Antischistosomal Properties of Mefloquine: From *in vitro* Studies to Drug Target Discovery

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Basel, den 24. Mai 2011

Prof. Dr. Martin Spiess
Dekan

Dedicated to my dear parents and my lovely brother

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Summary

Schistosomiasis is a chronic helminthic disease caused by fluke worms of the genus *Schistosoma spp*. The disease belongs to the so-called neglected tropical diseases (NTDs), a group of poverty-promoting chronic infectious diseases, which primarily occur in rural areas and poor urban areas of low-income and middle-income countries. Schistosomiasis is a major public health problem and endemic in approximately 78 countries of the world, but mainly in sub-Saharan Africa. It has been estimated that more than 207 million people worldwide are infected with schistosomiasis and 779 million people are at risk of contracting the disease. The annual mortality rate due to schistosomiasis in sub-Saharan Africa might be as high as 280,000.

Overall, there are five schistosome species parasitizing humans, with *Schistosoma haematobium* (Sub-Saharan Africa, Middle East and some islands in the Indian Ocean), *S. mansoni* (Sub-Saharan Africa, parts of South America and some Caribbean islands) and *S. japonicum* (China, the Philippines and Indonesia) being the three main species with the widest geographical distribution. The disease is transmitted by contact with infested fresh water, containing the infectious stage of the parasites, the cercariae, which were released by infected aquatic snails and penetrate human skin. During penetration cercariae develop to larval stages, which enter the blood and develop into adult worms. Female schistosomes begin laying eggs, which either trapped in the tissues of the infected host or are excreted with the faeces or the urine. Chronic pathology and morbidity is not due to the adult worms itself, but due to inflammatory and granulomatous reactions provoked by the parasite eggs. For example, *S. haematobium* affect the bladder and the vesical and urethral walls and causes e.g. haematuria, ulcera and fibrosis (urinary schistosomiasis), *S. mansoni* affect the large bowel, the liver and the mesenteric veins and causes e.g. abdominal pain, ulcera and fibrosis (intestinal schistosomiasis).

The WHO recommended strategy for control of schistosomiasis is morbidity control through regular treatment with praziquantel. Praziquantel is active against the adult stages of all five human schistosome species, however, it has only little or no effect on eggs and immature worms. Hence re-treatment is necessary to kill those parasites that have since matured. Overall, treatment is regarded as safe and generally well-tolerated. However, to date praziquantel is the only drug available for treatment and control of schistosomiasis. The dependence on only one drug for such an important helminthic infection and the massive use of praziquantel in mass drug administration programs is a considerable risk factor for the development of praziquantel resistant schistosome strains. In fact, resistance was already demonstrated in the laboratory and in some field studies. Furthermore, to date no vaccines

against schistosomiasis are available. Therefore there is a need to develop new drugs against schistosomiasis.
Recently the antimalarial drug mefloquine was found to have interesting antischistosomal activities *in vitro* and in the mouse model. For example, single oral doses of 200 or 400 mg/kg mefloquine administered to mice infected with juvenile and adult stages of *S. mansoni* and *S. japonicum* resulted in high or complete total and female worm burden reduction. Treatment with a lower dose (150 mg/kg) revealed significantly reduced egg production in *S. mansoni*-infected mice. Furthermore, a clinical trial conducted with school children in Côte d´Ivoire showed that a mefloquine/artesunate combination cured 11 out of 18 children infected with *S. haematobium*.

Based on these findings, the aim of my PhD thesis was to further assess the newly discovered antischistosomal properties of the antimalarial drug mefloquine and to get a first insight into the mode of action and the pharmacodynamics of mefloquine in schistosomes. Therefore different *in vitro* and *in vivo* studies, including drug target discovery studies, were conducted with *S. mansoni*.

By assessing the time-dependent drug effect of mefloquine on schistosomes we showed that mefloquine has a rapid onset of action on newly transformed schistosomula (NTS; artificially produced, non hematophagous) and a slightly slower onset of action on adult worms (recovered from infected mice) *in vitro*: 10 µg/ml killed adult worms within 24 h of incubation (LC_{50}= 1.9 µg/mL). In the presence of hemin drug activities against adult *S. mansoni* were enhanced. *In vivo* already 72 h post treatment more than 90% of schistosomes were shifted to the liver, a criterion for drug activity, which shows how quick after drug treatment worms are forced to migrate from the mesenteric veins to the liver (ED_{50}= 62 mg/kg). With scanning electron microscopy (SEM) studies, a common technique to document the efficacy of antischistosomal drugs on the parasites tegument, we confirmed our previous observations and the differences in the onset of action between *in vitro* and *in vivo* studies: SEM revealed extensive tegumental damages including blebbing, shrinking and sloughing on the tegument of NTS and adult worms, in particular following *in vitro* incubation and on the tegument of female worms.
Furthermore, we showed conducting a small structure-activity relationship study that the optical isomers and the racemic forms of the chiral drug mefloquine have only moderate stereoselectivity, in particular *in vivo*, since prominent activities were observed with all derivates in the *S. mansoni* mouse model.
To evaluate the potential of a mefloquine/praziquantel drug combination against *S. mansoni* we conducted combination studies and achieved encouraging results, since synergistic

effects *in vitro* and in the *S. mansoni* mouse model were seen, when both drugs were given simultaneously or when praziquantel treatment followed mefloquine. The latter administration regime achieved the best results and only moderate worm burden reductions were achieved when praziquantel was administered prior to mefloquine.
Finally, to get a better knowledge about the mechanism of action of mefloquine in schistosomes we launched drug target discovery studies using affinity chromatography. We identified enolase, a glycolytic enzyme, as a major mefloquine- binding protein in *S. mansoni* schistosomula. We demonstrated that mefloquine interacts with glycolysis in schistosomula.
To strengthen the field of antischistosomal drug discovery we assessed a new technique, isothermal microcalorimetry (IMC), to examine *in vitro* drug effects against *S. mansoni*. To date, the "gold standard" is microscopic reading, which is easy to handle and accomplishable in every laboratory, but difficult to standardize and subjective. We examined *in vitro* drug activities of three antischistosomal drugs and mefloquine against *S. mansoni* and compared the results to microscopic readouts. Both methods showed a good correlation, however, IMC identified drug effects that were not visible by microscopic evaluation and precisely determined the onset of action of the test drugs. Furthermore, we compared drug effects on NTS and adult schistosomes, because NTS are increasingly used in antischistosomal drug screening assays.

In conclusion, my work has confirmed that mefloquine possesses excellent antischistosomal properties and that the drug might be an interesting partner drug in combination chemotherapy.
We demonstrated that IMC is a useful tool for antischistosomal drug discovery and should therefore be assessed also for studying drug effects against other helminths. In addition, our data support the use of NTS in *in vitro* antischistosomal drug assays, since we observed similar drug sensitivities on both stages.

Zusammenfassung

Schistosomiasis (oder auch Bilharziose) ist eine chronische Wurmerkrankung, verursacht durch Saugwürmer (Trematoda) der Gattung *Schistosoma spp*. Sie gehört zu den sogenannten vernachlässigten Tropenkrankheiten, einer Gruppe von chronischen Infektionskrankheiten, die vor allem in armen Gebieten von Ländern mit mittlerem oder niedrigem Einkommen auftreten. Die Bilharziose ist endemisch in rund 78 Ländern der Welt, vor allem aber kommt sie in Afrika südlich der Sahara (Sub-Sahara) vor. Dort ist sie ein grosses Problem der allgemeinen Volksgesundheit. Es wird geschätzt, dass mehr als 207 Millionen Menschen weltweit mit Bilharziose infiziert und weitere 779 Millionen Menschen gefährdet sind. Die jährliche Todesrate aufgrund von Bilharziose wird allein im Endemiegebiet südlich der Sahara auf ungefähr 280.000 geschätzt.
Insgesamt gibt es fünf Arten von Schistosomen (Pärchenegel), die den Menschen als Parasiten befallen. Die drei wichtigsten (mit ihrer jeweiligen geographischen Verteilung) sind *Schistosoma haematobium* (Sub-Sahara, Naher Osten und einige Inseln im Indischen Ozean), *S. mansoni* (Sub-Sahara, Teile von Südamerika und einige karibische Inseln) und *S. japonicum* (China, die Philippinen und Indonesien). Die Krankheit wird durch Kontakt mit kontaminiertem Wasser übertragen, welches das infektiöse Entwicklungsstadium, die Zerkarien, enthält. Diese werden von infizierten Wasserschnecken (als Zwischenwirt) ausgeschieden und infizieren den Menschen durch Penetration der Haut. Dabei verlieren die Zerkarien ihren Ruderschwanz und entwickeln sich zu Schistosomula (Larvenstadium, junge Schistosomen), welche dann über die Lymph- und Blutgefässe in die Pfortader und die intrahepatischen Gefässverzweigungen der Leber wandern, wobei sie sich zu adulten Würmern entwickeln. Dort vereinigen sich die Männchen und Weibchen zu Paaren, welche in die Mesenterial- und Darmvenen (*S. mansoni*) bzw. das Venengeflecht der Blase und des Enddarms (*S. haematobium*) migrieren. Die weiblichen Schistosomen beginnen Eier zu legen, welche sich entweder in den entsprechenden Geweben des infizierten Wirtes einnisten oder mit dem Kot oder Urin ausgeschieden werden. Krankheitssymptome werden nicht durch die Würmer selbst ausgelöst, sondern durch die eingenisteten Eier, welche entzündliche und granulomatöse Gewebsreaktionen auslösen. *S. haematobium* zum Beispiel verursacht unter anderem Hämaturie, Ulcera und Fibrosen an Blasen- und Harnleiterwänden (Harn-Bilharziose) und *S. mansoni* befällt Dickdarm, Pfortader und die Mesenterialvenen und verursacht Abdominalschmerzen, Geschwüre und Leberfibrosen (Darm-Bilharziose).
Die WHO-Strategie zur Bekämpfung der Bilharziose besteht in der regelmässigen Behandlung mit Praziquantel. Praziquantel ist gegen alle adulten Stadien der fünf menschenpathogenen Schistosomaarten wirksam, wobei es nur geringe oder gar keine

Wirkung auf Eier und junge Würmer hat. Daher ist eine erneute Behandlung, nach Heranreifen dieser Stadien zu adulten Schistosomen, notwendig, um diese endgültig und vollständig abzutöten. Die Behandlung wird insgesamt als sicher und allgemein gut verträglich angesehen. Allerdings ist Praziquantel bisher das einzige zur Verfügung stehende Medikament zur Behandlung und Eindämmung der Bilharziose. Die Tatsache, dass nur ein einziges Medikament gegen eine so schwere und weit verbreitete Wurminfektion zur Verfügung steht und dass durch den dadurch bedingten massiven Einsatz von Praziquantel ein erhebliches Risiko für die Entstehung von Resistenzen besteht, veranlasst zu grosser Sorge. Tatsächlich wurden bereits im Labor und in einigen Feldversuchen Praziquantel resistente Schistosomastämme nachgewiesen. Darüber hinaus gibt es noch keine Impfstoffe gegen die Bilharziose. Aus diesen Gründen besteht ein grosser Bedarf, neue Medikamente gegen Schistosomaisis zu entwickeln bzw. bereits vorhandene auf deren mögliche Wirksamkeit zu prüfen.
Kürzlich wurde gezeigt, dass das Antimalariamittel Mefloquin sowohl *in vitro* als auch im Mausmodell interessante antischistosomale Wirkungen hat. Zum Beispiel führten orale Einzeldosen von 200 oder 400 mg/kg Körpergewicht bei Mäusen, welche mit jugen und adulten Stadien von *S. mansoni* und *S. japonicum* infiziert waren, zu einer starken Verringerung der gesamten Wurmlast oder sogar zur vollständigen Eliminierung der weiblichen Würmer. Die Behandlung mit einer niedrigeren Dosis (150 mg/kg Körpergewicht) bewirkte eine deutlich reduzierte Eierproduktion in *S. mansoni*-infizierten Mäusen. Eine klinische Studie an der Elfenbeinküste zeigte, dass 11 von 18 mit *S. haematobium* infizierten Schulkindern mit einer Kombination von Mefloquin und Artesunat, einem weiteren Antimalariamittel, geheilt werden konnten.

Basierend auf diesen Ergebnissen war das Ziel meiner Dissertation, die neu entdeckten antischistosomalen Eigenschaften des Antimalariamittels Mefloquin weiter zu untersuchen und somit einen Einblick in die Wirkungsweise von Mefloquin in Schistosomen zu bekommen. Dazu haben wir verschiedene *in vitro* und *in vivo* Studien, einschliesslich Target Discovery Studien mit *S. mansoni*, durchgeführt.

Wir konnten zeigen, dass Mefloquin *in vitro* einen sehr schnellen Wirkungseintritt bei Schistosomula hat (NTS; frisch im Labor aus Zerkarien hegestellt, nicht haematophag) und einen etwas langsameren auf adulte Würmer (durch Sektion aus infizierten Mäusen gewonnen), wobei eine Konzentration von 10 µg/ml adulte Schistosomen innerhalb von 24 Std. Inkubationsszeit töteten (LC_{50}= 1,9 µg/ml). In Anwesenheit von Hämin wurde die Wirksamkeit gegen adulte Formen noch verbessert. In *vivo* waren 72 Std. nach der Behandlung mehr als 90% der Schistosomen aus den Mesenterialvenen in die Leber

gewandert (ED_{50}= 62 mg/kg). Dieser Leber-Shift ist ein Kriterium zur Beurteilung der Wirksamkeit eines antischistosomalen Wirkstoffes. Mit der Rasterelektronenmikroskopie (REM), einer Technik um die Wirksamkeit von Arzneistoffen an der Wurmoberfläche (Tegument) zu untersuchen, bestätigten wir unsere bisherigen Beobachtungen und die Unterschiede im Wirkungseintritt zwischen *in vitro* und *in vivo* Studien. Wir konnten zeigen, dass Mefloquin sowohl bei NTS, als auch bei adulten *S. mansoni*, eine starke Zerstörung des Tegumentes mit Blasen- und Faltenbildung und sogar partiellem Ablösen der oberen Schicht hervorrief. Dies war insbesondere *in vitro* und bei den Weibchen zu beobachten.
In einer Studie zur Struktur-Aktivitäts-Beziehung zeigten wir, dass der chirale Wirkstoff Mefloquin nur eine mässige Stereoselektivität besitzt, weil im Mausmodell sowohl mit den einzelnen optischen Isomeren als auch mit beiden racemischen Formen sehr gute Wirkungen gegen *S.mansoni* beobachtet werden konnten.
Um das Potential einer Mefloquin/Praziquantel-Wirkstoffkombination gegen *S. mansoni* zu prüfen, führten wir Kombinationsstudien durch und erzielten ermutigende Ergebnisse. Sowohl *in vitro* als auch im *S. mansoni* -Mausmodell sahen wir synergistische Effekte, wenn beide Medikamente gleichzeitig gegeben wurden oder wenn Praziquantel der Mefloquinbehandlung folgte. Wenn umgekehrt Mefloquin zuerst verabreicht wurde, zeigte sich nur ein mässiger Effekt.
Um den Wirkmechanismus von Mefloquin in Schistosomen besser zu verstehen und mögliche Targetproteine zu identifizieren, führten wir Target Discovery Studien mit Hilfe der Affinitätschromatographie durch. In einem ersten Schritt, identifizierten wir Enolase, ein Enzym der Glykolyse, als ein wichtiges Mefloquin bindendes Protein in *S. mansoni* Schistosomula. In einem zweiten Schritt konnten wir zeigen, dass Mefloquin in die Glykolyse der Schistosomula eingreift und so wahrscheinlich den Tod der Würmer mit verursacht.
Um die antischistosomale Wirkstoffforschung voranzutreiben, untersuchten wir die Einsatzfähigkeit einer neuen Methode, der isothermalen Mikrokalorimetrie (IMC), zum *in vitro* Screening von Arzneimittelwirkungen gegen *S.mansoni*.
Bis heute ist die mikroskopische Beobachtung der "Goldstandard" in dieser Art der Wirkstoffforschung. Sie ist einfach zu handhaben und in jedem Labor durchführbar, allerdings schwierig zu standardisieren und immer von der subjektiven Bewertung des Beobachters abhängig. Wir untersuchten *in vitro* die Wirkungen von drei bekannten antischistosomalen Stoffen (Praziquantel, Oxamniquin und Artesunat) und Mefloquin gegen *S. mansoni* mit IMC und verglichen die Ergebnisse mit denen der mikroskopischen Untersuchungen. Beide Methoden zeigten eine gute Korrelation bei den Ergebnissen, jedoch lassen sich mit IMC Wirkungen nachweisen, die mit der mikroskopischen Auswertung entweder gar nicht oder erst viel später nachweisbar waren. Des Weiteren liess sich der Zeitpunkt des Wirkeintritts genauer feststellen.

Ausserdem verglichen wir Arzneimittelwirkungen auf NTS und adulte Schistosomen, weil NTS zunehmend in Screening-Assays zur Suche nach antischistosomalen Wirkstoffen verwendet werden.

Insgesamt konnte ich mit meiner Arbeit zeigen, dass Mefloquin ausgezeichnete antischistosomale Eigenschaften besitzt und ein interessanter Wirkstoff in einer Kombinations- Chemotherapie der Bilharziose sein könnte. Ausserdem haben wir gezeigt, dass IMC eine nützliche Methode in der antischistosomalen Wirkstoffforschung ist und daher auch bei der Untersuchung von weiteren Wirkstoffen gegen andere Wurmarten eingesetzt werden könnte. Zusätzlich unterstützen unsere Untersuchungsergebnisse die Verwendung von NTS in *in vitro* Assays in der antischistosomalen Wirkstoffforschung, weil sowohl NTS als auch adulte Schistosomen ähnliche Empfindlichkeiten gegen Testsubstanzen zeigten und erstere einfacher zu gewinnen sind.

1. Introduction

Schistosomiasis is a chronic helminthic disease and belongs to the so-called neglected tropical diseases (NTDs). The causative agents are parasitic fluke worms of the genus *Schistosoma spp*. In this introduction first, a brief overview of the umbrella term NTDs is given (Section 1.1). In Section 1.2 a summary of the disease schistosomiasis, including the taxonomy, the morphology, the life cycle, the global burden and the pathology is described. The current global strategy for schistosomiasis treatment and control is outlined in Section 1.3. Section 1.4 highlights research needs and Section 1.5 provides a background on the antischistosomal properties of mefloquine.

1.1. Neglected Tropical Diseases (NTDs)

Overall, there is no standard global definition for the term neglected tropical diseases (NTDs). The journal PLoS Neglected Tropical Diseases defines it as a group of poverty-promoting chronic infectious diseases, which primarily occur in rural areas and poor urban areas of low-income and middle-income countries. They are poverty-promoting because of their impact on child health and development, pregnancy, and worker productivity, as well as their stigmatizing features (http://www.plosntds.org/static/scope.action). Many parasitic and bacterial infections are included in the list of NTDs, within approximately 37 major diseases. One of these diseases is schistosomiasis, which is a major public health problem in sub-Saharan Africa.

Collectively, the NTDs represent one of the most serious burden to public health among the most deprived communities (Liese et al., 2010) estimated global burden due to the NTDs is > 50 million disability-adjusted life years (DALYs), which represent the fourth most important group of communicable diseases worldwide, behind lower respiratory infections, HIV/AIDS and diarrhoeal diseases (Hotez et al., 2006a). However, in the global health policy NTDs have been largely ignored; for example, only 0.6% of funding of official development assistance was spent on NTDs control projects during the last couple of years (Liese and Schubert, 2009).

1.2. Schistosomiasis

Taxonomy

Schistosomiasis is a chronic parasitic inflammatory disease caused by fluke worms of the genus *Schistosoma*. In Figure 1 the basic taxonomy of these parasites is shown. Overall, there are five schistosome species parasitizing humans, with *Schistosoma haematobium, S. mansoni* and *S. japonicum* being the three main species with the widest geographical distribution (Utzinger and Keiser, 2004, Gryseels et al., 2006)

Figure 1. Taxonomy of *Schistosoma spp.*

Morphology

Schistosomes have separate sexes and live as permanently embraced couples, with the thinner female in a slit in the male's body, in mesenteric veins (*S. intercalatum, S. japonicum, S. mansoni and S. mekongi*) or the vesical plexus and veins that drain the urether (*S. haematobium*). The name *schistosoma* is derived from Greek and is based on the special morphology of the male's body: *schistos* means "split", *soma* means "body".

Overall, worms vary in morphology, egg shape and size according to species and sex. For example, worm size range between 7-20 x 0.25-1 mm. However, one morphological characteristic, which all schistosomes (and digeneans in general) have in common, is the dorso-ventral flattening, bilaterally symmetrical body. Additionally, all species have an oral and ventral sucker and no circulatory system. An overview of the morphology of schistosomes is shown in Figure 2.

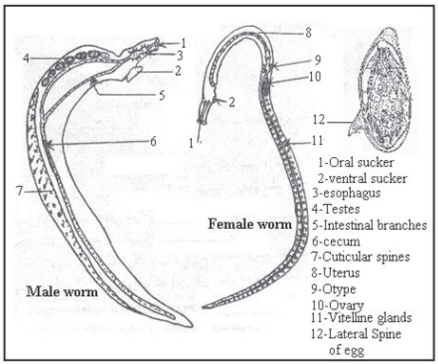

Figure 2. Schematic illustration of a male and female adult *S. mansoni* and a *S. mansoni* egg (source :http://course1.winona.edu/kbates/Parasitology/Images/Schistosomemorph.png)

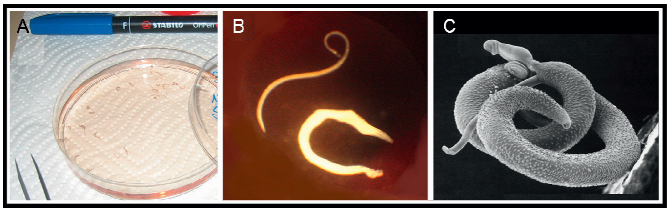

Figure 3. *S. mansoni* A) schistosomes in culture media, freshly removed from infected mice B) Light microscope picture of a female and male worm C) SEM picture of a *S. mansoni* couple

Life cycle

Schistosomiasis is transmitted through contact with infested fresh water, containing the infectious stage of the parasites, the cercariae, which were released by aquatic snails carrying the parasite. Playing activities such as swimming and bathing, occupational activities like fishing and irrigated rice farming as well as daily household activities can put people at risk of acquiring schistosomiasis (Keiser and Utzinger, 2011) (http://globalnetwork.org/files/press_releases/schistosomiasis.pdf). For a better understanding, the life cycle of schistosomiasis is illustrated in Figure 3. Briefly, people become infected when they come in contact with water containing cercariae which penetrate human skin. In this process cercariae lose their tails, develop to larval stages (schistosomula) and finally enter the blood vessels via the lymphatics. Schistosomula develop into adult worms, mate and female schistosomes begin laying eggs. The number of eggs passed daily into the venules depend upon the species of worm, which ranges between 300 (*S. mansoni*) to 3500 (*S. japonicum*) (Neva and Brown). Some of the eggs are trapped in the tissues of the infected host, and some of the eggs are excreted with the faeces (*S. intercalatum, S. japonicum, S. mansoni and S. mekongi*) or the urine (*S. haematobium and S. japonicum*). Once eggs reach a freshwater body, they develop, release miracidiae, which then search for the intermediate host, the aquatic snails. Within the snail, asexual multiplication takes place and resulting cercariae are released from infected snails into the water.

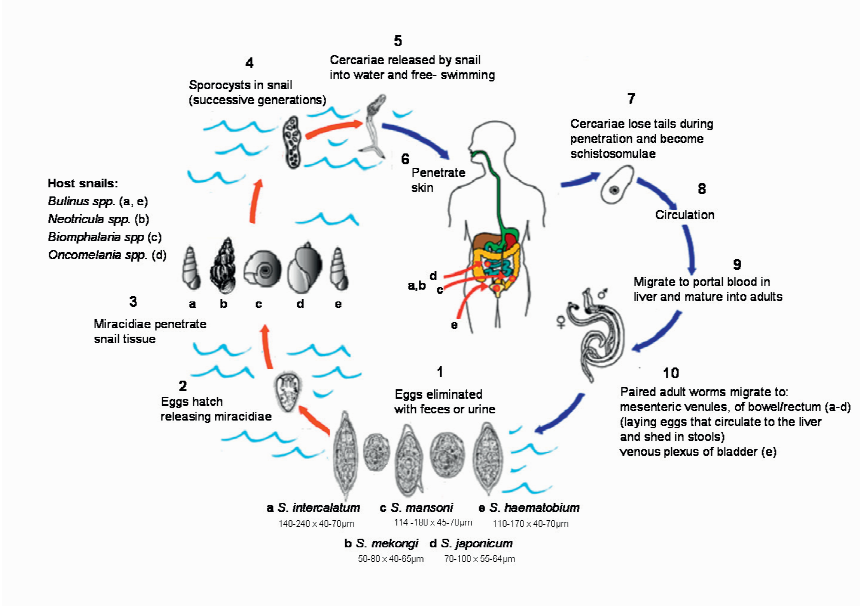

Figure 4. Life cycle of schistosomiasis (modified, according to CDC, Centers for disease control and prevention, http://www.cdc.gov/parasites/schistosomiasis/biology.html)

Global burden of schistosomiasis

Schistosomiasis is a major public health problem and endemic in approximately 78 countries of the world. *S. haematobium, S. mansoni* and *S. japonicum* have the widest geographical distributions and are of particular public health and economic significance (Steinmann et al., 2006). The global distribution of the disease is shown in Figure 4. It has been estimated that more than 207 million people worldwide are infected with schistosomiasis and 779 million people are at risk of contracting the disease (Steinmann et al., 2006). The highest infection prevalence is found in school-aged children (SAC) and young adults (Neva and Brown). The annual mortality rate due to schistosomiasis in sub-Saharan Africa might be as high as 280,000 (van der Werf et al., 2003). Estimates of the global burden of schistosomiasis range from 1.7 to 4.5 million disability adjusted life years (DALYs) (Hotez and Fenwick, 2009) through direct pathologies associated with the disease and consequences including impaired child growth, malnutrition and anaemia. But since the latter figure does not consider chronic disability (Hotez et al., 2006b), one can speculate that the number of DALYs is likely to be underestimated. Another calculation comes to the conclusion that estimates for schistosomiasis are at 3-70 million DALYs

(King and Dangerfield-Cha, 2008). Overall, the DALYs represent a summary measure of both mortality and disability to quantify and compare the health of populations (Mathers et al., 2007).

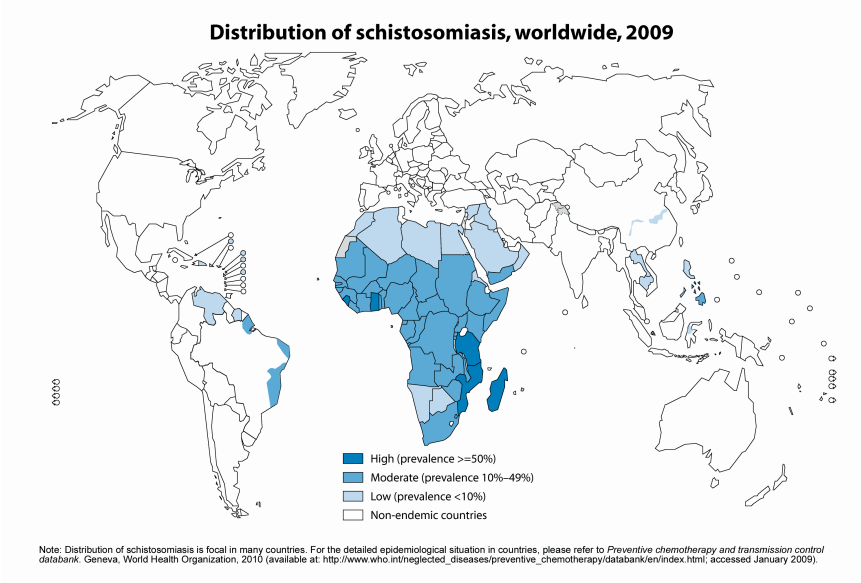

Figure 5. Global distribution of schistosomiasis 2009 (source: WHO (WHO, 2011))

Pathology

Pathology of schistosomiasis can be divided into two stages, acute and clinical pathology. Acute pathology develops following cercarial penetration of human skin and the subsequent maturation to adult worms. The clinical manifestations produced by the three main schistosome species are similar: due to penetration of cercariae a temporary urticarial rash or cercarial dermatitis might occur. Acute schistosomiasis (Katayama fever) is a systemic hypersensitivity reaction caused by the migrating schistosomula, which might result in fever, fatigue, myalgia and later abdominal symptoms (Gryseels et al., 2006, Neva and Brown). The second stage is the chronic pathology and morbidity, which depend upon the parasite species and is due to the parasite eggs. Systemic signs might occur in the period of active oviposition and egg excretion by the schistosomes. Further established and chronic infections develop when inflammatory and granulomatous reactions due to proteolytic enzymes secreted by the eggs appear (Neva and Brown). Clinical manifestations caused by the three main schistosome species and their global distribution are summarized table 1.

	Distribution	Affected organs	Symptoms
S. haematobium	Sub-Saharan Africa, Middle East, some islands in the Indian Ocean	Bladder, kidneys, vesical and urethral walls	Haematuria, ulcera, fibrosis, calcification ,bladder stones, carcinoma, bladder cancer, hydrourether, nephrosis, kidney failure,
S. mansoni	Sub-Saharan Africa, parts of South America, some Caribbean islands	Large bowel, rectum, liver, portal vein, mesenteric veins	Abdominal pain, discomfort, bloody diarrhoea, ulcera, hepatomegaly, fibrosis, portal hypertension, GI-varices, ascites
S. japonicum	China, the Philippines, and Indonesia	Circulation	Sudden and massive bleeding, abdominal pain, abscesses, bloody diarrhoea, ulcera, hepatomegaly, fibrosis, portal hypertension

Table1. Clinical manifestations caused by the three main schistosome species and their global distribution (source: (Gryseels et al., 2006))

1.3. Global strategy for schistosomiasis treatment and control

The WHO recommended intervention strategy for control of schistosomiasis is morbidity control through regular and targeted large scale preventive chemotherapy (WHO, 2006). People living in endemic areas or people defined as being at high risk are therefore treated without a previous individual diagnosis. Population considered to be at high risk are one the one hand school-age children and one the other hand adults from special groups, such as pregnant and lactating women and people who have regular contact with infested water, such as fishermen, farmers or women doing their daily laundry. The frequency of the treatment varies according to the risk of schistosomiasis. In high-risk communities treatment should take place once a year, in moderate-risk communities once every 2 years and in low-risk communities children will be treated twice during primary school (WHO, 2006) Chemotherapy is carried out with the drug praziquantel. The WHO recommended use is a single 40 mg/kg oral dose of praziquantel (marketed as 600 mg tablets). When high initial egg counts are recorded and therefore a risk for rapid re-infection exists, higher doses of 60 mg/kg (or even higher, maybe dose splitting) are recommended (WHO, 2006). Praziquantel has a broad spectrum of activity, because it is active against the adult stages of all five human schistosome species. Additionally, praziquantel is efficacious against soil-transmitted helminthiasis (some liver flukes, e.g. *Clonorchis sinensis* and *Opisthorchis viverrini* and lung flukes, e.g. *Paragonimus westermani*) (Cioli and Pica-Mattoccia, 2003). The drug acts within 1 h of ingestion by paralysing the worms and

damaging the tegument (Andrews, 1985). However, the drug has only little or no effect on eggs and immature worms. Hence re-treatment is necessary to kill those parasites that have since matured. The preferred time for follow up treatment is 4-6 weeks (Gryseels et al., 2006). Overall, treatment is regarded as safe and generally well-tolerated, and can be used in pregnant woman and in young children (note that no safety information is available for children under four years of age) (WHO, 2002). Only light and temporary side effects, including weakness, abdominal pain, dizziness, headache and nausea might occur (reported occurrence ≥ 10%) (Utzinger and Keiser, 2004). However, in heavy infections acute colic with bloody diarrhoea provoked by massive worm shifts and antigen release can occur (Stelma et al., 1995).

However, to date, praziquantel is the only drug available for treatment and control of schistosomiasis (Doenhoff et al., 2009, Utzinger and Keiser, 2004). Praziquantel originates from a spate of isoquinoline-pyrazino derivatives, which were synthesised as potential tranquilizer in the early 1970s (Bayer Schering Pharma and Merck KGaA, Germany) (Hotez et al., 2010). Further laboratory investigations with praziquantel in 1976/1977 showed its antihelminthic activity and the first clinical trials in humans were carried out in 1978/1979 (Utzinger and Keiser, 2004). There are presently no real alternatives to praziquantel, since metrifonate (against *S. haematobium*) has been withdrawn from the market and oxamniquine is only active against *S. mansoni* and difficult to obtain (Doenhoff et al., 2002, Utzinger and Keiser, 2004).

1.4. The need for novel antischistosomal drugs

Although praziquantel is a very efficacious and safe antischistosomal drug (as described in section 1.3), there are five reasons justifying the need to discover and develop new drugs against schistosomiasis (Caffrey, 2007, Ribeiro-dos-Santos et al., 2006): First, the global strategy for schistosomiasis control is based on morbidity control, but to date only one single drug, namely praziquantel, is deployable for antischistosomal chemotherapy (Keiser and Utzinger, 2007). The dependence on one drug for an important helminthic infection that affects approximately 207 million people in the poorest areas of the world is of great concern (Caffrey and Steverding, 2008). Second, the massive use of praziquantel in mass drug administration programs recommended by the World Health Organisation (Doenhoff et al., 2009) is a considerable risk factor for the development of resistant schistosome strains to praziquantel. In fact, resistance to praziquantel in *S. mansoni* infected mice was already demonstrated in laboratory studies in 1994 (Doenhoff et al., 2002) (Melman et al., 2009). Furthermore, in field studies conducted in Senegal and Egypt and

published in 2001 and 2005, respectively, treatment failures potentially explainable through praziquantel-tolerant schistosomes strains were observed: In the first case, treatment of *S. mansoni* infected patients achieved low cure rates, ranging from 18-39%. In the second study, patients repeatedly treated with praziquantel continued excreting viable parasite eggs (Utzinger and Keiser, 2004). Third, there are currently no vaccines available to prevent an infection with schistosomiasis, even if some potential vaccine candidates were identified (recombinant rShGST-28, phase I and II of clinical trials) it will take a long time before these vaccines will be commercially available (Bergquist and Colley, 1998, Zhang et al, Capron et al., 2005). Fourth, due to the narrow spectrum of activity of praziquantel a second dose of praziquantel has to be administered after a short interval, to eliminate parasites that have since matured. Observed treatment failures could possibly also be explained by this. The last reason, which justifies the development of a new antischistosomal drug is, that once an alternative drug is found (especially with another mechanism of action), the new drug could not only be used alone, but also in combination with praziquantel. Combination chemotherapies are recognised to be a viable therapeutic strategy to delay the development of drug resistance, because different drug targets are affected, thus increasing the life time of each individual drug. Additionally, combination chemotherapy ideally exceeds the individual effect of each drug alone and is often better tolerated (Utzinger et al., 2003).

1.5. The antischistosomal properties of the antimalarial mefloquine

In 1973 the WHO listed a group of new experimental antimalarial compounds, which were to be further tested in order to develop a new drug against chloroquine-resistant malaria (Sweeney, 1981, Trenholme et al., 1975). All compounds were developed by the U.S. Army at the Walter Reed Army Institute of Research. Out of the compounds assessed, mefloquine, a 4-quinoline-methanol and a synthetic analogue of quinine, was efficacious against drug-susceptible and multi drug resistant *P. falciparum* (Sweeney, 1981, Trenholme et al., 1975). In 1989 mefloquine was approved by the American Food and Drug Administration (FDA) for the prophylaxis and treatment of malaria (www.FDA.gov). Mefloquine is effective against all forms of malaria and is currently used either in combination with artesunate (as one possibility of the WHO recommended artemisinin- based combination therapy (ACT)) or as monotherapy for the treatment of malaria (WHO, 2010).

Now, around 20 years after its discovery, mefloquine has received renewed attention: In recently conducted *in vivo* studies in mice mefloquine showed interesting antischistosomal activities (Keiser et al., 2009). Single oral doses of 200 or 400 mg/kg of the antimalarial administered to mice infected with adult stages of *S. mansoni* and *S. japonicum* resulted in high or complete total and female worm burden reduction of 72.3-100%. High worm burden reductions were also achieved in mice infected with the juvenile form of both parasites

species (Keiser et al., 2009). Van Nassauw and colleagues showed that a treatment with a lower dose of mefloquine (150 mg/kg) revealed a significantly reduced egg production in *S. mansoni*-infected mice (Van Nassauw et al., 2008). *In vitro* studies with juvenile and adult *S. japonicum* and histopathological investigations of livers from *S. japonicum* infected mice confirmed the antischistosomal activities exhibit by mefloquine against both stages of *S. japonicum* (Xiao and Zhang, 2009, Xiao et al., 2009, Zhang et al., 2009)
Another *in vivo* study carried out with mefloquine revealed, that even in athymic and immunocompetent *S. mansoni* infected mice mefloquine has antischistosomal activities. An oral dose of 200 mg/kg resulted in total and female worm burden reductions of 80.4-87.3% against juvenile and adult worms in a mutant mouse strain with T-cell deprived mice (Keiser et al., 2010b).
To evaluate the effect of mefloquine against *Schistosoma* infections in humans a randomized, exploratory open-label trial was conducted in Cote d´Ivoire: the efficacy of mefloquine, artesunate and the combination mefloquine/artesunate (administered according to malaria treatment schemes) were investigated in *S. haematobium* infected school children (Keiser et al., 2010a). Mefloquine/artesunate combination chemotherapy reached high cure rates of 61% (11 out of 18 children) and egg-reduction rates of > 95% against *S. haematobium* infections. Praziquantel, the current treatment of choice, achieved cure and egg reduction rates of 88% and > 95%, respectively.

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2. Study aim, objectives and approaches

The overall aim of my Ph.D. thesis was to further assess the newly discovered antischistosomal properties of the antimalarial drug mefloquine and to get a first insight into the mode of action of mefloquine in schistosomes. This will be achieved on the one hand through drug efficacy and morphological studies with mefloquine and the isomeric and racemic forms of mefloquine, and on the other hand with combination chemotherapy studies. Additionally, drug target discovery by the means of affinity chromatography will be carried out. In addition, to strengthen the field of drug discovery, I assessed the use of isothermal microcalorimetry for antischistosomal drug screening by using schistosomula and adult worms.

Objective 1

To study morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult flukes of *S. mansoni*

Approach:

Dose-response relationships and temporal drug effects will be studied *in vitro* and *in vivo*. To examine drug effects in greater detail, the tegumental surface of parasites will be examined by the means of scanning electron microscopy (SEM). SEM studies will be done in collaboration with the Centre for Microscopy at the University of Basel, Switzerland.

***In vitro* studies:** Schistosomula (1-7 day-old schistosomes, newly transformed schistosomula (NTS)) and adult *S. mansoni* (49- day old schistosomes*)* will be incubated with different concentrations of mefloquine (100-1 µg/ml). NTS will be artificially produced by mechanical transformation in the laboratory. NTS are model parasites, whose use in antischistosomal drug screening assays is in line with the requirements of the 3R protection principles to reduce, refine and replace animal experiments, since NTS are produced without the need of any mice. Worms will be assessed microscopically (at least every 24 h) up to four days. Drug effects will be assessed with an emphasis on changes in worm motor activity and morphological alterations. Afterwards worms will be prepared for SEM examinations and assessed.

***In vivo* studies:** Laboratory mice will be infected with *S. mansoni* cercariae. Once worms have matured, mice will be treated orally with mefloquine (400 mg/kg). 24, 48, 72 and 96 h post treatment schistosomes will be recovered from infected mice and distribution of worms in the mesenteric veins and the liver will be recorded. Afterwards worms will be analyzed by SEM.

Objective 2

To compare the antischistosomal properties of the four mefloquine isomers and two racemates *in vitro* and *in vivo*

Approach:

To assess the antischistosomal properties of the four optical isomers and two racemates of the chiral drug mefloquine on *S. mansoni* NTS and adult *S. mansoni in vitro* and *in vivo* Additionally, we will assess, whether the antischistosomal efficacy of mefloquine depends on hemin.

***In vitro* studies**: The effect of the six different compounds on schistosomes will be analyzed microscopically and by the means of isothermal microcalorimetry (IMC). Schistosomes will be incubated in the presence and absence of hemin.

***In vivo* studies**: The effect of single oral doses of 200 mg/kg of the drugs will be assessed on adult *S. mansoni* harbored in mice.

Objective 3

To evaluate isothermal microcalorimetry (IMC) for antischistosomal drug screening

Novel tools should facilitate the readout of *in vitro* antischistosomal drug screening. IMC is a highly sensitive and accurate tool to detect heat produced by microorganisms. We will examine whether IMC is applicable to analyze antischistosomal activities of test drugs.

Approach:

We will study *in vitro* drug effects of praziquantel, oxamniquine, artesunate and mefloquine on the metabolic activity and the motility of *S. mansoni* NTS and adult worms by using IMC. Microcalorimetric results will be compared to results obtained by microscopic readouts of viability, the standard method for compound screening against schistosomiasis.

Objective 4

To assess the applicability of NTS for antischistosomal drug screening assays

Approach

The same experimental setting as explained in Objective 3 will be used to assess the applicability of *S. mansoni* NTS for antischistosomal drug screening assays. Both, the schistosomular stages as well as the adult stages of *S. mansoni* will be incubated with the test drugs and phenotypes of parasites will be assessed microscopically and by microcalorimetric measurements. Results of drug effects on NTS and the adult worms will be compared.

Objective 5

To investigate the antischistosomal properties of mefloquine/praziquantel combinations *in vitro* and in the mouse model

Approach:

Dose response relationships will be discovered following exposure of adult *S. mansoni* to mefloquine, praziquantel and fixed dose combinations of mefloquine/praziquantel *in vitro*. *S. mansoni*-infected mice will be treated orally with selected doses of single drugs and drug combinations 7 weeks post-infection.

Objective 6

To find a possible drug target of mefloquine in schistosomes

Approach:

In order to identify mefloquine binding proteins in extracts of *S. mansoni* schistosomula, affinity chromatography experiments will be performed using mefloquine-agarose. Since the mechanism of action and the molecular targets for mefloquine in schistosomes are not known and due to the fact that schistosomula are not blood feeders, this parasites stage allows identifying possible mefloquine targets besides heme degradation

3. Morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult flukes of *Schistosoma mansoni*

T. MANNECK, Y. HAGGENMÜLLER and J. KEISER

Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, CH-4002 Basel, Switzerland

Morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult flukes of *Schistosoma mansoni*

T. MANNECK, Y. HAGGENMÜLLER *and* J. KEISER*

Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, CH-4002 Basel, Switzerland

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SUMMARY

There is a pressing need to develop novel anti-schistosomal drugs, as current treatment relies largely on praziquantel (PZQ). To further strengthen current evidence of the anti-schistosomal properties of mefloquine (MQ), we studied the temporal effect of this compound *in vitro* and *in vivo*, and examined alterations on the tegumental surface of schistosomula and adults of *S. mansoni* by means of scanning electron microscopy (SEM). Schistosomula and adults were each incubated *in vitro* using MQ over a wide concentration range (1–100 μg/ml). In addition, mice infected with adult *S. mansoni* were treated with a single oral dose of 400 mg/kg MQ, and worms were recovered 24, 48, 72, 96 and 120 h following treatment. MQ showed a rapid onset of action on schistosomula *in vitro*; 100 and 75 μg/ml of MQ killed schistosomula immediately; the minimal lethal and effective concentrations of MQ on schistosomula after 1 h were 25 and 5 μg/ml, respectively. Adult worms incubated with 100 and 10 μg/ml of MQ were dead after 1 h and 24 h of incubation, respectively. A hepatic shift of adult schistosomes was observed in mice already 24 h after treatment, and 120 h following treatment >98% of all worms had translocated to the liver. SEM observations revealed extensive tegumental destruction, including blebbing, shrinking and sloughing, particularly following *in vitro* incubation and on the tegument of female worms.

Key words: *Schistosoma mansoni*, schistosomiasis, mefloquine, *in vitro*, *in vivo*, scanning electron microscopy.

INTRODUCTION

Schistosomiasis is a neglected tropical disease (NTD) caused by blood flukes of the genus *Schistosoma*. *Schistosoma haematobium*, *S. japonicum* and *S. mansoni* are the 3 main species parasitizing humans (Utzinger and Keiser, 2004; Gryseels *et al.* 2006). It has been estimated that there are more than 207 million people infected worldwide, with 779 million people at risk of infection (Steinmann *et al.* 2006). The annual mortality rate in sub-Saharan Africa is estimated to be as high as 280 000 (van der Werf *et al.* 2003). Estimates of the global burden of schistosomiasis range from 1·7 to 4·5 million disability-adjusted life years (DALYs) (WHO, 2002, 2004); the latter figure is likely to underestimate the true burden, as it does not consider chronic disability (Hotez *et al.* 2006). Revised estimates for schistosomiasis are at 3–70 million DALYs (King and Dangerfield-Cha, 2008).

Despite schistosomiasis being one of the most prevalent and debilitating NTDs, a devoted drug discovery and development programme does not exist (Ridley and Kita, 2007). Since the late 1970s, the treatment and control of schistosomiasis relies on a single drug, namely praziquantel (PZQ) (Gönnert and Andrews, 1977; Cioli and Pica-Mattoccia, 2003; Utzinger and Keiser, 2004; Doenhoff *et al.* 2008). However, the heavy reliance and application of PZQ within the frame of large-scale preventive chemotherapy treatment programmes might select for drug-resistant parasites (Botros and Bennett, 2007; Caffrey, 2007). In addition, PZQ has only moderate activity against juvenile worms, which is an important deficiency in its spectrum of activity, as it requires administration of a second PZQ dose after a short interval to eliminate the parasites that have since matured (Doenhoff *et al.* 2008).

Nonetheless, in spite of the lack of dedicated product development partnerships for helminth diseases, a number of compounds with interesting anti-schistosomal properties have been identified by various research groups in recent years; for example, peroxidic compounds (the semi-synthetic artemisinins and the synthetic trioxolanes (OZs)), 4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide and the cysteine protease inhibitor K11777 (Abdulla *et al.* 2007; Keiser and Utzinger, 2007; Utzinger *et al.* 2007; Xiao *et al.* 2007; Sayed *et al.* 2008; Caffrey *et al.* 2009). An important finding was that mefloquine (MQ), an arylamino alcohol (4-quinoline-methanol), developed by the Walter Reed Institute of Research

* Corresponding author: Tel: +41 61 284 8218. Fax: +41 61 284 8105. E-mail: jennifer.keiser@unibas.ch

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in the early 1970s (Sweeney, 1981) and now marketed for the prophylaxis and treatment of malaria, showed promising anti-schistosomal activity in mice. Single oral doses of 200–400 mg/kg to mice infected with either juvenile or adult stages of *S. mansoni* or *S. japonicum* resulted in high total or even complete reductions in female worm burdens (Keiser *et al.* 2009). Treatment with a lower dose of 150 mg/kg MQ revealed a significantly reduced egg production in *S. mansoni*-infected mice (Van Nassauw *et al.* 2008).

The aim of the present study was to further strengthen the current evidence base of the anti-schistosomal properties of MQ and to gain a first insight into the potential mode of action of the drug. The dose-response relationships were studied *in vitro*. The temporal drug effect was monitored *in vitro* and *in vivo*. In addition, alterations in the tegumental surface of schistosomula and adult flukes were studied by means of scanning electron microscopy (SEM).

MATERIALS AND METHODS

Experimental animals and parasites

All experiments were carried out at the Swiss Tropical Institute (Basel, Switzerland), in accordance with Swiss national animal welfare regulations. Female NMRI mice ($n=40$, age = 3 weeks, weight = 25 g) were purchased from Harlan (Horst, The Netherlands).

Cercariae of *S. mansoni* (Liberian strain) were obtained from infected intermediate host snails (*Biomphalaria glabrata*) maintained according to standard procedures (Keiser *et al.* 2009).

Drugs

Mefloquine ((*RS*)-(±)-α-(*2-Piperidinyl*)-*2,8-bis*(*trifluormethyl*)-*4-quinolinemethanol hydrochloride*, MW = 414.81 g/mol) (MQ) was kindly provided by Mepha AG (Aesch, Switzerland). For *in vitro* studies, stock solutions of MQ (10 mg/ml) were prepared in dimethyl sulfoxide (DMSO) (Fluka, Buchs, Switzerland). For *in vivo* studies, MQ (40 mg/ml free base) was suspended in 7% (v/v) Tween 80 and 3% ethanol shortly before oral administration to mice.

In vitro *studies on schistosomula*

Cercariae of *S. mansoni* were mechanically transformed into schistosomula (Ramalho-Pinto *et al.* 1974). Briefly, 50 ml of an ice-cold cercarial suspension were centrifuged and the packed volume of cercariae was re-suspended in 2 ml of Minimal Essential Medium (MEM) (Eagle, 1959) and vortexed for 2 min to trigger tail loss. For the isolation of cercarial bodies, cold Hank's Basal Salt Solution (HBSS) was added to the cercarial suspension up to a volume of 7 ml. After cooling on ice for 15 min, the tail-rich supernatant was decanted and the sedimented bodies were re-suspended in 7 ml of cold HBSS. This procedure was repeated twice. The schistosomula suspension was kept in Basch culture medium (Basch, 1981) in an incubator at 37 °C in an atmosphere of air, (95%) + CO_2 (5%) for 1–3 h until use.

To study the temporal drug effect and the concentration-response relationships, schistosomula were incubated in 24-well plates (Costar), with 500–1000 schistosomula per well, in the presence of different concentrations of MQ. Each well contained 1 ml of Basch medium supplemented with 5% heat-inactivated foetal calf serum (iFCS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, USA) and MQ at 100, 75, 50, 25, 15, 10, 5 or 1 μg/ml, respectively. Schistosomula incubated with 1% DMSO, the highest concentration of drug solvent, served as controls. The parasites were kept in an incubator at 37 °C in an atmosphere of air, (95%) + CO_2 (5%) for up to 96 h.

Immediately after adding (0·125), 0·5, 1, 6, 17, 24, 48, 72 and 96 h after incubation with MQ, all schistosomula were observed under a dissecting microscope and the effect of MQ was assessed, with an emphasis on changes in worm motor activity, morphological/tegumental alterations and the occurrence of death. These phenotypic changes were scored using a viability scale of 0–3: (3 = totally vital, normally active, no morphological changes, 2 = slowed activity, first morphological changes and granularity visible, 1 = minimal activity, severe morphological changes and granularity, 0 = all worms dead, severe granularity) based on standard procedures for compound screening at WHO-TDR (Ramirez *et al.* 2007) and the UCSF Sandler Center (Caffrey *et al.* 2009). The minimal lethal concentration (MLC), which is the minimum concentration needed to kill all schistosomula and the minimal effective concentration (MEC), which is the minimum concentration needed to observe any change in viability or morphology of schistosomula were determined after 1 h. All experiments were carried out in duplicate and were repeated at least 3 times.

For SEM studies, samples of schistosomula incubated with 3 different concentrations of MQ (100, 10 and 1 μg/ml) were taken at different time-points (after 0·5, 1, 6, 24, 72 and 96 h). Specimens were fixed with 2·5% (v/v) glutaraldehyde in a phosphate-buffered saline (PBS) (pH 7·4) for 24 h at 22–24 °C (room temperature). After rinsing 3 times with PBS, the specimens were stored in the same buffer at 4 °C until use. Before SEM examination, the flukes were washed twice with double-distilled water, dehydrated in ascending ethanol concentrations and then air-dried. Schistosomula were placed on aluminium stubs, sputter coated with 20 nm gold particles and

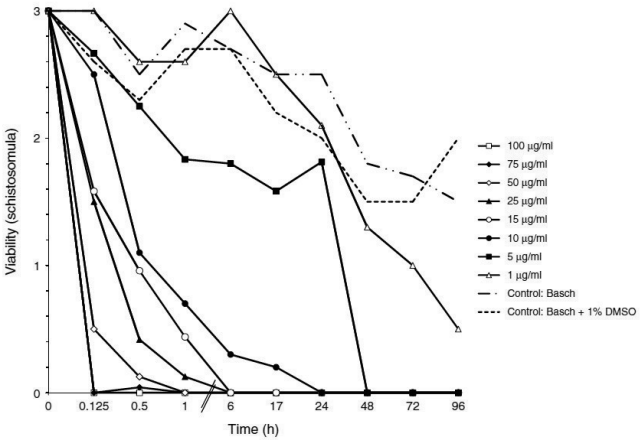

Fig. 1. Effect of different concentrations of MQ on the viability of *Schistosoma mansoni* schistosomula. Mean values of viability using a viability score. Numbers derived from a minimum of 6 experiments (500–1000 schistosomula per experiment were scored at each concentration). Open squares: 100 µg/ml; filled diamonds: 75 µg/ml; open diamonds: 50 µg/ml; filled triangles: 25 µg/ml; open circles: 15 µg/ml; filled circles: 10 µg/ml; filled squares: 5 µg/ml; open triangles: 1 µg/ml; dashed and dotted line: Control (Basch medium); dashed line: Control (Basch medium + 1% DMSO).

observed in a high-resolution SEM (Phillips XL30 ESEM) at an accelerating voltage of 5 kV.

In vitro *studies of adult schistosomes*

NMRI mice were infected subcutaneously with ~80 cercariae of *S. mansoni* and euthanized with CO_2 after 56 days. All adult schistosomes were collected from the hepatic portal system and mesenteric veins and removed from the liver by perfusion with ice-cold PBS supplemented with heparin (Yolles *et al.* 1947; Smithers and Terry, 1965). Schistosomes were incubated in 24-well plates (Costar), placing 2 male and 2 female worms in each well. Individual wells contained RPMI 1640 culture medium (Invitrogen, Carlsbad, USA) supplemented with 5% heat-inactivated calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, USA). Schistosomes were incubated in the presence of 100, 10 and 1 µg/ml MQ at 37 °C in an atmosphere of air (95%) + CO_2 (5%). Medium without and medium with 1% DMSO (the highest concentration of drug solvent used) served as controls. Before incubation with MQ and immediately after incubation (0·125), 1, 6, 24, 48 and 72 h with MQ, all *S. mansoni* were observed under a dissecting microscope. The effect of the drug was assessed with an emphasis on changes in worm motor activity, morphological/tegumental changes and death of worms. Death was defined as no movement observed for at least 2 min of examination and no movement at the other observation time-points. All experiments were carried out in duplicate and were repeated at least 3 times. For SEM examination, the worms were prepared as described above, with the exception that the worms were critical-point dried (Bomar SPC-900; Tacoma USA).

In vivo *studies*

NMRI mice were infected as described above. At 7–8 weeks after infection, mice were treated with a single oral dose of 400 mg/kg MQ, the most effective dose as established in our previous work (Keiser *et al.* 2009). At each time-point after treatment (24, 48, 72, 96 and 120 h), 3–6 mice were killed by cervical dislocation and the mesentery and the liver were collected. Schistosomes were removed from the mesenteric veins, sexed and counted using a binocular microscope. The liver was flattened and examined for the presence of worms. The distribution of schistosomes in the liver and mesenteric veins was recorded. Schistosomes recovered from the mesenteric veins were further examined, and changes in worm motor activity, morphological/tegumental changes and death were documented. Worms were then prepared for SEM examination.

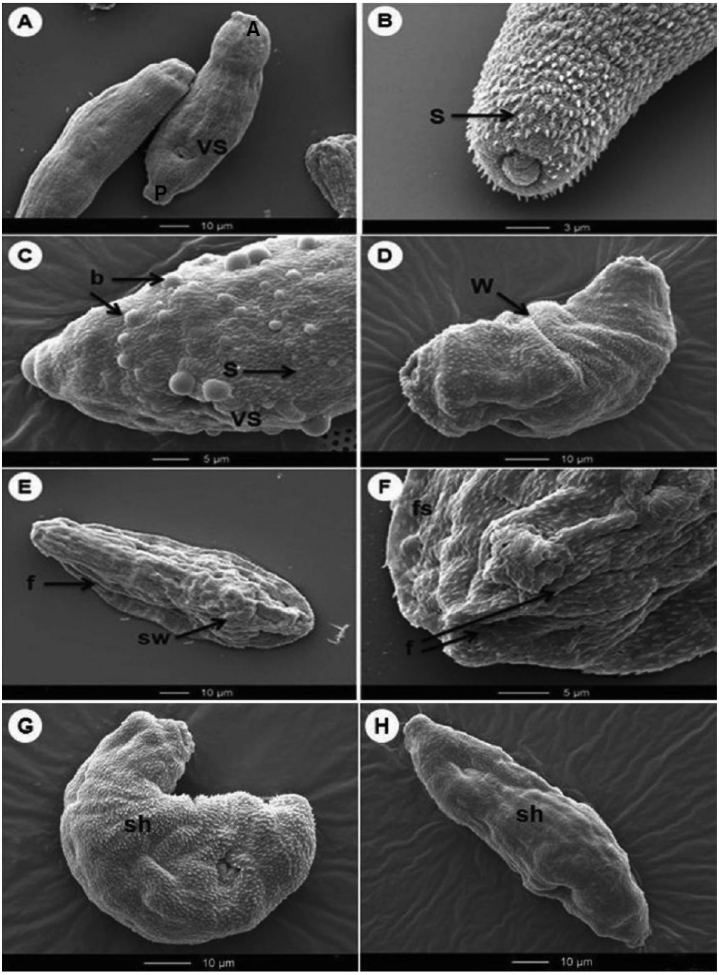

Fig. 2. SEM observations of *Schistosoma mansoni* schistosomula after *in vitro* incubation. (A) Control, showing the ventral sucker (VS) (=acetabulum), the anterior end (A) and the posterior end (P). (B) Control. Posterior region with clearly defined tegumental spines (S). (C) Thirty min after incubation with MQ (100 µg/ml). Shortened spines (S) and blebbing (b) near the ventral sucker (VS) visible. (D) Thirty min after incubation with MQ (10 µg/ml). Contraction and formation of wrinkles (W) visible. (E) Six h after incubation with MQ (10 µg/ml). A shrunken body with swelling (sw) and many furrows (f) observed. (F) Six h after incubation with MQ (10 µg/ml). The tegument shows deep furrows (f) and flattened spines (fs). (G) Twenty-four h after incubation with of MQ (1 µg/ml). Shrunken (sh) tegument and bending of the body. (H) Seventy-two h after incubation with MQ (1 µg/ml). A shrunken (sh) tegument visible.

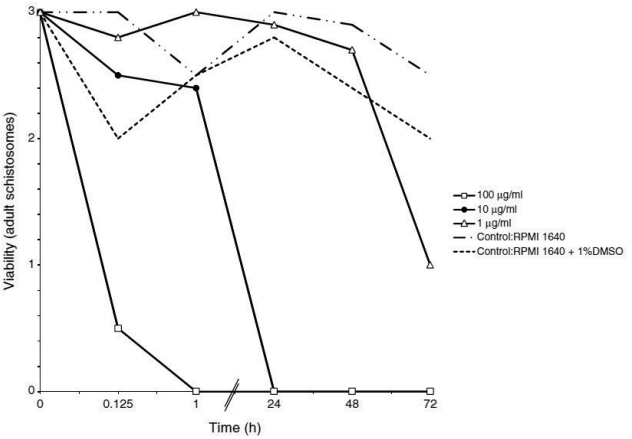

Fig. 3. Effect of different concentrations of MQ on the viability of adult worms of *Schistosoma mansoni*. Mean values of viability using a viability score. Numbers derived from a minimum of 6 experiments (in total 24 worms were scored at each concentration). Open squares: 100 μg/ml; filled circles: 10 μg/ml; open triangles: 1 μg/ml; dashed and dotted line: Control (RPMI 1640); dashed line: Control (RPMI 1640 + 1% DMSO).

RESULTS

In vitro *studies of schistosomula – dissecting microscopic and SEM evaluations*

The effect of incubation with different concentrations of MQ on the viability of schistosomula for up to 96 h is depicted in Fig. 1. In the absence of the drug, schistosomula showed normal viability without any morphological change for up to 24 h. Slight morphological changes such as granularity, a shorter body length with a falcate shape and reduced activity, were observed after 48 h of incubation. Schistosomula were viable for at least 96 h.

Following incubation with the 2 highest concentrations of MQ (100 and 75 μg/ml) schistosomula died rapidly; no movement was observed 3–4 min after incubation. At this time-point, schistosomula incubated with 1–10 μg/ml of MQ showed normal movements, while schistosomula incubated with 15–50 μg/ml of MQ showed slowed activity (Fig. 1).

At 1 h after incubation with 25 and 50 μg/ml of MQ all worms had died. Schistosomula incubated with 5, 10 and 15 μg/ml showed only minimal activity, including convulsions and contractions. Severe morphological changes, such as dark granular and crescent-shaped bodies associated with a reduction in body lengths were seen. No effect was observed at the lowest concentration of MQ (1 μg/ml) at the 1 h time-point. Hence, the MLC and MEC of MQ on schistosomula determined after 1 h were 25 and 5 μg/ml, respectively. In the presence of 1 μg/ml of MQ schistosomula revealed first morphological changes and a decreased motility 48 h post-incubation and 96 h after incubation most of the schistosomula were dead.

The structure and appearance of *S. mansoni* schistosomula, incubated for 1 h in the absence of MQ were similar to those described in the literature (Crabtree and Wilson, 1980; Basch and Basch, 1982). In brief, schistosomula had a round shape, clearly defined head and tail regions and an incompletely developed ventral (acetubulum) and oral sucker (Fig. 2A). The tegument was covered by tegumental spines, which were directed towards the posterior end (Fig. 2B).

Thirty min after incubation with 10 and 100 μg/ml of MQ, all schistosomula examined showed a change in shape and extensive tegumental alterations. Worms incubated with 100 μg/ml of MQ showed intensive blebbing and shortened spines on the entire tegumental surface (Fig. 2C). The incubation of schistosomula with 10 μg/ml of MQ showed contraction and bending of the worm body, resulting in the formation of wrinkles and a reduction of body length by ~50%. In addition, spines were shortened (Fig. 2D). Schistosomula exposed to 1 μg/ml of MQ showed no morphological changes 30 min after incubation, and their structure and appearance was similar to the controls.

At 6 h after incubation with 10 μg/ml MQ the tegument of schistosomula showed many deep furrows, swelling and flattened spines (Fig. 2E and F). At this same time-point, the lowest concentration of MQ induced no tegumental alterations on schistosomula.

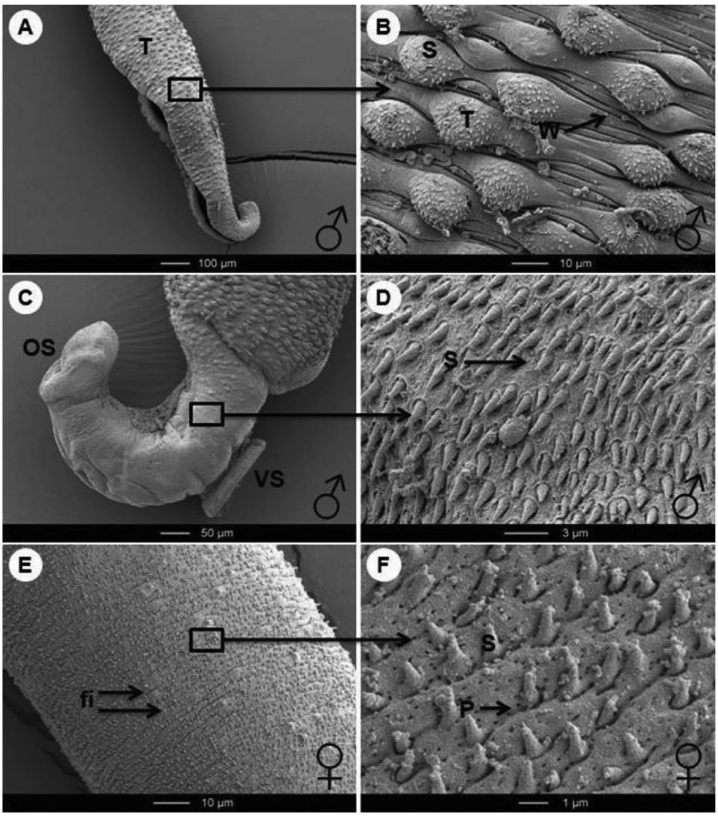

Fig. 4. SEM observations of untreated controls of adult *Schistosoma mansoni* recovered from a mouse 49 days post-infection by portal perfusion. (A) Tail region of male worm, showing tubercles (T). (B) Higher magnification of (A): tegument with tubercles (T), spines (S) and parallel-arranged wrinkles (W). (C) Head region of a male worm, showing the ventral (VS) and the oral sucker (OS). (D) Higher magnification of (C): tegument of the head region with spines (S) visible. (E) Female tegument of the mid-body region with parallel arranged fissures (fi). (F) Higher magnification of (E). Spines (S) and pores (P) visible.

Slight tegumental alterations were observed on worms incubated with 1 µg/ml of MQ after 72 h; schistosomula were bending backwards (Fig. 2G) and showed a shrunken tegument (Fig. 2G and H).

In vitro *studies of adult* S. mansoni – *dissecting microscopic and SEM evaluations*

The dose-response relationships of MQ on adult schistosomes for up to 72 h are given in Fig. 3. Control female and male schistosomes showed normal viability for up to 72 h. Egg production was observed starting 24 h post-incubation. After 3–4 min of incubation with 100 µg/ml of MQ, schistosomes displayed only minimal activities, including slow convulsions and contractions. At 1 h after incubation, adult worms exposed to this concentration were dead (Fig. 3). Worms incubated with 10 and 1 µg/ml of MQ showed normal activity at the 1 h time-point. After 24 h, all schistosomes incubated with 10 µg/ml of MQ were dead. In the presence of 1 µg/ml of MQ, there was a reduction in

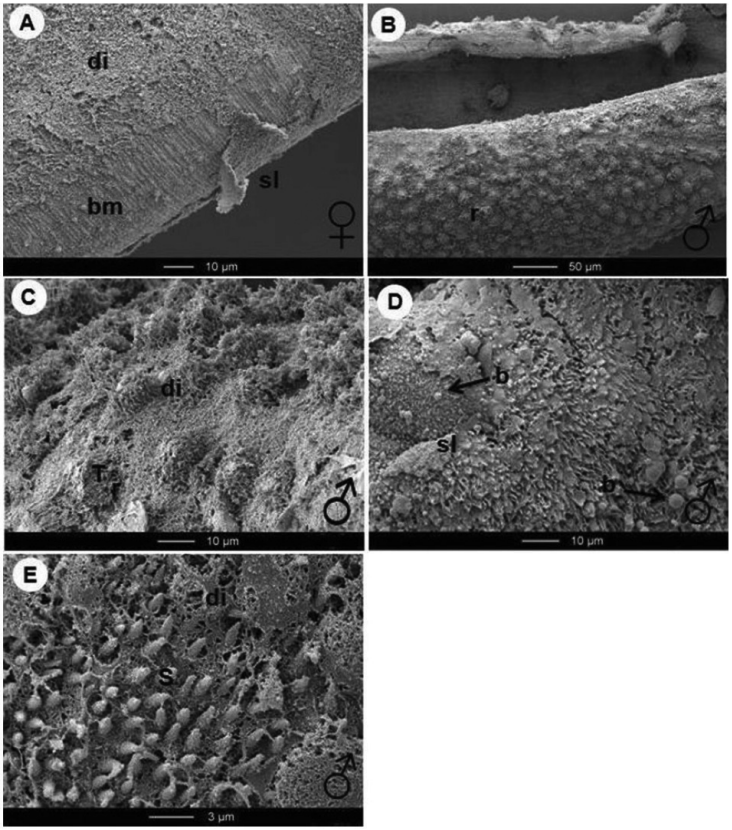

Fig. 5. (Cont.)

the viability of female worms after 72 h compared with untreated schistosomes. MQ suppressed egg production in female worms incubated with all 3 concentrations used.

The tegumental surface of adult *S. mansoni* has been described in a number of SEM studies (Miller *et al*. 1972; Hockley, 1973; Hockley and McLaren, 1973). Briefly, the surface of female schistosomes is simpler and more uniform when compared with the male worm (Basch and Basch, 1982; Gupta and Basch, 1988). The latter sex showed many tubercles on the dorsal body surface of the tail region (Fig. 4A). At a higher magnification, spines on the top of the tubercles and parallel-arranged wrinkles on the tegument were visible (Fig. 4B). The head region of both sexes of *S. mansoni* is characterized by an oral and a ventral sucker (Fig. 4C) and a smooth tegument with numerous tegumental spines (Fig. 4D). The mid-body region of female adults had parallel arranged fissures (Fig. 4E) and, at a higher magnification, tegumental spines and pores were visible (Fig. 4F).

By 30 min after incubation with 100 μg/ml of MQ extensive destruction was apparent on the entire tegument of all adult schistosomes examined. For example, on the mid-body region of female worms, extensive sloughing was visible, leaving the basal membrane exposed. In addition, the tegument showed a roughened appearance and started to disintegrate (Fig. 5A). Figure 5B depicts a roughened surface of a male specimen; at a higher magnification, disintegration of the tegument, resulting in a fibrous

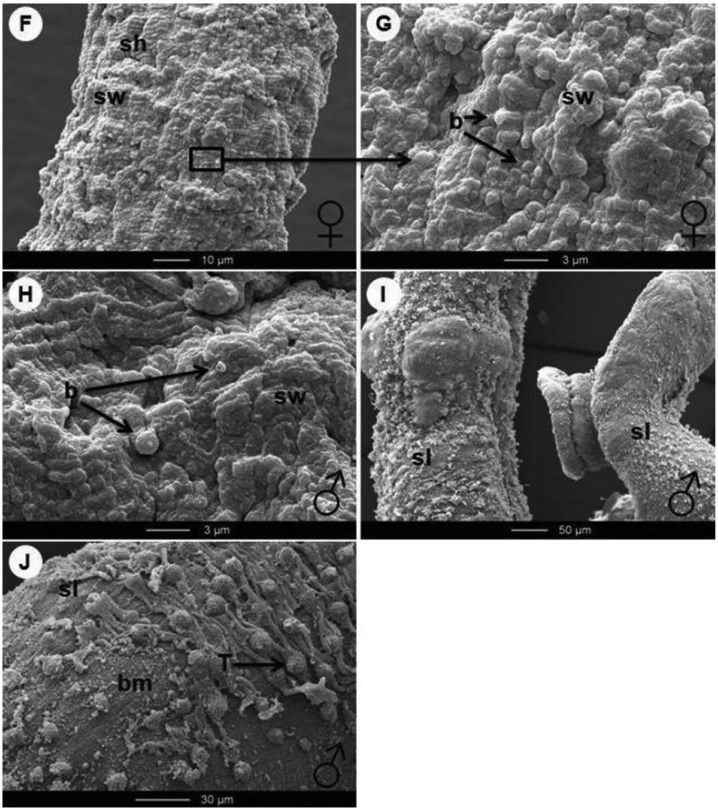

Fig. 5. (Cont.)

appearance, loss of tubercles, spines and the parallel-arranged wrinkles, was visible (Fig. 5C). The whole tegumental layer was destroyed.

After 1 h of incubation with 10 μg/ml of MQ, tegumental alterations were observed on the oral suckers of male worms and on the anterior and mid-body regions of both males and females. For example, blebbing and sloughing were observed on the head region of a male worm. Even the inner surface of the oral sucker of this worm was affected (Fig. 5D). Higher magnification revealed a disintegration of the interspinal tegument of the oral sucker, resulting in a roughened and fibrous appearance, with a loss of regularly arranged spines (Fig. 5E). A roughening of the tegument was also observed on the ventral suckers of male worms (not shown). While part of the female mid-body regions showed no damage, other mid-body regions revealed heavy sloughing, leaving the underlying membrane exposed.

After incubation for 24 h with 10 μg/ml of MQ, massive shrinking and swelling of the tegument was observed on all female worms examined (Fig. 5F). At a higher magnification of the ventral mid-body region, numerous blebs and swelling of the tegument, associated with a loss of spines, fissures and pores were seen (Fig. 5G). Similar observations were made when the tegument of the male flukes was examined. The male tegument was swollen and showed some blebs (Fig. 5H).

By 48 h after incubation with 10 μg/ml of MQ, sloughing affected the whole bodies of male and female worms. The ventral suckers were also affected

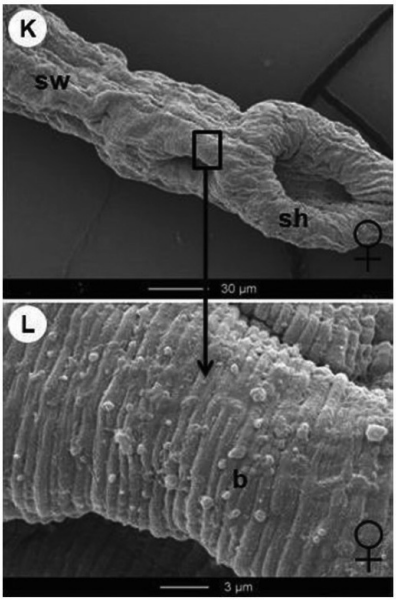

Fig. 5. SEM observations of adult *Schistosoma mansoni* after *in vitro* incubation with MQ. (A) Thirty min after incubation with MQ (100 µg/ml). Tegument of the mid-body region of a female worm. Extensive sloughing (sl) exposing to view the basal membrane (bm). Tegument shows roughening and disintegration (di). (B) Thirty min after incubation with MQ (100 µg/ml). Lateral and dorsal tegument around the gynecophoral canal of a male worm depicting a roughened (r) surface. (C) Higher magnification of (B). Tubercles (T) and the lateral tegument disintegrate (di) resulting in disappearance of the knobs, spines and the parallel-arranged wrinkles. (D) One h after incubation with MQ (10 µg/ml). Blebbing (b) and sloughing (sl) on the inner surface of the oral sucker of a male specimen is visible. (E) Higher magnification of (D) reveals disintegration (di) of the tegument. Spines (S) are still apparent, but not as clear and regular as in the control. (F) Twenty-four h after incubation with MQ (10 µg/ml). The tegument of the ventral mid-body region of a female worm is shrunken (sh) and swollen (sw). (G) Higher magnification of (F) shows many blebs (b) and swelling (sw) of the tegument. (H) Twenty-four h after incubation with MQ (10 µg/ml). Swollen (sw) male tegument of the ventral mid-body region with many blebs (b) seen. (I) Forty-eight h after incubation with MQ (10 µg/ml). Sloughing (sl) on the anterior region and at the ventral sucker of a male worm visible. (J) Forty-eight h after incubation with MQ (10 µg/ml). Mid-body region of a male worm: tegument with tubercles (T). The tegument shows extensive sloughing (sl). In some parts it lifted away leaving the basal membrane (bm) exposed. (K) Forty-eight h after incubation with MQ (1 µg/ml). Female with a shrunken (sh) and swollen (sw) mid-body tegument. (L) At higher magnification of this body region numerous blebs (b) were seen.

(Fig. 5I). In many parts, the tegument lifted away and left the basal membrane exposed. In addition, tubercles had lost their spines (Fig. 5J). Additionally, many blebs of different sizes were visible on the basal membrane. At the same time-point, worms incubated with the lowest concentration of MQ (1 µg/ml) also revealed tegumental alterations with a more severe tegumental response observed on female worms. The female body was shrunken and formed many wrinkles and deep furrows. In addition, the tegument was swollen in some parts and blebbing was observed (Fig. 5K and L). On the other hand, the teguments of male worms were only slightly roughened.

In vivo *studies of adult* S. mansoni – *hepatic translocation*

The distribution of adult worms in the mesenteric veins and the liver of *S. mansoni* infected mice 24, 48, 72, 96 and 120 h post-treatment with MQ (400 mg/kg) is summarized in Table 1. A hepatic shift was already observed 24 h post-treatment with MQ, with 61·8% of *S. mansoni* harboured in the liver. At 72 h and 120 h post-treatment >92% and >98%, respectively, of all worms had shifted to the liver.

In vivo *studies of adult* S. mansoni – *dissecting microscope and SEM evaluations*

All adult flukes recovered from the mesenteric veins of mice at 24, 48, 72, 96 and 120 h after MQ treatment were alive and viable. Worms which had shifted to the liver could not be examined.

Twenty-four h after *S. mansoni*-infected mice had received a single oral dose of MQ (400 mg/kg) localized blebbing on the tegument was the main response to drug action observed on worms recovered from the mesenteric veins (Fig. 6A). By 48 h post-treatment, blebbing increased, affecting the entire tegumental surface of the worms (Fig. 6B).

At 72 h after treatment, tegumental damage was more severe and several worms, in particular the females, were now affected. For example, the mid-body tegument of a female *S. mansoni* became shrunken (Fig. 6C). Again, many blebs were visible on the teguments as depicted in Fig. 6D. The oral sucker of a male specimen had a shrunken appearance resulting in formation of deep furrows. Moreover, some parts of the oral sucker around the pharyngeal opening were swollen. Small spines, which cover the inner surface and the outer margin of the oral sucker were not affected (Fig. 6E).

Table 1. Hepatic shift test following a single 400 mg/kg oral dose of MQ administered to mice infected with *Schistosoma mansoni*

Time-point of analysis (h post-treatment)	No. of mice investigated	No. of worms in mesenteric veins		No. of worms in liver		Total worm burden
		Mean (+/−S.D.*)	%	Mean (+/−S.D.)	%	Mean (+/−S.D.)
Control	6	42·6 (11·7)	97·3	1·2 (1·0)	2·7	43·8 (12·0)
24	4	14·3 (11·2)	37·9	23·3 (11·8)	61·8	37·7 (9·2)
48	4	29·5 (14·2)	56·3	25·5 (7·9)	46·3	55·0 (10·6)
72	4	5·7 (9·8)	7·6	69·0 (13·5)	92·4	74·7 (4·7)
96	4	3·0 (3·8)	6·3	44·8 (10·8)	93·7	47·8 (11·3)
120	3	0·5 (0·5)	1·1	44·0 (9·0)	98·9	44·5 (9·5)

* S.D., standard deviation.

At 96 h after treatment a slightly increased tegumental response to drug treatment was observed on female worms when compared to previous time-points. For example, the mid-body region of a female worm examined showed a shrunken appearance with deep furrows (Fig. 6F).

DISCUSSION

Recently, promising anti-schistosomal properties of MQ against both adult and juvenile schistosomes harboured by mice were found (Keiser *et al.* 2009) and proof-of-concept trials have been launched with MQ in *S. mansoni* or *S. haematobium*-infected children in geographical regions in which malaria and schistosomiasis are co-endemic. To further deepen our understanding of the activity of this anti-malarial drug against schistosomes, we investigated morphological effects and tegumental alterations of MQ on *S. mansoni* juveniles (schistosomula) and adults *in vitro* and *in vivo*. Dissecting microscopic investigations demonstrated the temporal effect of MQ on viability and morphology as well as dose-response relationships on schistosomula and adult *S. mansoni*. In addition, SEM examinations revealed the progressive stages of damage to and disruption of the tegumantal surface of the worm.

MQ had a very fast onset of action on schistosomula *in vitro*. In the presence of 100 and 75 μg/ml worms died immediately. Concentrations of $\geqslant 25$ μg/ml killed all schistosomula after 1 h of incubation. Interestingly, a slightly slower onset of action was observed when adult worms were incubated with MQ *in vitro*. For example, while 10 μg/ml of MQ killed schistosomula already after 6 h, the adults did not die until 24 h of exposure *in vitro*. A much slower onset of action was observed on adult worms collected following *in vivo* treatment with MQ. Worms collected from the mesenteric veins were still alive up to 96 h following treatment, and only moderate tegumental disruption, such as blebbing and a shrunken body, was observed. However, severely damaged worms might have already been expelled and worms that had translocated to the liver could not be examined. Hence, the evidence presented might have underestimated the amount of damage inflicted by the drug to the worm population as a whole. Nonetheless, the discrepancy between the effect of the drug and the onset of action of MQ *in vitro* and *in vivo* might be related to lower MQ concentrations present in the livers and mesenteric veins in mice compared with *in vitro* results. Pharmacokinetic (PK) studies, measuring drug concentrations in the body and the target organs, might aid elucidation of these differences observed *in vitro* and *in vivo*. In addition, the effect as well as onset of action of MQ on different stages of juvenile worms (e.g. 21-day-old worms) *in vitro* and *in vivo* remains to be elucidated.

Interestingly, worms incubated with MQ *in vitro* died without the presence of haemin or red blood cells. In contrast, no apparent effect was observed when schistosomes were incubated with the anti-malarial artemether *in vitro* in the absence of haemin, suggesting that artemether interacts with haemin to exert a toxic effect on the worms (Xiao *et al.* 2001). Though the exact mechanism of action of MQ on malaria parasites has not yet been entirely elucidated, a disturbance of the haemoglobin metabolism due to inhibition of the haematin polymerization and formation of an insoluble polymer, termed haemozoin, has been suggested (Dorn *et al.* 1998). A recent morphological study of *S. japonicum* in mice, showed a pronounced dilatation of the gut of adult worms, accompanied by focal or extensive peeling of gut epithelial cells, or even focal collapse of the gut wall (Zhang *et al.* 2009). Erythrocytes with accumulated MQ entering the worm gut might inhibit haem detoxification, resulting in gut damage (Zhang *et al.* 2009). Hence, while an interference of MQ with the haemoglobin digestion of schistosomes might play a role *in vivo*, MQ seems to attack additional drug targets *in vitro*. Further studies are necessary to elucidate the multiple mechanisms of action of MQ, which seems to be involved in the killing of schistosomes. It might also be useful to investigate whether

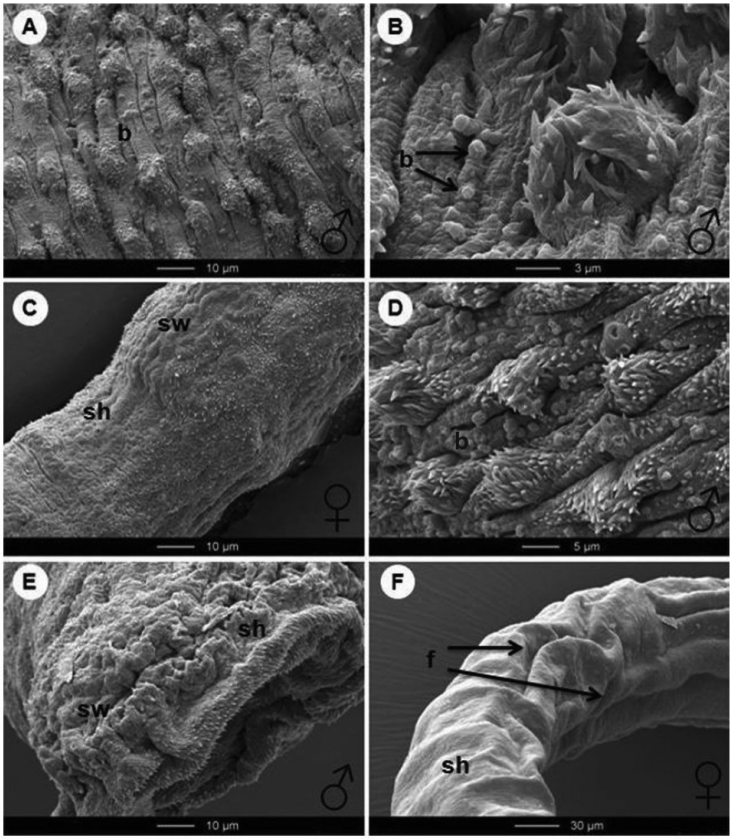

Fig. 6. SEM observations of adult *Schistosoma mansoni* recovered from mice treated with a single oral dose of MQ (400 mg/kg). (A) Twenty-four h after treatment. Blebbing (b) on the dorsal mid-body tegument of a male worm is visible. (B) Forty-eight h after treatment. Male dorsal tegument showing blebs (b). (C) Seventy-two h after treatment. Tegument of the mid-body region of a female has a shrunken (sh) and swollen (sw) appearance. (D) Seventy-two h after treatment. Dorsal mid-body male tegument reveals many blebs (b). (E) Seventy-two h after treatment. Oral sucker (OS) of a male specimen shows a shrunken (sh) and swollen (sw) tegument. (F) Ninety-six h after treatment. The shrunken (sh) mid-body region of a female *S. mansoni* with many furrows (f) is visible.

MQ acts synergistically with the host immune response, similar to the chemotherapeutic effect of PZQ, which has been shown to be dependent on the host antibody response (Brindley and Sher, 1987).

SEM is often used for documenting the efficacy of anti-schistosomal drugs. The 10–20 μm thick tegument (Neves *et al*. 2001) that covers the entire surface of schistosomes represents an important drug target. It consists of a double outer membrane (2 lipid bilayers), has no glycocalyx (Hockley, 1973; Wilson and Barnes, 1974; McLaren and Hockley, 1977), is responsible for absorptive activities (Rogers *et al*. 1983) and is a protection barrier between the parasite and the host (Hoffmann and Strand, 1996). In addition, it contains numerous ciliated or unciliated bulb-shaped tubercles, which are sensory receptors and associated with ring-shaped actin filaments (Zhou and Podesta, 1992). Also, the spines of the tubercles contain bundles of highly packed actin filaments (Zhou and Podesta, 1992).

We observed extensive tegumental damage on the larvae and adult developmental stages of *S. mansoni*, in particular following *in vitro* incubation, which intensified progressively as the incubation period and the concentration of MQ increased. Blebbing was visible on the tegument of both treated juvenile and adult flukes. Blebbing is an indicator for stress and has been observed in previous SEM studies evaluating anti-schistosomal drugs (Jiraungkoorskul *et al.* 2005). Nonetheless, vesicle formation induced by MQ might also occur due to the focal lysis of muscles (Zhang *et al.* 2009). Interestingly, while in the present study blebbing was observed on the tegument of both male and female adult *S. mansoni*, vesiculation was absent from female *S. japonicum* (Zhang *et al.* 2009). Flattened spines were observed on the tegument of schistosomula and roughening, sloughing and disintegration of the tegument were other typical features observed on the tegument of adult worms in the present investigation. In addition, extensive swelling and furrowing were observed on both development stages.

The effect of PZQ on the tegument of *S. mansoni* shows distinct differences, when compared to MQ, though related tegumental alterations such as sloughing, swelling, and loss or shortening of spines have also been documented for PZQ (Xiao *et al.* 2000). First, in contrast to small- and also medium-sized blebs observed in the present study a typical feature seen on the tegument of *S. mansoni* treated with PZQ was the presence of large-sized vesicles protruding from the tegumental surface, most likely due to swelling of the cytoplasm (Xiao *et al.* 1981). Second, in the current study, the tegument of female worms was slightly more affected and females died more rapidly when compared to male worms, in particular following *in vitro* exposure to MQ, male worms had a higher sensitivity to PZQ than female worms *in vitro* (Pica-Mattoccia and Cioli, 2004) and exhibited more extensive tegumental damage than female worms following PZQ treatment (Shaw, 1990). The greater MQ susceptibility of females is consistent with results from a previous *in vivo* study (Keiser *et al.* 2009), in which greater reductions in the female worm burden were observed compared with total worm burden reductions. We speculated that either a sex-specific interference of the drug with the target might occur or that there are different targets for MQ in females compared with males (Keiser *et al.* 2009). Alterations induced by artemether on the tegument of *S. mansoni* have also been well studied. Briefly, severe tegumental damage was observed on juvenile schistosomes, correlating with the high efficacy of artemether in killing 21-day-old schistosomes (Xiao *et al.* 2000). Focal swelling and fusion of tegumental ridges and occasionally sloughing was observed on adult *S. mansoni* recovered from mice. Similar to the present results obtained using MQ, female worms were more affected following treatment with artemether (Xiao *et al.* 2000).

We documented distinct morphological characteristics of schistosomes incubated with MQ. Schistosomula treated with MQ had a crescent-shaped body and showed contraction resulting in a reduction of the body length by approximately half. In addition, a dark granular body was seen. Adult worms showed convulsions after incubation with MQ *in vitro*. In contrast, rapid spasmodic contractions of the worm body are the most prominent morphological alterations induced by PZQ (Xiao *et al.* 1984). Finally, *in vivo* studies showed that extensive structural changes to worms occurred within 15 min of treatment, and both female and male adult worms died within 24 h following treatment with PZQ (Shaw, 1983; Xiao *et al.* 1983). In contrast, in our study worms were still alive several days post-treatment of mice with MQ and nearly all worms had shifted to the liver after 72 h. Interestingly, a more rapid hepatic translocation was observed with MQ on *S. japonicum*, all worms had moved to the liver 48 h after treatment (Zhang *et al.* 2009).

In conclusion, we demonstrated that MQ induces extensive morphological and tegumental alterations on both *S. mansoni* schistosomula and adults *in vitro* and *in vivo*. Importantly, given the distinct morphological and tegumental changes caused by MQ compared with PZQ, the only other effective anti-schistosomal drug available, we speculate that both drugs attack different targets; hence, it is suggested that no cross-resistance will occur. Thus, the present findings provide a sound basis for further in-depth studies of the anti-schistosomal properties of MQ, both in the laboratory and in the field.

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4. *Schistosoma mansoni*: Antischistosomal activity of the four optical isomers and the two racemates of mefloquine on schistosomula and adult worms *in vitro* and *in vivo*

Theresia Manneck[1, 2], Olivier Braissant[3], William Ellis[4] and Jennifer Keiser[1, 2]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH–4002 Basel, Switzerland,
[2] University of Basel
[3] Laboratory of Biomechanics and Biocalorimetry, Biozentrum/Pharmazentrum, University of Basel, Basel, Switzerland
[4] Department of Chemical Information, Walter Reed Army Institute of Research, Washington, DC 20314, USA

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Schistosoma mansoni: Antischistosomal activity of the four optical isomers and the two racemates of mefloquine on schistosomula and adult worms *in vitro* and *in vivo*

Theresia Manneck [a,b], Olivier Braissant [c], William Ellis [d], Jennifer Keiser [a,b,*]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH–4002 Basel, Switzerland
[b] University of Basel, Petersplatz 1, CH–4003 Basel, Switzerland
[c] Laboratory of Biomechanics and Biocalorimetry, Biozentrum/Pharmazentrum, University of Basel, Basel, Switzerland
[d] Department of Chemical Information, Walter Reed Army Institute of Research, Washington, DC 20314, USA

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ABSTRACT

Recent studies have shown that mefloquine (MQ) reveals interesting antischistosomal properties. We examined the antischistosomal activities of the erythro and threo isomers and racemates of MQ on newly transformed schistosomula (NTS) and adult *Schistosoma mansoni in vitro* and in mice harbouring adult *S. mansoni*. The *in vitro* effects in the presence and absence of haemin were monitored by means of microcalorimetry, scanning electron microscopy and phenotypic evaluation. Incubation of NTS with the erythro derivatives at concentrations of 3 μg/ml and above resulted in convulsions, granularity, decrease in heat flow, and death while NTS incubated with the threo derivatives were only affected at high concentrations (100 μg/ml). Extensive tegumental alterations, decrease in metabolic activity, viability, and death were observed when adult schistosomes had been exposed to 10 μg/ml of the erythro compounds. Moderate tegumental and viability changes but reduced heat production rates were observed with the threo derivatives at 10 μg/ml. In the presence of haemin, all MQ derivatives showed pronounced antischistosomal properties against adult *S. mansoni in vitro*. *In vivo*, MQ derivatives achieved statistically significant total and female worm burden reductions ranging between 65.4% and 100%. The highest total worm burden reductions of 93.4% and 90.2% were observed following treatment with the erythro and threo racemates, respectively. In conclusion, the optical isomers and racemates of MQ show only moderate stereoselectivity, in particular *in vivo*. Our results may enhance our understanding of the mechanism of action and therapeutic profile of MQ derivates on schistosomes.

1. Introduction

Schistosomiasis is a chronic inflammatory disorder caused by fluke worms of the genus *Schistosoma*, which affects the world's poorest people. It has been estimated that more than 207 million people are infected and 779 million people are at risk of infection (Steinmann et al., 2006). The disease is associated with pathologies that relate to the site of adult infections, the liver (*Schistosoma mansoni* and *Schistosoma japonicum*) or the bladder and kidneys (*Schistosoma haematobium*) impaired child growth and development, anemia, and malnutrition and inflicts a burden of up to 70 million disability-adjusted life years (DALYs) lost annually (King and Dangerfield-Cha, 2008).

Treatment and control of schistosomiasis relies almost exclusively on praziquantel (Utzinger and Keiser, 2004; Doenhoff et al., 2008). However, drug resistance is an imminent threat, particularly in the light of large-scale administration of praziquantel (Botros and Bennett, 2007; Caffrey, 2007). Indeed, *S. mansoni* isolates with reduced susceptibilities to praziquantel have already been identified (Melman et al., 2009). In addition, praziquantel shows a deficiency in its spectrum of activity, revealing only moderate activity against juvenile worms (Doenhoff et al., 2008). Hence, the discovery and development of new antischistosomal drugs should be pursued.

In recent laboratory studies, mefloquine (MQ), an arylamino alcohol, discovered by the Walter Reed Army Institute of Research (Trenholme et al., 1975; Sweeney, 1981) and approved by the U.S. Food and Drug Administration (FDA) in 1989 for the prophylaxis and treatment of malaria (www.fda.gov) showed promising antischistosomal activity *in vitro* and in mice infected with *S. mansoni* and *S. japonicum* (Van Nassauw et al., 2008; Keiser et al., 2009; Manneck et al., 2010). Furthermore, a MQ-artesunate combination, administered over three days following the recommended malaria treatment schedule, showed a cure rate of 61% and an egg reduction rate of 95.9% against *S. haematobium* in school-aged children

* Corresponding author at: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box CH–4002 Basel, Switzerland. Fax: +41 61 2848105.
E-mail address: jennifer.keiser@unibas.ch (J. Keiser).

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in a recently conducted randomized, exploratory open-label trial in Côte d'Ivoire (Keiser et al., 2010).

MQ, a chiral drug with two dissimilar asymmetric centres, exists in two racemic forms (erythro and threo), each of which is composed of a pair of optical isomers, i.e., (±)-erythro-enantiomers and the (±)-threo-epimers (Sweeney, 1981). The chemical structures of the four optical isomers are shown in Fig. 1 (Carroll and Blackwell, 1974; Basco et al., 1992; Xie et al., 2008). Clinically, the racemic mixture of the erythro isomers is used.

Each enantiomer of a chiral drug may differ significantly in its pharmacological profile, including efficacy, toxicity, bioavailability, metabolism, and excretion (Brocks and Mehvar, 2003). For example, while dextro-praziquantel is almost ineffective, levo-praziquantel has excellent antischistosomal activity (Liu et al., 1993).

The aim of the present study is to compare the antischistosomal properties of the four MQ isomers and two racemates *in vitro* and *in vivo*. The *in vitro* studies were carried out utilizing 1–7 day-old *S. mansoni* schistosomula (newly transformed schistosomula, NTS) and 49 day-old adult *S. mansoni* in the presence and absence of haemin. We monitored phenotypic and tegumental changes and evaluated microcalorimetric curves, which give a direct indication of metabolic heat production. *In vivo*, the effect of single oral doses (200 mg/kg) of the drugs was assessed on adult *S. mansoni* harboured in mice.

2. Materials and methods

2.1. Animals and parasites

All experiments were carried out at the Swiss Tropical and Public Health Institute (Basel, Switzerland), in accordance with Swiss National animal welfare regulations.

Female NMRI mice (n = 100, age = 3 weeks, weight~35 g) were purchased from Harlan (Horst; The Netherlands).

Cercariae of *S. mansoni* were harvested from infected intermediate host snails (*Biomphalaria glabrata*) maintained at our laboratories after exposure to light for 4 h.

2.2. Drugs

Racemic mefloquine hydrochloride [(±)-erythro-α-(2-piperidinyl)-2,8-bis(trifluormethyl)-4-quinolinemethanol hydrochloride] (rac-erythro (MQ)), the racemic mixture of the threo-epimers [(±)-threo-α-(2-piperidinyl)-2,8-bis(trifluormethyl)-4-quinolinemethanol hydrochloride] (rac-threo), the (+)- and (−)- erythro enantiomers of MQ, and the (+) and (−)- threo epimers of the threo racemate were kindly provided by the Walter Reed Army Institute of Research (Washington DC, USA). The molecular weight of all isomers is MW = 414.81 g/mol.

For *in vitro* studies, drugs were dissolved in dimethyl sulfoxide (DMSO, Fluka, Buchs, Switzerland) to obtain stock solutions of 10 mg/ml. For *in vivo* studies, MQ isomers were suspended in 7% (v/v) Tween 80 and 3% ethanol with final concentrations of 40 mg/ml (free base).

2.3. In vitro phenotype-based drug screening assay with NTS

Cercariae of *S. mansoni* were mechanically transformed into NTS based on a method by Ramalho Pinto and colleagues (Ramalho-Pinto et al., 1974) as described previously (Keiser, 2010; Manneck et al., 2010). NTS were kept in TSS 199 culture medium supplemented with 5% heat-inactivated foetal calf serum (iFCS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, USA) at 37 °C in an atmosphere of 5% CO_2 for a minimum of 3–12 h before use to make sure that the process of metamorphosis from cercariae to NTS was completed.

Stock solutions of the drugs were diluted in 24-well plates (Costar) in TSS 199 culture medium, and 100 μl NTS-suspension containing 500–1000 NTS was added to each well. The following drug concentrations were studied: 100, 10, 5, 3, and 1 μg/ml, corresponding to 241, 24.1, 12.05, 7.23, and 2.41 μM. NTS incubated in medium with 1% DMSO, the highest concentration of drug solvent used, served as controls. Parasites were kept in an incubator at 37 °C, 5% CO_2 for up to 96 h.

Before incubation with the drugs and after 3, 17, 24, 48, 72 and 96 h of incubation, all worms were observed and characterized using a dissecting microscope (8–40-fold magnification, Carl Zeiss AG, Germany). Phenotypic changes were determined and scored using a viability scale as described in a previous publication (Keiser, 2010; Manneck et al., 2010) (Box 1). All experiments were carried out in duplicates and were repeated at least three times.

Box. 1

Scale	Phenotypic characterization
3	• normal activity • no morphological changes

2	• slowed activity
	• first morphological changes
	  • granularity*
	  • blebs

1	• minimal activity
	• severe morphological changes
	  • severe granularity*
	  • blebs
	  • dark body colour
	  • irregular shape

0	• all worms dead
	  • total absence of motility
	  • severe morphological changes

* characterization only for NTS

2.4. In vitro phenotype-based drug screening assay with adult S. mansoni

NMRI mice were infected subcutaneously with approximately 200 *S. mansoni* cercariae. Forty-nine days post-infection, animals were sacrificed with CO_2, dissected, and all schistosomes were removed from the hepatic portal system and the mesenteric veins. Worms were washed three times, and two male and two female schistosomes were placed in each well containing RPMI 1640 culture medium supplemented with 5% iFCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The drug dilutions were prepared as described above. Adult *S. mansoni* were incubated in the presence of 100, 10, or 1 μg/ml corresponding to 241, 24.1, or 2.41 μM of each isomer or racemate. To study the influence of haemin on drug efficacy in a second set of experiments, schistosomes were incubated in the presence of 166 μg/ml (255 μM) haemin in PBS (Dalton et al., 1995) and 100, 10, or 1 μg/ml of the drugs.

Schistosomes incubated with the highest concentration of drug solvent or haemin-supplemented medium served as controls. Worms were kept in an incubator at 37 °C, 5% CO_2 for up to 96 h. Phenotypic changes were recorded as described above (Box 1). All experiments were carried out in duplicates and were repeated three times.

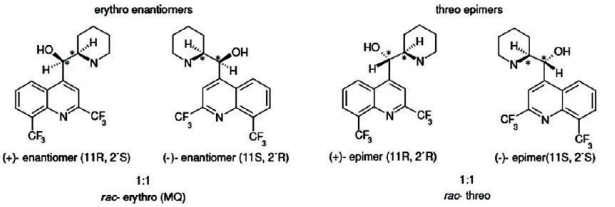

Fig. 1. Chemical structures of the four optical isomers of MQ.

2.5. Scanning electron microscopy with adult S. mansoni

Forty-eight hours after incubation of adult worms with 10 μg/ml of the drugs, worm samples were processed for scanning electron microscopy (SEM). Specimens were fixed with 2.5% (v/v) glutaraldehyde in a phosphate-buffered saline (PBS, pH 7.4) for 24 h at 22–24 °C (room temperature). After rinsing three times with PBS, they were stored in PBS at 4 °C until use. Before SEM examination, flukes were washed twice with double-distilled water, dehydrated in ascending ethanol concentrations, and critically point dried. Schistosomes were placed on aluminium stubs, sputter coated with 20 nm gold nanoparticles, and observed in a high-resolution SEM (Phillips XL30 ESEM) at an accelerating voltage of 5 kV.

2.6. In vitro drug screening assay based on microcalorimetry

A multi-channel isothermal microcalorimeter (Model "TAM 48", TA Instruments, New Castle, DE) equipped with 48 measuring channels was used to monitor the heat production by schistosomes as a result of their metabolism over time. The isothermal microcalorimeter was thermostated to 37 °C, at least two days before experiments to achieve maximum stability. We determined the effect of the four MQ isomers and the two racemates on the motor activity of adult worms and metabolic activity of NTS and adult *S. mansoni* by measuring the heat flow before and after addition of the MQ derivates. We analysed drug effects on the metabolic activity by comparing the heat flow curves of medium only or medium containing dead worms (positive controls), worms alive without treatment (negative control, 1000 NTS or four adults), and drug-treated schistosomes. Mortality rates of adult *S. mansoni* were calculated by comparing noise amplitudes which derive from worm motor activities (Fig. 2A–C) of drug-treated and control schistosomes. Previous experiments performed with snails and insects have clearly linked movements to very sharp peaks in the heat flow pattern (Lamprecht and Becker, 1988; Kuusik et al., 1995; Harak et al., 1996).

Glass ampoules were filled with either 800 μl TS 199 culture medium and 100 μl NTS suspension (containing 1000 NTS) or with either 900 μl RPMI 1640 culture medium or 734 μl medium and 166 μl haemin solution (1 mg/ml in PBS) and two adult male and two adult female worms. All materials used were autoclaved at

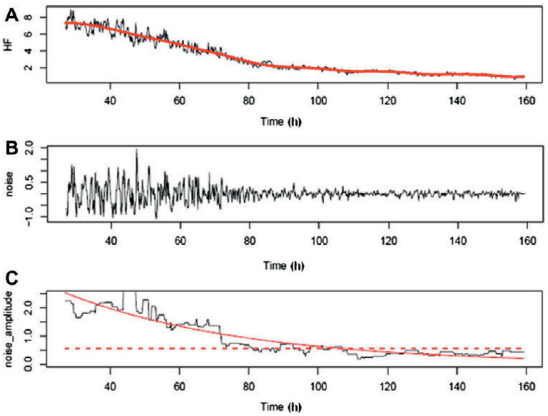

Fig. 2. Heat flow pattern of adult *S. mansoni*. (A) Heat flow curve of adult *S. mansoni* ($n = 4$). (B) Magnification of noise derived from (A). (C) Absolute values of the amplitudes of noise obtained for 20 min over the entire course of the experiment (144–159 h). Noise amplitude values follow exponential decay (solid line). The following equation was applied: $H_0e^{-\lambda t} + 3e^{-t}$, where λ represent the decay rate of noise amplitudes (motor activity), $H0$ is the initial starting point and t is time. Background noise of the calorimetric system (amplitude values, 0.55 μW) is shown as dotted line. The intersection of the sample curve (solid line) with the background curve (dotted line) is the endpoint of worm motility and corresponds to the death of worms.

121 °C for 20 min, and all media and drug solutions sterile-filtered (0.2 μm). Ampoules were sealed and put into the measuring channels. After an initial equilibration time (until a stable signal was obtained), drug dilutions were injected into the ampoules using a sterile 0.5 ml insulin syringe (Becton Dickinson, NJ, USA) reaching final concentrations of 100, 10, and 1 μg/ml. Blank medium was injected into the controls. The heat flow was recorded every 10 min for a minimum of 96 h (NTS) and 144 h (adult *S. mansoni*). All samples were assayed in triplicates and were repeated twice.

2.7. Efficacy studies of 6 MQ-isomers/racemates on adult S. mansoni in vivo

Fifty-six NMRI mice were infected subcutaneously with approximately 90 *S. mansoni* cercariae. Seven weeks post-infection, groups of mice (4–7) were treated with single 200 mg/kg oral doses of the drugs, based on previous efficacy data (Keiser et al., 2009). One untreated group of mice ($n = 7$) served as controls. Three weeks post-treatment, mice were killed by cervical dislocation, and the mesenteric tissue and the liver were collected. Schistosomes were removed from the hepatic portal system and the mesenteric veins, sexed, and counted using a binocular microscope. The liver was flattened and examined for the presence of worms. Distribution of schistosomes in the liver and mesenteric veins was recorded.

3. Results

3.1. Antischistosomal activity of mefloquine isomers/racemates on NTS in vitro

3.1.1. Phenotypic screening

In the presence of 100 μg/ml of the different isomers or racemates, all NTS died immediately. At concentrations of 10 μg/ml and below, differences in the antischistosomal activities of the drugs were observed. The temporal drug effects of all MQ derivatives at concentrations of 10 and 3 μg/ml on NTS are depicted in Fig. 3A and B, respectively. At a concentration of 10 μg/ml, both erythro enantiomers and the erythro racemate (MQ) showed a rapid onset of action. Three hours after incubation with the erythro drugs, NTS showed only minimal activities including convulsions and contractions and granularity. These NTS were characterized by shorter body lengths and a sickle shape. Seventeen hours

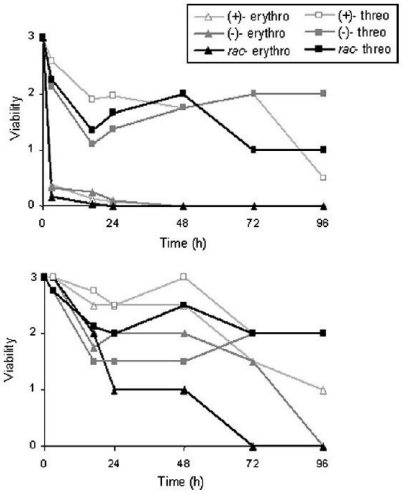

Fig. 3. Viability of *S. mansoni* NTS following exposure to 10 (A) and 3 μg/ml (B) of four MQ isomers and two racemates *in vitro*. Mean values of viability obtained using a viability score. Numbers derived from a minimum of six experiments.

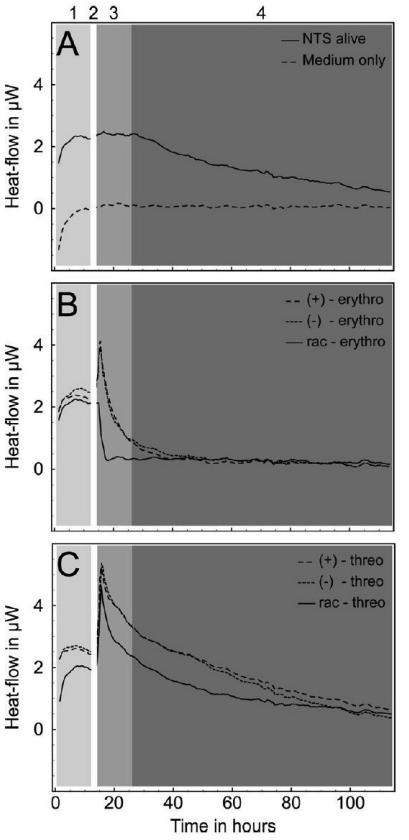

Fig. 4. Smoothed heat flow curves of *S. mansoni* NTS: (A) Positive (medium only) and negative controls (NTS without treatment). (B) NTS treated with the erythro derivates. (C) NTS treated with the threo derivates. Initial equilibration phase (1), medium/drug injection (10 μg/ml of the four MQ derivates and two racemates) (2), immediate drug reaction and re- equilibration phase (excluded from analysis) (3), analysis of heat flow over the course of the experiment (4).

(rac-erythro, MQ) and 48 h (erythro isomers) post-incubation with 10 μg/ml all NTS had died. Incubation with the threo epimers and the threo racemate at this concentration resulted in a decreased viability observed at all examination points, but all NTS survived drug exposure for 96 h (Fig. 3A).

Only a very moderate effect on NTS was observed following exposure to 3 μg/ml of the threo isomers and threo racemate. Incubation with the erythro racemate and (−)- erythro isomer at 3 μg/ml resulted in death of NTS between 72–96 h, while exposure to the (+)- erythro isomer resulted in decreased viability. No effect on NTS was observed at 1 μg/ml with any of the derivatives tested.

3.1.2. Microcalorimetry of NTS

The smoothed thermogenic power-time curves following incubation of *S. mansoni* NTS with MQ derivates (c = 10 μg/ml) are depicted in Fig. 4, highlighting the equilibration phase (15 h); (1), drug/medium injection (2), immediate drug/injection reaction, re-equilibration phase (3) and heat flow over the entire course of the experiment (4).

Constantly low signals of 0–0.25 μW were measured for the positive controls over the entire study period including medium injection. The negative control had a stable signal (2.3 μW) until 30 h post-incubation; towards the end of the experiment lower values (0.9 μW at 96 h), possibly due to lack of oxygen, were observed.

Following exposure to 100 μg/ml of any MQ derivative tested, heat production of NTS rapidly decreased and returned to baseline (0–0.25 μW) within one hour (data not shown); hence all NTS had died. At 10 μg/ml, the erythro derivatives showed superior activity over the threo derivatives. NTS incubated with the erythro racemate (MQ) showed a rapid decrease in heat flow, reaching the value of the medium blank within 2 h after drug injection. Worms treated with either of the erythro enantiomers showed a 50% and 90% reduction in their metabolism 8 and 38 h after drug injection, respectively. No effect on heat production was observed with the threo epimers or the threo racemate at this concentration. At 1 μg/ml, no effect on heat production rate of NTS was observed with any of the MQ derivatives tested (data not shown).

3.2. Antischistosomal activity of mefloquine isomers/racemates on adult S. mansoni in vitro

3.2.1. Phenotypic screening

Incubation with 100 μg/ml of the drugs resulted in death of all schistosomes. At 10 and 100 fold lower concentrations, *S. mansoni* exhibited differences in sensitivity depending on the drug used and whether haemin was added to the medium. In the absence of haemin and at a concentration of 10 μg/ml, only worms exposed to either of the erythro enantiomers or the erythro racemate (MQ) died (Fig. 5A). At the same concentration, but in the presence of

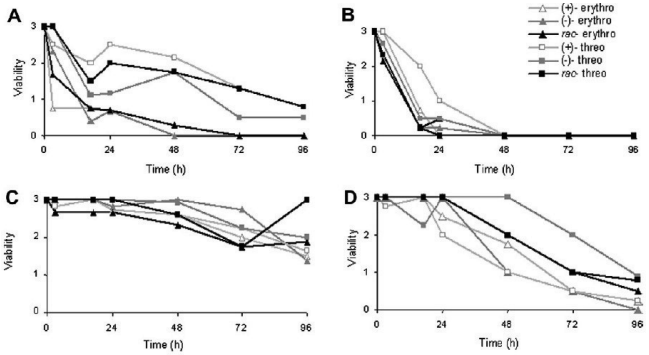

Fig. 5. Viability of adult *S. mansoni* following exposure to 10 and 3 μg/ml of four MQ isomers and two racemates *in vitro* in the presence or absence of haemin (A) 10 μg/ml (B) 10 μg/ml + haemin (C) 3 μg/ml (D) 3 μg/ml + haemin. Mean values of viability obtained using a viability score. Numbers derived from a minimum of six experiments.

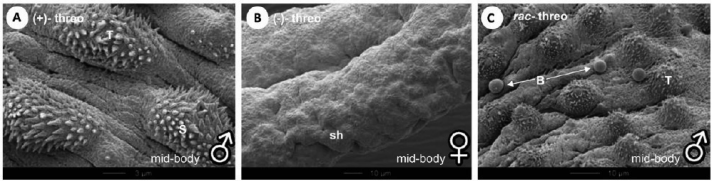

Fig. 6. SEM observations on adult schistosomes 48 h post-incubation with 10 μg/ml of the threo epimers and the threo racemate *in vitro*. (A) Mid body tegument with tubercles (T) and spines (S) of a male worm following incubation with the (+)- threo isomer. (B) A slightly shrunken (sh) mid-body region of a female worm following incubation with the (−)- threo isomer. (C) Scattered blebs (B) around the tubercles (T) of a male tegument visible following exposure to the threo racemate.

haemin, schistosomes were affected rapidly by all drugs and died within 48 h (Fig. 5B). Only moderate activities were observed when schistosomes were incubated with 1 μg/ml of the drugs (Fig. 5C). However, when the culture medium was supplemented with haemin, lethal effects were observed. Worms incubated with 1 μg/ml of the (−)- erythro isomer and haemin died after 96 h, and incubation with the other threo and erythro derivates resulted in a marked decrease of activity of *S. mansoni* (Fig. 5C). All control worms were alive for at least 96 h.

3.2.2. SEM studies

Tegumental alterations observed on adult schistosomes following incubation with 10 μg/ml of MQ derivatives for 48 h are presented in Fig. 6A–C and Fig. 7A–D. Schistosomes incubated for 48 h in the absence of drugs showed an intact tegument (no figure shown) as described in a previous publication (Manneck et al., 2010). When adult worms were incubated with 10 μg/ml of the threo epimers for 48 h, the tegument of male worms was not affected (Fig 6A), while the female worms showed a slightly shrunken tegument (Fig. 6B). Incubation with the threo racemate revealed several scattered blebs on the mid body region of male and female worms (Fig. 6C). In contrast, extensive tegumental changes were observed when schistosomes had been exposed to the erythro isomers. Fig. 7A and B depict typical tegumental alterations observed on male and female worms such as shrinking, furrowing (Fig. 7A), and swelling (Fig. 7B). The most pronounced tegumental damage was observed on schistosomes exposed to the erythro racemate (MQ). All worms examined showed shrinking with furrowing (Fig. 7C), extensive sloughing around the tubercles on the tegument exposing subtegumental tissues, and blebbing (Fig. 7D).

3.2.3. Microcalorimetry of adult worms

Smoothed thermogenic curves of adult *S. mansoni* following incubation with the erythro enantiomers (100, 10, and 1 μg/ml) are presented in Fig. 8. The effects of 10 and 1 μg/ml of four MQ isomers and two racemates (with and without addition of haemin) on the mortality rates of adult *S. mansoni* (mortality rates) are presented in Fig. 9A and B.

The positive controls showed continuous heat flows of 0.5 μW during the entire experiment. The negative control showed heat values of ~7 μW during the first 10 h of the experiment, which decreased to 2.6 μW 72 h after incubation, due to reduced oxygen levels.

No difference was seen in the thermograms of adult *S. mansoni* following injection with 100 μg/ml of the different MQ derivates. All heat flow curves decreased rapidly reaching baseline values within 1 h.

Exposure to 10 μg/ml of the erythro derivates resulted in a heat reduction of 50% after 10 h ((+)- and (−)- enantiomer) and 18 h (rac- erythro; MQ), respectively. The total heat production rates decreased gradually, and baseline levels were reached after 61–135 h. The threo derivates (figure not shown) showed a 50% decrease in their metabolic activity 28 h ((+)- threo), 32 h ((−)- threo), and 35 h (rac-threo), following drug injection, respectively. Analysis of the noise amplitudes, which reflect worm motor activity, showed 100% mortality rates for worms treated with the three erythro derivatives and the threo racemate (Fig. 9A). For the threo epimers, mortality rates were 83.7 ((+)- threo) and 65.4% ((−)- threo), respectively.

In the presence of haemin (no figure shown) and 10 μg/ml of the drugs, the metabolic rate of *S. mansoni* treated with the erythro derivates decreased slightly faster. A 50% reduction in heat

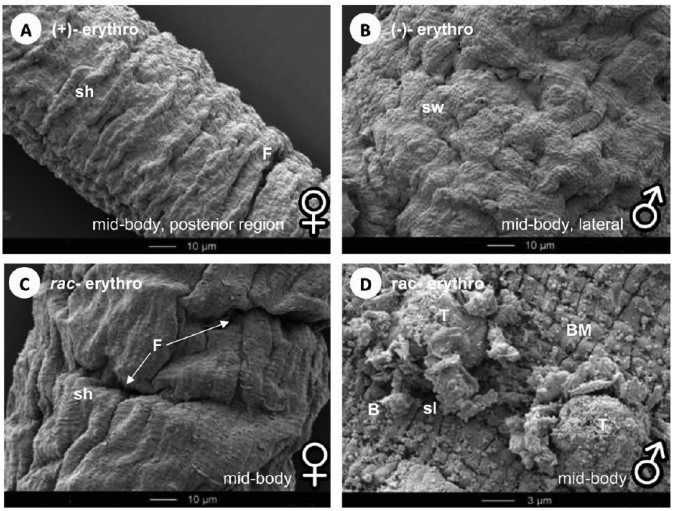

Fig. 7. SEM observations on adult schistosomes 48 h post-incubation with 10 μg/ml of the erythro enantiomers of MQ and the erythro racemate (MQ) *in vitro*. (A) Incubation with the (+)- erythro isomer. Shrunken (sh) mid-body region of a female tegument with many furrows (F). (B) Incubation with the (−)- erythro showing a swollen (sw) tegument of a male worm. (C) Incubation with the erythro racemate (MQ) revealed a shrunken (sh) female tegument with many deep furrows (F). (D) Incubation with the erythro racemate (MQ). Extensive sloughing (sl) around the tubercles (T) on the tegument of a male *S. mansoni* exposing the basal membrane (BM). Blebs (B) were also visible.

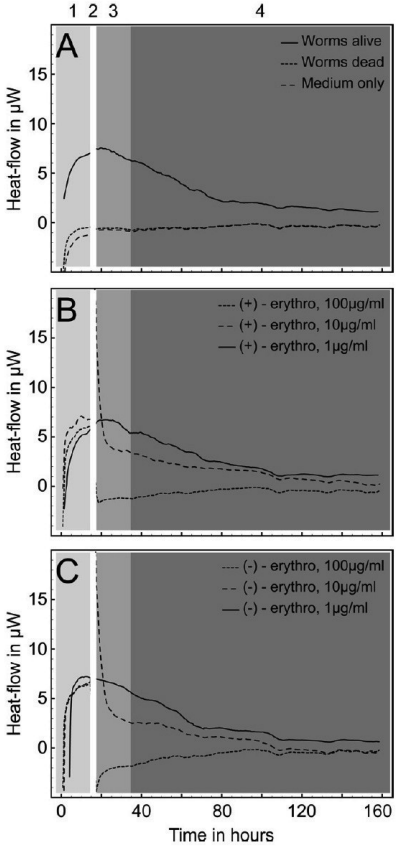

Fig. 8. Smoothed heat flow curves of adult *S. mansoni*: (A) Positive (medium only or medium containing dead worms) and negative controls (worms alive without treatment). (B) Schistosomes treated with the (+)- erythro enantiomers of MQ. (C) Schistosomes treated with the (−)- erythro enantiomers of MQ. Initial equilibrating time (1), medium/ drug injection (100, 10 and 1 µg/ml of the erythro isomers) (2), immediate drug reaction (excluded from analysis) (3), examination period (4).

production rate was observed after 9 h ((−)- and (+)- erythro enantiomer) and 16 h (rac- erythro; MQ) post-incubation, respectively. Based on noise analysis, worms incubated with the (+)- erythro enantiomer and the erythro racemate (MQ) had died. The (−)- erythro isomer reduced worm survival by 87.2%. For the threo derivates, slower drug response was observed in the presence of haemin: a 50% reduction in heat production was determined 34 h ((+)- epimer) – 41 h (rac- threo) following treatment. Worm survival rates ranging from 14.9% (rac- threo) to 24.4% ((+)- threo) were determined for the threo compounds in the presence of haemin (Fig. 9B).

Incubation of *S. mansoni* with 1 µg/ml of erythro derivatives showed thermogenic curves comparable to those of the controls. In the presence of haemin and 1 µg/ml of erythro drugs, a 50% decrease in metabolic heat was observed in the calorimetric curves after 26 h ((+)- and (−)- enantiomer) and 32 h (rac- erythro), respectively. Mortality rates of 82.6% (rac- erythro (MQ)) and 50% (both erythro isomers) were calculated. A concentration of 1 µg/ml of the threo derivatives had no effect on the thermodynamics on adult *S. mansoni* (data not shown), and mortality rates were low even in the presence of haemin.

3.3. Efficacy studies in vivo

Table 1 shows the ***in vivo*** antischistosomal efficacies of the optical isomers and racemates of MQ. Drugs were administered at 200 mg/kg to mice harbouring an adult *S. mansoni* infection. All drugs achieved statistically significant total and female worm burden reductions ranging between 65.4% and 100% ($p < 0.01$). Changes in the worm distribution were observed. The highest activities were documented following single oral doses of the erythro and the threo racemates (total and female worm burden reductions of 93.4% and 97.1% (erythro) and 90.2% and 100% (threo), respectively). The total worm burden reduction of MQ (93.4%) was significantly higher ($p < 0.05$) than the total worm burden reduction of the (+)- erythro isomer (65.4%).

4. Discussion

The role of stereochemistry in the pharmacokinetics, efficacy, and toxicity of drugs is receiving high attention in the discovery and development of drugs today (Brocks and Mehvar, 2003). Several marketed racemic drugs exhibit stereoselectivity in their pharmacokinetic and pharmacodynamic profile. Therefore, single-enantiomer formulations might provide greater selectivity for their biological targets, less adverse events, increases in therapeutic indices, or better drug disposition (Brocks and Jamali, 1995).

In this study, we investigated the antischistosomal activities of four MQ isomers and two racemates. ***In vitro***, the erythro derivatives showed superior activity when compared to the threo derivatives, while ***in vivo*** both racemates achieved the highest worm burden reductions.

As in our previous study which compared the ***in vivo*** activity of the erythro enantiomers (Keiser et al., 2009), the (+)- and (−)- erythro isomer showed comparable antischistosomal properties ***in vitro*** against NTS and adults and ***in vivo***. Hence, no stereoselectivity of interaction was observed. This finding is in line with results from an ***in vitro*** study with *Plasmodium falciparum*, which showed that both MQ erythro enantiomers were equally active (Basco et al., 1992). However, this finding was not confirmed by a second ***in vitro*** study against *P. falciparum* (Karle et al., 1993), which reported that the (+)- isomers (both erythro and threo) were almost twice as active as the (−)- isomers. Since different strains of *P. falciparum* were used in the individual studies, a strain-specific stereoselective behaviour of the MQ enantiomers might have been observed (Brocks and Mehvar, 2003).

Interestingly, in our study marked differences were observed between the ***in vitro*** and ***in vivo*** activities with the threo derivatives. While the threo isomers as well as the racemate were less active than the erythro derivatives against *S. mansoni* ***in vitro***, in particular in the absence of haemin, all threo derivatives achieved high worm burden reductions ***in vivo***. Similarly, the threo isomers were less active than the erythro isomers against *P. falciparum*

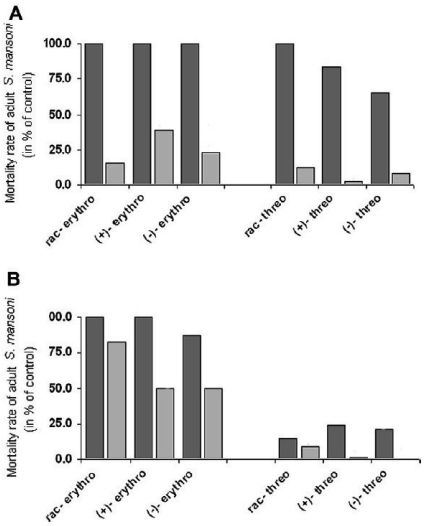

Fig. 9. Mortality rates induced by the four MQ isomers and two racemates (10 and 1 μg/ml) in the absence (A) and presence of haemin (B) on adult *S. mansoni*. Dark grey columns: $c = 10$ μg/ml, light grey columns: $c = 1$ μg/ml. Effects shown were recorded 24 h after incubation (10 h after drug injection) until the end of the experiment.

Table 1
Total and female worm burden reductions and changes in the worm distribution of the optical isomers and racemates of MQ against 49 day-old *S. mansoni* in mice.

Drug tested	No. of mice cured/ investigated	Number of worms							Total worm burden reduction (%)	KW	*p*	Female worm burden reduction (%)	KW	*p*
		Mesenteric veins		Liver		Total worm burden	Females							
		Mean (±SD)	%	Mean (±SD)	%	Mean (±SD)	Mean (±SD)	%						
Control	0/7	24.9 (4.9)	97.8	0.6 (0.5)	2.2	25.4 (5.0)	11.6 (2.9)	45.5	–			–		
(+)- erythro	0/5	0.8 (1.8)	9.1	8.0 (5.4)	90.9	8.8 (7.0)	2.2 (2.2)	25.0	65.4	8.19	0.004	81.0	8.16	0.004
(−)- erythro	0/4	0 (0)	0	7.5 (5.2)	100	7.5 (5.2)	1.8 (2.1)	23.3	70.5	7.16	0.007	84.9	7.10	0.008
rac- erythro	0/6	0 (0)	0	1.7 (2.3)	100	1.7 (2.3)	0.3 (0.8)	20.0	93.4*	9.15	0.003	97.1	9.58	0.002
(+)- threo	0/6	0.2 (0.4)	2.9	5.7 (3.2)	97.1	5.8 (3.1)	0.7 (0.8)	11.4	77.1	9.13	0.003	69.7	9.18	0.003
(−)- threo	0/6	0.2 (0.4)	3.6	4.5 (4.9)	96.4	4.7 (5.0)	0.3 (0.8)	7.1	81.6	9.13	0.003	97.1	9.58	0.002
rac- threo	0/6	0.2 (0.4)	6.7	2.3 (1.8)	93.3	2.5 (1.9)	0 (0)	0	90.2	9.10	0.003	100.0	10.02	0.003

KW, Kruskal Wallis *t*-value; SD, standard deviation.
* Total worm burden reduction significantly higher ($p < 0.05$) than the total worm burden reduction of the (+)- erythro enantiomer.

in vitro (Karle et al., 1993), whereas *in vivo* similar activities were observed (unpublished observation).

A possible explanation for these findings might be that the threo compounds undergo a metabolic chiral inversion *in vivo* to the erythro forms. These conversions are known for many drugs, for example ibuprofen (Adams et al., 1976; Geisslinger et al., 1989; Müller et al., 1990; Reddy et al., 2007). While (−) R ibuprofen is less active than the antipode and the racemate *in vitro*, no differences in activities were observed *in vivo*, which has been explained by an enzymatic inversion of the (−) R to the (+)- S isomer (Knihinicki et al., 1989).

In *Plasmodium* spp., the mode of action of MQ has been described as a disturbance of haemoglobin metabolism due to inhibition of the haematin polymerisation and formation of haemozoin (Dorn et al., 1998). The process of haemozoin crystallization in order to detoxify haem, which derived from haemoglobin digestion, was also demonstrated in *S. mansoni* and might be an interesting target for antischistosomal chemotherapy (Oliveira et al., 2000). Our results point to two different modes of action of MQ isomers: a haemin independent pathway, since high activities on *S. mansoni*, in particular NTS, were observed in the absence of haemin *in vitro*, and a haemin-dependent mechanism of action,

explained by the more pronounced activity *in vitro* once haemin is added and *in vivo*.

In the present study, microcalorimetry has been used for the first time to examine drug effects on schistosomes. This technique has been previously applied to investigate the metabolism of microorganisms, insects, snails, small reptiles like lizards and the effects of several types of antimicrobial agents (Lamprecht and Becker, 1988; Braissant et al., 2009), and biological, physical, or chemical processes (Koenigbauer, 1994). The results presented here show that microcalorimetry can be a valuable tool to study antischistosomal drugs. The microcalorimetric measurements confirmed the results of the phenotypic evaluation, i.e., that the erythro derivatives possess prominent activities against *S. mansoni in vitro*, which are even further exacerbated in the presence of haemin. It is noteworthy that contradictory results were obtained with the threo derivatives in the presence of haemin between microscopic and calorimetric evaluation. While microscopic evaluation showed faster death of schistosomes with these drugs in this medium, microcalorimetry showed a slower reduction of heat production and decreased mortality rates of worm survival in the presence of haemin. These contradictory findings might be due to false interpretation of worm death, defined as absence of movement for 2 min by microscopy, in particular as in our study only one reader examined the drug-incubated worms. Microcalorimetry, on the other hand, had measured the heat flow quantitatively every 10 min during the whole course of the experiment and is thus characterized by high sensitivity. The level of agreement between microscopy and microcalorimetry warrants further investigation; hence further studies are ongoing in our laboratories evaluating the use of microcalorimetry in antischistosomal drug discovery including reference drugs, i.e., praziquantel.

In conclusion, the optical isomers and racemates of MQ show only moderate stereoselectivity, in particular *in vivo* since prominent activities were observed with all derivatives in the *S. mansoni* mouse model. None of the MQ derivatives tested offers a therapeutic advantage over MQ. Indeed, the highest *in vivo* antischistosomal activities were observed with the erythro-racemate, the marketed drug MQ. Since the pharmacodynamic effect of both erythro isomers are similar and might contribute to overall antischistosomal activity a separation of the enantiomers is not needed from a therapeutical point of view. In addition, a recent Phase I study has shown a similar overall safety and tolerability profile of the (+)- erythro enantiomer and the racemate MQ and terminated the development of (+)- erythro MQ (http://www.treague.com/MMV_Treague_Web_Announcement%20_Jan10_Final.pdf). Further laboratory and clinical studies on the antischistosomal properties of MQ are warranted.

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5. Isothermal Microcalorimetry To Study Drugs against *Schistosoma mansoni*

Theresia Manneck[1,2], Olivier Braissant[3], Yolanda Haggenmüller[1,2] and Jennifer Keiser[1,2]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH–4002 Basel, Switzerland,
[2] University of Basel, Petersplatz 1, CH-4003 Basel, Switzerland
[3] Laboratory of Biomechanics and Biocalorimetry, c/o Biozentrum/Pharmazentrum, University of Basel, Basel, Switzerland

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Isothermal Microcalorimetry To Study Drugs against *Schistosoma mansoni*[▽]

Theresia Manneck,[1,2] Olivier Braissant,[3] Yolanda Haggenmüller,[1,2] and Jennifer Keiser[1,2]*

Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland[1]; University of Basel, Petersplatz 1, CH-4003 Basel, Switzerland[2]; and Laboratory of Biomechanics and Biocalorimetry, c/o Biozentrum/Pharmazentrum, University of Basel, Klingelbergstrasse 50-70, 4056 Basel, Switzerland[3]

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Alternative antischistosomal drugs are required since praziquantel is virtually the only drug available for treatment and morbidity control of schistosomiasis. Manual microscopic reading is the current "gold standard" to assess the *in vitro* antischistosomal properties of test drugs; however, it is labor-intensive, subjective, and difficult to standardize. Hence, there is a need to develop novel tools for antischistosomal drug discovery. The *in vitro* effects of praziquantel, oxamniquine, artesunate, and mefloquine on metabolic activity and parasite motility of *Schistosoma mansoni* (newly transformed schistosomula [NTS] and 49-day-old adult worms) were studied using isothermal microcalorimetry (IMC). Results were compared to morphological readouts of viability. Results obtained for the four drugs tested with phenotypic evaluation by microscopy and IMC showed a good correlation, but IMC also identified drug effects that were not visible by microscopic evaluation, and IMC precisely determined the onset of action of the test drugs. Similar sensitivities on NTS and adult schistosomes were observed for praziquantel and mefloquine, while slight differences in the drug susceptibilities of the two developmental stages were noted with oxamniquine and artesunate. IMC is a useful tool for antischistosomal drug discovery that should be further validated. In addition, our data support the use of NTS in *in vitro* antischistosomal drug assays.

Schistosomiasis, caused by blood flukes of the genus *Schistosoma*, is an important health problem affecting 779 million people mainly in sub-Saharan Africa (36). Praziquantel is virtually the only drug available and is increasingly deployed in mass drug administration programs, advocated by the World Health Organization (11). The annual estimated need for praziquantel in Africa exceeds 400 million tablets (http://www.who.int/wormcontrol/newsletter/PPC7_Eng_min.pdf). Two alternative drugs, oxamniquine and metrifonate, exist; however, they are rarely used today as they have a rather narrow activity profile and multiple doses have to be administered (12, 37). The extensive use and reliance on one single agent have raised concerns about the emergence of praziquantel resistance (3, 8), and indeed, schistosome strains with increased drug tolerance have already been isolated from patients as well as selected in the laboratory (9, 26). Therefore, there is growing consensus that novel antischistosomal drugs should be discovered and developed (8, 15, 33).

Antischistosomal drug discovery at many academic institutions (e.g., the Special Programme for Research and Training in Tropical Diseases; screening centers in London, United Kingdom, and Cairo, Egypt; the University of California, San Francisco, Sandler Center in San Francisco, CA; and the Swiss Tropical and Public Health Institute [Swiss TPH] in Basel, Switzerland) is based on *in vitro* whole-organism drug screening assays followed by *in vivo* tests in infected mice (15, 31). Stages used for *in vitro* assays are 1- to 7-day-old schistosomula (newly transformed schistosomula [NTS]) and juvenile and adult schistosomes.

The current "gold standard" is to assess worm viability microscopically and to evaluate drug effects with regard to death, changes in motility, viability, and morphological differences (15). However, microscopic techniques are often very labor-intensive and hence do not allow medium or high throughput, have to be carried out by well-trained personnel, and have a subjective nature. In addition, they are difficult to standardize and do not provide any information about the possible drug target and/or the mechanism of action of the drugs (1). Several studies have therefore been launched in the recent past. These have explored novel tools to facilitate the readout of *in vitro* drug screening on schistosomes, such as fluorescence-labeled albumin (14), fluorophores (propidium iodide and fluorescein diacetate) (29), and colorimetric assays with alamarBlue (25).

In the present work, we studied the effects of the 4 schistosomicidal compounds, praziquantel, oxamniquine, artesunate, and mefloquine, on the metabolic activity and parasite motility of *Schistosoma mansoni* (NTS and 49-day-old adult worms) using isothermal microcalorimetry (IMC). IMC measures the heat flow of biological processes (endo- or exothermic reactions) over time and has already been applied in various disciplines, including studies on food deterioration, drug shelf life (34), microbial processes, and the effects of different antimicrobial agents (6, 39). We have already demonstrated the applicability of this method evaluating the antischisto-

* Corresponding author. Mailing address: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. Phone: 41 61 284 8218. Fax: 41 61 284 8105. E-mail: jennifer.keiser@unibas.ch.

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somal properties of mefloquine isomers (23). The whole-organism microscopic assay served as a reference.

MATERIALS AND METHODS

Parasites. Cercariae of *S. mansoni* were harvested from infected intermediate-host snails (*Biomphalaria glabrata*) maintained at laboratories at the Swiss TPH after exposure to light for 3 h. Female NMRI mice ($n = 20$; age, 3 weeks; weight, ~35 g) were purchased from Harlan Laboratories (Horst, Netherlands).

Drugs. Praziquantel was purchased from Sigma, oxamniquine was obtained from Q. Bickle (London School of Hygiene and Tropical Medicine, London, United Kingdom) and M. J. Doenhoff (University of Nottingham, Nottingham, United Kingdom), artesunate was the product of Dafra Pharma (Turnhout, Belgium), and mefloquine hydrochloride was provided by Mepha Pharma AG (Aesch, Switzerland).

Drugs were dissolved in dimethyl sulfoxide (DMSO; Fluka, Buchs, Switzerland) to obtain stock solutions of 10 mg/ml. Stocks were kept at 4°C for a maximum of 6 months.

Preparation of NTS. Cercariae of *S. mansoni* were mechanically transformed into NTS (30) as described previously (15, 24). NTS were kept in medium 199 (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (iFCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO_2 in ambient air for a minimum of 3 to 12 h until use. This incubation period ensured that conversion from cercariae to NTS was completed.

Preparation of adult *S. mansoni*. NMRI mice were infected subcutaneously with approximately 200 *S. mansoni* cercariae. After 7 to 8 weeks, mice were killed with CO_2 and dissected, and all schistosomes were removed from the hepatic portal system and the mesenteric veins. Worms were washed 3 times with phosphate-buffered saline (PBS; pH 7.4), placed in RPMI 1640 culture medium, and kept at 37°C in an atmosphere of 5% CO_2 until use. All culture media used were supplemented with 5% iFCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Drug sensitivity assay with microscopic evaluation. Stock solutions of praziquantel, oxamniquine, artesunate, and mefloquine were diluted (Costar) in culture medium (medium 199 for NTS and RPMI 1640 medium for adult worms supplemented with 5% iFCS, 100 U/ml penicillin, and 100 μg/ml streptomycin) in 24-well plates, yielding final concentrations of 100, 10, and 1 μg/ml. One hundred microliters of an NTS suspension containing 100 to 1,000 NTS or 2 adult male and 2 adult female worms were added to each well. Worms incubated in medium containing 1% DMSO served as controls. Parasite fitness was assessed before and 3, 17, 24, 48, and 72 h after drug incubation at 37°C in 5% CO_2 using a dissecting microscope (magnification, 8- to 40-fold; Carl Zeiss AG, Germany). Drug effects were evaluated using a viability scale, as described previously (15, 24), which classifies parasite fitness and motility with scores ranging from 4 (hyperactive, increased motility) and 3 (normal activity, no morphological changes) to 0 (all worms dead). All experiments were carried out in duplicate and were repeated at least three times.

Microcalorimeter. A 48-channel isothermal microcalorimeter (model TAM 48; TA Instruments, New Castle, DE) was used to measure the heat production of schistosomes over 96 h. The instrument was thermostated at 37°C at least 2 days before the experiment to achieve maximum stability. A simple drug injection system (Fig. 1) designed and manufactured by the Laboratory of Biomechanics and Biocalorimetry (University of Basel, Basel, Switzerland) was employed. It offers the advantage of minimal thermal disturbance of the sample and minimal loss of data compared to those that occur by removing the ampoule from the microcalorimeter, injecting the drug, and reintroducing the ampoule into the microcalorimeter.

Sample preparation. Glass ampoules were filled with 800 μl medium 199 and 100 μl NTS suspension containing 100 to 1,000 NTS for initial validation experiments and 400 to 1,000 NTS for the main studies. For adult worm assay, ampoules were filled with 900 μl RPMI 1640 medium and 3 to 9 schistosomes for preliminary validation studies and 3 or 4 parasites for the main experiments. All materials used were autoclaved at 121°C for 20 min, and media and drug solutions were sterile filtered (pore size, 0.2 μm). Ampoules were sealed and placed into the measuring channels. After a stable signal (equilibration) was obtained, medium or drug dilutions (reaching final concentrations of 100, 10, and 1 μg/ml) were injected into the ampoules. Heat flows were recorded at a frequency of 1 data point per 10 min for a minimum of 96 h. All samples were assayed in triplicate.

Heat-flow analysis. To investigate drug effects on the metabolic activity of schistosomes, heat-flow curves of NTS and adult *S. mansoni* were examined before and after drug addition. The levels of heat production of treated and untreated parasites (adjusted to their corresponding starting values) were compared at 24 and 72 h after drug injection.

FIG. 1. Injection system for IMC. 1, microcalorimeter tube; 2, rod with insulating rings; 3, prefilled 0.5-ml syringe (medium/drugs) (Becton Dickinson, NJ); 4, ampoule with schistosomes; 5, thermoelectric module.

Motor activity analysis. We analyzed the amplitudes of the random oscillations occurring in heat-flow curves of adult *S. mansoni* to study drug effects on the motor activity of adult worms. Increased amplitudes compared to the machine average signal noise reflect worm motor activities (20, 23). The endpoint of worm motility (reflecting death of worms) was determined by the intersection of the sample amplitude curve (exponential decay) with the background signal noise of the microcalorimeter (0.4 to 0.55 μW). Since 3 or 4 schistosomes were present in each ampoule, the heat flow obtained and the motor activity observed in the calorimetric data reflect the overall motor activity of these worms, which is presented as delayed response due to the calorimeter time constant (21).

Statistics. Heat-flow values and parasite motility (heat-flow random oscillations) of treated and untreated worms were calculated as means (± standard deviations) using Microsoft Excel software. The statistical significance of the means was assessed using the parametric paired *t* test at a 5% level of significance (StatsDirect statistical software, version 2.7.2.; StatsDirect Ltd., Cheshire, United Kingdom). Analyses of noise amplitudes were performed by using R software (32) and Microsoft Excel.

RESULTS

Drug sensitivity assay with NTS and microscopic evaluation. The time-effect curves of praziquantel, oxamniquine,

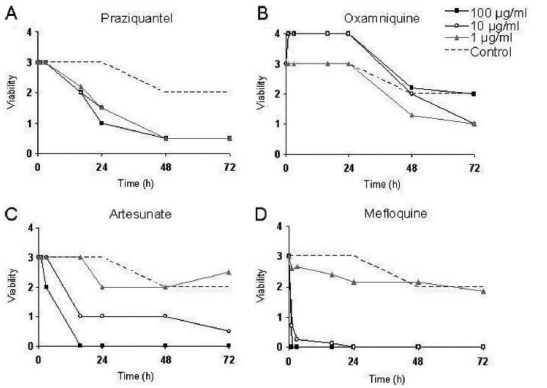

FIG. 2. Viability of *S. mansoni* NTS following *in vitro* treatment with praziquantel (A), oxamniquine (B), artesunate (C), and mefloquine (D) (100, 10, and 1 μg/ml) over time. Mean values of viability (viability score) were derived from a minimum of 6 experiments.

artesunate, and mefloquine against NTS evaluated microscopically for up to 72 h are shown in Fig. 2A to D. Morphological changes induced by these drugs are depicted in Fig. 3. Untreated control NTS were viable for at least 72 h. In the presence of praziquantel (Fig. 2A and 3A), NTS showed decreased levels of viability, granularity, and tegumental alterations, such as extensive blebbing, at all concentrations tested (100, 10, and 1 μg/ml), but none of the concentrations tested resulted in the complete absence of motility. Treatment of NTS with 100 and 10 μg/ml oxamniquine (Fig. 2B and 3B) increased worm motility for 24 h; at later examination points, motility was similar to that of the controls (100 μg/ml) or slightly reduced (10 μg/ml). Moderate granularity and swollen bodies were observed. Incubation with 1 μg/ml oxamniquine resulted in slightly decreased viability of NTS. None of the oxamniquine concentrations were lethal over the examination period of 72 h. NTS exposed to 100 μg/ml of artesunate (Fig. 2C and 3C) died within 17 h. Incubation with 10 μg/ml artesunate steadily reduced parasite viability and caused granularity and swollen bodies, resulting in minimal viability after 72 h. At 1 μg/ml, artesunate showed no effect. In the presence of 100 μg/ml mefloquine (Fig. 2D and 3D), all NTS died immediately. Incubation with a 10-fold lower concentration of mefloquine caused granularity and decreased viability and death of NTS at 24 h postincubation. No effect was observed at 1 μg/ml of mefloquine (23).

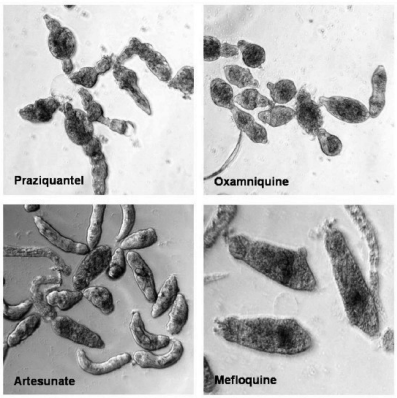

FIG. 3. Light microscopic observations of *S. mansoni* NTS after *in vitro* incubation with 10 μg/ml praziquantel, oxamniquine, and artesunate for 72 h and 10 μg/ml mefloquine for 24 h.

Adult *S. mansoni*. Temporal effects of praziquantel, oxamniquine, artesunate, and mefloquine on adult *S. mansoni* are depicted in Fig. 4A to D. Control schistosomes remained viable over the entire observation period of 72 h. Schistosomes treated with praziquantel (Fig. 4A) showed immediate contractions at any of the concentrations tested (100, 10, and 1 μg/ml) and the absence of motility, and worms had a dark, shrunken appearance. Only basic movements such as occasional twitching of the female sucker were observed. Worms exposed to 100 μg/ml of oxamniquine (Fig. 4B) showed increased motor activity for 48 h, while concentrations of 10 and 1 μg/ml oxamniquine did not influence parasite viability. The highest artesunate concentration (100 μg/ml) tested caused death of adult worms after 72 h; however, worms treated with 10 and 1 μg/ml artesunate were vital without any morphological changes for up to 72 h following incubation (Fig. 4C).

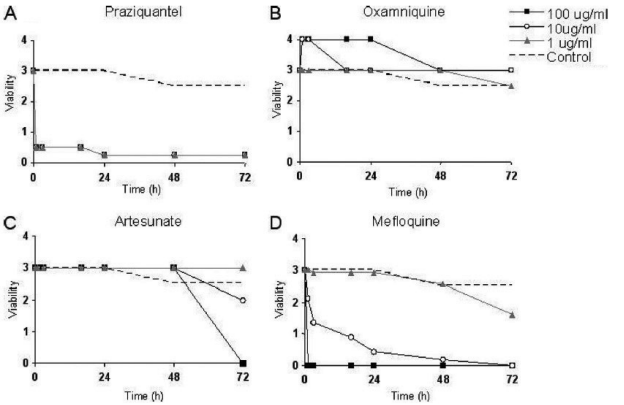

FIG. 4. Viability of adult *S. mansoni* following *in vitro* treatment with praziquantel (A), oxamniquine (B), artesunate (C), and mefloquine (D) (100, 10, and 1 μg/ml) over time. Mean values of viability (viability score) were derived from a minimum of 6 experiments.

Exposure to 100 μg/ml of mefloquine (Fig. 4D) resulted in immediate death of all schistosomes. Mefloquine at 10 μg/ml highly affected worm viability within the first 24 h, and all worms were dead after 72 h of incubation. Incubation with the lowest mefloquine concentration (1 μg/ml) resulted in decreased activity after 72 h (23).

Comparison of drug susceptibilities of NTS and adult schistosomes by microscopic evaluation. Since antischistosomal drug discovery often relies on the schistosomular stage, we compared the *in vitro* responses (viability) of NTS and adult schistosomes to praziquantel, oxamniquine, artesunate, and mefloquine at 24 h and 72 h after drug incubation (Fig. 5). This comparison shows that drug sensitivities of NTS and adult worms were similar following praziquantel incubation, though praziquantel showed a slightly faster effect on adults. Similarly, comparable *in vitro* responses of NTS and adults were obtained following exposure to mefloquine. On the other hand, NTS were more affected by oxamniquine (hypermotility after 24 h, viability decrease after 72 h) and artesunate than adult worms.

IMC. (i) Preliminary studies. To determine whether IMC is able to monitor the heat flow of schistosomes over time and therefore can be used for antischistosomal drug screen-

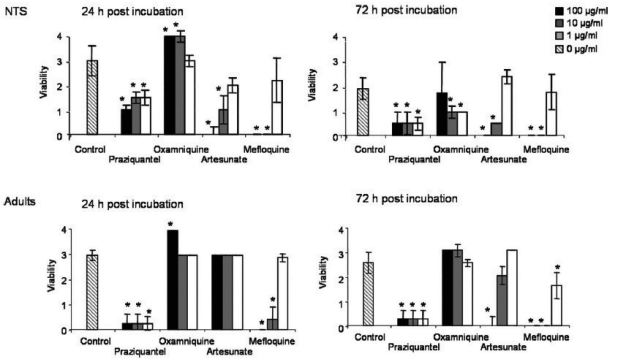

FIG. 5. *In vitro* response (viability) of 2 developmental stages of *S. mansoni* (NTS and adults) to praziquantel, oxamniquine, artesunate, and mefloquine 24 h and 72 h after drug incubation. Asterisk, significant difference between control and drug of interest ($P < 0.05$).

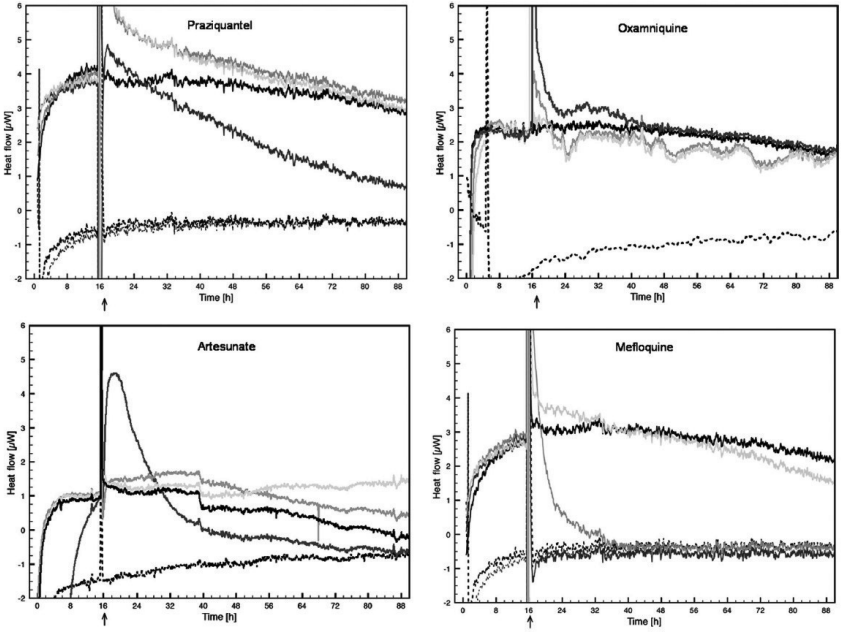

FIG. 6. Heat-flow curves of *S. mansoni* NTS exposed to 3 different concentrations of praziquantel, oxamniquine, artesunate, and mefloquine *in vitro*. Black lines, negative control (untreated NTS); dark gray dotted lines, background control (culture medium); light gray dotted lines, positive control (dead NTS); dark gray lines, 100 μg/ml; medium gray lines, 10 μg/ml; light gray lines, 1 μg/ml. Data shown are mean values from 3 experiments. Arrows indicate the time of medium or drug injection.

ing, initial experiments with different numbers of NTS and adult *S. mansoni* worms were carried out (in duplicate). Ampoules containing culture medium only or dead worms showed low signals of −1.4 μW to 0.4 μW. No difference in the thermogenic profiles of culture medium and medium containing dead worms was observed. One hundred to 200 NTS did not yield any detectable signal (comparable to that for medium alone). Increasing the number of NTS to 400 to 1,000 worms per vial resulted in heat-flow values of 0.4 to 4.3 μW (mean, 2.4 μW), respectively. NTS did not show any motility-related random oscillations in their thermogenic curves. Consistently higher heat-flow values of 5.4 to 9.6 μW were obtained following the examination of 3 to 9 adult *S. mansoni* worms. A 50% decrease in the heat flow of adult *S. mansoni* worms was observed after about 46 h, possibly due to the lack of oxygen (23). Adult worms showed random oscillations in the heat-flow signals for 88 h. After injection of medium to NTS or adult worms, heat flow as well as motility-related random oscillations remained stable. A low interindividual variability was observed. Based on these results, we decided to run experiments for 72 h after drug injection and to use 400 to 1,000 NTS or 3 or 4 adult worms per sample, as these numbers produced readily detectable signals and were in the same range as those in our microscope assays.

(ii) IMC of NTS. Heat-flow curves of culture medium (background control), dead NTS (positive controls), untreated NTS (negative controls), and NTS exposed to praziquantel, oxamniquine, artesunate, and mefloquine (100, 10, and 1 μg/ml) are shown in Fig. 6.

Exposure to praziquantel at any of the 3 concentrations tested (100, 10, and 1 μg/ml) resulted in an immediate increase of heat flow (metabolic activation). At 72 h after injection of praziquantel, the metabolic activity of NTS was still detectable, with worms exposed to 100 μg/ml characterized by a decreased heat flow and worms treated with 10 and 1 μg/ml showing increased heat flow in comparison to that for control worms. When 100 μg/ml of oxamniquine was added to NTS, metabolic activation was observed for approximately 24 h; at later examination points, no effect on the heat flow was observed. Exposure to 10 and 1 μg/ml oxamniquine caused an alternating

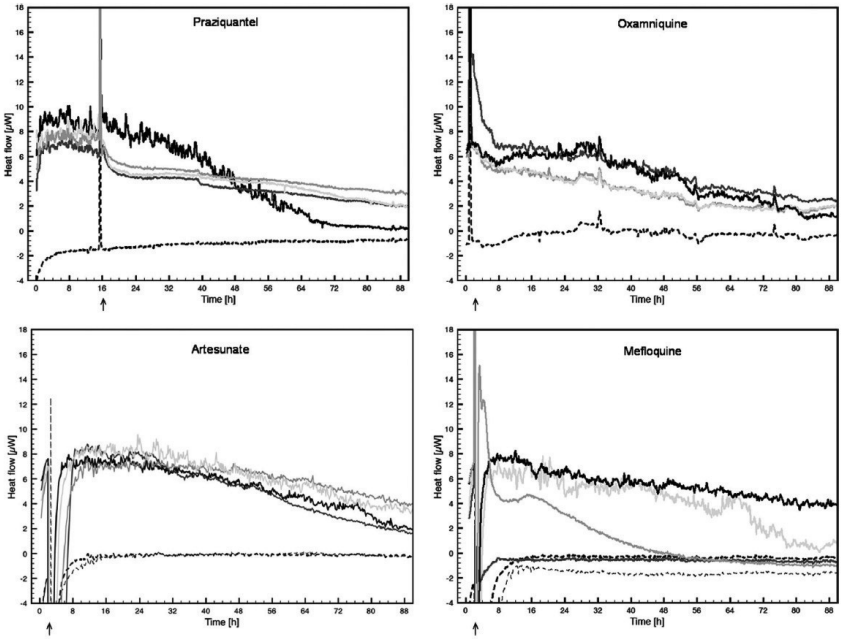

FIG. 7. Heat-flow curves of adult *S. mansoni* worms treated with 3 different concentrations of praziquantel, oxamniquine, artesunate, and mefloquine *in vitro*. Black lines, negative control (untreated schistosomes); dark gray dotted lines, background control (culture medium); light gray dotted lines, positive control (dead schistosomes); dark gray lines, 100 μg/ml; medium gray lines, 10 μg/ml; light gray lines, 1 μg/ml. Data shown are mean values from 3 experiments. Arrows indicate the time of medium or drug injection.

increase and decrease of heat production of approximately 0.7 μW over the entire course of the experiment. The heat flow of NTS increased significantly for about 5 h after injection of 100 μg/ml artesunate. From this time point onwards, the heat flow decreased rapidly, and at approximately 24 h after drug addition, only 10% metabolic heat production was detectable. Heat-flow curves of NTS treated with 10 and 1 μg/ml artesunate showed slightly higher signals than control curves. Following the addition of 100 μg/ml mefloquine to NTS, the heat production of NTS decreased immediately and worms died within 1 h. Metabolic activity of NTS was rapidly reduced by 50% at 2 h postexposure to 10 μg/ml of mefloquine. At 8 h following incubation with 10 μg/ml of mefloquine, NTS did not reveal any heat production (23). When 1 μg/ml of mefloquine was added to NTS, a slight decrease of heat production was observed, in particular, at 48 to 72 h after drug injection.

(iii) IMC of adult *S. mansoni*. Heat-flow curves of medium (background control), dead schistosomes (positive controls), adult control worms (negative controls), and *S. mansoni* worms treated with praziquantel, oxamniquine, artesunate, and mefloquine (100, 10, and 1 μg/ml) are presented in Fig. 7.

No differences in the heat profiles of adult *S. mansoni* worms were seen following exposure to 100, 10, and 1 μg/ml of praziquantel. Heat-flow random oscillations, which reflect parasite motility, stopped immediately ($P < 0.001$) after praziquantel addition, and during the first 8 to 10 h after injection, the heat flow was reduced by approximately 30%. Afterwards the heat flow continued to decrease slowly. When 100 μg/ml oxamniquine was injected into the ampoules, metabolic activation was observed for approximately 4 h, followed by a steady decrease of heat production. Decreased heat flow was also observed for schistosomes exposed to 10 and 1 μg/ml oxamniquine. Oscillation analysis revealed a significant difference ($P < 0.01$) between 1 μg/ml oxamniquine-treated and control worms. Worms treated with 100 μg/ml artesunate were characterized by an absence of detectable random oscillations after 21 h ($P < 0.01$). Heat-flow curves of schistosomes treated with 10 and 1 μg/ml artesunate showed the same heat and oscillation profiles as the controls.

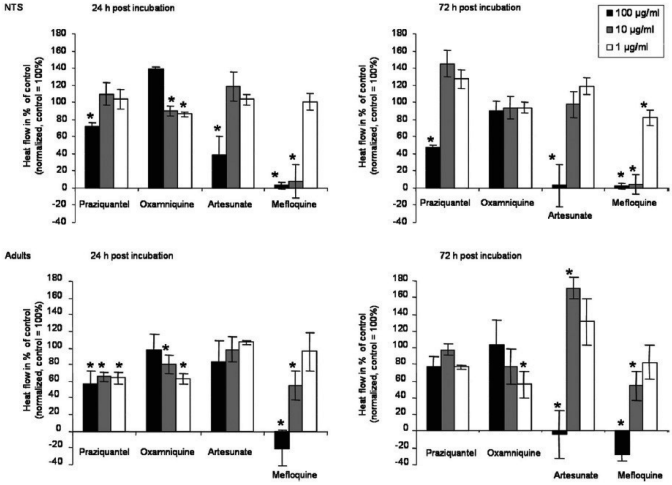

FIG. 8. *In vitro* response (heat flow) of two developmental stages of *S. mansoni* (NTS and adults) to praziquantel, oxamniquine, artesunate, and mefloquine at 24 h and 72 h after drug addition. Asterisk, significant difference between control and drug of interest ($P < 0.05$).

Following injection of 100 µg/ml mefloquine, heat-flow curves decreased rapidly, reaching baseline values within 1 h. Random oscillations were not detectable ($P < 0.001$). Mefloquine at 10 µg/ml provoked a 50% heat-flow reduction during the first 24 h postexposure, whereas oscillations disappeared immediately after drug addition ($P < 0.001$). No effect on the heat flow of adult *S. mansoni* was observed with 1 µg/ml of mefloquine (23), while parasite motility was affected significantly ($P < 0.01$).

Comparison of drug susceptibilities of NTS and adult schistosomes by IMC. Figure 8 illustrates the heat production of NTS and adult worms at 24 h and 72 h following incubation with the four drugs tested. Heat production of control worms was set to 100%. This comparison shows that praziquantel influenced the metabolic activity of NTS and adult schistosomes at all concentrations tested. However, differences in drug response were observed: while heat production of adult parasites decreased significantly ($P < 0.05$) by 43.1% (100 µg/ml), 34.5% (10 µg/ml), and 35.6% (1 µg/ml) during the first 24 h, the heat flow of NTS decreased only in the presence of 100 µg/ml praziquantel ($P < 0.01$). Lower praziquantel concentrations had no significant effect on the metabolic heat of NTS. Both developmental stages of *S. mansoni* also showed variations in the drug response toward oxamniquine: at 24 h postincubation, activated heat production was observed for NTS in the presence of 100 µg/ml and significant decreases were observed in the presence of 10 µg/ml ($P < 0.05$) and 1 µg/ml ($P < 0.01$). At 72 h, the metabolic activities were comparable to that of the controls. On the other hand, reduced heat flow was observed for adult worms in parallel to decreasing oxamniquine concentrations used. Differences between NTS and adult worms treated with artesunate were also observed and were particularly visible with the 2 lowest concentrations tested and at the 72-h examination point: while NTS showed only slight increases in heat production, adult *S. mansoni* worms revealed a strong increase in heat flow, which was significant for 10 µg/ml ($P < 0.05$). Finally, mefloquine showed a similar antischistosomal effect on NTS and adults using IMC, with NTS being slightly more susceptible to the drug.

DISCUSSION

Microscopy is the current gold standard to evaluate drug effects on schistosomes. However, the method is rather labor-intensive and less accurate due to the fact that it is prone to subjective results and interpretations (15). In the present study, we evaluated whether IMC, a highly sensitive, accurate, and simple tool to detect heat produced by microorganisms (6), could be used to study the *in vitro* activity of antischistosomal compounds. We were motivated to examine well-described antischistosomal drugs with IMC based on promising preliminary findings obtained with this technique analyzing the antischistosomal properties of 6 mefloquine isomers and racemates (23). In addition, in the framework of the present work, we thoroughly compared drug effects on the schistosomular and adult stages, as the former developmental stage is increasingly being used in antischistosomal drug screening assays (1, 15).

Comparing the performance of the two diagnostic techniques, IMC showed two key advantages over microscopy. First, the onset of action of all drugs tested could precisely be determined with IMC. For two of the drugs (mefloquine, artesunate), the onset of action on adult schistosomes was detected earlier by IMC than by microscopic evaluation. Heat-flow random oscillation analysis revealed an immediate parasite immobility following exposure of adult *S. mansoni* worms to 10 μg/ml mefloquine and after 21 h following exposure to 100 μg/ml artesunate. By microscopy, decreased viabilities were observed only after 24 h for mefloquine and after 72 h for artesunate. Second, drug effects, which were not visible by microscopic evaluation, were recorded by calorimetric measurements. For example, while an absence of motility of adult schistosomes was recorded by IMC 7 h after oxamniquine treatment (1 μg/ml), no effect was observed with microscopy. The faster and more accurate detection of drug activity by IMC is not surprising, as IMC provides a continuous real-time electronic signal proportional to the amount of heat produced (38), while worms are monitored by microscopy at selected time points only (in our study, 1 or 2 times per day). Third, IMC is characterized by a high sensitivity (200 nW) (5) and is therefore able to capture small changes in the thermal output of schistosomes, which corresponds to metabolism and motility, while morphological damages and death are the key criteria assessed by microscopy (31). Hence, while microscopy reliably detects severely damaging and lethal effects of drugs, IMC also readily detects more subtle drug effects, as demonstrated for oxamniquine and for low concentrations of mefloquine. Finally, another advantage of IMC, though not done in the present study, is that IC_{50}s can be exactly determined by analyzing parasite motility (amplitudes in thermogenic curves), which would facilitate the comparison of antischistosomal activities of different standard and experimental drugs.

Although IMC seems to have many advantages in the evaluation of antischistosomal drugs, potential drawbacks have to be noted. First, all materials used need to be sterile, since contamination (e.g., bacterial growth) would be recorded by the calorimeter (6). Second, heat-flow curves obtained by IMC reflect the overall activity of the worms. It is not possible to distinguish between worm contractions and increased movement, because both physiological processes result in increased metabolic activity with heat production. Third, the present cost of a microcalorimeter is much higher than that of a regular microscope. Therefore, unless substantially less expensive instruments become available, it is unlikely that IMC will be widely used, and its use will be restricted to well-equipped laboratories in high-income countries. Fourth, in order to achieve analyzable signals, a large number of NTS (at least 400) were required per sample. Finally, the IMC used in this study allows the independent evaluation of just 48 measuring channels, and several hours of data must be accumulated. Hence, IMC does not allow high-throughput evaluation of drugs, i.e., thousands of samples per week.

An interesting difference observed between the two methods was that microscopy revealed more drug effects on NTS than calorimetric assessment. This finding might be explained by the nonspecificity of IMC, as described above, on the one hand (the net signal corresponds to the sum of all processes occurring in the ampoule), and the procedure of cercaria transformation into NTS, with a certain amount of cercariae and tails remaining, on the other hand. The impurities of cercariae and tails do not affect microscopy, as they can be disregarded during observation. However, cercariae and tails also produce heat-flow signals, which might disturb the heat-flow signal of the parasites of interest. Furthermore, the drug susceptibilities of cercarial stages can be different from those of schistosomula (13, 28), resulting in false-positive or -negative signals. Another explanation might be that the drugs analyzed impact motility and morphology (which are not detectable on NTS by IMC) rather than the metabolic activity of NTS.

Today, schistosomula are often used in *in vitro* studies, as large numbers of this developmental stage can be obtained in a cost-effective manner and the use of live animals is not required (1, 15). The transformation from cercariae to schistosomula by the vortexing (30) used in our labs is convenient and easy to perform. Similar advantages have been described for the syringe transformation (1). It is interesting to note that schistosomula show different viabilities depending on the transformation method (whether cercariae are mechanically transformed or actively penetrate skin) applied (data not shown). Schistosomula derived by vortex transformations lose some of their fitness and become granular and less active within 24 h posttransformation, but afterwards the viability remains relatively steady (Fig. 2). It is believed that the application of mechanical stress as the only key transformation factor might not be sufficient to induce different biochemical modifications, such as an emptying of acetabular glands or shedding of the glycocalyx (7, 35), which might be crucial for the further survival of the parasite. In our study, regardless of whether IMC or microscopy was used, the overall sensitivities to 2 of the 4 drugs tested (praziquantel and mefloquine) were similar on schistosomula produced by vortex transformation and adult *S. mansoni* worms. Differences in the drug susceptibilities were noted with oxamniquine and artesunate. NTS seemed to be slightly more susceptible to these drugs than adults. The slightly lower susceptibility of adult *S. mansoni* worms than NTS to artesunate might be due to the lack of hemin, a primary activator of the peroxide group, in the incubation medium (41). Overall, the findings on the individual parasite stages are consistent with previous *in vitro* studies with these drugs (2, 10, 24, 27, 40).

In conclusion, in line with previous studies (1), our data support the use of NTS in antischistosomal drug screening assays and indicate that IMC is a useful tool for antischistosomal drug discovery. IMC may be particularly helpful in the characterization of new hits. The method should be further validated using additional antischistosomal drugs, which ideally are characterized by distinct mechanisms of action (e.g., nilutamide or related hydantoin derivatives) at different concentrations (18). In order to overcome the limitation of the low throughput of the widely used 48-channel IMC, either chip calorimeters (4, 22) or prototypes of 96-well-plate calorimeters should be further developed and adapted. These calorimeters are presently used for different biophysical applications. IMC might also be useful to distinguish drug effects on resistant and sensitive schistosome strains, in line with a recently developed bioassay based on the praziquantel susceptibility of miracidiae (19). Finally, it is likely that IMC is widely applicable to a range of helminths and their developmental stages. Hence, drug effects on other helminths, for example, hookworms, should be

monitored with IMC, as the number of drugs currently available to treat these infections is too small and drug screening approaches are limited (16).

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We declare that we have no competing interests. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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6. Interactions of mefloquine with praziquantel in the *Schistosoma mansoni* mouse model and *in vitro*

Jennifer Keiser[1,2] , Theresia Manneck[1,2] and Mireille Vargas[1,2]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, CH-4002 Basel, Switzerland;
[2]University of Basel, CH-4003 Basel, Switzerland

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Interactions of mefloquine with praziquantel in the *Schistosoma mansoni* mouse model and *in vitro*

Jennifer Keiser[1,2]*, Theresia Manneck[1,2] and Mireille Vargas[1,2]

[1]*Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, CH-4002 Basel, Switzerland;* [2]*University of Basel, CH-4003 Basel, Switzerland*

*Corresponding author. Tel: +41-61-284-8218; Fax: +41-61-284-8105; E-mail: jennifer.keiser@unibas.ch

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Objectives: Mefloquine has interesting antischistosomal properties, hence it might be an attractive partner drug for combination treatment with praziquantel. The aim of this study was to evaluate activities of mefloquine/praziquantel combinations against *Schistosoma mansoni in vitro* and *in vivo*.

Methods: Dose–response relationships were established following exposure of adult *S. mansoni* to mefloquine, praziquantel and fixed dose combinations of mefloquine/praziquantel *in vitro*. *S. mansoni*-infected mice were treated orally with selected doses of single drugs and drug combinations 7 weeks post-infection.

Results: We calculated *in vitro* LC_{50} values of 0.024 and 1.9 μg/mL for praziquantel and mefloquine, respectively. Mefloquine/praziquantel combinations showed synergistic effects, with combination index (CI) values <1 when adult *S. mansoni* were simultaneously incubated with both drugs *in vitro*. Reduced viabilities were also observed when schistosomes were first exposed to mefloquine followed by praziquantel *in vitro*. ED_{50}s of 62 mg/kg and 172 mg/kg were determined for mefloquine and praziquantel against adult *S. mansoni in vivo*, respectively. Combinations of praziquantel (50 or 100 mg/kg) followed the next day by mefloquine (50 or 100 mg/kg) treatment revealed only moderate total worm burden reductions of 47.8%–54.7%. On the other hand, when both drugs (100 mg/kg each) were either given simultaneously or mefloquine was given prior to praziquantel, high total and female worm burden reductions of 86.0%–93.1% were observed. For the later treatment regimen, synergistic effects (CI < 1) were calculated when mefloquine and praziquantel were combined using a fixed dose ratio based on their ED_{50}s.

Conclusions: Combinations of mefloquine and praziquantel may have clinical utility in the treatment of schistosomiasis.

Keywords: schistosomiasis, combination chemotherapy, activity, combination index, isobolography

Introduction

In the treatment of tuberculosis, cancer or malaria, drugs are often given in combination to increase their therapeutic advantages.[1–3] The clinical effect of a combination of two drugs should either be the sum (additive behaviour) or ideally even exceed (synergy) the individual effect of each drug. On the other hand, for adverse events, antagonism (the effect of the two drugs being less than the effect of each drug) is preferable. In addition, combination chemotherapy is a viable therapeutic strategy to delay the development of drug resistance.[4]

There is scarce information available as to whether antischistosomal drug combinations provide an increased therapeutic efficacy over monotherapy. A few clinical trials have evaluated praziquantel plus oxamniquine combinations and combinations of praziquantel with an artemisinin derivative.[5,6] In the laboratory, combinations of 'something old' (praziquantel) with 'something new', e.g. novel experimental drugs such as Ro 15-5458[7] or nilutamide,[8] were studied. However, the great disadvantage of these combinations (involving novel drug candidates) is the long drug development process (12–15 years), and therefore the associated high costs, in the order of $1 billion.[9]

Another possibility for an antischistosomal drug combination might be a polytherapy with praziquantel and the antimalarial drug mefloquine. Several laboratory studies have demonstrated interesting antischistosomal properties of mefloquine. For example, a single 200 mg/kg oral dose of mefloquine achieved a worm burden reduction of 72% in mice harbouring a chronic *Schistosoma mansoni* infection.[10] In addition, in a randomized, exploratory open-label trial in Côte d'Ivoire in *Schistosoma*

haematobium-infected schoolchildren, a mefloquine/artesunate combination achieved a cure rate of 61% and an egg reduction rate of 95%.[11]

The aim of the present study was to evaluate the effect of mefloquine/praziquantel combinations against *S. mansoni in vitro* and *in vivo*. A preliminary study has already pointed to significant worm burden reductions following treatment with praziquantel plus mefloquine in mice infected with *Schistosoma japonicum*.[12] We determined whether the potency of this drug combination is additive, antagonistic or synergistic.[13] In addition, we analysed whether mefloquine/praziquantel combinations should be given simultaneously or in sequence.

Materials and methods

Animals and parasites

Female NMRI mice ($n=125$, age = 3 weeks, weight ~35 g), obtained from Harlan Laboratories (Horst, The Netherlands), were kept under standard conditions (temperature, ~25°C; humidity, 70%; 12 h light and 12 h dark cycle) with free access to water and rodent diet in accordance with the Swiss national and cantonal regulations on animal welfare. Experiments were approved by the local veterinary agency (permit 2070). *S. mansoni* cercariae (Liberian strain) were collected after exposing *Biomphalaria glabrata* to light for 3 h. Mice were infected subcutaneously with ~80 cercariae.

Drugs

Praziquantel was purchased from Sigma (Buchs, Switzerland), and mefloquine hydrochloride was kindly provided by Mepha Pharma AG (Aesch, Switzerland). For *in vitro* studies, drugs were dissolved in 100% DMSO (Fluka, Buchs, Switzerland) to obtain stock solutions of 10 mg/mL. For *in vivo* studies, drugs were prepared as suspensions in 7% (v/v) Tween 80 and 3% (v/v) ethanol before oral administration to mice (10 mL/kg).

In vitro assay procedures

Preparation of adult S. mansoni *and culture conditions*

Forty-nine-day-old adult schistosomes, removed by picking from the hepatic portal system and mesenteric veins from infected NMRI mice, were washed with PBS (pH 7.4) and kept in RPMI 1640 culture medium [supplemented with 5% inactivated fetal calf serum (iFCS) and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA)] at 37°C in an atmosphere of 5% CO_2 until use.

Combination chemotherapy studies on adult S. mansoni *in vitro*

In a first step, the lethal concentrations (LC_{100}) that kill all schistosomes within 72 h of *in vitro* drug exposure and the median effect concentrations (LC_{50}) were determined for praziquantel and mefloquine. Drugs were serially diluted in 24-well plates (Costar) in RPMI 1640 culture medium and 2 male and 2 female worms were added to each well. Praziquantel concentrations of 1, 0.5, 0.2, 0.1, 0.05, 0.01 and 0.001 μg/mL and mefloquine concentrations of 10, 9, 6, 5, 2, 1, 0.5, 0.2, 0.1 and 0.01 μg/mL were tested. Each drug concentration was assessed in duplicates and repeated once ($n=12$ worms/drug concentration). For the interaction studies, mefloquine and praziquantel were added simultaneously in a first experiment at a fixed dose ratio based on the calculated LC_{50} values (1.9 μg/mL for mefloquine and 0.024 μg/mL for praziquantel) and 2-fold dilutions were carried out up and down (7.6 and 0.1 μg/mL; 3.8 and 0.05 μg/mL; 1.9 and 0.024 μg/mL; 0.95 and 0.0125 μg/mL; 0.475 and 0.006 μg/mL; and 0.238 and 0.003 μg/mL of mefloquine and praziquantel, respectively). In addition, we studied a 5-fold dilution of the LC_{50} value (combination of 0.03 μg/mL mefloquine and 0.0004 μg/mL praziquantel). Worms were incubated at 37°C and 5% CO_2 for 72 h, their viabilities recorded using a microscope (8–40-fold magnification; Carl Zeiss AG, Germany) and the mean viability of the 12 examined worms calculated as described previously.[14] Worms were classified as dead if no movement was observed for 2 min and worms had a dark colour. In a second experiment, schistosomes were exposed to LC_{50}:LC_{50} (1.9 μg/mL for mefloquine and 0.024 μg/mL for praziquantel) and 0.5 LC_{50}:0.5 LC_{50} (0.95 μg/mL for mefloquine and 0.0125 μg/mL for praziquantel) and drug addition was spaced by the respective half-life of the drugs in mice.[15,16] In more detail, schistosomes were exposed to (i) praziquantel followed by mefloquine 1 h post-incubation and (ii) mefloquine followed by praziquantel 17 h post-exposure. The viabilities of these worms were assessed 72 h after drug incubation. Worms incubated in medium containing the highest solvent concentration used (1% DMSO) served as controls in all experiments.

In vivo studies

Monotherapy

Forty-nine days post-infection, groups of 6–11 mice were treated orally with subtherapeutic single oral doses of mefloquine (50 and 100 mg/kg) and praziquantel (50, 100, 150 and 200 mg/kg). Untreated mice served as controls. At 21 days post-treatment, animals were killed by the CO_2 method and dissected. Worms were removed by picking, then sexed and counted as described in previous publications.[17] For the calculation of the ED_{50} values, worm burden reductions obtained in recent experiments with effective doses of mefloquine (200 mg/kg) and praziquantel (400 mg/kg) were included.[10,17]

Effect of treatment schedule

We evaluated whether the administration schedule has an influence on the activity of the drug combination. Six groups of mice were treated with combinations of mefloquine and praziquantel (50 mg/kg mefloquine plus 50 mg/kg praziquantel, 100 mg/kg mefloquine plus 100 mg/kg praziquantel) administered either simultaneously or on subsequent days (mefloquine followed by praziquantel or praziquantel followed by mefloquine). Untreated mice were included as controls. Worms were recovered as described above.

Effect of drug interactions

To determine the combination dose effect, four groups of mice were treated with combinations based on their ED_{50}s (1:2.8 ratio and 2-fold dilutions up and down). The treatment was administered on subsequent days (mefloquine followed by praziquantel), as this regimen has shown the highest activity in our experiments *in vitro* and *in vivo*. Three weeks post-treatment, mice were killed and processed as described above.

Scanning electron microscopy study

We collected adult *S. mansoni* 72 h post-treatment from three mice as described above, which had been treated with (i) mefloquine (60 mg/kg), (ii) praziquantel 170 mg/kg, and (iii) mefloquine (60 mg/kg) followed on the next day by praziquantel (170 mg/kg). Worms were fixed with 2.5% (v/v) glutaraldehyde in PBS (pH 7.4) for several hours. The schistosomes

were then washed twice with double-distilled water, dehydrated in ascending ethanol concentrations and critically point dried (Bomar SPC-900; Tacoma, WA, USA). Finally, worms were sputter coated with 20 nm gold particles and observed using a high-resolution scanning electron microscope (Phillips XL30 ESEM) at an accelerating voltage of 5 kV.

Statistical analysis

LC_{50} and ED_{50} values, combination index (CI), dose reduction index (DRI) and isobologram plots were calculated using the CompuSyn software package (ComboSyn, Paramus, NJ, USA). LC_{50} plots were drawn using *XLfit*® (xlfit5, IDBS, Guildford, UK). We used the Kruskal–Wallis (KW) test to compare the medians of the worm burdens in the monotherapy versus combination chemotherapy treatment groups [version 2.4.5 Statsdirect (Cheshire, UK)]. A difference in median was considered to be significant at a significance level of 5%.

Results

In vitro studies

Determination of LC_{50} values of monotherapy

The dose–response curves of mefloquine and praziquantel are depicted in Figure 1. LC_{50} values of 0.024 and 1.9 μg/mL were calculated for praziquantel and mefloquine, respectively. The corresponding LC_{75} and LC_{95} values are 0.04 and 0.11 μg/mL for praziquantel and 3.4 and 9.2 μg/mL for mefloquine, respectively.

Simultaneous drug administration

In Figure 1, the dose–response curve of adult *S. mansoni* exposed simultaneously to praziquantel and mefloquine (LC_{50}:LC_{50}) *in vitro* is shown. Figure 2 illustrates the combination dose effect using an isobologram.

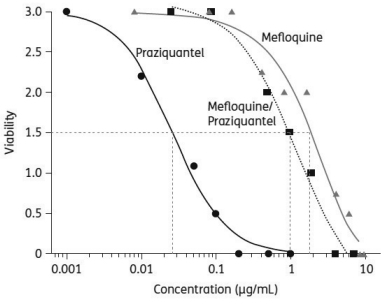

Figure 1. Dose–response curves of mefloquine and praziquantel and combined mefloquine/praziquantel (LC_{50}:LC_{50}) against adult *S. mansoni* *in vitro* using a viability score.[17] The broken line represents the concentration required to achieve a medium effect level (LC_{50}). Data points represent mean values of viabilities of three assay wells (12 schistosomes) for each drug concentration and combination.

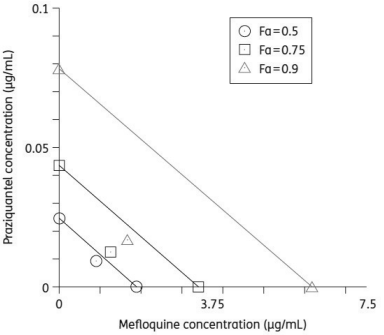

Figure 2. Isobologram showing the synergistic interaction of a mefloquine/praziquantel combination using the LC_{50}:LC_{50} ratio *in vitro* at the LC_{50}, LC_{75} and LC_{90}. Fa, fraction affected.

Praziquantel/mefloquine combinations showed a synergistic effect on adult *S. mansoni*, with CI values <1 (CI 0.87 at the LC_{50} and 0.40 at the LC_{95}). To achieve a 50% reduction of schistosome viability the praziquantel and mefloquine concentrations could be reduced 2.6- and 2.1-fold, respectively.

Spaced drug administration

When praziquantel and mefloquine were added to the *in vitro* cultures with a time lag corresponding to their respective half-lives, only schistosomes exposed first to mefloquine and 17 h later to praziquantel were affected and showed reduced viabilities (a viability of 1 for the combination of 1.9 μg/mL mefloquine and 0.024 μg/mL praziquantel and a viability of 1.5 for the combination of 0.95 μg/mL mefloquine and 0.0125 μg/mL praziquantel) within 72 h. Parasites exposed to praziquantel for 1 h followed by mefloquine revealed only a slight loss of viability (viability of 2.0 for both combinations tested) in comparison with the untreated controls.

In vivo studies

ED_{50} calculation for mefloquine and praziquantel

For the ED_{50} calculation we also included results obtained from previous experiments in our laboratories.[10,17] Treatment of *S. mansoni*-infected mice with 50, 100 or 200 mg/kg mefloquine resulted in total worm burden reductions of 44.1, 64.0 (Table 1) and 93.4%,[17] respectively. We calculated an ED_{50} of 62 mg/kg and an ED_{95} of 262 mg/kg.

Worm burden reductions of 13% (50 mg/kg; Table 1) up to 96% (400 mg/kg)[10] were observed following treatment with praziquantel. Praziquantel given at 172 mg/kg and 592 mg/kg is estimated to achieve worm burden reductions of 50% and 95%, respectively.

Table 1. Effect of praziquantel and mefloquine monotherapies administered at single oral doses of 50–100 mg/kg and praziquantel/mefloquine combinations (50/50 and 100/100 mg/kg) following three different treatment schedules to mice harbouring a 49-day-old *S. mansoni* infection

	Drug	Dose	No. of mice investigated	No. of mice cured	Mean number of worms (SD)			Total worm burden reduction (%)	Female worm burden reduction (%)
					total	males	females		
Control	—	—	20	—	24.7 (14.7)	14.1 (7.6)	10.7 (7.6)		
Monotherapy	praziquantel	50	4	0	21.5 (10.5)	10.0 (3.4)	11.5 (7.2)	13.0	0
		100	6	0	21.0 (9.5)	11.5 (5.8)	9.5 (3.8)	15.0	11.2
	mefloquine	50	11	0	13.8 (9.4)	8.0 (11.6)	5.8 (4.2)	44.1*	45.8
		100	9	0	8.9 (3.9)	6.4 (3.4)	2.4 (1.4)	64.0*	77.6*
Combination chemotherapy, simultaneous application	praziquantel/mefloquine	50/50	8	0	18.3 (8.5)	10.6 (5.7)	7.6 (3.1)	25.9	29.0
	praziquantel/mefloquine	100/100	8	1	3.5 (3.9)	2.1 (2.9)	1.4 (1.1)	86.0*	86.9
Combination chemotherapy, mefloquine followed by praziquantel	praziquantel/mefloquine	50/50	8	0	9.5 (6.8)	5.0 (3.6)	4.5 (3.3)	61.5	58.0
	praziquantel/mefloquine	100/100	9	4	1.7 (2.0)	0.9 (1.1)	0.8 (1.0)	93.1**	93.0*
Combination chemotherapy, praziquantel followed by mefloquine	praziquantel/mefloquine	50/50	8	0	12.9 (7.8)	6.6 (4.3)	6.3 (3.7)	47.8	41.1
	praziquantel/mefloquine	100/100	9	0	11.2 (8.8)	8.2 (6.6)	3.0 (3.1)	54.7	72.0

*$P<0.05$.
**$P<0.001$.

Effect of combination treatment regimen on efficacy

Based on the results obtained in our *in vitro* experiments, which showed differences depending on the treatment schedule used (simultaneous or spaced incubation), we were interested whether these findings could also be documented *in vivo*. Mice were divided in three groups. Group 1 was treated with both drugs simultaneously, group 2 was treated with mefloquine followed by praziquantel 24 h later, and group 3 was treated with praziquantel followed by mefloquine 24 h later. We used subtherapeutic doses of 50 and 100 mg/kg for each of the drugs.

The results are summarized in Table 1. When mefloquine and praziquantel were administered simultaneously at doses of 50 mg/kg each, low total (25.9%) and female (29.0%) worm burden reductions were observed, which were even lower than worm burden reductions observed with mefloquine (50 mg/kg) alone. When both drugs were given at 100 mg/kg simultaneously, high total and female worm burden reductions of 86.0% ($P=0.014$) and 86.9% ($P=0.091$), respectively, were calculated.

High significant worm burden reductions (total worm burden reduction of 93.1% ($P<0.001$) and female worm burden reduction of 93.0% ($P=0.008$) were observed when mice were treated with 100 mg/kg mefloquine followed by 100 mg/kg praziquantel 24 h later. When half of these doses were given, total and female worm burden reductions decreased to 61.5% and 58.0%, respectively.

Combinations of praziquantel (50 or 100 mg/kg) followed the next day by mefloquine (50 or 100 mg/kg) treatment revealed only moderate total worm burden reductions of 47.8%–54.7%, which were similar to the results obtained with mefloquine monotherapy.

Combination dose–effect analysis

We used a constant ratio design based on the ED_{50}s of both drugs (1:2.8) to analyse whether a mefloquine/praziquantel combination reveals additive, antagonistic or synergistic effects. Since the highest worm burden reductions were obtained when praziquantel followed mefloquine administration, this treatment schedule was employed to determine the combination dose effect. Significant total worm burden reductions of 91.8% and 97.8% were observed at the two highest concentrations tested (Table 2). At a dose of 30 mg/kg mefloquine and 85 mg/kg praziquantel, total and female worm burden reductions of 51.6% and 48.8%, respectively, were observed, which were not statistically significant. At the lowest dose tested (15 mg/kg mefloquine, 42.5 mg/kg praziquantel), low total and female worm burden reductions of 19.2% and 19.8%, respectively, were achieved. The ED_{50} dose of the combination was calculated as 101.8 mg/kg (26.8 mg/kg mefloquine and 75.1 mg/kg praziquantel, corresponding to a 2.3-fold dose reduction for each drug). A CI of 0.87 was determined at the median dose–effect level. At the ED_{75}, ED_{90} and ED_{95}, CI values were below 0.8. Hence, at the dose ratio for combined praziquantel and mefloquine (1:2.8), synergistic interactions were observed.

Scanning electron microscopy study

As expected, only very mild tegumental alterations (a handful of blebs, swelling and fusion of the tegumental ridges) were

Table 2. Effect of praziquantel/mefloquine combinations using a constant ratio design based on the ED_{50}s administered to mice harbouring a 49-day-old *S. mansoni* infection

Dose (mg/kg)	No. of mice investigated	No. of mice cured	Mean number of worms (SD)			Total worm burden reduction (%)	Female worm burden reduction (%)
			total	males	females		
Control —		—	15.2 (6.3)	8.0 (2.9)	7.2 (3.5)	—	—
Combination chemotherapy, mefloquine followed by praziquantel 15 (mefloquine) and 42.5 (praziquantel)	4	0	12.3 (3.3)	6.7 (1.3)	5.5 (2.1)	19.2	19.8
30 (mefloquine) and 85 (praziquantel)	4	0	7.3 (1.2)	3.7 (0.6)	3.7 (0.6)	51.6	48.8
60 (mefloquine) and 170 (praziquantel)	4	2	1.3 (1.5)	0 (0)	1.3 (1.5)	91.8*	82.6*
120 (mefloquine) and 340 (praziquantel)	4	3	0.3 (0.6)	0.3 (0.6)	0 (0)	97.8*	100*

*$P < 0.05$.

observed on adult schistosomes collected from mice treated with 60 mg/kg mefloquine and 170 mg/kg praziquantel, respectively (Figure 3a–d). Many worms revealed no tegumental damage. On the other hand, the majority of worms had already been expelled from a mouse treated with a combination of mefloquine and praziquantel 72 h post-treatment. Only a single worm could be recovered that showed extensive blebbing on its mid-body (Figure 3e).

Discussion

In several medical fields the search for effective drug combinations has been recognized as an important strategy for a successful treatment outcome and to delay drug resistance.[1–3] To our knowledge, we have performed the first analysis of the pharmacodynamic interactions of mefloquine/praziquantel combinations against *S. mansoni*. *In vitro* and *in vivo* studies were conducted, which allow assessment of drug combinations in much more detail than do clinical studies. We used isobologram and CI analyses, which are popular methods to analyse drug interactions of combination chemotherapy.[13] It is interesting to note that although several studies have analysed the *in vitro* and *in vivo* antischistosomal efficacy of praziquantel combinations, including the effect of combined treatment of mefloquine and praziquantel against *S. japonicum*,[12] in depth modelling of dose–effect relationships, defining additive effects, synergy or antagonism of these combinations have not been carried out to date.

Synergistic interactions were observed in the *S. mansoni* mouse model and *in vitro* when praziquantel was combined with mefloquine. This finding is encouraging since the control of schistosomiasis, a chronic and debilitating disease, relies on a single drug, praziquantel.[6,18] The need to develop alternative treatment options, including drug combinations, has been repeatedly emphasized because the development of a praziquantel-resistant schistosome strain is a threat.[6,19]

It has been suggested to analyse a series of different fixed dose ratios in combination treatment experiments to confirm whether two drugs behave additively or synergistically, since it has been shown that the effect might depend on the ratio of the drugs used.[20] For the evaluation of mefloquine/praziquantel combinations *in vivo* we used the median effect analysis only.[13] *In vitro*, however, a range of different dose ratios (including fixed ratios based on the LC_{50} presented here) were tested and analyses of these data confirmed the synergistic properties of the mefloquine/praziquantel combinations (data not shown).

It is interesting to note that the best results were achieved in the *S. mansoni* mouse model when praziquantel treatment followed mefloquine. On the other hand, only moderate worm burden reductions were achieved when praziquantel was administered prior to mefloquine. These findings were also observed *in vitro*. The effects of praziquantel on schistosomes might play a role in the antagonistic effects observed when praziquantel was administered first. Exposure of schistosomes to praziquantel results in a calcium-dependent contraction of the musculature, an increase in tension and a disrupted tegument of the worms.[21,22] One could speculate that due to the paralysis and damage of worms caused by praziquantel, the uptake of mefloquine by schistosomes might be decreased, resulting in lower

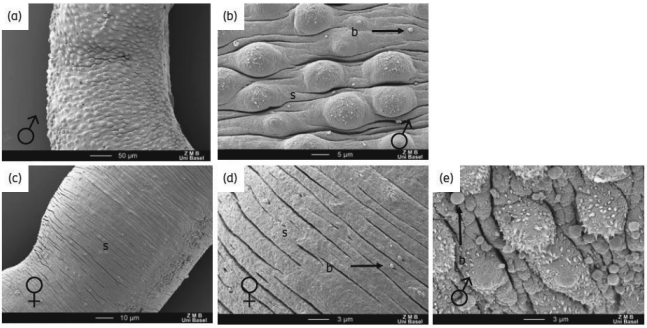

Figure 3. Scanning electron microscopy of adult *S. mansoni* recovered from mice 72 h after the administration of a single 170 mg/kg oral dose of praziquantel (a and b) and 60 mg/kg mefloquine (c and d). (a) Mid-body of a male specimen treated with 170 mg/kg praziquantel. (b) Higher magnification of (a). Blebbing (b) and swelling (s) observed. (c) Mid-body of a female specimen treated with 60 mg/kg mefloquine. (d) Higher magnification of (c). Blebbing (b) and swelling (s) visible. (e) Extensive blebbing observed on a male worm following a combination of 60 mg/kg mefloquine and 170 mg/kg praziquantel administered on subsequent days.

activities of the drug combination. On the other hand, the mechanism of action of mefloquine on schistosomes has not yet been elucidated. However, *in vivo* and scanning electron microscopy studies have shown that adult schistosomes exposed to mefloquine were not affected immediately and only died after 24–72 h.[23] Hence, mefloquine-treated schistosomes might still be able to interact with praziquantel, resulting in increased activities of mefloquine/praziquantel combinations.

Since our *in vitro* and *in vivo* findings are encouraging, exploratory randomized open-label trials have been launched to investigate the effect of mefloquine/praziquantel combinations in schistosome-infected children. We will treat children first with mefloquine or mefloquine/artesunate (3 day regimen, in line with the common malaria treatment schedule), followed by praziquantel on day 4, since this treatment schedule achieved the highest activity *in vitro* and *in vivo*. In addition, the advantage of a spaced application of the drugs (in contrast to simultaneous administration) is that this treatment regimen does not raise regulatory and review challenges that combination products would require.[24] In a first step we will administer the standard doses (mefloquine 25 mg/kg, artesunate/mefloquine 300/250 mg and praziquantel 40 mg/kg); however, since synergistic interactions were observed in the present study *in vivo*, we are hoping in a next step to be able to lower the doses of these drugs to decrease the prevalence of adverse events and also costs. Of note, we have not included artesunate in this *in vitro* and *in vivo* study, as a preliminary experiment in our laboratory has shown similar worm burden reductions (both 86%) of mefloquine/praziquantel/artesunate (all 100 mg/kg administered simultaneously) compared with mefloquine/praziquantel (both 100 mg/kg simultaneously) in mice harbouring adult *S. mansoni*. Our result is in line with numerous experiments, which have documented a greater sensitivity of juvenile schistosomes towards the artemisinins than the adult worm in laboratory animals.[25] However, why increased cure rates have been observed with mefloquine/artesunate over mefloquine in our previous study in *S. haematobium*-infected children cannot be explained at the moment, as only moderate cure rates were observed in the group of children treated with artesunate.[11]

In conclusion, we have demonstrated that a combination of mefloquine/praziquantel reveals synergistic behaviour in the treatment of *S. mansoni*-infected mice and *in vitro*. The effect of mefloquine/praziquantel combinations on *S. haematobium* should be also studied in detail. To assess the clinical utility of this drug combination in the treatment of schistosomiasis, proof-of-concept studies in schistosome-infected children have been launched.

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Transparency declarations

None to declare.

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7. Mefloquine interferes with glycolysis in schistosomula of *Schistosoma mansoni* via inhibition of enolase

Theresia Manneck[1,2], Jennifer Keiser[1,2], Joachim Müller[3]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, 4002 Basel, Switzerland
[2] University of Basel, Petersplatz 1, 4003 Basel, Switzerland
[3] Institute of Parasitology, University of Berne, Länggass-Strasse 122, 3012 Berne, Switzerland

Mefloquine interferes with glycolysis in schistosomula of *Schistosoma mansoni* via inhibition of enolase

THERESIA MANNECK[1,2], JENNIFER KEISER[1,2]* *and* JOACHIM MÜLLER[3]

[1] *Department of Medical Parasitology and Infection Biology, Socinstrasse 57, Swiss Tropical and Public Health Institute, CH-4051 Basel, Switzerland*
[2] *University of Basel, Petersplatz 1, CH-4003 Basel, Switzerland*
[3] *Institute of Parasitology, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland*

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SUMMARY

The antimalarial drug mefloquine has promising antischistosomal properties killing haematophagous adult schistosomes as well as schistosomula. The mode of action and involved drug targets of mefloquine in *Schistosoma mansoni* schistosomula are unknown. In order to identify mefloquine-binding proteins and thus potential drug targets, mefloquine affinity chromatography with *S. mansoni* schistosomula crude extracts was performed. We found one specific mefloquine-binding protein that was identified by mass spectrometry as the glycolytic enzyme enolase (Q27877). Enolase activity assays were performed on schistosomula crude extracts and on the recombinant enolase Q27877 expressed in *Escherichia coli*. In schistosomula crude extracts enolase activity was inhibited by mefloquine and by the enolase inhibitor sodium fluoride, while activity of the recombinant enolase was not affected. In contrast to enolase from crude extracts, recombinant Q27877 did not bind to mefloquine-agarose. Using isothermal microcalorimetry, we next investigated the metabolic inhibition of mefloquine and 3 known glycolytic inhibitors in *Schistosoma* spp., namely sodium fluoride, 3-bromopyruvate and menadione on schistosomula in the presence or absence of glucose. We found that in the presence of glucose, schistosomula were less affected by mefloquine, sodium fluoride and 3-bromopyruvate, whereas glucose had no protective effect when schistosomula had been exposed to menadione. These results suggest a potential role of mefloquine as an inhibitor of glycolysis, at least in stages where other targets like haem degradation are not relevant.

Key words: *Schistosoma mansoni*, affinity chromatography, mefloquine, drug target, enolase, metabolic inhibition, isothermal microcalorimetry, glycolysis.

INTRODUCTION

Antischistosomal chemotherapy is still restricted to the use of praziquantel. The antimalarial drug mefloquine, a synthetic analogue of quinine has promising antischistosomal activity *in vitro* and *in vivo* (Keiser *et al*. 2009, 2010; Xiao *et al*. 2011). The mode of action and the target of mefloquine in *Schistosoma* spp. are, however, not known. Knowledge of the drug target might, however, aid in the design and discovery of novel antischistosomal drugs (Renslo and McKerrow, 2006; Sleno and Emili, 2008).

There is good evidence that in *Plasmodium* spp. mefloquine interferes with haemoglobin degradation. Toxic haem is released when the parasite feeds on haemoglobin. The haem is rapidly oxidized to haemin and subsequently converted to haemozoin. In the presence of mefloquine the conversion of free haem to haemozoin is inhibited. As a consequence, toxic haem and haemin complexes accumulate and kill the parasite (Dorn *et al*. 1998; Zhang *et al*. 1999; Pasternack *et al*. 2010).

Schistosomes feed on blood and haem is detoxified into large insoluble crystals identical to *Plasmodium* haemozoin (Oliveira *et al*. 2000). Therefore, a similar mode of action of mefloquine in both parasites is probable. A recently conducted *in vitro* study suggests that mefloquine interferes with the haem detoxification pathway in *Schistosoma mansoni*, since a more pronounced activity of the drug was observed against *S. mansoni* in the presence of haemin (Manneck *et al*. 2011*a*). However, mefloquine also shows high *in vitro* activities on 1-day-old *S. mansoni* schistosomula and on adult schistosomes without addition of haemin or red blood cells. In fact, RNAi studies have shown that schistosomula show an extensive uptake of exogenous material into the gut immediately after transformation of cercariae into schistosomula. Haemoglobin-derived pigment is present in the gut of 2-day-old schistosomula, but there is no evidence for blood uptake at earlier time-points (Stefanic *et al*. 2010). This suggests that the drug may have targets independent of haemoglobin digestion.

The purpose of the present work was to identify targets for mefloquine in 1-day-old, most likely

* Corresponding author: Department of Medical Parasitology and Infection Biology, Socinstrasse 57, Swiss Tropical and Public Health Institute, CH-4051 Basel, Switzerland. Tel: +41 61 284 8218. Fax: +41 61 284 8105. E-mail: jennifer.keiser@unibas.ch

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the suspension was washed once in coupling buffer ($NaHCO_3$ 0·1 M, pH 9·5). After the last wash, epoxy-agarose was suspended in 2 ml of coupling buffer. Twenty mg mefloquine were dissolved in 2 ml of dimethyl-formamide and added to the agarose. The mixture was incubated for 3 days at 37 °C under slow but continuous shaking in order to allow coupling of the epoxy group to mefloquine via the OH- group of the piperidine-2-yl-methanol part of the molecule. For the negative control a mock column was prepared by blocking a column with ethanol-amine only (Müller *et al*. 2008). Prior to the runs, both columns (bed volumes ca. 1 ml each) were combined in a tandem (mock column first, then mefloquine column) and washed with 50 ml of PBS equilibrated at 20 °C. Then 3 ml of schistosomula crude extract was loaded onto the column tandem at a flow rate of 0·25 ml/min. The columns were washed with PBS until a flat baseline was detected (more than 10 column volumes). The columns were separated, and proteins binding to the columns were eluted with 1 mM mefloquine in PBS followed by elution with a pH shift (glycine Cl^- 100 mM, pH 2·9) in order to remove non-specifically bound proteins. Moreover, fractions were taken before elution with mefloquine (pre-mefloquine) and following a pH shift (pre-pH shift). Sizes of these fractions ranged between 3 and 5 ml. Aliquots from each fraction (200 μl) and from the crude extract (50 μl) were taken and analysed by SDS-PAGE. SDS-PAGE and silver staining were performed as summarized in a recent publication (Müller *et al*. 2008).

The protein identified in the mefloquine eluate only was then further analysed by mass spectrometry. For protein sequencing by mass spectrometry, protein was prepared (Müller *et al*. 2008) and sequenced by the Proteomics Core Facility, Faculty of Medicine, University of Geneva, Switzerland.

Cloning and heterologous expression of recombinant S. mansoni *enolase*

Cloning, heterologous expression and His-tag purification of recombinant enolase were carried out as described (Müller *et al*. 2008). Briefly, in order to clone enolase into the His-tag-expression vector pET151 directional TOPO (Invitrogen, Carlsbad, CA), the primers pENOfor (CACCATGTCCATTTTTAACGATCCAC) and pENOrev (TTATACTTTGGGATGGCGGAAG) were created for the amplification of a 1260 base pair product encoding the enolase (Q27877) polypeptide with 4 additional bases at the 5′end allowing directional cloning (MWG Biotech, Ebersberg, Germany). cDNA was created from adult *S. mansoni* following a protocol by Lochmatter *et al*. (2009) and PCR was performed using Phusion Polymerase and the appropriate protocol (Phusion TM DNA Polymerase, High Fidelity PCR KIT, Finnzymes). The resulting product was cloned into pET151 TOPO vector and transformed into *E. coli* TOP 10 cells (Invitrogen). Heterologous expression of enolase in positive transformants of *Escherichia coli* (BL21 Star) and subsequent his-tag purification of recombinant enolase was performed as previously described (Müller *et al*. 2008). Purified protein was stored in 50% glycerol at −20 °C.

Enolase assay

Enolase activity was determined using an enzymatic assay coupled to pyruvate kinase and L-lactate dehydrogenase by measuring the conversion of NADH to NAD by the latter enzyme according to a protocol of Sigma-Aldrich. The assay was performed in a 96-well-plate containing a total volume of 250 μl in each well and 80 mM triethanolamine buffer (pH 7·4), 0·12 mM ß-NADH, 25 mM magnesium sulfate, 100 mM potassium chloride solution, 1·3 mM ADP, 7 U/ml pyruvate kinase, 10 U/ml of lactate dehydrogenase and enolase (crude extracts or recombinant, 2–5 μl). The reaction was initiated by adding the enolase substrate 2-phosphoglycerate (0–1·8 mM). Blanks without enolase and blanks without substrate served as controls.

For inhibition studies, mefloquine and sodium fluoride were added as indicated in Fig. 3 of the results section (24–240 μM). All samples were assessed in triplicate. The decrease of absorbance of NADH was recorded for 20 min at 340 nm by continuous photometric rate determination (VersaMax™ Absorbance Microplate Reader, Molecular Devices, Sunnyvale, California, USA). Effects of mefloquine on pyruvate kinase and lactate dehydrogenase were monitored by adding phosphoenolpyruvate (final concentration of 1·8 mM) instead of enolase and 2-phosphoglycerate to the reaction mix.

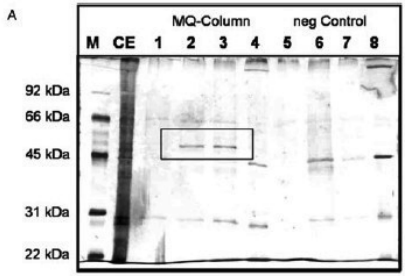
Statistics

Pairwise *t*-tests were performed using the StatsDirect statistical software package (version 2.7.2., StatsDirect Ltd; Cheshire, UK). K_m and V_{max} values were determined after regression analysis by the corresponding software tool contained in the Excel software package (Microsoft, Seattle, Washington, USA).

RESULTS

Enolase is a major mefloquine-binding protein in S. mansoni *schistosomula*

Since mefloquine was found to affect the non-haematophagous schistosomula (Manneck *et al*. 2010) we aimed to identify the cellular target of this



B

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SCHJA  MAIIAIHARQIFDSRGNPTVEVDLKTAKGLFRAAVPSGASTGVHEALELRDTNSKAYMCKGVLTAVSNVNNIIAPALLKKQIPVTHQSEVDQFMIELDGKENKGNLGANA 110
FASHE  MAIKAIHARQIFDSRGNPTVEVDVTTAKGLFRAAVPSGASTGVHEALELRDG-PPGYMGKGVLKAVANVNSQIAPNLIKSGINVTDQAAVDKFMLDLDGTPNKEKLGANA 110
HUMAN  MSILKIHAREIFDSRGNPTVEVDLFTSKGLFRAAVPSGASTGIYEALELRDNDKTRYMGKGVSKAVESHINKTIAPALVSKKLNVTEQEKIDKLMIEMDGTENKSKFGANA 110

SCHMA  ILGVSLAVCKAGAAEAGLPLYRYIARLAGHEDVIMPVPAFNVINGGSHAGNKLAMQEFMILPTGASSFTEAMQIGTEVYHNLKAVIKEKEYGLDACNVGDEGGFAPNIQDN 220
SCHJA  ILGVSLAVCKAGAAELNLPLYRYIAKLAGHKDVIMPVPAFNVINGGSHAGNKLAMQEFMILPTGASSFTEAMQMGSEVYHNLKAVIKEKEFGLDACNVGDEGGFAPNIQDN 220
FASHE  ILGVSLAXCKAGAAEKGLPLYKYIATLAGNKEVIMPVPSFNVINGGSHAGNKLAMQEFMIMPTGASSFTEAMKIGSEVYHNLRAVIKSKYGLDACNVGDEGGFAPSIQDN 220
HUMAN  ILGVSLAVCKAGAVEKGVPLYRHIADLAGNSEVILPVPAFNVINGGSHAGNKLAMQEFMILPVGAANFREAMRIGAEVYHNLKNVIKEKYGKDATNVGDEGGFAPNILEN 220

SCHMA  MKGLQLLEEAIKIAGYTGKVEIGMDCAASEFHKNGKYDLDFKNPHSAESTWLSPDAMANMYKQMISKFPIVSIEDPFDQDDWETWPKLTSSTNIQIVGDDLTVTNPKRIK 330
SCHJA  MKGLQLLEEAIKIAGYTGKVEIGMDCAASEYYKKGKYDLDFKNPQSAESHWLSPDEMANVYKEMIQKYPIVSIEDPFDQDDWDAWPKLTASTNIQIVGDDLTVTNPKRIE 330
FASHE  LEGLELLRTAIDKAGYTGKVXIAMDCAASEFYKEGKYDLDFKNPKSQASSWITSDAMADVYKKMMSTYPIVSIEDPFDQDDWPAWTKLTGECKIQIVGDDLTVTNPLRVQ 330
HUMAN  KEGLELLKTAIGKAGYTDKVVIGMDVAASEFFRSGKYDLDFKSP-DDPSRYISPDQLADLYKSFIKDYPVVSIEDPFDQDDWGAWQKFTASAGIQVVGDDLTVTNPKRIA 330

SCHMA  QAIASKACNCLLLKVNQIGSLTESIEACKLAQDSGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSDRLAKYNQLLRIEEELGTAAKYAGKNFRHPKV- 434
SCHJA  KAIKVKACNCLLLKVNQIGSITESIEACKMAQKAGWGVMVSHRSGETEDNFIADLVVGLCTGQIKTGAPCRSERLAKYNQLLRIEEELGSTAKYAGKHFRHPQI- 434
FASHE  KAIDQKACNCLLLKVNQIGSVSESIKACKMAQEAGWGVMVSHRSGETEDNFIADLVVGLRTGQIKTGAPCRSERLAKYNQLLRIEEDLGGAAKYAGENFRRP--- 431
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Fig. 1. (A) SDS-PAGE of affinity chromatography eluates of *Schistosoma mansoni* schistosomula extracts. Eluates were obtained by mefloquine-agarose affinity chromatography (MQ-column; lanes 1–4) and by an ethanolamine coupled mock column (negative control; lanes 5–8). Marker (M), crude extract (CE), Wash plus pre-mefloquine (1 and 5), eluates with 1 mM mefloquine (2 and 6 and 3 and 7), pH-shift with 0·1 M glycine at pH 2·9 (4 and 8). Bands were visualized by silver staining. (B) Protein sequence of *S. mansoni* enolase Q27877 (SCHMA) and alignment with other enolases, namely P33676 from *S. japonicum* (SCHJA), Q27655 from *Fasciola hepatica* (FASHE) and P06733 from human (HUMAN). Peptides identified by mass spectrometry are highlighted in grey.

drug in schistosomula. Mefloquine was coupled to epoxy-agarose, a mock-column was prepared, and affinity chromatography was performed with schistosomula crude extracts on both columns mounted in tandem. Both columns were washed and separately eluted with mefloquine or a low-pH buffer. The elution with low pH of both columns yielded bands of ca. 40 and 30 kDa. These bands were also present to various extents in mefloquine elutions. In addition, only elution with mefloquine of the mefloquine-column yielded a band of ca. 50 kDa (Fig. 1A, rectangle). This band was subjected to mass spectrometry analysis. Eight peptides aligned with 100% protein probability to *S. mansoni* enolase Q27877 (identical to AAC46884, AAC46886, CBN61518.1) and to *S. mansoni* enolase C4Q3S7 (identical to XP_002573848.1, CAZ30081.1) with 23% coverage of the sequence (Fig. 1B). C4Q3S7 differed in 2 amino acids only from Q27877, namely an isoleucine instead of a threonine in position 52 and a phenylalanine instead of a tyrosine in position 93. Q27877 was identified through an analysis of trans-spliced mRNAs (Davis *et al*. 1995), C4Q3S7 via the genomic sequencing effort (Berriman *et al*. 2009). Analysis by BLAST (Altschul *et al*. 1997) revealed that the coding sequences for both proteins were identical (data not shown). One peptide aligned also to enolases from *S. japonicum* (P33676) and *F. hepatica* (Q27655), 3 aligned with the *S. japonicum* enolase only, 4 were unique to *S. mansoni*. The protein with the highest similarity in humans is alpha-enolase (P06733; Fig. 1B).

Enolase activity from schistosomula crude extract is inhibited by mefloquine

Enolase activity in parasite crude extracts was inhibited by mefloquine in a concentration-dependent manner at 24 μM and above. At 240 μM, both mefloquine and sodium fluoride, a well-known enolase inhibitor (Warburg and Christian, 1941), nearly completely inhibited enolase activity in crude extracts (Fig. 2). The inhibition by mefloquine was not due to inhibition effects on the coupled reactions catalysed by pyruvate kinase and lactate dehydrogenase. This was shown by control assays where the product of the reaction catalysed by enolase, namely

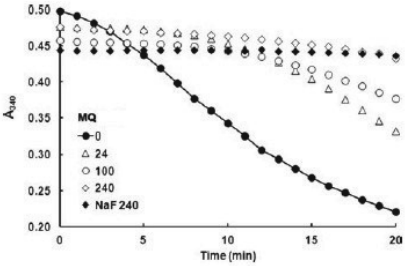

Fig. 2. Mefloquine inhibits enolase activity in *Schistosoma mansoni* crude extracts. Enolase assays were performed with *S. mansoni* schistosomula crude extracts in the presence of different concentrations of mefloquine (MQ 0–240 μM) or sodium fluoride as a positive control for inhibition (NaF 240 μM). Data are expressed as the decrease of absorption at 340 nm over time.

phosphoenol-pyruvate, was added to the reaction mix instead of enolase and the substrate 2-phosphoglycerate. Activities of the coupled reactions performed without mefloquine (Control, 60·6 ΔmA_{340}/min ±3·5) or with 240 μM mefloquine (59·3 ΔmA_{340}/min ±4·6) were identical.

Subsequently, Q27877 was over-expressed as a His-Tag protein in *E. coli* and affinity purified under non-denaturing conditions (Fig. 3A). The recombinant protein had enolase activity, but was not inhibited, either by fluoride or by mefloquine at 240 μM (Fig. 3B). In order to see whether the recombinant enzyme had the same catalytic properties as the enolase activity in crude extracts, we performed enolase assays with both enzymes at various substrate concentrations and determined K_m and V_{max} values according to Lineweaver-Burke. Enolase from crude extracts had nearly 1 magnitude higher K_m and about 5 times lower V_{max} values than the recombinant enzyme (Table 1). Moreover, we performed a pull-down assay on mefloquine agarose with the recombinant enolase. The protein was detected quantitatively in the flow-through, hence the recombinant protein did not bind to the column (Fig. 3C).

Glucose interferes with the antischistosomal activity of mefloquine

Based on previously published results on metabolic inhibition of schistosomula by mefloquine, we compared the effect of mefloquine to compounds well known for interfering with glycolysis, namely sodium fluoride (Warburg and Christian, 1941) and 3-bromopyruvate (Pelicano *et al.* 2006). We hypothesized that a supplementation of the medium with glucose may counteract inhibition by these compounds. Additional glucose would offer more substrate for each reaction during glycolysis, increase the rate of glycolysis and might decrease the inhibitory effect of the tested glycolytic inhibitors and of mefloquine. As a control, we included menadione (Bueding, 1950) interfering with glycolysis via induction of oxidative stress (Verrax *et al.* 2006).

Figure 4 illustrates the relative heat production (metabolic activity of untreated control worms in the absence of glucose was set to 100%) of schistosomula incubated with the 4 tested compounds in the presence or absence of glucose (0 and 20 mM) 24 h post-incubation. The addition of glucose caused no significant increase as compared to the control group. After incubation with 24 μM mefloquine in glucose-free medium, metabolic activity of schistosomula dropped to nearly one fifth of the control values. Schistosomula incubated with 20 mM glucose showed a significantly higher metabolic activity after exposure to mefloquine as compared to schistosomula incubated in plain medium. The same effect was seen for schistosomula incubated with 48 μM mefloquine. By 24 h post-incubation, metabolic activity of parasites incubated in glucose-free medium had decreased to less than 20% compared to control values, whereas worms exposed to mefloquine incubated in medium containing glucose had a significantly higher activity. When schistosomula were incubated with a lower concentration of 10 mM glucose (data not shown) again a significantly higher heat-flow after exposure to mefloquine was seen, when compared to schistosomula incubated in medium without glucose. The effects of higher mefloquine concentrations could not be evaluated since these doses resulted in a rapid decrease of metabolic activity of schistosomula, which was too fast to be recordable by our system (Manneck *et al.* 2011*b*).

The addition of sodium fluoride caused a concentration-dependent decrease of the heat-flow. Incubation with 20 mM sodium fluoride resulted in death of all schistosomula. In the presence of 20 mM sodium fluoride plus glucose metabolic activity was still detectable. Both parasites incubated with and without additional glucose had similar metabolic activities 24 h after incubation with 50 μM 3-bromopyruvate (data not shown). In the presence of 0·5 mM 3-bromopyruvate metabolic activity was nearly at background levels. In the presence of glucose and 0·5 mM 3-bromopyruvate metabolic activity was detectable at nearly 10% of the control levels. Finally, menadione (50 μM) killed schistosomula 24 h post-incubation regardless of glucose supplementation.

DISCUSSION

Isothermal microcalorimetry, which measures the heat produced by microorganisms has proven to be a

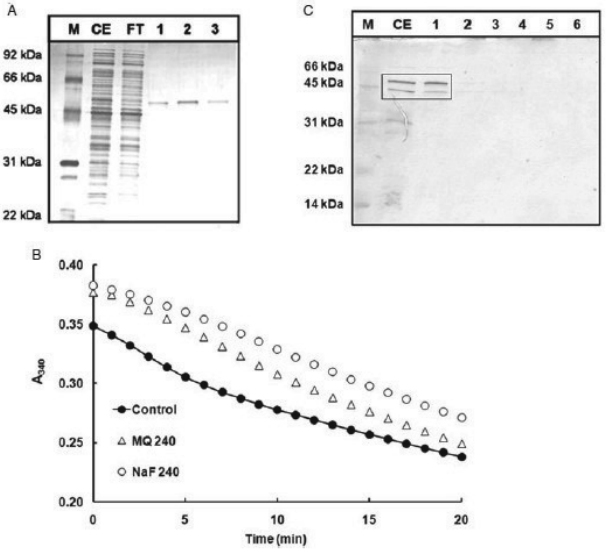

Fig. 3. Recombinant enolase Q27877 is not inhibited either by fluoride or by mefloquine. (A) SDS-PAGE of his-tag purified recombinant enolase. Marker (M), crude extract (CE), flow through (FT), eluates (1–3). (B) Enolase activity of recombinant Q27877 in the presence of 240 μM mefloquine (MQ 240) or 240 μM sodium fluoride (NaF 240). (C) SDS-PAGE of mefloquine agarose affinity chromatography of recombinant enolase Q27877. Crude extract (CE), flow-through (1), wash (2), pre-mefloquine (3), mefloquine (4), mefloquine pre pH-shift (5), pH-shift (0·1 M glycine, pH 2·9) (6). Bands were visualized by silver staining.

Table 1. Kinetic parameters of enolase activity from *Schistosoma mansoni* schistosomula crude extracts and of recombinant enolase Q27877

(To determine K_m and V_{max} values, enolase reactions were performed with substrate concentrations ranging from 0·1 to 1·8 mM. The reciprocals of the resulting reaction rate (v_i) values and the substrate concentrations were plotted and K_m and V_{max} values and the corresponding errors were calculated by regression analysis.)

Parameter	Crude extract	Recombinant
K_m (mM)	3·22 ± 0·21	0·21 ± 0·05
V_{max} (mkat/mg prot)	95·2 ± 26·2	485·2 ± 81·5

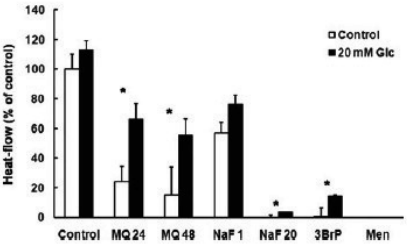

Fig. 4. Heat-flow of *Schistosoma mansoni* schistosomula 24 h post-incubation with 24 μM (MQ 24) and 48 μM mefloquine (MQ 48), 1 mM or 20 mM sodium fluoride (NaF 1, NaF 20), 0·5 mM 3-bromopyruvate (3-BP), or with 50 μM menadione (Men) in the absence of glucose (Control) or with glucose (20 mM Glc). Mean values ± S.E. are indicated for 4 replicates. Values with significant differences (*t*-tests; $P < 0·05$) between samples with and without additional glucose are labelled with asterisks.

suitable method to evaluate the activity of antischistosomal compounds (Manneck *et al.* 2011*b*). Energy acquisition in schistosomes is provided mainly via glycolysis (Bueding, 1950; Van den Bossche, 1985; Yang *et al.* 2010). Compounds interfering with glycolysis thus rapidly block muscular contraction and heat development. We have investigated a series of compounds directly interfering with glycolysis, namely sodium fluoride, 3-bromopyruvate, and menadione on schistosomula using microcalorimetry. Sodium fluoride is a well-characterized inhibitor of enolase, the enzyme catalysing the

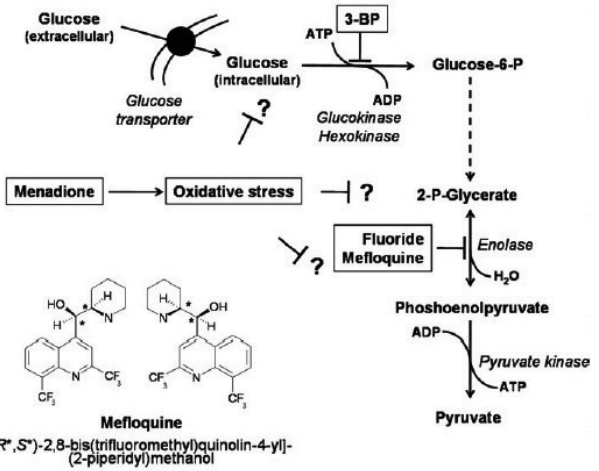

Fig. 5. Scheme summarizing glycolysis in *Schistosoma* and targets for inhibitors (in rectangles) as used in the present study. A structural formula of mefloquine is shown.

conversion of phosphoglycerate to phosphopyruvate (Warburg and Christian, 1941). However, sodium fluoride is not selective to enolase and inhibits a variety of other enzymes such as phosphatases or ATPases. Therefore, the reduction of glucose consumption in schistosomula may also be due to effects of sodium fluoride on other metabolic pathways. In adult schistosomes, sodium fluoride reduces the consumption of glucose and the production of lactate (Bueding, 1950). Menadione is a naphthoquinone with structural similarities to mefloquine. Similar to sodium fluoride, it reduces glucose consumption and lactate production in adult schistosomes (Bueding, 1950), but most likely not by an inhibition of glycolysis but instead by an induction of oxidative stress (Verrax *et al*. 2006). 3-bromopyruvate inhibits hexokinase in the glycolytic pathway and decreases the ATP content in cells by inhibition of ATP production and depletion of cellular ATP. Due to its cytotoxic activity against cancer cells, pre-clinical trials have been launched to confirm its activity for anticancer treatment (Pelicano *et al*. 2006). To our knowledge, the antischistosomal activity of 3-bromopyruvate has not been described so far. Our microcalorimetric studies reveal that simultaneous addition of glucose attenuates the metabolic inhibition by sodium fluoride, 3-bromopyruvate and mefloquine, but not by menadione. Note that, in the case of sodium fluoride, the effective concentration is very high (above 1 mM), which is most likely due to a reduced uptake of this compound by schistosomula. This phenomenon has also been described for adult schistosomes with at least 20 mM sodium fluoride being necessary to inhibit glucose utilization in the parasites by 50% (Bueding, 1950). Our findings suggest that mefloquine directly interferes with glycolysis similar to the two inhibitors of glycolytic enzymes rather than acting indirectly as menadione. This assumption is corroborated by biochemical data.

Using affinity chromatography followed by mass spectrometry, we have identified the glycolytic enzyme enolase as a major mefloquine-binding protein in *S. mansoni* schistosomula. Enolase activity in schistosomula crude extracts is inhibited by mefloquine in a similar concentration range as by sodium fluoride. The recombinant enolase Q27877 is, however, not inhibited by either mefloquine or sodium fluoride. Moreover, the recombinant enolase has different kinetic parameters from the activity in crude extracts.

At first glance, these results suggest that mefloquine may inhibit an enolase isoform other than our recombinant Q27877. Mammals have 3 enolase isoforms, alpha, beta and gamma with multiple functions beside glycolysis (Pancholi, 2001) and for *S. mansoni* to date 2 isoforms of enolase, namely Q27877 and C4Q3S7, are listed in genomic databases. However, although these 2 isoforms are provided in genomic databases, it is likely that enolases other than Q27877 do not exist in *S. mansoni*. The small differences between Q27877 and C4Q3S7, the protein derived from the genomic sequencing effort, are most likely due to errors, because the corresponding coding sequences are identical. A BLAST analysis showed that Q27877

has 75% identity to human alpha-enolase, 72% to beta-enolase, and 71% to gamma-enolase and may thus fulfil – to a certain extent – the functional requirements for all classes in mammals.

More likely, enolase from crude extracts has been subjected to post-translational modifications and/or interacted with other proteins present in its natural cellular environment resulting in catalytic properties differing from the recombinant enzyme. For example, in mammalian cells such as human muscle fibres, enolases may be phosphorylated (Hojlund *et al*. 2009). The functional implication of this phosphorylation is unknown so far. In yeast, enolase is bound to vacuoles (Decker and Wickner, 2006) and may be part of a large macromolecular complex associated with mitochondria (Brandina *et al*. 2006). In *Plasmodium*, enolase has been described to have undergone post-translational modifications that are associated to different subcellular fractions (Pal-Bhowmick *et al*. 2007). Taken together, these results indicate that a native enolase may be different from the recombinant enolase produced in *E. coli*. Results obtained with the latter may thus not reflect the original situation.

Moreover, there is increasing evidence that in *S. bovis* (Ramajo-Hernandez *et al*. 2007) and *S. japonicum* (Yang *et al*. 2010) enolase is localized not only intracellularly but also at the cell surface. The role of this localization is unclear, but extracellular enolase may be involved in the interaction with host proteins such as plasminogen or the immune system (Pancholi, 2001). It is possible that an extracellular, extensively modified enolase is the target for mefloquine. In *S. japonicum*, enolase is expressed in all developmental stages, but has higher expression levels in schistosomula than in adult worms suggesting that enolase is critical for growth, migration and adaption of the young schistosomes (Yang *et al*. 2010).

Overall, our results suggest, that in *S. mansoni* schistosomula, mefloquine inhibits enolase resulting in an interference with glycolysis and/or in debilitating the parasite via an unknown mechanism in the case of extracellular enolase. Since mefloquine has 3 fluorine residues, it is possible that it blocks enolase at the same site as fluoride ions as suggested in the scheme presented in Fig. 5. Fluoride forms a complex with magnesium and phosphate at the active site (Bunick and Kashket, 1982; Qin *et al*. 2006). There is, however, a non-deniable discrepancy between the concentrations of mefloquine inhibiting enolase activity in crude extracts and the nearly 1 magnitude lower concentrations needed for metabolic inhibition measured by microcalorimetry and by drug sensitivity assays (Manneck *et al*. 2011*b*). This suggests the existence of other molecular targets for mefloquine. Further work should focus on this aspect as well as on the role of enolase in the mode of action of mefloquine in *Schistosoma* spp.

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8. Overall discussion and conclusions

The overall aim of this Ph.D. thesis was to further assess the newly discovered antischistosomal properties of the antimalarial drug mefloquine (Keiser et al., 2009). The finding that mefloquine possess antischistosomal activities is the result of a successful applied “piggy back” strategy. This strategy is often used when two diseases share the same target and thus a chemical ‘starting point’ for subsequent investigations is provided (Nwaka and Hudson, 2006). In our case, *Plasmodia spp*. and *Schistosoma spp* both degrade hemoglobin and additionally, have a geographical overlap (Sangweme et al., 2010, Hotez et al., 2006). Therefore marketed antimalarials, which have hemoglobin as putative target, were assessed on their effect against schistosomes. Mefloquine was found to be a potential drug candidate for schistosomiasis with activities against all stages of *S. mansoni* and *S. japonicum in vivo* (Keiser et al., 2009). This drug discovery strategy is fast and cost-effective, since for established drugs, clinical trials including toxicological studies have already been conducted and clinical experience exist (Dissous and Grevelding, 2011).

Therefore different *in vitro* and *in vivo* studies were conducted with *Schistosoma mansoni*. We have performed *in vitro* studies using adult schistosomes (recovered from infected mice) and newly transformed schistosomula (NTS; artificially produced, non hematophagous) investigating drug effects on motility and morphology (light and electron microscopy) and on metabolism (microcalorimetry). Moreover, we have used the mouse model to study drug effects *in vivo* (Keiser, 2010).
Findings should feed into a better understanding of the activity profile of mefloquine and the possible mechanism of action in schistosomes. An understanding of these parameters is crucial for the potential clinical utility of mefloquine in the treatment and control of schistosomiasis. In addition, the field of antischistosomal drug discovery was strengthened by development of a novel screening method.

In the first part of the discussion I will summarize the key facts of my research. In the second part of the discussion, results obtained in drug efficacy and mode of action studies are reviewed and critically assessed. In the third part, findings of drug discovery and methodological issues of my studies are discussed. The fourth section comments on new treatment options. The fifth part provides implications and suggestions for further research and finally, in the last part of the discussion I will draw a conclusion.

8.1. Research highlights

The results described in detail in the previous chapters confirm the recently discovered antischistosomal properties of mefloquine *in vitro* and *in vivo*. Furthermore, they give an

insight into the spectrum of activity and the pharmacodynamics of mefloquine on *S. mansoni*. Below I summarize the key results:

1. Mefloquine has a rapid onset of action on NTS and a slightly slower onset of action on adult worms *in vitro* (10 µg/ml killed adult schistosomes within 24 h of incubation, LC_{50}=1.9 µg/mL).
2. *In vivo* already 72 h post treatment with mefloquine more than 90% of schistosomes were shifted to the liver (ED_{50}= 62 mg/kg), a criterion for drug activity, which shows how quick after drug treatment worms are forced to migrate from the mesenteric veins to the liver (Buttle and Khayyal, 1962). In *S. japonicum* infected mice worms shifted within 48 h post mefloquine treatment to the liver (Zhang et al., 2009). For comparison, praziquantel, the standard drug for treatment and control of schistosomiasis killed worms *in vivo* within 24 h.
3. Activities against adult *S. mansoni* are enhanced in the presence of hemin.
4. Scanning electron microscopy (SEM) studies revealed extensive disruption on the tegument of *S. mansoni* schistosomula and adult worms after mefloquine treatment, in particular following *in vitro* incubation and on the tegument of female worms. In contrast, praziquantel provokes more extensive tegumental damage to the male worms
5. The four optical isomers and the two racemic forms of the chiral drug mefloquine showed only moderate stereoselectivity in their antischistosomal activity *in vivo*.
6. Isothermal microcalorimetry (IMC) is a useful tool to study drug effects on schistosomes.
7. Mefloquine interacts with the glycolytic enzyme enolase in *S. mansoni* schistosomula and thus with glycolysis.
8. Mefloquine/praziquantel combinations reveal synergistic effects *in vitro* and in the *S. mansoni* mouse model when both drugs are given simultaneously or when praziquantel treatment followed mefloquine.
9. Our studies support the use of NTS in *in vitro* antischistosomal drug assays, since similar sensitivities on NTS and adult schistosomes were observed with test drugs.

8.2. Mefloquine: Antischistosomal properties and mode of action in *S. mansoni*

Investigations on the temporal drug effect of mefloquine revealed a very fast onset of action on the schistosomular stage (NTS) and a slightly slower onset of action on adult schistosomes *in vitro*. However, 10 µg/ml mefloquine (~24 µM) killed adult worms within 24 h of incubation.

In vivo 72 h after treatment more than 90% of schistosomes were shifted to the liver, which was also seen in the previous published *in vivo* study (Keiser et al., 2009). In *S. japonicum* infected mice, a slightly faster hepatic shift was observed after mefloquine treatment, since all worms were shifted to the liver within 48 h (Zhang et al., 2009). For comparison, praziquantel, the standard drug for treatment and control of schistosomiasis killed worms *in vivo* within 24 h (Shaw, 1990).

Although mefloquine kills schistosomes in our *in vitro* study without the addition of hemin, a heme dependency study revealed that in the presence of hemin the antischistosomal activities of mefloquine are further pronounced. The latter result is in line with the mechanism of action of mefloquine in malaria parasites. It was shown that in *Plasmodia spp*. mefloquine and also other 4- aminoquinoline antimalarial drugs interfere with the hemoglobin pathway by inhibiting the essential formation of toxic hem into the untoxic hemozoin pigment (Sweeney, 1981, Correa Soares et al., 2009). This detoxification process was also shown for *S. mansoni* to be the main heme degradation pathway and it was demonstrated, that the antimalarial quinoline methanols interfere with the hemozoin formation of *S. mansoni*, which is suggested to represent the mechanism of schistosomicidal action of these compounds. Interestingly, the hemozoin content in female worms was more impaired after treatment with these drugs (Gönnert and Andrews, 1977). Overall, these studies confirm our results of a heme- dependent mechanism of mefloquine in schistosomes. However, in contrast to the antimalarial drug artemether, which only possess antischistosomal activities *in vitro* in the presence of hemin (Xiao et al., 2001), mefloquine seems to display its schistosomicidal activities with at least one other mechanism.

SEM studies, a common technique to document efficacy of antihelminthic drugs (Neves et al., 2001), confirmed the schistosomicidal action of mefloquine and the observed difference in the onset of action between *in vitro* and *in vivo* studies. Mefloquine induces progressive and time-dependent alterations on the tegument of *S. mansoni* NTS and adult worms with extensive tegumental damages including blebbing, shrinking and sloughing. Severe damages were observed in particular following *in vitro* incubation and on the tegument of female worms. The observed discrepancy between *in vitro* and *in vivo* studies might be due to absorption and distribution effects of the drug in the mouse organism

resulting in lower drug concentrations at the target organs *in vivo*. Our observation that the tegument of females was more affected by mefloquine is in line with the results of the *in vivo* study of Keiser and colleagues, where mefloquine reached higher female worm burden reductions compared to total worm burden reductions (Keiser et al., 2009). In addition, this result is suitable to the findings of the above described hemozoin study, since female *S. mansoni* were found to be more affected after antimalarial drug treatment (Correa Soares et al., 2009). In contrast, praziquantel provokes more extensive tegumental damages to the male worms (Shaw and Erasmus, 1983).
In addition to our result, an *in vitro* study (Holtfreter et al., 2011) as well as an *in vivo* stage specific susceptibility study showed, that mefloquine is effective against all parasite stages of *S. mansoni* and *S. japonicum* (Keiser et al., 2009). In contrast, praziquantel is only active against the adult blood flukes and has a lack of efficacy against juvenile schistosomes (Cioli and Pica-Mattoccia, 2003, Pica-Mattoccia and Cioli, 2004).

Taken together the following findings let us conclude that both drugs may differ in their modes of action and act on different target proteins: First, mefloquine and praziquantel have sex-specific differences and induce different types of morphological changes and tegumental alterations on *S. mansoni*. Second, mefloquine and praziquantel have different spectra of activity. Third, mefloquine seems to have a hemin dependent and hemin independent pathway in schistosomes, since not only heme feeding adults, but also the non hematophagous schistosomula are susceptible to that drug.

The mode of action of mefloquine is thus different from praziquantel and is not restricted to the presence of heme. In order to discover drug targets for mefloquine in schistosomes, we have performed affinity chromatography with schistosomula crude extracts, and have identified a *S. mansoni* enolase (EC 4.2.1.11; Q27877), a glycolytic enzyme, as a major mefloquine- binding protein in schistosomula. Moreover, results obtained with IMC suggest that mefloquine interferes with glycolysis thus causing rapid death of the worms. For *S. japonicum* it was shown that the enolase gene was significantly higher expressed in the schistosomula stage as well as during development phase into adult worms. This finding that enolase is critical for the rapid growth, migration and survival of the worms (Yang et al., 2010). This function of the enzyme may confirm our hypothesis that mefloquine exert its antischistosomal activity (at least partly) via enolase, since mefloquine disturbs an important and life-supporting regulating mechanisms of the parasite. Already in 1950 it was shown that glycolysis is essential for the survival of *S. mansoni*, but the respiratory metabolism is not (Bueding, 1950). In the same study it was shown that also funadin (antimonial) and naphthoquinone (structural similarities to mefloquine) inhibit glycolysis in schistosomes and showed chemotherapeutic effects in experimental schistosomiasis. Further investigations with adult schistosomes, sex-specific interactions (female schistosomes tend have a lower

rate of glycolysis than males, because they contain less glycogen as males), and drug screening studies with funadin, naphthoquinone, fluoride (selective effect on enolase) and other known inhibitors of glycolysis should be undertaken to deepen our understanding on glycolysis as potential target pathway for drugs.
Since mefloquine and naphthoquinone have structural similarities, another approach might be to determine which structure is required for antischistosomal activity.

We already demonstrated in a structure-activity study that the optical isomers and the racemic forms of the chiral drug mefloquine are characterized by only moderate stereoselectivity, in particular *in vivo* since prominent total and female worm burden reductions were observed with all derivates in the *S. mansoni* mouse model. The role of stereochemistry in the pharmacokinetics, efficacy and toxicity of drugs is very important for discovery and development of drugs today (Brocks and Jamali, 1995, Brocks and Mehvar, 2003). However, we showed that none of the derivatives tested offers a therapeutic advantage over mefloquine. Indeed, the highest *in vivo* antischistosomal activities were observed with the erythro-racemate, the marketed form of the drug mefloquine. Furthermore, the (+) and (-) form of the erythro isomers showed the same overall activity in the *S. mansoni* mouse model and *in vitro*. It was hypothesized that (+) mefloquine might cause less side effects in the treatment of malaria, but interestingly a recent Phase I study has shown similar safety and tolerability profiles for the (+)- erythro isomer and the racemate (http://www.sosei.com/pdf/press_en_20100106_265.pdf). Therefore, since no advantages in the pharmacodynamics affect and in the safety profile of the (+) - erythro isomer were seen, a separation of the enantiomers is not needed from a therapeutical point of view. These facts facilitate the potential clinical use of mefloquine against schistosomiasis, since further studies can be undertaken with the marketed drug mefloquine.

Our combination chemotherapy studies with mefloquine plus praziquantel against *S. mansoni* revealed synergistic effects *in vitro* and in the mouse model. Hence, the combination yields a result which is not independently obtainable by each drug alone. This can occur when drugs do not inhibit each other in pharmacokinetics or pharmacodynamics and act on different targets (ref). In our case this suggests that mefloquine has a different mode of action than praziquantel, which would also confirm our previous hypothesis described above. Both drugs, however, are only able to display their entire mechanism of action when mefloquine is administered 24 h prior to praziquantel, since the synergistic behavior of the combination in the mouse model was observed when both drugs were given simultaneously or when praziquantel treatment followed mefloquine. On the other hand only moderate worm burden reductions were achieved when praziquantel was administered prior to mefloquine.

Our previous findings that mefloquine disturbs the glycolytic pathway in schistosomes and the mode of action of praziquantel may explain the synergism between mefloquine and praziquantel. By giving mefloquine prior to praziquantel, the energy yield due to glycolysis is lowered and thus ATP depleted (Pelicano et al., 2006, Lithaw, 2009). If in a next step praziquantel is added, praziquantel exerts its mode of action and causes muscle contraction by opening of the calcium channels. Furthermore, due to the action of praziquantel against the tegument of the parasites, changes in permeability of tegumental membrane can be observed (Mehlhorn, 2001). Therefore parasites loose glucose and ATP pools of the schistosomes are further depleted until a point of no return, even with drug dosages/concentrations that are not effective if administered alone. These mechanisms can proceed in parts, when drugs are given simultaneously. On the other hand, if praziquantel is given first, an increase in tension and a disrupted tegument would decrease the uptake of mefloquine by schistosomes, resulting in lower activities of the drug combination (Andrews, 1985).

8.3. Antischistosomal drug discovery and methological issues

Throughout the entire framework of my studies I evaluated *in vitro* drug effects against *S. mansoni* by manual microscopic reading, since this screening method is the current “gold standard” to assess the *in vitro* antischistosomal properties of test drugs (Keiser, 2010, Ramirez et al., 2007). Although the use of microscopy is easy to handle and accomplishable in every laboratory, microscopic reading is subjective and time extensive. Therefore, to improve antischistosomal drug screening, we have assessed a new technique, namely isothermal microcalorimetry (IMC), to examine drug activities against adult *S. mansoni*, and NTS. In general IMC measures the heat- flow of biological processes (endo- or exothermic reactions) over time and has already been applied for various studies of food deterioration, drug shelf life or the effects of antimicrobial agents.

Our studies on *in vitro* effects of well-described antischistosomal drugs (praziquantel, oxamniquine and artesunate), and of the optical isomers and racemates of mefloquine against *S. mansoni* showed a good correlation between assessment by microscopy and IMC. Additionally, we found two key advantages for IMC. First, IMC allows identifying drug effects that were not visible by microscopic evaluation and second, IMC precisely determines the onset of action of the test drugs. On the other hand, microscopy allows investigating drug effects on morphology. Overall, we could show that IMC is a useful tool for antischistosomal drug discovery and helpful in the characterization of new hits. IMC is also applicable to monitor drug effects against a range of other helminths and their developmental stages where drug screening approaches are limited, for example, to study activities against

Fasciola hepatica or *Echinostoma caproni* (Kirchhofer et al., 2011). Results obtained with IMC, however, should be confirmed by phenotypic microscopic evaluation.
There are also a few drawbacks for IMC. A big drawback is that the use of IMC is associated with high costs, since there are high initial costs for the microcalorimeter and other expenses for the appropriate material (e.g. glass ampoules). Therefore its use will be restricted to well-equipped laboratories in high-income countries. Another drawback is that IMC does not allow high-throughput evaluation of drugs, since the IMC used in this study possess only 48 individual measuring channels.

Moreover, we also compared drug effects on the schistosomular (NTS) and adult stages, as the former developmental stage is increasingly being used in antischistosomal drug screening assays and by our laboratory (Abdulla et al., 2009, Keiser, 2010, Mansour and Bickle, Holtfreter et al., 2010). Our data support the use of NTS in *in vitro* antischistosomal drug assays, since we observe similar susceptibilities on NTS and adult schistosomes for praziquantel and mefloquine. Slight differences in the drug susceptibilities of the two developmental stages are, however, noted with oxamniquine and artesunate.

8.4. Antimalarials against schistosomiasis: comments on new treatment options

In our studies we have shown through several approaches that mefloquine and praziquantel have different spectra of activity and different mechanisms of action. Therefore a mefloquine/praziquantel combination chemotherapy might offer the following advantages:

First, an improved treatment outcome compared to praziquantel monotherapy might occur. However, this has not been assessed in detail yet. Second, the threat of resistance is decreased. Third, less “re- infections” might occur. Usually, with praziquantel monotherapy, a follow up treatment after four to six weeks is necessary, because unaffected immature worms would mature to adult schistosomes (Gryseels et al., 2006). On the other hand mefloquine exhibit activities against juvenile schistosomes. The next step is to assess, if encouraging results obtained in the mouse model can be confirmed in patients. Proof of concept trials in schistosome infected children to evaluate the use of the mefloquine/praziquantel combination in cure, prophylaxis and safety have already been launched.

Overall, the mouse is the best model organism for understanding human biology as well as disease mechanisms (genome is closely related to humans) and is the most commonly used mammalian host for *in vivo* drug discovery studies (including *S. mansoni*) (Keiser, 2010).

However, we do not know, whether synergistic effects observed with the combination mefloquine/praziquantel in the mouse model will also occur in patients, since not the entire complexity of human life, including pharmacokinetic and pharmacodynamic is reflected (Proetzel and Wiles, 2010) (http://genome.wellcome.ac.uk/doc_WTD023552.html)

Another group of antimalarials with antischistosomal activities (mainly active against juvenile schistosomes) are the artemisinins. This was demonstrated *in vitro*, *in vivo* and in several clinical trials (Utzinger et al., 2010b, Borrmann et al., 2001, Utzinger et al., 2007, Utzinger et al., 2002). Therefore another possibility for a combination therapy against *Schistosoma* infection is the use mefloquine plus the artemisinin derivate artesunate. Indeed, a randomized, explatory open-label trail, which tested the efficacy and safety of a mefloquine/artesunate combination (artemisinin- based combination therapy (ACT)) in schistosome infected school children showed, that 11 out of 18 *S. haematobium* infected children were cured (Keiser et al., 2010). Praziquantel monotherapy achieved cure rates of 88%. Egg reduction rates were for both treatment regimes > 95%. A handful of patients were concurrently infected with *S. haematobium* and *S. mansoni*. In this case, high cure rates were also achieved against *S. mansoni*. Drugs were administered in accordance with current malaria treatment schedule. The effect of a praziquantel/mefloquine/artesunate combination, which will also be assessed in the framework of the above mentioned proof of concept study, will therefore be interesting to note.

However, the use of antimalarials for the treatment and control of schistosomiasis have to be regarded with extreme caution because of the concern of antimalarial resistance. That is also one reason why the artemisinins, the most powerful antimalarials discovered so far, are used in combination therapy for malaria treatment. Therefore the ACTs must be reserved for prevention and control of malaria (Utzinger et al., 2010b). However, since the ACTs are already used against malaria in sub Saharan Africa where malaria and schistosomiasis co exist, a potential ancillary effect against schistosomiasis might be obtained. One the one hand, people who will be treated with mefloquine plus artesunate would be cleared from malaria parasitemia and on the other hand schistosomiasis related morbidity would be reduced.

8.5. Implications and suggestions for further research

- To conduct structure activity relationship studies with structural derivates of mefloquine to elucidate which structure is required for activity and therefore to increase efficacy and decrease side effects.

- To conduct proof of concept trials with mefloquine/praziquantel combinations.

- To investigate the antischistosomal properties of mefloquine, single and in combination, against *S. haematobium*, since *S. haematobium* is an important and neglected parasite.

- To assess, whether mefloquine also interferes with glycolysis in adult schistosomes.

- To search for non-toxic inhibitors of glycolysis in schistosomes, since we documented an interaction of mefloquine with glycolysis, an essential metabolic reaction in *S. mansoni* schistosomula. For example, by testing marked drugs against schistosomes, which interact with glycolysis in parasites, protozoa or bacteria as a kind of “piggy back” strategy.

- Since liver infections often strongly influence kinetics and metabolism, the pharmacokinetic of mefloquine should be studied in infected hosts. It will be interesting to note, whether $t_{1/2}$, c_{max} and AUC will be altered in infected mice.

8.6. Conclusion

In conclusion, my work has confirmed that mefloquine has excellent antischistosomal properties *in vitro* and *in vivo*. Furthermore, mefloquine might be an interesting partner drug in combination chemotherapy against schistosomiasis. We demonstrated that IMC is a useful tool for antischistosomal drug discovery and should be therefore be assessed also for studying drug effects against other helminths. In addition, our data support the use of NTS in *in vitro* antischistosomal drug assays.
However, although chemotherapy is the key factor to control and reduce morbidity due to schistosomiasis, and thus there is a pressing need to develop new antischistosomal drugs, safe water supply and sanitation should also not be neglected
(Utzinger et al., 2010a, Sarvel et al., 2011).

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9. Appendix: Impressions from the laboratory

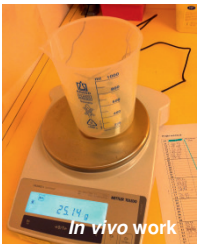

Entrance into the „wormy world“

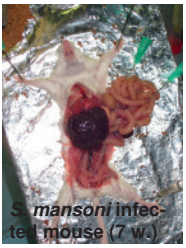

Chemicals


Tank with host snails of *S. mansoni*

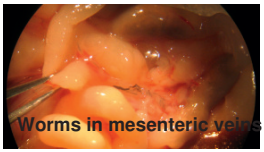

Biomphalaria glabrata

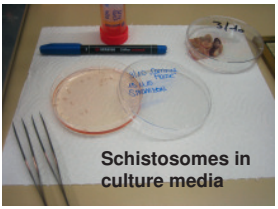

NTS pellet


In vivo work

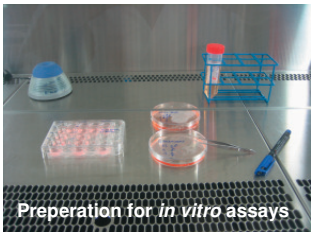

S. mansoni infected mouse (7 w.)

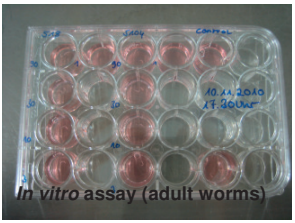

S. mansoni couple in liver

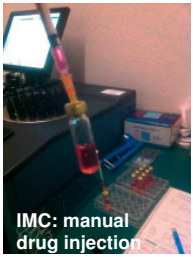

Worms in mesenteric veins


Schistosomes in culture media


Light microscope picture


Preperation for *in vitro* assays


In vitro assay (adult worms)


IMC: manual drug injection

10. Bibliography

CURRICULUM VITAE

Theresia Barbara Manneck

Personal Details

Forenames	Theresia Barbara
Surname	Manneck
Date of birth	5 June 1982
Place of birth	D- Lingen
Nationality	German
Country of residence	Switzerland
E-mail address	t.manneck@gmail.com, theresia.manneck@unibas.ch

Education

June 2008 – May 2011	**PhD in Microbiology** Swiss Tropical and Public Health Institute (Swiss TPH), Basel, Switzerland and University of Basel Supervisor: Prof. Dr. Jennifer Keiser (cf. training and work experiences section below)
Oct 2002 – Feb 2008	**Study of Pharmacy and Pharmaceutical Sciences** Goethe-Universität Frankfurt am Main, Germany
Sep 1995 – May 2002	**Grammar school** Windhthorst-Gymnasium, Meppen, Germany

Training and Work Experience

Sep 2010:	**Advanced Course in Laboratory Animal Science** "Schweizer Tierschutzgesetz und Anästhesie bei Labortieren" „Health Monitoring und Zoonosen bei Labortieren" Charles River Laboratories Germany GmbH, Swiss TPH, Basel, Switzerland
June 2008:	**Introducy Course in Laboratory Animal Science** University of Zürich, Institute of Laboratory Animal Science
Sep 2008 – Dec 2010:	**Work experience as pharmacist** Parttime job, Apotheke im Rheincenter, Weil am Rhein, Germany
July 2007 – Dec 2007**:**	**Internship at the Hoffman-La Roche Ltd, Basel, Switzerland (Parenterals Manufacturing)** Assistance of the GMP Training Manager, to contribute to manufacturing process for aseptic production (GLP) of parenterals, participated in many aspects of daily project work and lab activities

Nov 2006 – June 2007: **Internship at the Pharmacy**
Centrum Apotheke, Frankfurt am Main, Germany

Publications

Manneck T, Haggenmüller Y, Keiser J. Morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult flukes of Schistosoma mansoni. *Parasitology* 2010; **137**: 85-98.

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Poster

Mefloquine- a new antischistosomal drug?
September 2009, 6th European Congress on Tropical Medicine and International Health, Verona, Italy

Oral presentation

Mefloquine and schistosomes: Invetigations for possible drug targets
March 2011, Monday Seminar, Swiss TPH, Basel, Switzerland

Mefloquine and schistosomes: from *in vitro* studies to drug target discovery
April 2010, MPI Ressearch Seminar, Swiss TPH, Basel, Switzerland

Microcalorimetry: a new drug screening method for antischistosomal drugs
April 2010, Seminar of the Laboratory Of Biomechanics & Biocalorimetry (LOB^2), University of Basel, Switzerland

Current state of my PhD research
Swiss Society of Tropical Medicine and Parasitology (SSTMP) Student meeting
2008: Vevey; 2009: Basel; 2010: Spiez

Languages

German and English (fluent)
French and Spanish (basic knowledge)

References

Jennifer Keiser — PhD thesis supervisor
Swiss TPH, Basel; Switzerland
Phone: +41 61 284-8218
Fax: +41 61 284-8105
Email: Jennifer.Keiser@unibas.ch