs in the rodent model</li><li>• Determination of the pathway of drug entry into soil-transmitted helminths to elucidate the compartment (blood or gastrointestinal content) responsible for drug efficacy.</li><li>• Quantification of active pharmaceutical ingredient in tablets to treat helminthiases</li></ul>
2007 - 2011	<b>Bachelor and master in pharmaceutical sciences,</b> University of Basel

### Work experience

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Oct 2012 - Sept 2016	<b>PhD studies, helminth drug discovery and development unit,</b> Swiss Tropical and Public Health Institute (SwissTPH), Basel <ul style="list-style-type: none"><li>• Elaboration, management and execution of scientific projects</li><li>• Providing colleagues scientific expertise and support</li><li>• Writing peer-reviewed articles</li><li>• Purchase and organization of laboratory equipment</li></ul>
May - Dec 2011	<b>DSM branch Pentapharm,</b> Supplier of natural products, Aesch, Switzerland Master thesis: „Optimization of the RVV-V purification for application in the diagnostic blood clotting kit Pefakit® PiCT®“ <ul style="list-style-type: none"><li>• Protein purification of viper venom using size exclusion and ion exchange chromatography</li><li>• Assessment of protein purity using enzymatic and protein quantification assays</li></ul>



- Successful production of three Pefakit® PiCT® batches, meeting the company's quality standards

### Mentoring experience

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Jan – May 2015	<p><b>Master theses</b></p> <p>Supporting students of the pharmaceutical sciences and infection biology during their master theses:</p> <ol style="list-style-type: none"> <li>1. “Establishment of a human liver microsome assay”</li> <li>2. “Preclinical studies on <i>Trichuris muris</i>: from structure-activity relationship to drug uptake studies”</li> <li>3. “Drug uptake of albendazole, mebendazole, and oxantel pamoate into <i>T. muris</i> and <i>Heligmosomoides polygyrus</i>”</li> </ol> <ul style="list-style-type: none"> <li>• Introducing the students to the research field, as well as to biological and analytical methods</li> <li>• Provide guidance and supervision for the work</li> </ul>
Dec 2014	<p><b>Bachelor block course</b></p> <p>Giving students a taste of scientific work</p> <ul style="list-style-type: none"> <li>• Organize and conduct a short but interesting laboratory based project for students</li> <li>• Provide students with essential background information</li> <li>• Intensive assistance of their practical work and analysis of results</li> </ul>

### Other work experience

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2009 - 2011	<p><b>KidsLab</b>, children's program of the Café Scientifique in collaboration with the University of Basel</p> <p>Explaining scientific and political topics to 6-12-year-old children</p> <ul style="list-style-type: none"> <li>• Planning and executing scientific workshops for children</li> </ul>
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### Languages

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German	Mother language
English	Proficient user, C1 (European reference; 2012)
French	Intermediate
Italian, Spanish, Japanese	Basic

### Software skills

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Microsoft office: Very good  
 Chemstation for HPLC: Routine usage  
 Prism: Basic knowledge  
 Biograf<sup>®</sup>, Quasar, Raptor, und Yeti (for testing target affinity *in silico*): basic knowledge

## Oral presentations at international conferences

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April 2015 “On the way to new antischistosomal drugs”, British Society of Parasitology Spring meeting 2015

## Peer-reviewed publications

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Approved oncology drugs lack in vivo activity against *Trichuris muris* despite in vitro activity.

**Cowan N**, Raimondo A, Keiser J.

Parasitol Res. 2016 Nov;115(11):4443-4446.

In vitro and in vivo drug interaction study for two lead combinations, oxantel pamoate plus albendazole and albendazole plus mebendazole, for the treatment of human soil-transmitted helminthiasis.

**Cowan N**, Vargas M, Keiser J

Antimicrob Agents Chemother. 2016 Sep 23;60(10):6127-33.

Treatment of human and livestock helminth infections in a mobile pastoralist setting at Lake Chad: Attitudes to health and analysis of active pharmaceutical ingredients of locally available anthelmintic drugs.

Greter H, **Cowan N**, Ngandolo BN, Kessely H, Alfaroukh IO, Utzinger J, Keiser J, Zinsstag J

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