Investigations on potential drug candidates and metabonomics-based diagnostic biomarker discovery for human soil-transmitted helminthiases

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Summary

Soil-transmitted helminthiases (STHs) are tropical diseases caused by nematode worms. The most common species affecting humans are *Ascaris lumbricoides*, the hookworms (*Necator americanus* and *Ancylostoma duodenale*), *Trichuris trichiura* and *Strongyloides stercoralis*. More than 1 billion people are infected worldwide. Most at risk are the 3 billion poorest people of the world, and particularly children. Heavy infections cause iron-deficiency anemia, malnutrition, growth stunting and intellectual retardation. STHs occur often concomitantly with other infections such as malaria, adding to the disease burden. At present, STHs morbidity control relies on chemotherapy, mainly administered within the frame of regular mass drug administration campaigns. A tiny panel of five drugs (albendazole, mebendazole, levamisole, pyrantel pamoate and ivermectin) is available to combat STHs. These present limited efficacies, especially when administered in single dose against *T. trichiura* and hookworm infections. Although anthelmintic drug resistance has not yet appeared as a major public health problem, emergence of drug resistance may be inevitable in the future. Therefore, the situation is precarious and there is an urgent need for new drugs. This has been largely ignored by research. In addition, existing tools for *in vitro* drug sensitivity testing are based on a subjective viability assessment assay which lacks convenience and does not allow high-throughput screening rates. Also, the lack of accurate diagnostic tools to detect STHs and malaria infections hampers an optimal management of these diseases, when it comes to control and eventually eliminate them.

This work aimed on the one hand to set up nematode-rodent life cycles at the Swiss Tropical and Public Health Institute (Swiss TPH), improve drug screening assays and evaluate potential new treatments for human STHs. Prior to this thesis, monepantel (AAD 1566), tribendimidine, nitazoxanide and oxantel pamoate had been identified as potential drug candidates for STHs. On the other hand, we aimed to strengthen our understanding of the impact of a murine malaria and hookworm co-infection on the host's metabolism and explore the potential of metabolic profiling as multiplexing diagnostic tool.

Once the animal models corresponding to human helminthiases have been introduced at the Swiss TPH, namely *Ancylostoma ceylanicum* (at a later stage also *N. americanus*) and *Trichuris muris*, the Alamar Blue assay, the MTT assay, the acid phosphatase assay, the xCELLigence System, isothermal microcalorimetry, and the feeding-inhibition assay (*A. ceylanicum* only) were tested and compared to the current assay of choice, the larval/adult motility assay. No assay performed better than the motility assay for drug testing on *A. ceylanicum* third-stage larvae (L_3), whereas the xCELLigence System was found as good as the adult motility assessment. Using *T. muris* fourth-stage larvae (L_4) or adult worms, the

Alamar Blue Assay, the xCELLigence System and isothemal microcalorimetry were found at least as useful as the motility assay.

The potential of monepantel was assessed against *A. ceylanicum*, *N. americanus*, *T. muris*, *Ascaris suum* and *Strongyloides ratti*. *In vivo*, the veterinary drug showed good and moderate activities respectively, against *A. ceylanicum* (10 mg/kg: 100% worm burden reduction) and *N. americanus* (10 mg/kg: 58.3% worm burden reduction), but failed to show sufficient anthelmintic properties in the other three models.

Tribendimidine, a Chinese anti-hookworm drug, and its metabolites (dADT and AdADT) were tested *in vitro* and *in vivo* using the hookworm models *A. ceylanicum* and *Heligmosomoides bakeri*. Tribendimidine and dADT were found efficacious in both models. In *A. ceylanicum*-infected hamsters, single oral doses of 10 mg/kg resulted in 74.8% and 87.4% worm burden reduction, respectively. In the *T. muris* mouse model, single oral doses of 2 mg/kg achieved worm burden reductions of 100% and 97.1%, respectively. The second metabolite, AdADT, displayed moderate activity against both parasites. The combination tribendimidine-levamisole displayed an additive to synergistic behavior in the *A. ceylanicum* model *in vivo* (combination index at ED_{90} = 0.19).

Nitazoxanide, an anti-cestodal and anti-protozoal drug was evaluated against *A. ceylanicum* and *T. muris*. *In vitro*, it had a marked effect on *A. ceylanicum* adult worms (IC_{50} = 0.74 µg/ml) and on *T. muris* L_3 and adult worms (IC_{50}s = 0.27 and 12.87 µg/ml, respectively). However, in both models *in vivo* no anthelmintic effect was observed.

The “old” anthelmintic oxantel pamoate was studied in the *A. ceylanicum*, *N. americanus* and *T. muris* models. The drug lacked anti-hookworm activity *in vivo* (10 mg/kg), but showed promising trichuricidal properties *in vitro* and *in vivo* (ED_{50} = 4.7 mg/kg). Moreover, the combination oxantel pamoate-mebendazole revealed highly synergistic properties.

Murine *H. bakeri* and *Plasmodium berghei* single and co-infection (delayed and simultaneous) models were established for metabolic analysis. Urine and plasma samples were subjected to ^{1}H nuclear magnetic resonance (NMR) spectroscopy and subsequent multivariate analysis in order to identify infection-discriminating metabolic fingerprints. Characteristic metabolic fingerprints have been found for each of the infection scenarios. We detected two unknown metabolites and confirmed the accumulation of urinary pipecolic acid in *P. berghei*-infected mice, reported in a previous study. Pipecolic acid may therefore represent a candidate for human malaria diagnostics.

In conclusion, for *T. muris*, one alternative *in vitro* assay, the Alamar Blue assay, compared most favorably to the standard motility assay since it is precise and cost-effective. For

A. ceylanicum, no alternative assay was found better than the motility assay for testing on L_3, whereas the xCELLigence System was found accurate and convenient for adult worms.
Importantly, out of four examined compounds, one drug has been positively evaluated for its trichuricidal properties (oxantel pamoate), one for promising anti-hookworm effects (tribendimidine) and two potent drug combinations have been uncovered (tribendimidine-levamisole and oxantel pamoate-mebendazole), which should be studied in further detail. Two drugs failed to show sufficient activity (monepantel and nitazoxanide).
^{1}H NMR spectroscopy was found powerful for detecting metabolic changes in a co-infection model, but still presents some drawbacks as diagnostic tool in its actual form.

Table of Abbreviations

^{1}H NMR	^{1}H (proton) nuclear magnetic resonance
3R	Reduce, replace, refine
AAD	Amino-acetonitrile derivatives
APR	Aspartic protease
ASP	*Ancylostoma*-secreted protein
CI	Combination index
dADT	Deacylated acetylated amidantel
DND*i*	Drugs for Neglected Diseases initiative
ED_{50}	Effective dose 50%
ELISA	Enzyme-linked immunosorbent assay
GABA	Gamma aminobutyric acid
GluCl	Glutamate-gated chloride channel
GST	Glutathione-*S*-transferase
HIV	Human immunodeficiency virus
HPLC-MS	High performance liquid chromatography coupled to mass spectroscopy
IC_{50}	Inhibitory concentration 50%
L_3	Third-stage larva(e)
L_4	Fourth-stage larva(e)
LC-MS	Liquid chromatography coupled to mass spectroscopy
malERA	Malaria Eradication Research Agenda
MDA	Mass drug administration
nAChR	Nicotinic acetylcholine receptor
NTD	Neglected tropical disease
O-PLS-DA	Orthogonal projection to latent structure-discriminant analysis
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase-chain reaction
PLS	Projection to latent structure
PLS-DA	Projection to latent structure-discriminant analysis
PPPs	Public-private partnerships
R&D	Research and development
STH/STHs	Soil-transmitted helminthiasis/-ses
SVM	Support vector machine
Swiss TPH	Swiss Tropical and Public Health Institute
TSP	3-(trimethysilyl) [2,2,3,3-$^{2}H_4$] propionate
WHO	World Health Organization

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Chapter 1

General Introduction

1. Introduction

1.1. Soil-transmitted helminthiases

1.1.1. Epidemiology

Soil-transmitted helminths are gastrointestinal nematode worms that parasitize humans, and cause important chronic and debilitating diseases. The most common species are the roundworm *Ascaris lumbricoides*, the hookworms *Necator americanus* and *Ancylostoma duodenale*, and the whipworm *Trichuris trichiura*. The threadworm *Strongyloides stercoralis* is the fourth most prevalent soil-transmitted helminth. The global burden of soil-transmitted helminthiases (STHs) was revised in 2001, and estimated as high as 1 billion people infected [1]. As illustrated in Figure 1, STHs occur primarily in sub-Saharan Africa, Asia, Latin America and in the Caribbean [2].

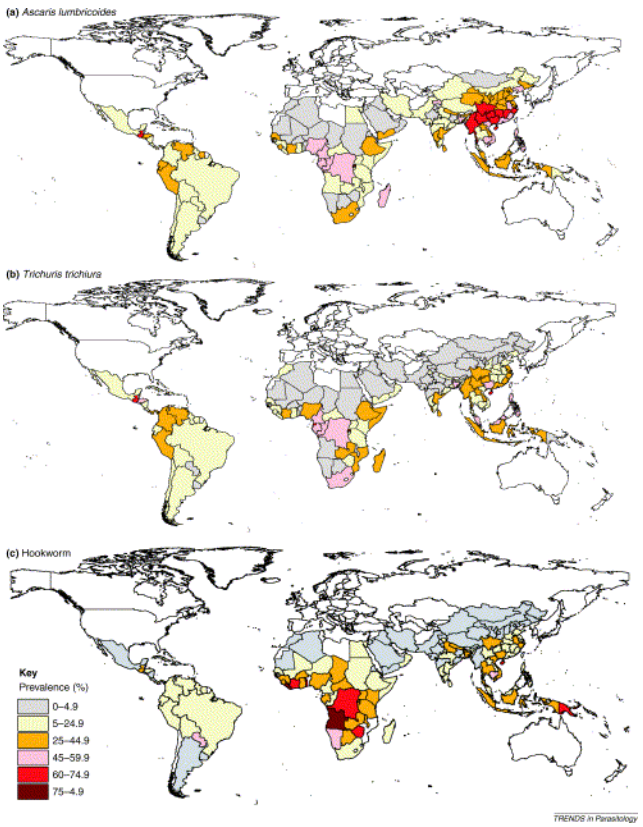

Figure 1. The global distribution of *Ascaris lumbricoides* (a), *Trichuris trichiura* (b) and hookworm (c). White areas represent countries not included in the present analysis. From [2].

Most at risk are the roughly 3 billion most impoverished people living on less than US$ 2 per day, particularly school-aged children [3,4]. *A. lumbricoides* accounts for 807-1221 million

infections, *T. trichiura* for 604-795 million, the hookworms for 576-740 million and *S. stercoralis* for 30-100 million [1]. In 2010, it was estimated that 5.2 billion people are at risk of ascariasis, 5 billion at risk of trichuriasis and 5 billion at risk of hookworm infections [5]. Heavy soil-transmitted helminth infections lead to serious health conditions such as iron-deficiency anemia, malnutrition, growth stunting and intellectual retardation [6-9]. STHs are estimated to result in approximately 135,000 deaths annually [10]. Despite their alarmingly high impact on socio-economic life in the affected countries, STHs have remained substantially neglected by the medical and international communities, contributing to an entrenched cycle of poverty [1].

1.1.2. Biology

Soil contains the infective stages (eggs or larvae) of STH. Upon infection with *Ascaris* or *Trichuris* spp., embryonated eggs are ingested, i.e. via soil-contaminated food or hands, whereas hookworm and *Strongyloides* third-stage (filariform) larvae (L_3) either penetrate the skin, most often at the feet or hands, or are ingested (*Ancylostoma* spp. only) (Figures 2 and 3).

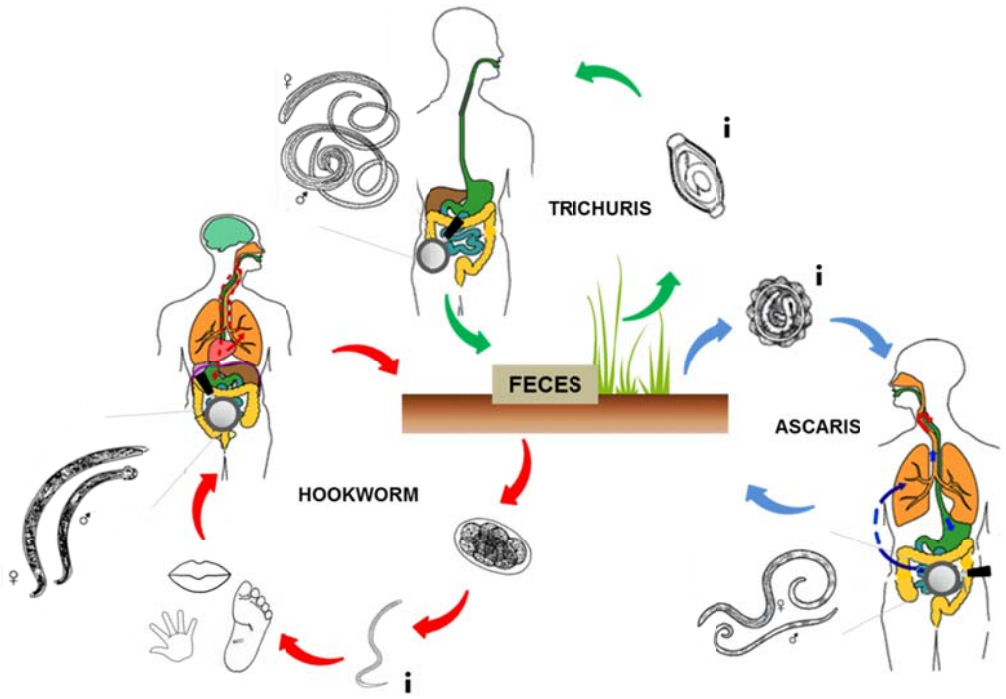

Figure 2. Life cycles of *Trichuris*, *Ascaris* and the hookworms. i: infective stage. Adapted from [11-13].

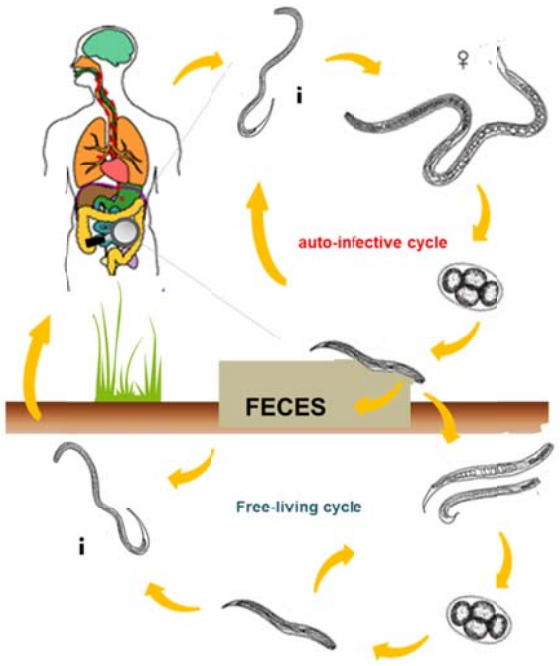

Figure 3. Life cycles of *Strongyloides stercoralis*. i: infective stage. Adapted from [14].

Ascaris larvae hatch in the intestine and penetrate the duodenal mucosa to enter the blood stream and are carried to the heart and to the lungs. Larvae mature further in the lungs for 10-14 days, penetrate the alveolar walls and move towards the throat, where they are swallowed. In the small intestine, they develop into adult worms and reside in the lumen. *Ascaris* spp. feed on digesta [15]. Two to 3 months post-infection and onwards, female worms release as many as 200,000 eggs per day. The lifespan of an adult worm in a human host is one year [1]. Fertilized eggs mature in the soil within 3 weeks or longer under favorable conditions (humid and warm) [12].

Trichuris larvae hatch in the small intestine, develop embedded in the intestinal mucosa and mature into adult worms, which remain attached to the caecum and colorectal mucosa. There, they mate and females start producing 3,000-5,000 eggs per day from day 60-70 post-infection. The eggs are then released in the feces. *Trichuris* spp. feed essentially on the intestinal mucosa [15]. Adults can remain in the large intestine for up to 2 years [1]. Eggs slowly embryonate in the soil within one month [11].

After penetrating the host skin, hookworm larvae enter the blood stream and reach the heart, from where they move to the lungs. They ascend the bronchial tree to the throat, and are swallowed. Larvae reach the small intestine where they mature into adults that can remain in the host for 5-7 years. Hookworms feed mainly on host's blood, but also on the intestinal mucosa [15]. Daily, 9,000-30,000 eggs are passed in the stools [1]. Larvae rapidly hatch and molt twice. L_3 can survive in the soil for 3-4 weeks under optimal conditions [13].

The threadworm *S. stercoralis* infects between 30 and 100 million people worldwide. Its parasitic life cycle shares most features with the hookworm's (except the passage through

the heart). However, *S. stercoralis* larvae (not eggs) are released with the stools, and the parasite has the capacity to re-infect its host (autoinfection). In the host, only female worms are present and reproduce by parthenogenesis. Another great difference is that the threadworm has a free-living life cycle and can alternate between parasitic and free-living life modes [14].

1.1.3. Diagnosis

Infection with *Ascaris*, *Trichuris*, or hookworm is diagnosed by detecting helminth eggs (or *Strongyloides* larvae) in the patient's stools, using several possible approaches. The method recommended by the World Health Organization (WHO) is the Kato-Katz thick smear technique [16]. More sensitive and resource-demanding possibilities include the Koga agar plate method [17] and the Baermann method [18], especially for the tricky detection of *S. stercoralis* larvae. The ether-concentration method allows the use of larger amounts of stools (and detection of concomitant intestinal protozoan infections) [19]. FLOTAC is a recent technology, requiring relatively simple laboratory equipment [20]. These techniques can also be combined to increase sensitivity. Recent field work identified FLOTAC as more sensitive than multiple Kato-Katz thick smears for *A. lumbricoides*, *T. trichiura* and hookworm eggs [21]. Moreover, FLOTAC can be used for detection of concomitant infections by several helminth species and intestinal protozoan parasites, such as *Giardia intestinalis* and *Entamoeba* spp. [22]. Alternative approaches such as serologic tests (enzyme-linked immunosorbent assays, ELISA) have shown risk of cross-reactivity and are suboptimal when antibodies persist [23-25]. Polymerase-chain reaction (PCR) methods for detecting intestinal helminths are also available and exhibit superior sensitivity compared to microscopy [26,27]. However PCR, the method of choice in industrialized countries, has not been well-accepted yet, because it necessitates well-equipped laboratories and is less field-applicable than microscopy.

1.2. Chemotherapy for the treatment and control of soil-transmitted helminthiases

To date, STH control relies essentially on preventive chemotherapy, mainly administered to school-aged children or other at-risk groups within the frame of periodic mass drug administration (MDA) campaigns [28]. In 2011, a tiny pharmacopeia of five drugs (Figure 4) was recommended by the WHO [29]. The benzimidazoles albendazole and mebendazole are the first line drugs against ascariasis, trichuriasis and hookworm infections. Pyrantel and

levamisole, although not playing prominent roles in preventive chemotherapy, display different degrees of efficacy against these three diseases. Ivermectin is used against strongyloidiasis, as well as albendazole [28]. In many cases, the drugs are donated by pharmaceutical companies, and public-private partnerships (PPPs) [4,30,31].

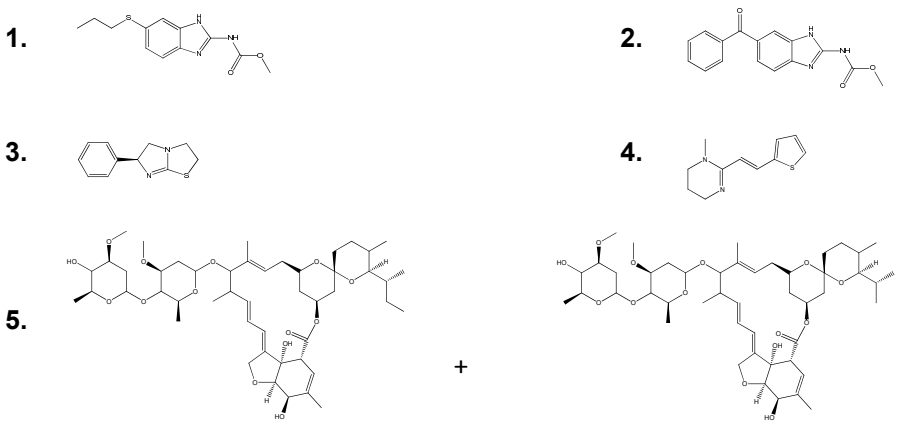

Figure 4. Chemical structures of the standard anthelmintic compounds for treatment of STHs.
1. Albendazole; 2. Mebendazole; 3. Levamisole; 4. Pyrantel; 5. Ivermectin.

All of the drugs were initially developed for veterinary needs and have been in use in humans for decades (Table 1). However, the transitioning into human medicine took place without proper adaptation. It has been suggested that the currently applied dosages are suboptimal [31]. Many gaps remain in our understanding of the drugs routinely used in MDA programs, and in the long term, these knowledge gaps might impair the sustainability of treatment efficacy [31]. Moreover, none of these drugs present satisfactory efficacy using single doses on all soil-transmitted helminth species, and particularly low efficacy is observed against *T. trichiura* [32].

There is currently no new drug in the anthelmintic pipeline for treatment of STHs. In light of the alarming status in livestock, there is rising concern about emergence of drug resistance in human helminth populations. Relying on only a handful of anthelmintics is a precarious situation, and the need for new anthelmintic treatments is acute (new agents or combinations of existing drugs).

STHs are associated with extreme poverty, poor environmental and personal hygiene, and therefore other important means to control these diseases exist besides chemotherapy by reducing transmission [33]. Health education, access to clean water and sanitation are key aspects for moving towards (at least local) STH elimination, as part of socio-economic development [34,35]. There is evidence suggesting that urban areas experience significantly lower hookworm prevalence, while the situation is reversed for *A. lumbricoides* and *T. trichiura* [5].

Current anthelmintic treatment options against STHs in preventive chemotherapy programs					
Drug	**Discovery**	**Incentive**	**Target organisms**	**Adult dosage (oral, single)**	**Reference**
Albendazole	1976	Veterinary	*Ascaris*, *Trichuris*, hookworms, *Strongyloides*	400 mg	[28,29,36-39]
Mebendazole	1972	Veterinary	*Ascaris*, *Trichuris*, hookworms	500 mg	[28,29,37-40]
Levamisole	1969	Veterinary	*Ascaris*, (*Trichuris*), hookworms	2.5 mg/kg	[28,29,38,39,41]
Pyrantel pamoate	1966	Veterinary	*Ascaris*, (*Trichuris*), hookworms	10 mg/kg	[28,29,38,39,42]
Ivermectin	1970s	Veterinary	*Strongyloides*	3 or 6 mg	[28,29,38,39,43,44]

Table 1. Current anthelmintic drugs for treatment of STHs in preventive chemotherapy programs.

1.2.1. Benzimidazole carbamates: albendazole and mebendazole

The benzimidazoles have proven to be very useful in both veterinary and human medicine. They are relatively inexpensive, safe and have a broad spectrum of activity against nematodes and other helminths [38]. Currently, albendazole and mebendazole are the two most widely used drugs for control and treatment of STHs [32]. As studied for albendazole, the benzimidazoles are thought to bind to the free β-tubulin subunits, thus interfering with helminth tubulin polymerization [45,46], and thereby leading to progressive cell function disruption. Albendazole is largely converted into its more hydrophilic metabolite, albendazole sulfoxide, by liver microsomes, and possibly also in the intestine [47,48]. Lipophilicity has been shown to facilitate drug diffusion through the nematode cuticle [49,50]. Hence, it has been suggested that most nematocidal activity is exerted by the parent drug albendazole, complemented by the sulfoxide form [51]. This is in line with the idea that the main route of anthelmintic uptake occurs rather by transcuticular diffusion than by oral ingestion by the worms [52]. Overall cure rates of ≥94% have been reported after treatment of *A. lumbricoides* infection with 400 mg albendazole or 500 mg mebendazole using single doses [39]. The same doses achieved cure rates of 43.6% and 23.0%, respectively, in *T. trichiura*-infected patients [39]. Mebendazole administered in multiple doses (2x100 mg over 3 days) has been reported to achieve a median cure rate of approximately 75% [53]. Against hookworms, cure rates of 78.4% were observed for 400 mg albendazole, cure rates of only 22.9% were detected for 500 mg mebendazole [39], and cure rates were approximately 80% following treatment with multiple doses of mebendazole (2x100 mg over 3 days) [53]. Indications of teratogenicity and embryotoxicity in animal models are the basis for contra-indications for treatment of children under one year and women in the first trimester of pregnancy [48].

1.2.2. Nicotinic cholinergic agonists: levamisole and pyrantel pamoate

Levamisole, an imidazothiazole, and pyrantel pamoate, a tetrahydropyrimidine, act as nematode nicotinic acetylcholine receptor (nAChR) agonists. They produce muscle contraction and spastic paralysis as a consequence of membrane depolarization by opening non-selective cation-channels that are permeable to both Na^+ and K^+ [54]. The nematode muscular AChR are classified into three pharmacological categories, each presenting a different channel conductance. The *L*-subtype is preferentially activated by levamisole, even at low concentrations. It has been shown that resistance to levamisole in parasitic nematodes is associated with a decrease in response of the *L*-subtype receptors [55]. Levamisole administered at a single oral dose of 2.5 mg/kg, achieved cure rates of 91.5% against *A. lumbricoides*, only 8.6% against *T. trichiura*, and 38.2% against hookworm [39]. Overall cure rates of 87.9%, 28.1% and 87.9% were observed respectively, in *A. lumbricoides*-, *T. trichiura*-, or hookworm-infected patients, following a single oral dose of 10 mg/kg pyrantel pamoate [39].

1.2.3. Macrocyclic lactones: ivermectin

Ivermectin, the only drug of this class approved for humans, is a potent agent against a wide range of nematodes as well as arthropod ectoparasites [31,43]. It is mostly used for the treatment and control of infections with filarial parasites (lymphatic filariasis and onchocerciasis) and strongyloidiasis [28,32]. Ivermecin displays a low efficacy against hookworms, but significantly impacty on *Trichuris*, whereas controversial findings have been reported regarding *Ascaris* [56-58]. The most likely physiological target is a nematode-specific glutamate-gated chloride receptor (GluCls) [59]. Ivermectin acts as a potentiator and increases the worm muscle Cl^- permeability, leading to paralysis and finally, starvation of the parasite. At high concentrations, these GluCls are irreversibly and directly opened by the drug [54,60]. Although it is the drug of choice for treatment of strongyloidiasis, the treatment schedule for ivermectin still has to be defined [44,61]. Ivermectin is rapidly metabolized by liver microsomes to several metabolites, and is relatively safe in humans. In vertebrates, ivermectin can trigger release of neuronal gamma aminobutyric acid (GABA), but is likely prevented from crossing the blood-brain barrier by a P-glycoprotein efflux pump [48].

1.3. Drug resistance and lessons from veterinary medicine

1.3.1. Drug resistance

Drug resistance is defined as an increased proportion of individuals in a parasite population that show reduction in sensitivity to a given drug, compared to a normal population [62,63]. It can arise following a genetic mutation. Changes at different levels can occur: in the molecular target, leading to, for example, a less efficient drug binding; in the metabolism resulting in drug detoxication or removal; in the distribution of the drug in the parasite, preventing contact between the drug and its target; or amplification of target genes to overcome the drug effect [63]. These mutations are inherited, conferring resistance to the next generations and no evidence suggests that the phenomenon is reversible [64]. Inappropriate treatment use can result in drug resistance. Contributing factors are multiple, from under-dosing, sub-optimal targeting and timing of mass treatments, to high treatment frequency [65]. Moreover, the treatment frequency, efficacy and pharmacokinetics of the drug (fast-acting drugs are safer), as well as the worm's biology (life-span, fecundity etc.) and the environmental conditions, play determining roles in the development of resistance [66]. The emergence of drug resistance is no inescapable fate and depends on the relative fitness costs attributed to the resistance alleles at the level of drug use [63]. In the case of periodic mass treatment, worms that have been excluded from treatment with a given drug (refugia) are important in that they "dilute" the resistance alleles with their susceptible ones. This is why, according to the prevalence of an infection, it is preferable not to treat the entire population [66].

1.3.2. Drug resistance in veterinary medicine

Drug resistance has evolved as a serious problem against a wide range of parasitic nematodes of livestock (i.e. *Haemonchus* spp., *Teladorsagia* spp., *Trichostrongylus* spp.). In some countries such as Australia, with high parasite prevalence in sheep, drug resistance has arisen against several broad-spectrum drugs of all major families (benzimidazoles, nicotinic cholinergic agonists, macrocyclic lactones) [63,67]. Other resistant cases against narrow-spectrum drugs (closantel) have also been reported [63]. Drug resistance inflicts significant (yet hard to quantify) economic losses to the animal production industry [68]. Pasture management and efforts to limit resistance selection pressure are part of the strategy to combat these infections, but new drugs are still needed. Monepantel, adding a novel mode of action to the arsenal of veterinary anthelmintics, has been the only treatment introduced to the market since ivermectin in the late 1970s [69]. However, it is unlikely that novel drug classes alone solve the problem of drug resistance, now grown too big [70].

Moreover, with the limited revenues from veterinary drugs, the cost-benefit relationship is often seen as financially unrewarding by pharmaceutical companies [71].

1.3.3. Drug resistance in human soil-transmitted helminth populations

The recent scale up in chemotherapy programs in numerous geographical regions might exert significant drug pressure on soil-transmitted helminth populations [72]. Drug resistance in *A. lumbricoides* populations has not been suspected yet [72]. A few cases of low cure rates in human hookworm infections have been recently reported, suggesting for the development of drug resistance, but no conclusive evidence is available [65].

In Mali, *N. americanus* failed to respond as expected to a single dose of 500 mg mebendazole or to 400 mg albendazole, with reported cure rates of 22.9% and 51.4%, respectively [73,74]. Surprisingly low efficacies for mebendazole were also reported in Vietnam and on Pemba Island (Zanzibar) against hookworms [75,76]. In 2011, albendazole showed a high treatment failure rate against hookworms in Ghana [77]. Finally, in Australia, a 10 mg/kg pyrantel treatment failed to effectively cure *Ancylostoma*-infected patients (cure rate of 13%) [78]. The number of reports is very limited and based on small groups of patients. In addition, without assessing the potential confounding factors, the nature of the problem remains uncertain, and could be reflecting either real selection for resistance alleles or just reduced efficacy. While likely resistance alleles have been detected in human STHs, the frequency of resistance mutations following drug pressure has not been investigated yet [72]. An important limiting factor to monitoring emergence of drug resistance in human soil-transmitted helminth populations is the lack of sensitive diagnostic tools and standardized procedures.

1.4. The need for new treatments

1.4.1. Challenges in anthelmintic drug discovery

Drug discovery and development is a complicated, costly, risky, time-consuming and highly regulated process. The clinical development being the most expensive step, the decision to move a compound from discovery into the development phase must be justified by sound scientific data and cost considerations [79,80].

As listed in a proposed target product profile for drugs for STHs, the main characteristics of an ideal drug are that it should be cheap, safe and active against at least eggs and adult worms of all major geohelminth species and that it should present a novel mode of action. It

should be best administered in a single oral dose (or maximum over 2 days) and the resulting cure rates should be >90% against adult worms [79].

The original strategy to discover new anthelmintic compounds was based on direct whole-organism screening on cultured parasites, or in animals, which presents significant drawbacks such as the high cost of random screening relying on animals, or the incompatibility with combinatorial chemistry [38]. Therefore, the pharmaceutical industry attempted to replace it by an approach named mechanism-based screening [52]. The high costs of the equipment needed and the paucity of well-characterized and validated drug targets necessary to this screening approach render mechanism-based screening even less suitable for anthelmintic research [52,71]. The standard methods for drug sensitivity testing on soil-transmitted helminths therefore rely mainly on microscopy for phenotypic assessment of a given stage's viability *in vitro* and on a range of helminthiases animal models for further *in vivo* investigations. The egg hatch assay tests the ovicidal properties of tested compounds [81]. The viability of adult worms or free-living infective larvae is assessed using the motility assay, in which the capacity to move is rated on a viability scale or the percent survival is calculated [82,83]. These assays are labor-intensive, subjective, and require killing of the animal host, for testing on adult worms. Little attention has been given to optimize the compound screening strategies, and to date, no assay meets the ideal profile: cheap, simple, user-friendly, with an automatized reading. It has to be emphasized that so far, it is not possible to culture any parasitic helminth *in vitro* throughout all the life cycle stages.

Helminthiases are among the most neglected diseases in terms of drug research and development. It has been proposed, that in this situation of very limited resources, few candidate drugs and few development partners, a global research and development pipeline would be a more efficient strategy than several uncoordinated initiatives. This is why the interested public and not-for-profit organizations should build platforms to consistently concert their efforts [79].

1.4.2. Animal models for human soil-transmitted helminthiases

Animal models have been used extensively to evaluate the potential of drug candidates, creating more real-life conditions relating closer reality than in culture systems. A handful of rodent models for human STHs have been used in the past decades for drug screening. In the frame of this work, we used three hookworm models (*Necator americanus*, *Ancylostoma ceylanicum* and *Heligmosomoides bakeri*), a trichuriasis model (*Trichuris muris*), an ascariasis model (*Ascaris suum*) and a strongyloidiasis model (*Strongyloides ratti*). A human *N. americanus* and a canine *A. ceylanicum* have been adapted to hamster hosts over decades [84-87]. *T. muris* is a well-established mouse model [88,89]. *A. suum* is a murine model for the larval stages only and the complete life cycle is maintained in the swine.

S. ratti is a rat parasite [90]. Except for *A. suum*, all the above-mentioned parasites can be maintained continuously in the laboratory.

1.4.3. Possible future anthelmintics

Because veterinary drugs are selected according to very stringent criteria, robust safety, pharmacokinetic and efficacy data are usually available and essentially equivalent to human requirements. A recent work screened for potential development candidates in the animal health arsenal for rapid transitioning into human medicine [79]. Using different publicly available information platforms, emodepside, and monepantel have been identified. The old drug oxantel, used in the past against *T. trichiura*, as well as the antiprotozoal drug nitazoxanide deserved renewed attention and careful examination of its potential in our animal models [91].

1.4.3.1. Cyclooctadepsipeptides

PF1022A and its semisynthetic derivative emodepside are fungal derivatives displaying anthelmintic activity against a range of gastrointestinal nematodes of veterinary importance. Both PF1022A and emodepside showed promising efficacy in the threadworm and hookworms models *S. ratti* and *H. bakeri* after oral treatment, emodepside being slightly better [92-94]. The modes of action of the cyclooctadepsipeptides are thought to be both interaction with the latrotoxin-induced Ca^{2+} influx at the latrophilin receptor and relaxation of nematodes via Ca^{2+}-activated K^{+} channels [95]. Treated worms show flaccid paralysis followed by death. Importantly, livestock nematode strains that were resistant to the currently available drug classes were sensitive to these compounds [95]. In 2005, Bayer Animal Health developed emodepside for use in pets against hookworms and ascarids [79]. PF1022A has recently been tested against *H. bakeri* in our laboratory [94].

1.4.3.2. Tribendimidine

Tribendimidine, a derivative of the drug amidantel (Bayer) was discovered in the 1980s by the Institute of Parasitic Diseases in Shanghai, China [96]. Its anthelmintic properties have been determined against numerous laboratory and human helminths. In addition, a rapid onset of action and a good reported tolerability contributed to its promising profile [96,97,98]. The drug has been approved for human use in China in 2004 to treat hookworm infections (*N. americanus* and *A. duodenale*) as it performed better with a single oral dose than the drug of choice albendazole [39]. In addition, *A. lumbricoides* in humans as well as *S. ratti* in rodents revealed marked sensitivity [96,98]. Tribendimidine is thought to belong to the *L*-subtype nAChR agonists, sharing the same mode of action as levamisole [99].

1.4.3.3. Monepantel

Monepantel (AAD 1566) is an amino-acetonitrile derivative drug developed by Novartis Animal Health and was first registered in 2009. Primarily used to treat sheep abomasal and intestinal parasites, monepantel is a safe broad-spectrum agent, showing high cure rates on fourth stage larvae and adult worms [79]. It has been proposed that the drug interferes with nematode-specific nAChR subunits, causing somatic muscle paralysis and thereby resulting in death of the worms [69]. It is an agonist of the nematode-specific DEG-3 subtype of nAChR, specifically targeting the protein MPTL-1 in *H. contortus* (or homologs) [69,100]. *S. ratti* lacks this protein and the drug was therefore found inefficacious in this model for STH [100].

1.4.3.4. Nitazoxanide

Initially described in the 1980s, nitazoxanide is a broad spectrum thiazolide drug with anthelmintic, antiprotozoal and antiviral properties [101-103]. It was commercialized more than 10 years ago because of its activity against the protozoa *Cryptosporidium parvum* and *Giardia intestinalis*. A few human clinical trials demonstrated potent nematocidal activity following multiple doses of the drug against *A. lumbricoides*, *T. trichiura*, *A. duodenale*, *Enterobius vermicularis* and *S. stercoralis* [104,105,106]. Nitazoxanide has been shown to be a noncompetitive inhibitor of the pyruvate oxydoreductases in protozoa and anaerobic bacteria [107]. However, alternative targets such as nitroreductases and protein disulphide isomerases have been proposed [103,108]. Nitazoxanide presents an excellent safety and bioavailability profile [48,79].

1.4.3.5. Oxantel

Oxantel is a meta-oxyphenol derivative of the nicotinic cholinergic agonist pyrantel. Discovered in the 1970s by Pfizer, it has been used for treatment of trichuriasis in children, while pyrantel lacked efficacy [91]. Experiments with *A. suum* lead to the classification of oxantel as an *N*-subtype nAChR agonist, like nicotine but unlike levamisole and pyrantel [109]. The combination oxantel-pyrantel-praziquantel is widely administered to canines against nematodes and cestodes [110]. The combination displayed a poor efficacy (<40%) in *T. trichiura*- and hookworm-infected children in Pemba Island (Zanzibar) [111]. Combinations of oxantel with other marketed drugs have not been tested.

1.5. Drug combinations

Mathematical modeling revealed a risk reduction of drug resistance development by administering several drugs in combination [66]. Combination chemotherapy is now an acknowledged strategy against a wide range of pathogens and cancer. In human clinical trials, eleven drug combinations of already marketed drugs have been tested so far against soil-transmitted helminths and often increased efficacies were obtained (For details, see [32]). Combinations of readily approved drugs for human use present the advantage of being a “new” treatment, for which introduction to the market might be greatly facilitated. In addition, drug combinations help cover a greater range of helminth species, and thus may prove to be very useful to treat commonly observed soil-transmitted helminth multiple infections [112].

1.6. The hookworm vaccine

Periodic MDA does not interrupt STHs transmission. In addition, the fact that these worms persist in their host for years and that both the prevalence and infection intensity do not decrease with age despite regular exposure (at least for hookworms) indicate that humans fail to develop protective immunity [113]. In 2000, the Human Hookworm Vaccine Initiative was created following concerns about mebendazole drug failure against hookworms and potential emergence of drug resistance to the benzimidazoles as well as the rapid re-infection after treatment [114]. Two human hookworm vaccines have been tested in clinical trials. A first recombinant candidate vaccine was based on *N. americanus* ASP2 *Ancylostoma*-secreted protein 2, an antigen secreted by third-stage larvae upon invasion of the host [114,115]. It was found safe and immunogenic in unexposed volunteers, but lead to adverse events in some patients in a field study. These pre-exposed patients presented high levels of IgE against ASP2 [114]. Trials with ASP2 as vaccine antigen were therefore discontinued. Since then, hookworm vaccine preclinical research has focused on the antigens GST1 (a glutathione-*S*-transferase) and APR1 (an aspartic protease) used in combination in a bivalent product. Both proteins play a role in the adult worms nutritional and metabolic requirements [114]. Such a vaccine, conferring modest protection in animal models could be cost-effective, even if periodic anthelmintic administration would need to be continued [115-117]. To date, no vaccine against other STHs is in development.

1.7. Co-infection of soil-transmitted helminths with *Plasmodium falciparum* malaria

It is widely known that helminth infections often occur concomitantly with other endemic diseases. Co-infections with several soil-transmitted helminth species are very frequent. Extremely common as well, especially in sub-Saharan Africa, is the co-infection of hookworms and the malaria causative agent *Plasmodium falciparum* (Figure 5) [118]. This co-infection will be discussed in greater detail here as investigated in the framework of this PhD thesis. Among parasitic diseases, *P. falciparum* inflicts the heaviest burden [119]. In 2010, it accounted for more than 600,000 deaths globally, mostly among African children [120]. Of the approximately 180 million children living on the African continent, an estimated 50 million are infected with hookworms, and 45 million are considered at risk of concomitant infection with malaria (Figure 5) [118]. Besides a documented increase in anemia caused by both parasites [121], the rare published reports on the interactions between both infections are controversial. Hookworm infections were often associated with increased *P. falciparum* malaria [122-128], but a lack of association was also described [129]. Despite these conflicting findings, a tendency for hookworm infections to worsen the pathogenesis of malaria has been identified [130]. More specifically, hookworm infections were mostly associated with negative effects such as increased incidence and prevalence of malaria [122,131] and increased anemia [121]. It has been hypothesized that besides immunomodulation, hookworm-induced blood loss might increase the attracting signals to malaria mosquito vectors (lactate, increased respiratory rate etc.), resulting in a greater probability of getting infected [131]. At the molecular level, both pathogens are thought to induce significant immunomodulation, affecting the Th1/Th2 balance of immune responses and the capacity to respond to single parasites [127,132]. At present, much remains to be discovered about the complex hookworm-malaria-human host interactions. In this work, we used a ^{1}H NMR-based metabolic profiling approach to characterize single- and co-infection-related biomarkers in plasma and urine from a murine model.

Figure 5. Predicted prevalences for hookworm (left), climatic suitability for *P. falciparum* malaria transmission (middle) and overlap of both (right). From [118].

1.8. Introduction to ^{1}H NMR-based metabolic profiling

Metabonomics is commonly defined as the "quantitative measurement of time-related multiparametric metabolic responses of multicellular systems to pathophysiological stimuli or genetic modifications" [133]. The combination of spectroscopic methods such as ^{1}H nuclear magnetic resonance (NMR) spectroscopy, or mass spectrometry with multivariate analysis, have allowed the overall metabolic characterization of any given condition (disease, aging, toxicology, nutritional deviations etc.), in a powerful and reproducible manner [134-136]. The metabolic profiling approach, as depicted in Figure 6 aids in understanding the relationships and interactions between metabolic variation and environmental triggers to an observed condition- information which is complementary to other -omics approaches, such as genomics or proteomics [134,137]. Spectral data can easily be integrated with measures derived from other analytical platforms such as ELISA or PCR, in order to explain an observed metabolic phenotype and obtain a more systemic view on a given effect. A metabolic profile can be established from any biological compartment whereby the most widely assessed matrices include plasma, urine, and tissue samples [135,138,139]. The development of mathematical tools and softwares enabled analysis, interpretation and visualization of the data and thus contributed to the applicability of metabolic profiling to parasite research [140].

^{1}H NMR has proven to be a technique that is highly efficient and reproducible. In combination with multivariate statistical methods, metabolites (biomarkers) can be identified that are discriminating between two given conditions, e.g. (infected and control) and also allow molecular structural identification in the case of unknown biomarker identity [141]. In

this context, a biomarker refers to a substance that is changing in response to an infection (in quantity or presence/absence). The metabolic fingerprint is defined as a selection of several biomarkers (that characterize a condition).

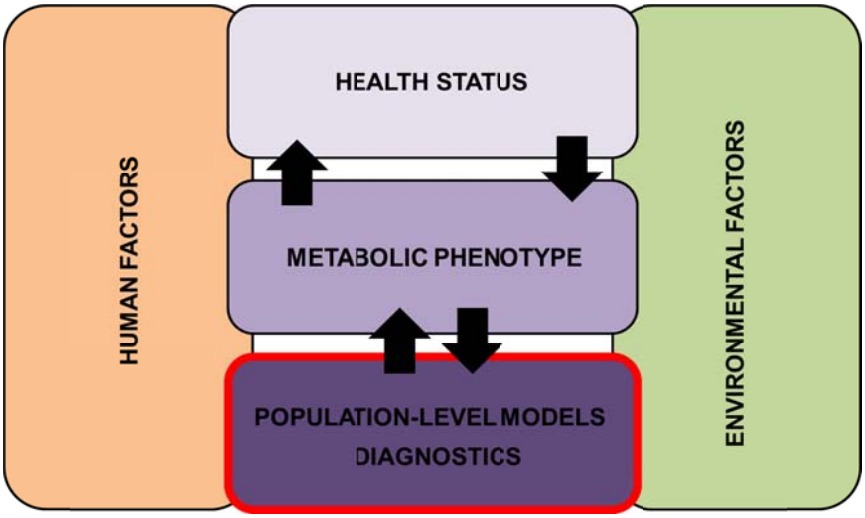

Figure 6. Interactions between human and environmental factors and their effect on individual or population health. Metabolic phenotype is influenced by both genetic and environmental factors, influencing in turn the health status. The characterization of a disease-related metabolic phenotype enables modeling for population-level disease surveillance/monitoring programs and diagnostics. Adapted from [134].

Some isotopes, such as ^{1}H, possess an inherent magnetic moment called spin. When an external magnetic field is applied, these nuclei absorb and re-emit this energy in the range of radio wavelengths. The interactions between the spin and the magnetic field determine the radio frequency at which energy is absorbed and depend on the field strength, the isotope type, and the electronic environment surrounding the isotope. ^{1}H nuclei have a spin of I = ½, which can demonstrate at two different energy levels. Following application of a high power magnetic field, most of the spins will align with it, producing an energetically favorable state. The sample is placed into the center of the spectrometer surrounded by a static magnetic field, whereby the nuclei align with or against it. A radiofrequency pulse is applied, which flips the magnetization vector by 90°. Nuclear resonance results from the relaxation of the nuclei back to their original state and is recorded as free induction decay, a transient signal that needs to be recorded several times and averaged to optimize the signal-to-noise ratio [142,143]. A Fourier transformation converts the time measurements into frequency and allows data visualization in the form of a spectrum.

NMR spectra provide 3 categories of structural information:

1) Electron density and the degree of electron shielding: Each CH group within a molecule is represented on a particular position on a chemical shift scale (in parts per million) which is dependent on the electronic environment i.e. the electron density surrounding the CH group and the resulting degree of shielding, but independent of the field strength [142,143]. The more exposed (unshielded) a CH group is, the higher up the chemical shift scale it will be found.

2) Neighboring CH group: All CH groups within a molecule are represented by a distinctive spectral pattern. The spin-spin coupling (or J coupling) is mediated by chemical bonds between CH groups and is indicative of the amount of protons in the neighbor CH group [142]. The peak multiplicity represents the number of neighbor ^{1}H groups plus one.

3) CH ratio: NMR spectroscopy can be a quantitative method, and the peak intensities are proportional to the number of ^{1}H within one molecule.

A reference compound is usually included, such as sodium 3-(trimethylsilyl) [2,2,3,3-$^{2}H_4$] propionate (TSP) which is located at the chemical shift position 0 ppm.

Multivariate analysis (or chemometrics) allows for the identification of inter-group differences [144], whereby principal component analysis (PCA) is the most widely applied method for initial screening of a data set. As an unsupervised method, it is used to visualize data clustering, systematic variation (i.e. over time) and outliers by projecting a cloud of coordinates into an n-dimensional space [139,144]. To do so, all information from one spectrum is compressed into a single data point placed and projected onto a two-dimensional plane whereby each axis or principal component (PC) represents a linear combination of the original spectral descriptors [143]. Projection to latent structure (PLS) is a supervised method and is the method of choice for identifying those metabolites which are responsible for class separation (i.e. biomarkers represented by loadings). Here, the concept of the statistical analysis is to relate the spectral information in an x-matrix (descriptor) to a second matrix (y, the response matrix) containing class information or other, continuous measures such as weight, cytokine levels, etc. [145]. Projection to latent structure-discriminant analysis (PLS-DA) enables class separation and effective subsequent biomarker recovery [146]. The orthogonal projection to latent structure-discriminant analysis (O-PLS-DA) contains an orthogonal data filter to eliminate systematic variation, such as

noise, unrelated to infection status. The method produces correlation coefficient plots (Figure 7) enabling identification of the discriminating features in the spectral information [145].

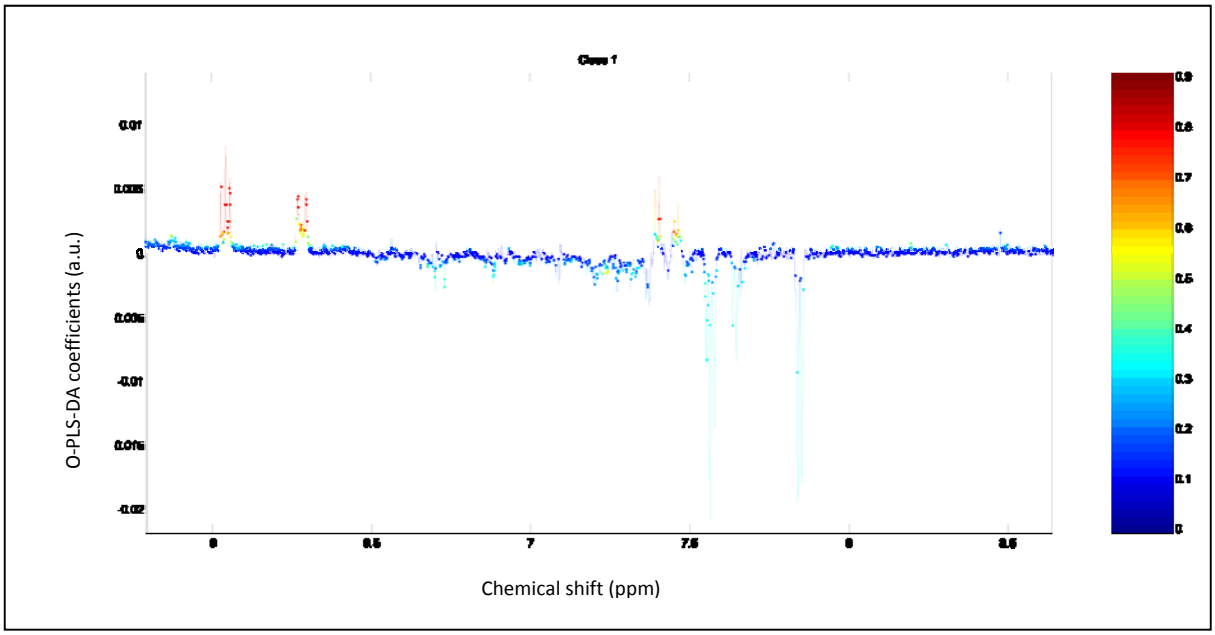

Figure 7. O-PLS-DA coefficient plot derived from ^{1}H NMR spectra of urine individually collected from mice 16 days post-infection with *P. berghei* (upper part of the graph) versus uninfected controls (lower part of the graph).

Metabolic profiling has already been broadly used to gain knowledge about host-parasite interactions, with the aim of discovering infection-related specific metabolic markers (< 1 kDa) and improving diagnostic and disease monitoring capacities [135,138,147-149]. However, the use of metabolic profiling for disease monitoring is not yet established and further solid animal data are needed [150]. Metabolic profiles from a malaria murine model and an *N. americanus* hamster model have already been studied [138,151], but no co-infection model between them has been established yet. ^{1}H NMR-based metabonomics represents an ideal approach to study the malaria-hookworm-host interactions.

1.9. Aim and objectives

Novel anthelmintics are urgently needed for treatment of STHs. Only a handful of drugs are recommended by the WHO (albendazole, mebendazole, levamisole, pyrantel pamoate and ivermectin), most often with insufficient efficacy when administered in single dose. Although anthelmintic drug resistance has not yet appeared as a major public health problem,

emergence of drug resistance may be inevitable in the future. The need for drugs has been largely ignored by research and existing tools for *in vitro* drug sensitivity testing lack convenience and high-throughput screening ability.
This work aimed on the one hand to improve drug screening capacities and identify potential new treatments for STHs. On the other hand, we aimed to strengthen our understanding of the impact of a murine malaria and hookworm co-infection on the host's metabolism and identify potential candidate diagnostic biomarkers.

The following objectives were accomplished:

1. To establish the life cycles and nematode rodent models of *A. ceylanicum*, *N. americanus* and *T. muris* at the Swiss TPH.

2. To test and optimize a range of different *in vitro* drug sensitivity assays for *A. ceylanicum* (chapter 2a) and *T. muris* larvae and adult worms (chapter 2b).

3. To evaluate the potential of the veterinary anthelmintic monepantel (AAD 1566) in five animal models for human helminthiases: *A. ceylanicum*, *N. americanus*, *T. muris*, *A. suum* and *S. ratti* (chapter 3).

4. To compile comprehensive efficacy data on the Chinese anti-hookworm tribendimidine against *A. ceylanicum* and *H. bakeri*, alone or in combination with the five standard drugs (chapter 4).

5. To assess the nematocidal properties of the broad-spectrum drug nitazoxanide against *A. ceylanicum* and *T. muris*, singly or combined with the five marketed anthelmintics (chapter 5).

6. To spotlight the forgotten properties of the old drug oxantel by generating data in the hookworm (*A. ceylanicum* and *N. americanus*) and whipworm (*T. muris*) rodent models, alone or as a partner drug in several combinations with standard anthelmintics (chapter 6).

7. To identify plasma and urinary metabolic fingerprints related to *P. berghei* and *H. bakeri* single and co-infections, in a murine model (chapter 7).

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Chapter 2a

Comparison of novel and existing tools for studying drug sensitivity against the hookworm *Ancylostoma ceylanicum in vitro*

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SUMMARY

The motility assay is the current gold standard for evaluating drug effects on hookworm larvae and adults, however, among other drawbacks the assay is time consuming, and prone to individual subjectivity. We evaluated six alternative *in vitro* assays, namely the feeding inhibition assay, the colourimetric AlamarBlue®, MTT formazan and acid phosphatase activity assays, as well as isothermal calorimetry and the xCELLigence System using *Ancylostoma ceylanicum* third-stage larvae, stimulated third-stage larvae and adults. The performances of the assays were compared to the motility assay using three standard drugs: albendazole, levamisole and ivermectin (100–1 μg/ml). None of the assays investigated offered an advantage over the motility assay, because they were all inapplicable to third-stage larvae, which were presumably metabolically and physically too inactive. Among all assays tested the xCELLigence System performed best on adult worms as the test was accurate, simple, required a minimal number of worms and offered the possibility for conducting a medium-throughput screening.

Key words: hookworm, *Ancylostoma ceylanicum*, *in vitro* assays, motility assay, feeding inhibition assay, acid phosphatase, Alamar Blue®, MTT, xCELLigence System, calorimetry.

INTRODUCTION

The hookworms, *Ancylostoma duodenale* and *Necator americanus* infect an estimated 600 million people worldwide and are major causes of global morbidity accounting for as many as 22·1 million disability-adjusted life years (DALYs) lost annually (Bethony *et al.* 2006; Chan, 1997). Hookworm infections belong to the so-called neglected tropical diseases and occur mostly in the poorest regions of tropical and subtropical countries (Bethony *et al.* 2006; Hotez *et al.* 2008). School-age children, pregnant women and the developing foetuses are particularly at risk of suffering severe morbidities from hookworm infections (Brooker *et al.* 2004).

Currently, control of hookworms relies essentially on regular anthelmintic treatment, mostly in the framework of mass drug administration campaigns (Harhay *et al.* 2010; Hotez, 2008; Hotez and Pecoul, 2010). Despite this, only five drugs (the two benzimidazoles albendazole and mebendazole, pyrantel pamoate, levamisole and ivermectin) are recommended by the World Health Organisation (WHO), which have all been used since decades (WHO, 2011). In terms of cure rates only albendazole is highly effective against hookworms (Keiser and Utzinger, 2010). In addition, resistance to these drugs is already widely spread among livestock (Wolstenholme *et al.* 2004) and therefore there are concerns about the emergence of drug resistance among human helminth populations. Hence, there is a pressing need to discover and develop alternative anthelmintics (Geerts and Gryseels, 2001; Kaplan, 2004) and robust tools to monitor helminths drug susceptibility.

Today, the few ongoing drug discovery efforts on human nematodes rely on low- to medium-throughput *in vitro* whole organism drug screening. In more detail, the current assay of choice, the motility assay, is based on the microscopic evaluation of the worms' viability, following drug exposure and subsequent stimulation of worms (Gill *et al.* 1991; Kotze *et al.* 2004; Satou *et al.* 2001). The motility assay can be performed with different developmental stages, and requires little laboratory equipment. However, it is time consuming and prone to subjectivity upon reading. The development of a novel medium- to high-throughput *in vitro* assay with a simple readout would therefore be a great step forward in the field of nematocidal drug discovery.

In this work, we aimed to evaluate several potential alternative *in vitro* assays to evaluate drug effects on

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A. ceylanicum. We assessed two viability markers, Alamar Blue®, an indicator for metabolic cell function (Räz *et al*. 1997) and the MTT reduction assay, which is based on a colourimetric reaction involving pyridine nucleotides cofactors (Berridge and Tan, 1993; Mosmann, 1983). In addition, we measured acid phosphatase, which is secreted in large amounts by several nematode species in feeding stages, possibly to promote extracorporeal digestion and tissue penetration (Maki and Yanagisawa, 1980; Martinez-Grueiro, 2002) and which is a good indicator of the worms' fitness. We also evaluated the feeding inhibition assay (Hawdon and Schad, 1990; Kopp *et al*. 2008), and two fairly new technologies, namely isothermal calorimetry (Braissant *et al*. 2010) and the xCELLigence System (Roche Inc.) (Smout *et al*. 2010). We analysed the effect of albendazole, levamisole and ivermectin on L3, stimulated L3 and adults in these assays and compared findings with results obtained with the current method of choice, the motility assay. Finally, advantages and disadvantages of the individual assays are highlighted.

MATERIALS AND METHODS

Drugs

Albendazole and ivermectin were purchased from Sigma-Aldrich (Buchs, Switzerland), and levamisole-hydrochloride was obtained from Fluka (Buchs, Switzerland). Stock solutions (5 mg/ml) were prepared for all drugs in 100% DMSO (Fluka, Buchs, Switzerland) and stored at 4 °C.

Animals and parasite infections

Three-week-old male Syrian Golden hamsters were purchased from Charles River (Sulzfeld, Germany). The *A. ceylanicum* life cycle has been maintained at the Swiss TPH since June 2009. Briefly, hamsters were immunosuppressed (0·5 mg/l dexamethasone (dexamethasone water-soluble, Sigma-Aldrich) in the drinking water from one day before infection onwards. They were orally infected with 150 *A. ceylanicum* L3, which had been assessed for viability. Animals were kept in groups of 5 in macrolon cages under environmentally-controlled conditions (temperature: 25 °C, humidity: 70%, light/dark cycle 12/12 hours) and had access to water and rodent food (Rodent Blox, Eberle NAFAG, Gossau, Switzerland). The current work was approved by the local veterinary agency based on Swiss cantonal and national regulations (permission no. 2070).

In vitro assays

General procedure

L3: In 96-well plates, 30 L3 per well were incubated in 200 µl HBSS supplemented with 25 µg/ml amphotericin B (Sigma-Aldrich), 10 000 U/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich) and drug dilutions ranging from 100 to 1 µg/ml, final concentration. The plates were incubated for 72 hours at room-temperature, in dark and humid conditions.

Stimulated L3: Third-stage larvae are in "dauer" stage and do not feed or show any metabolic activity. In the feeding inhibition assay, L3 are brought into the fourth stage, where they resume feeding, and are then exposed to drugs. We used a modified protocol from Kopp and colleagues for this transformation (Kopp *et al*. 2008).

L3 were pelleted (3000 × g, 3 minutes) and cleaned in 1% HCl 1 M in tap water for 10 minutes at 37 °C and then washed twice in RPMI medium (RPMI 1640 (Gibco) supplemented with 0·5% ALBUMAX II (Gibco), 25 mM HEPES (Sigma-Aldrich), 25 mM $NaHCO_3$ (Sigma-Aldrich), 25 µg/ml amphotericin B (Sigma-Aldrich), 10 000 U/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich), 15% v/v foetal calf serum (FCS, Gibco), and 20 mM reduced L-glutathione (Fluka)). Thirty L3 per well were incubated in 200 µl RPMI medium for 24 hours at 37 °C. In a next step 100 µl medium were removed and replaced with 100 µl of the respective drug dilution in RPMI medium. The plates were incubated for another 72 hours at 37 °C, 5% CO_2.

Adult stage: In 48-well plates, 3–4 adult worms of both sexes were incubated per well in 1 ml HBSS medium supplemented with 25 µg/ml amphotericin B (Sigma-Aldrich), 10 000 U/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich), 10% v/v FCS (Gibco), and drug dilutions (100 to 1 µg/ml). The plates were incubated for 72 hours at 37 °C, 5% CO_2.

Larval and adult motility assay

Following incubation of L3 and adults with drugs as described above, larval and adult motilities were evaluated under an inverted microscope (magnification 20 ×, Carl Zeiss, Germany), following addition of hot water (~ 80 °C, [100 µl] L3, [500 µl] adult worms) and exposure to the microscope light. Larvae showing no movement after stimulation were classified as dead and the percentage survival was established for each well. The viability of adult worms was assessed using a motility scale from 2 (normal movements, worms healthy) to 0 (no movements, death) and converted into percentage viability. Motility assays were conducted at least twice in triplicate.

Feeding inhibition assay

Following incubation of stimulated L3 (as described above), 100 µl medium were removed from each well

and replaced with bovine albumin conjugated with fluorescein isothiocyanate (1·25 mg/ml, final concentration, Sigma-Aldrich) and the plate was incubated for 3 hours at 37 °C. Larvae were washed 3 times in PBS, filled in individual 1·5 ml Eppendorf tubes and distributed again into wells. Using an inverted fluorescence microscope (Carl Zeiss, Germany, magnification 100–200×, excitation: 450–490 nm, emission: 520 nm), larvae whose intestinal tract was stained by more than 50% in the length were considered fit and counted (Kopp *et al*. 2008). The percentage survival was calculated for each well. The motility of worms was assessed in parallel without adding hot water. The assay was conducted three times.

Alamar Blue® assay

The Alamar Blue assay was adapted from the protocol developed by Räz *et al*. (Räz *et al*. 1997). After 72 hours of incubation with drugs (as summarized above), the worms (adults, L3 and stimulated L3) were transferred into 200 µl freshly supplemented HBSS medium (2 adult worms, or 30 L3 per well) in a 96-well plate. Ten µl resazurin (Sigma-Aldrich, 125 mg/l) were added to each well. The plates were then incubated at 37 °C, 5% CO_2 for 5 hours (up to 24 hours for adults). The resulting absorbance was measured every hour for 5 hours (and once after 24 hours for adults) in each well (excitation: 536 nm, emission: 588 nm, Spectramax® GeminiXS, Molecular devices, USA). The assay was carried out at least twice.

MTT reduction assay

The MTT assay was run according to James and colleagues with slight modifications (James and Davey, 2007). Drug activity was tested using a single adult worm, or 30 L3 per well (in duplicate) in 96-well plates (total volume 200 µl). After 72 hours of incubation, the worms' viability was assessed microscopically (inverted microscope, magnification 20×) without adding hot water. One hundred µl were removed from each well and replaced with 100 µl MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich) solution (2·5 mg/ml final concentration). The plates were incubated for up to 3 hours at 37 °C, 5% CO_2. The worms were washed once in sterile PBS, transferred to 50 µl DMSO 100% (Fluka, Buchs, Switzerland) and allowed to de-stain for 20 minutes at 37 °C. The resulting absorbance was measured at 588 nm, Versamax® (Molecular Devices, USA). The assay was performed at least two times.

Acid phosphatase activity assay

The assays were carried out as described by Martinez-Grueiro *et al*. (Martinez-Grueiro, 2002) with small changes in the protocol. After 72 hours of incubation, adult worms were transferred into 1 ml supplemented HBSS medium and incubated for another 5 hours at 37 °C, 5% CO_2. The motility was assessed before adding reagents, without adding hot water. The supernatant was frozen at −20 °C or directly used for the following steps. Two times 100 µl of the supernatant were removed and incubated for either 1 or 2 hours at 37 °C, 5% CO_2 with 50 µl fresh ρ-nitrophenyl phosphate (pNPP, Sigma-Aldrich) solution (10 mM, in sodium acetate buffer pH 4). The reaction was blocked with 70 µl NaOH 0·3 M and the absorbance measured at 405 nm (Versamax®, Molecular Devices, USA). For L3 and stimulated L3, 100 µl supernatant were removed from the drug assay plate directly and incubated for 2 hours with pNPP as described above. The assay was conducted three times.

Isothermal micro- and nanocalorimetry

Isothermal calorimetry measures quantitatively exo- and endothermic heat flow of metabolic processes, and has already shown applicability using different helminth models (Braissant *et al*. 2010; Kirchhofer *et al*. 2011; Manneck *et al*. 2011). The endpoint used in this study was the time of death of the worms. A thermostated (37 °C) 48-chanel microcalorimeter (TAM 48, TA Instruments, New Castle, Delaware, USA) or a 3-channel nanocalorimeter (TAM-III, TA Instruments, New Castle, Delaware, USA) were used to measure the heat flow of *A. ceylanicum* over at least 3 days. Briefly, 100, 200 or 500 L3 (microcalorimetry) or 8 adult worms (micro- and nanocalorimetry) were distributed into sterile glass ampoules containing 2·7 ml of supplemented HBSS medium. The system was allowed to equilibrate for at least 12 hours before injection of 0·3 ml pre-warmed drug solutions (or DMSO solutions as control conditions) and allowed to equilibrate again for around 12 hours. The heat flow was continuously recorded in each ampoule and summarized to obtain an effective sampling rate of one data point every 10 minutes. Noise amplitudes observed in heat flow curves reflect the worms' motor activity. The noise amplitude curve, following exponential decay, is based on absolute values of the amplitudes of noise obtained for 20 minutes over the entire course of the experiment. The intersection of the sample curve with the background line defines the death of worms (Kirchhofer *et al*. 2011; Manneck *et al*. 2011). The nanocalorimeter offers a greater sensitivity, in the range of 20 nW. The microcalorimetry assay was performed three times, the nanocalorimetry assay was performed once.

xCELLigence System

The xCELLigence System (Roche Inc.) offers an automated real-time measurement of the cellular

electrical impedance (Xing *et al.* 2005) across interdigitated micro-electrodes integrated on the bottom of the 96-well plates (E-plates). An innovative application of the system for nematode worms was recently described, monitoring motilities of *Haemonchus contortus*, *Strongyloides ratti*, *Ancylostoma caninum* and *Schistosoma mansoni* (Smout *et al.* 2010). Measurements were displayed in the RTCA controller software (Roche Inc.) The xCELLigence System was equilibrated either to room-temperature or to 37 °C for 2 hours before the experiment was started. Then 100 L3 or stimulated L3 or one adult worm (in a volume of 100 μl) were added to the wells. Measurements started 10 minutes before the pre-warmed drugs were added (100 μl). The impedance was recorded in each well every minute for 72 hours post-incubation with drugs. The signal amplitudes (noise amplitudes) were analysed from each well. As described above, the intersection of the sample motility curve (exponential decay) with the background curve is the endpoint of the analysis and represents death of worms. The assay was performed three times.

Statistical analysis

All data were analysed and plotted in Excel (Microsoft Office 2003). The motility data were analysed with the Fisher's exact test (L3 survival), and Mann-Whitney U test (adults' viability), using StatsDirect (version 2.4.5; StatsDirect Ltd; Cheshire, UK). The correlations between the feeding inhibition assay and the acid phosphatase assay with the corresponding motility assay data were tested using the Spearman rank correlation coefficient, in StatsDirect. Microcalorimetry and xCELLigence raw data were plotted using R (R 2.12.0, R Development Core Team). The exponential decay curve was defined by the equation $H_0e^{-\mu t}+c$, where μ represents the decay rate of noise amplitudes (motor activity), H_0 is the initial starting point, t is time and c is the short term noise of the calorimeter. The repartition of death time points over the course of the experiment was assessed using the Fisher's exact test (StatsDirect).

RESULTS

Larval and adult motility assay

The activities of albendazole, levamisole and ivermectin on L3 and adult worms using the motility assay are summarised in Fig. 1. Briefly, ivermectin showed the best activity against both hookworm stages 72 hours after incubation start, with 100 μg/ml of the drug killing 86·1 and 100% of larvae and adults (both *P*-values $\leqslant$0·001), respectively. Surviving larvae showed spastic movements, sometimes just at one end. Levamisole exhibited a high activity with

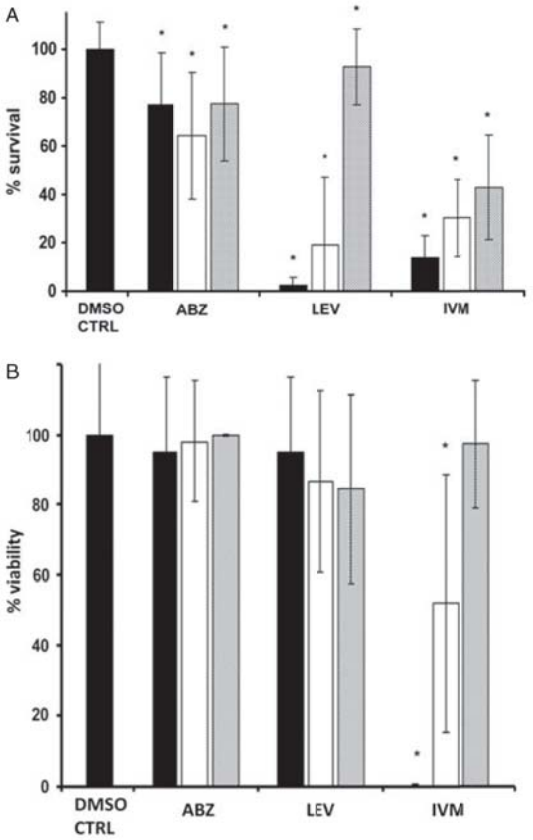

Fig 1. Anthelmintic activity of albendazole, levamisole and ivermectin against *Ancylostoma ceylanicum* L3 (A) and adult worms (E). The percentage survival or the viability scores are expressed as percentage of the controls. DMSO CTRL = DMSO control, ABZ = albendazole, LEV = levamisole, IVM = ivermectin. Black bars show 100 μg/ml; white bars show 10 μg/ml; striped bars show 1 μg/ml. The assays were conducted at least twice in triplicates. * p $\leqslant$ 0·05 (A: Fisher's exact test; B: Mann-Whitney U test).

only 2·4% of L3 surviving following incubation with 100 μg/ml of the drug for 72 hours ($P<0·001$). On the other hand adults were nearly unaffected by levamisole (viability >84%, all *P* values >0·05). Albendazole displayed a moderate effect against L3 (survival of 64·0–77·4% at 1–100 μg/ml, all *P*-values <0·001), but showed no effect against adults (all *P* values >0·05).

Feeding inhibition assay

Levamisole and ivermectin were tested against stimulated L3 in the feeding inhibition assay (Fig. 2). On average, 84·9% of the larvae in the control wells displayed a fluorescent intestinal tract, which fulfils the criteria set by Moser *et al.* for a successful activation (Moser *et al.* 2005). Levamisole-treated parasites exhibited between 49·0 and 66·0% gut staining, whereas survival was 0% at all concentrations using the motility assay (not shown).

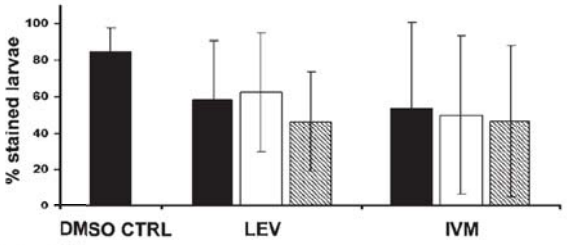

Fig. 2. Feeding inhibition assay. Percentage of stimulated L3 exhibiting fluorescent intestinal tract following treatment with levamisole or ivermectin. DMSO CTRL = DMSO control, LEV = levamisole, IVM = ivermectin. Black bars show 100 μg/ml; white bars show 10 μg/ml; striped bars show 1 μg/ml. Data points derive from three independent experiments.

Fluorescent guts were observed in 49·3 to 56·8% of larvae following incubation with ivermectin, while 47·3 to 74·4% still showed movement in the corresponding motility assay (not shown). With both treatments, no concentration-dependency and a poor reproducibility were observed. No correlation between the feeding inhibition assay and the corresponding motility data was found (Spearman rank correlation coefficient, $\rho = 0{\cdot}15$, $P = 0{\cdot}233$ for positive correlation).

Alamar Blue® assay

None of the tested stages (L3, stimulated L3 and adult worms) was able to convert resazurin into resorufin within 5 hours. The medium turned slowly violet only after 5 hours. Twenty-four hours post-incubation, the fluorescence intensity measured was not homogeneously distributed between wells, with emissions ranging from 3059–7427, compared to 2497 on average for the medium only.

MTT reduction assay

Following incubation of L3 and stimulated L3 with MTT, no reduction of the marker occurred and therefore neither the larvae nor the medium changed colour. Adult worms' internal tissues turned violet but their teguments remained colourless. In general, no reproducible staining pattern was observed within one treatment group (and among controls), and staining did not correspond to their capacity to move as observed with the motility assay. No reproducible absorbances could be measured at 588 nm.

Acid phosphatase activity assay

Neither L3 nor stimulated L3 triggered a colourimetric reaction following addition of pNPP (10 mM solution). Adult worms produced measurable amounts of the enzymes. The absorbance measured

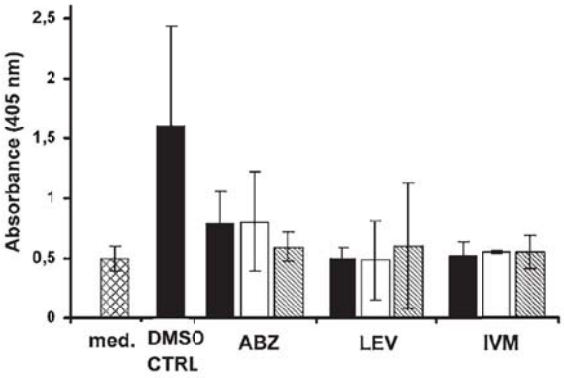

Fig. 3. Acid phosphatase activity assay for sensitivity of *Ancylostoma ceylanicum* adult worms to albendazole, levamisole and ivermectin. Med = medium only, DMSO CTRL = DMSO control, ABZ = albendazole, LEV = levamisole, IVM = ivermectin. Black bars show 100 μg/ml; white bars show 10 μg/ml; striped bars show 1 μg/ml. Data points derive from three independent experiments.

for control adult worms reached 1·6. However, the signal to noise ratio was low. The background noise produced by medium alone was high (0·5), similar to the absorbance of treated worms (0·48–0·81). In addition, no concentration-dependency was observed (Fig. 3). Finally, no correlation (Spearman rank correlation coefficient, $\rho = 0{\cdot}24$, $P = 0{\cdot}11$ for positive correlation) was determined between the results of the acid phosphatase secretion assay and the motility of the worms (assessed before the acid phosphatase activity assay was started).

Isothermal micro- and nanocalorimetry

Microcalorimetry: Neither L3 nor stimulated L3 (up to 500 per ampoule) exhibited sufficient heat production for a stable signal. Preliminary experiments revealed that 8 worms was the minimum number of worms to measure a sufficiently large signal, in the range of 2–3 μW. Eight adult worms per ampoule were treated with 100 μg/ml levamisole, 100 μg/ml ivermectin, or the corresponding amount of DMSO. Mortality rates of adult worms were determined by comparing noise amplitudes in their heat flows, which derive from worm motor activities of drug-treated and control worms. The thermogenic noise of 8 dead worms was used as background noise (dotted line) and defined as 0·13 μW (not shown). Fig. 4 shows the heat flow pattern produced by control worms over 72 hours. The time-points of the death of worms are summarized in Table 1. Two control worms died between 24 and 72 hours post injection of DMSO, while 2 control worms were still alive at the end of the experiment. The noise signals were reduced to a level not different than that of dead control worms within 12 hours following addition of

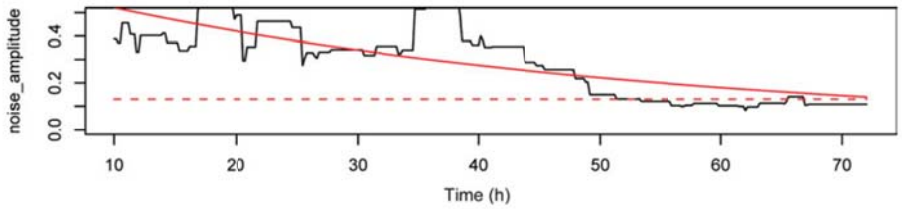

Fig. 4. Heat flow pattern of untreated adult *Ancylostoma ceylanicum* worms (n = 8). The produced noise obtained for 20 minutes is averaged over 72 hours. Noise amplitude values follow exponential decay (solid line). The following equation was applied: $H_0e^{-\mu t}+c$, where μ represents the decay rate of noise amplitudes (motor activity), H_0 is the initial starting point, t is time and c the short term noise of the microcalorimeter. The system background noise is shown as dotted line, defined as 0·13 μW. The intersection of the sample curve (solid line) with the background line (dotted line) is the endpoint of worm motility and corresponds to the death of worms.

Table 1. Time-points of worms' death measured by isothermal microcalorimetry, over 72 h after treatment with 100 μg/ml levamisole, ivermectin or DMSO (8 worms per ampoule)

(Death in the ampoule is defined as the intersection between the noise amplitude in the heat flow curve and the background noise line. Death was determined in 3 (ivermectin) or 4 ampoules for each treatment. The repartition of death time points is shown by the proportion and analysed using the Fisher's exact test (* $P \leqslant 0{\cdot}05$), cumulating the time ranges.)

Time-point of death	Control (death/total ampoules)	Levamisole (death/total ampoules)	Ivermectin (death/total ampoules)
0–12 h	0	4/4*	3/3*
12–24 h	0	—	—
24–48 h	1/4	—	—
48–72 h	1/4	—	—
>72 h	2/4	—	—

levamisole and ivermectin, significantly differently from the controls (both $P = 0{\cdot}03$).

Nanocalorimetry: The absolute heat flow obtained from control worms after equilibration of the system (about 12 hours) remained in the range of 1·5–2·5 μW during the whole experiment (Fig. 5). Heat flows recorded from levamisole- and ivermectin-treated worms were not higher than about 1·5 μW, reflecting the drug effects. Signals from control and drug-treated worms remained stable over the whole experiment duration. Great noise amplitudes were observed in the control ampoule, whereas they were flattened in the treated ones, reflecting the loss of motor activities. The dead worms data were recorded in a separate experiment and no drug or medium was injected. The absolute heat flow from this ampoule was higher, in the range of 2·5 μW, but stable over time and displaying minimal noise amplitudes.

xCELLigence System

Electrical impedance was measured from adults but was not detected in the 100 L3 nor in the stimulated

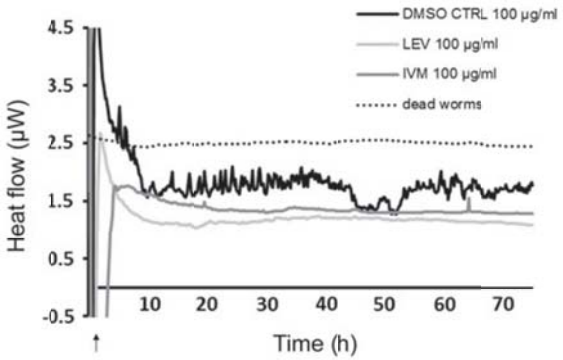

Fig. 5. Absolute heat flow of *Ancylostoma ceylanicum* adult worms recorded with an isothermal nanocalorimeter over 72 hours. 100 μg/ml levamisole, ivermectin or DMSO were injected at time = 0, indicated by the arrow.

L3. One adult worm per well was treated with 100 μg/ml albendazole, 100 μg/ml levamisole, 100 μg/ml ivermectin, or the corresponding amount of DMSO. The noise produced by one dead worm was used as background noise (dotted line) and was 0·0044 (cell index, not shown). Out of 7 control worms analysed, 1 worm died between 24–48 hours and 2 worms died between 48–72 hours post-incubation with drugs. Four worms remained alive over the entire examination period of 72 hours (Table 2). Fig. 6 shows the impedance signal curve produced by a representative control worm, still alive 72 hours after addition of DMSO. Ivermectin reduced the impedance signal to a level not different than that of dead control worms within 12 hours following addition of the drug ($P \leqslant 0{\cdot}01$). No clear activity pattern was observed for albendazole and levamisole. Worms died at different time points following incubation with both drugs (Table 2) (both P-values >0·05).

DISCUSSION

An ideal *in vitro* assay for drug sensitivity testing should be precise, sensitive, simple, fast and cost effective. Many *in vitro* assays used to study drug

Table 2. Time-points of worms' death measured by the xCELLigence System, over 72 h after treatment with 100 μg/ml levamisole, ivermectin or DMSO (1 worm per well)

(Death in the well is defined as the intersection between the noise in the impedance curve and the background noise line. Death was determined in 3–7 wells for each treatment. The repartition of death time points is shown by the proportion and analysed using the Fisher's exact test (* $P \leqslant 0{\cdot}05$), cumulating the time ranges.)

Time-point of death	Control (death/total wells)	Albendazole (death/total wells)	Levamisole (death/total wells)	Ivermectin (death/total wells)
0–12 h	0	1/7	2/3	7/7*
12–24 h	0	1/7	0	—
24–48 h	1/7	0	0	—
48–72 hours	2/7	2/7	1/3	—
> 72 h	4/7	3/7	—	—

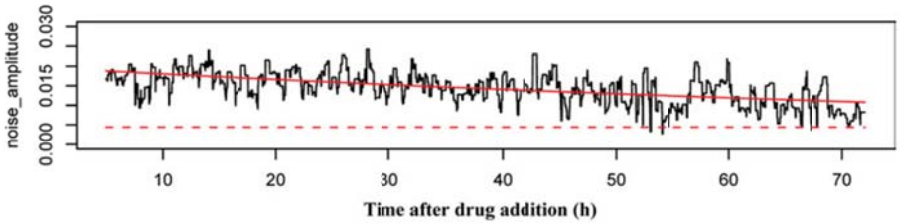

Fig. 6. Impedance pattern of adult *Ancylostoma ceylanicum* worms (n = 1) measured with the xCELLigence System. The produced noise obtained for 20 minutes is averaged over 72 hours. Noise amplitude values follow exponential decay (solid line). The following equation was applied: $H_0e^{-\mu t} + c$, where μ represents the decay rate of noise amplitudes (motor activity), H_0 is the initial starting point, t is time and c – the short term noise of the xCELLigence System. The system background noise is shown as dotted line, defined as 0·0044 (cell index). The intersection of the sample curve (solid line) with the background line (dotted line) is the endpoint of hookworm motility and corresponds to the death of worms.

effects on nematodes have drawbacks (Taylor *et al.* 2002). For instance, the egg-hatch test (Le Jambre, 1976) is only suitable for the benzimidazoles, since most drugs have no effect on *A. ceylanicum* egg hatching (Tritten *et al., manuscript submitted*). The motility meter was found to lack sufficient sensitivity to detect drug susceptibility of bovine intestinal nematode larval stages (Demeler *et al.* 2010). Although cheap and simple, the motility assay is time consuming and prone to individual subjectivity. An improved drug sensitivity *in vitro* assay might therefore aid the development of novel hookworm drug therapies. In the present work, we studied the applicability of six alternative *in vitro* assays using both *A. ceylanicum* larval and adult stages, based on well-established methods and more recent technologies.

Our findings, summarized in Table 3, show that the xCELLigence System was the only method comparing favourably to the motility assay, whereas isothermal microcalorimetry, the colourimetric assays as well as the fluorescence-based test presented no advantage over the motility assay.

None of the tested assays was suitable for stimulated or normal L3, the preferred stage for *in vitro* testing, since they survive for several weeks after collection, and the use of adult worms requires killing of the hamster host. Third-stage larvae are in the developmentally arrested non-feeding stage, where physiological processes are greatly reduced (Burnell *et al.* 2005; O'Riordan, 1989; O'Riordan, 1990), which might explain this result. Furthermore, their mouth and anus are sealed off (Cassada and Russell, 1975), limiting contacts with the environment. Several genes are differentially expressed upon feeding resumption by stimulated L3 (Moser *et al.* 2005), allowing a parasitic metabolic life, and adults are considered fully metabolically active (O'Riordan, 1989, 1990). However, it has been shown that the onset of development upon feeding resumption leads to more gene repressions than up-regulations (Moser *et al.* 2005).

The xCELLigence System offered the advantage of the format (96-well plate) and sufficiently strong signals could be obtained with only one adult worm per well. On the other hand, one hundred larvae did not yield a signal that could be detected and measured. Smout *et al.* showed that a large number of *Haemonchus contortus* or *Strongyloides ratti* larvae were necessary to exhibit measurable cell indices

Table 3. Summary of the tested assays and of their performance in testing drug susceptibility against *A. ceylanicum* larval and adult stages

(Tests evaluated unfavourably are scored with "−", those evaluated favourably, with a "+".)

Assays								
		Motility	Acid phosphatase	MTT	Feeding inhibition	Alamar Blue	Micro-calorimetry	xCELLigence System
Tested stage	L3	Gold standard	−	−	−	−	−	−
	Stimulated L3	Not tested	−	−	−	−	−	−
	Adult worms	Gold Standard	−	−	−	−	−	+

(Smout *et al.* 2010), which is a great disadvantage for medium- to high-throughput assays. The cost of the xCELLigence apparatus and the E-plates might also be a drawback. In addition, untreated control worms died faster than in other *in vitro* assays, presumably due to incubation in a small amount of medium (200 µl) for 72 hours. Hence, with the xCELLigence it is not possible to run drug assays for long periods.

It is interesting to note that according to xCELLigence, death occurred later in one third of the levamisole-treated worms, compared to data from microcalorimetry. It would be necessary to investigate a larger sample size to find out whether a variation in responsiveness to levamisole exists. However, it is possible that hookworms analysed were not equally fit at the start of the assay. In both assays, the results obtained following treatment with levamisole do not correspond to the motility data.

Although isothermal microcalorimetry is generally accepted as sensitive and accurate, several limitations were noted analysing drug effects on *A. ceylanicum*. First, it was necessary to place several adult hookworms per ampoule to obtain a sufficiently strong signal, a significant disadvantage in terms of costs and ethical requirements for research involving animal hosts. Similarly to the other assays analysed, it was not possible to run the assay with larval stages. Second, the injection of the drugs triggered a perturbation of the system with great noise amplitudes being recorded for at least 8 hours and it was therefore necessary to re-equilibrate the system for several hours before drug effects on the noise amplitudes produced by the worms could be measured. For fast acting drugs it is therefore not possible to determine the exact time point of death. Our findings are in contrast to studies using isothermal microcalorimetry with the helminths *Schistosoma mansoni*, *Fasciola hepatica* or *Trichuris muris*, where few worms generated sufficiently stable heat flows and quickly re-equilibrated following drug injections (Kirchhofer *et al.* 2011; Manneck *et al.* 2011; Silbereisen *et al.* 2011). Since the same machine was used in all of these experiments, the observed insensitivity of hookworms compared to whipworms cannot be explained by equipment differences.

Increased sensitivity and signal stability was obtained using nanocalorimetry. However, the observed heat flows in control versus treated worms were very similar and the baseline signal seems to shift from an experiment to the next, as it was observed with the higher absolute heat flow of dead worms, recorded separately. It is not unlikely that microcalorimetry might record some non-biological processes contributing to the higher baseline of dead worms. Possible processes might include chemical/enzymatic reaction initiated by the killing of the worms (performed using ethanol or freezing) or microcalorimeter baseline shift. As observed with microcalorimetry, the motor activity reflected by the noise amplitudes measured in the nanocalorimeter constituted the largest difference between control and treated conditions.

Our results obtained with the motility assay are in agreement with previous findings (Misra *et al.* 1981; Richards *et al.* 1995; Kotze *et al.* 2004). Briefly, albendazole had a weak effect against L3 and a moderate effect against adult worms. The low activity observed against larvae at 100 µg/ml albendazole might be explained by precipitation of the drug in the medium. Levamisole showed excellent efficacy at 100–10 µg/ml against larvae, but lacked activity against adults. Ivermectin strongly affected larvae and adult worms.

The feeding inhibition assay was first described by Hawdon and Schad (Hawdon and Schad, 1990, 1992), and proved useful in studies with *A. caninum* (Kopp *et al.* 2008). However, Kopp and colleagues identified lack of sufficient sensitivity with this assay, while testing resistance to pyrantel (Kopp *et al.* 2008). We found this assay to be time-consuming and tricky because of the numerous preparation steps, where larvae can be lost or damaged. In addition, the staining intensity of worms was weak, although the exsheathment did occur. The feeding inhibition assay therefore does not offer an advantage over the motility assay.

Also none of the tested colourimetric assays was found to perform satisfactorily. In general, colourimetric assays are difficult to carry out with organisms as large as *A. ceylanicum* adult worms, whose size can vary, because the endpoints depend directly on the number of living cells (Mosmann, 1983; Räz *et al.* 1997). The Alamar Blue assay, which determines the metabolic mitochondrial biotransformation of resazurin into resorufin, was not suitable for larval stages. Furthermore, the conversion of resazurin into resorufin by adult worms was very slow (no fluorescent emission compared to medium within 5 hours), hence the applicability of the Alamar Blue assay was considered limited, in particular since this assay is highly sensitive to contaminations. We expected adult worms to reduce the dye much more quickly, as it is the case for *Trichuris muris* (Silbereisen *et al.* 2011) and therefore cannot explain this finding. It might be possible that either resazurin is not taken up sufficiently, or the mitochondrial metabolic activity is lower in *A. ceylanicum* than in other nematode species.

Upon incubation with MTT, larval stages did not detectably reduce MTT to formazan and revealed no staining. Formazan formation relies on pyridine nucleotides cofactors (Berridge and Tan, 1993), and as suggested before, larval and adult hookworm metabolisms might greatly differ. It was reported elsewhere that L3 of *Caenorhabditis elegans* were able to take up MTT, whereas those of *H. contortus* were not (James and Davey, 2007). In the same report, the authors suggested that the protective sheath of the L3 might prevent dye uptake, presumably occurring by diffusion. In our experiments, adults got stained, however only in an inhomogeneous and non-reproducible manner, not supporting the hypothesis of dye diffusion. It is worth mentioning that ivermectin-treated worms, paralysed if not dead, were less intensively stained than control or levamisole-treated worms. Overall, our results support the suggestion that MTT uptake occurs via pharyngeal pumping (Smith *et al.* 2009).

Finally, neither L3 nor stimulated L3 were able to trigger any colourimetric reaction in the acid phosphatase assay. Results from the adult acid phosphatase activity assay showed no correlation with the adult motility assay. For example, while according to the motility assay, ivermectin-treated worms were dead, the acid phosphatase production by ivermectin-treated worms was still high at the highest concentration tested. It is possible, that ivermectin-treated worms might not be dead, but paralysed instead (Martin, 1997), with some metabolic activity still taking place. In addition, no concentration dependency effect was observed for ivermectin using the acid phosphatase activity assay. In contrast to our study, Martinez-Grueiro showed a clear inhibition of *Heligmosomoides bakeri* adult worms' acid phosphatase production following treatment with ivermectin.

In conclusion, in the present work seven test systems were examined for their usefulness in investigations of drug effects on hookworms. With exception of the motility assay, the gold standard, none of the presented assays was applicable to L3. Using adult worms, the xCELLigence System compared favourably to the motility assay. It was found convenient especially for fast-acting drugs, required a minimal amount of worms and would offer the possibility to conduct a medium-throughput screening. The results are highly accurate, since a precise endpoint can be obtained. However, additional drugs should be tested to confirm the potential advantage of the xCELLigence System over the motility assay in hookworm assays.

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Chapter 2b

Exploration of novel *in vitro* assays to study drugs against *Trichuris* spp.

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Exploration of novel in vitro assays to study drugs against *Trichuris* spp.

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ABSTRACT

Though trichuriasis is a significant public health problem, few effective drugs are available underscoring the need for new drug therapies. For the evaluation of trichuricidal activity of test compounds in vitro an accurate, reliable, sensitive, fast and cheap drug sensitivity assay is essential. The aim of the present investigation was to evaluate the performance of different in vitro drug sensitivity assays in comparison to the standard motility assay. *Trichuris muris* L4 larvae or adult worms were isolated from the intestinal tract from infected female C57BL/10 mice and incubated in the presence of ivermectin, levamisole and nitazoxanide (200, 100 and 50 μg/ml) for 72 h. The health status of the worms was either evaluated microscopically using a motility scale from 0 to 3 (motility assay), by examination of absorbance or emission in response to metabolic activity (MTT (Thiazolyl Blue Tetrazolium Bromide) and Alamar Blue assay), through analysis of absorbance of an enzyme-substrate reaction (acid phosphatase activity assay), by measuring the noise amplitudes (isothermal microcalorimetry and xCELLigence System) or the heat flow (isothermal microcalorimetry) of *T. muris*. The Alamar Blue assay, xCELLigence and microcalorimetry compared favorably to the standard motility assay. These three assays precisely determined the trichuricidal activity of the three test drugs. The acid phosphatase and the MTT assays showed a poorer performance than the motility assay. In conclusion, the colorimetric Alamar Blue in vitro assay is a good alternative to the motility assay to study drug effects against *T. muris* L4 and adults, since it is easy to perform, precise and of low cost.

1. Introduction

The gastrointestinal nematode *Trichuris trichiura* (whipworm) belongs to the soil-transmitted helminths and is a truly neglected parasite though infecting an estimated 604–795 million people worldwide (Bethony et al., 2006; Hotez et al., 2009). In highly endemic areas, children between 5 and 15 are the main age group at risk with a prevalence of up to 90% (Stephenson et al., 2000). Trichuriasis is prevalent in many countries in the tropics and subtropics, especially East Asia, the Pacific islands, sub-Saharan Africa, Latin America and the Caribbean (Bethony et al., 2006). Light *T. trichiura* infections are often asymptomatic, but abdominal pain, bloody diarrhea, anemia and rectal prolapses are common symptoms occurring in severe and chronic stages (Stephenson et al., 2000). The two benzimidazole drugs, albendazole and mebendazole, are intensively used for the treatment and control of soil-transmitted helminth infections, commonly in the framework of mass drug administration programs (Keiser and Utzinger 2010). The World Health Organization recommends a single-dose treatment with either 400 mg albendazole or 500 mg mebendazole, whereas the frequency of treatment intervention depends on the prevalence of the disease (WHO 2006). Due to the fact that the cure rates of both drugs against infections with *T. trichiura* are only moderate (Keiser and Utzinger 2010), there is a need to develop novel therapies.

In order to identify novel trichuricidal drug candidates a reliable, sensitive, fast and accurate in vitro test system is preferred. The current strategy to analyze drug sensitivity in vitro against different nematodes involves using the larval/adult motility assay (Kotze et al., 2004, 2005) which characterizes the worm viability under the microscope by using a motility scale. However, microscopic techniques are often time-consuming, low throughput, labor intensive, have to be carried out by experienced personnel and have a subjective nature. The aim of this study was therefore to evaluate several in vitro drug sensitivity assays using the mouse strain *Trichuris muris* and to compare the performance of these assays with the motility assay. We selected two standard drugs, ivermectin and levamisole, as well as the antiprotozoal drug nitazoxanide for our studies since this drug showed a high trichuricidal activity in previous in vitro tests we performed (Tritten et al., manuscript submitted for publication). Three colorimetric assays (Alamar Blue, MTT (Thiazolyl Blue Tetrazolium Bromide) and acid phosphatase activity assay) as well as two assays based on real-time monitoring of viability and heat flow (xCELLigence System and isothermal microcalorimetry) were included in our study.

Abbreviations: Lev, levamisole; Iver, ivermectin; NTZ, nitazoxanide; HF, heat flow; HFR, heat flow reduction; a.l., amplitude length.

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Briefly, viability marker assays are non-radioactive colorimetric assays based on the color change of dyes such as Alamar Blue (Raz et al., 1997; O'Brien et al., 2000) and MTT (Hordegen et al., 2006; Townson et al., 2006) to determine the health status of parasites, cells or bacteria. Healthy parasites ingest the dye resulting in a reduction process and color change of the dye due to chemical or enzymatic reactions by the parasite. The underlying principle of the acid phosphatase activity assay is the high acid phosphatase activity of gastrointestinal nematodes within their intestines. Acid phosphatase has been detected in excretory/secretory products of several nematode species, released in the culture medium (Maki and Yanagisawa 1980a, 1980b, 1980c; Moulay and Robert-Gero 1995; Fetterer and Rhoads 2000). The acid phosphatases react with the substrate pNPP (p-nitrophenyl phosphate) present in the medium, forming a yellow water-soluble product with a strong absorbance. The xCELLigence System, mainly used for cell-based assays, has recently been described to be a suitable technique to measure the motility of different helminths in real time in a fully automated high-throughput manner (Smout et al., 2010). Microelectrodes at the bottom of each well of the E-plate measure the impedance, which can be used to express the worm's viability as noise amplitudes. Finally, isothermal microcalorimetry (Yan et al., 2008) examines the heat flow (endo- or exothermic reactions) of biological processes. This method has recently proven to be an excellent tool to study drug effects on *Schistosoma mansoni* and *Fasciola hepatica* (Manneck et al., 2010; Kirchhofer et al., 2011).

2. Materials and methods

2.1. Drugs, chemicals, reagents and media

Ivermectin was purchased from Sigma-Aldrich (Buchs, Switzerland) and levamisole from Fluka (Buchs, Switzerland). Nitazoxanide was kindly obtained from Laboratoria Wolfs (Zwijndrecht, Belgium). Drug stocks (10 mg/ml) were prepared in 100% DMSO (dimethylsulfoxid, Sigma-Aldrich, Buchs, Switzerland) and stored at 4 °C pending use.

Alamar Blue (resazurin sodium salt, 125 mg/l), MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), p-nitrophenyl phosphate (pNPP), sodium acetate anhydrous (NaAc), amphotericin B (250 µg/ml) and penicillin-streptomycin (10,000 units penicillin + 10 mg streptomycin per ml) were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium hydroxide (NaOH) was purchased from Fluka (Buchs, Switzerland). Hank's Balanced Salt Solution (HBSS 1X, [−] phenol red, [−] calcium, [−] magnesium) and Fetal Calf Serum (FCS) from Gibco (Basel, Switzerland). RPMI medium was prepared using 10.44 g RPMI 1640 (Gibco), 5 g albumax II (Gibco), 5.94 g hepes (Sigma-Aldrich) and 2.1 g sodium bicarbonate (Sigma-Aldrich) in 1 l deionized water.

2.2. Animals and parasite

Three to five weeks old female C57BL/10 mice were purchased from Harlan Laboratories (Blackthorn, England). Mice were allowed to acclimatize for one week before infection. All animals were kept in groups of 10 mice in macrolon cages with free access to water and rodent food pellets (Rodent Blox from Eberle NAFAG, Gossau, Switzerland) and with a 12 hour light/dark cycle according to Swiss Animal Welfare guidelines.

We used the mouse strain *T. muris*, which is an excellent experimental model for whipworm infections (Worley et al., 1962). Embryonated eggs from *T. muris* were kindly provided by Prof. Jerzy Behnke (University Park of Nottingham, United Kingdom) and Prof. Heinz Mehlhorn (University of Düsseldorf, Germany).

Unembryonated eggs released in the feces of infected mice were isolated and purified through flotation (Ghiglietti et al., 1995) with saturated NaCl (359 g/l in distilled water) and cultured in tap water at room temperature. Embryonation of eggs was controlled under the microscope (magnification 80x, Carl Zeiss, Germany). Each mouse was treated with 4 mg/l dexamethasone (Sigma-Aldrich) supplied with the drinking water two days prior to infection until the end of the experiment (Campbell 1968; Wakelin 1970). All animals were orally infected with 400 embryonated *T. muris* eggs (Wakelin 1970; Stepek et al., 2006). The development of L4 larvae in the gastrointestinal tract was completed between days 26 and 28 after infection (p.i.). Adult *T. muris* worms were present in the mice's gut from day 35 p.i. onwards (Fahmy 1954; Stepek et al., 2006). We decided to use in the present work adult worms as well as the larval stage 4 (L4) of *T. muris* because both parasite stages have an ideal size, are robust and easy to handle.

2.3. In vitro studies

Mice were euthanized by exposure to CO_2 on days 26–28 or from day 35 onwards, respectively. The entire intestine was dissected and placed in a Petri dish containing 0.9% NaCl. Next, it was opened longitudinally using forceps under the binocular microscope (magnification 16x) and isolated parasites were transferred into pre-warmed RPMI medium containing 5–10% amphotericin B and 1% penicillin-streptomycin and maintained at 37 °C and 5% CO_2. Unless otherwise mentioned, RPMI medium supplemented with antibiotics was the medium of choice. Ivermectin, levamisole and nitazoxanide were tested in concentrations of 200, 100 and 50 µg/ml (motility assay, Alamar Blue assay, MTT assay and acid phosphatase assay). Drug concentrations of 50 µg/ml were used in the xCELLigence and microcalorimetry assays. In all assays at least three worms incubated in the highest DMSO concentration used in the test and medium blanks served as controls. All experiments were carried out at least in duplicate. The in vitro assays were performed either in 96-well plates, incubating worms in a total volume of 200 µl (100 µl drug solution) or in 48-well plates and 1 ml medium (500 µl drug solution). Parasites were incubated for at least 72 h at 37 °C and 5% CO_2.

2.3.1. Motility assay

Following incubation with the test drugs as described above, the motility of L4 larvae was evaluated under the microscope (magnification 20–80x) after periods of 24, 48 and 72 h using a motility scale from 0 to 3 (0 = dead, 1 = very low motility, 2 = low motility, 3 = normal motility) (Stepek et al., 2006). Worms classified as 1 (very low motility) showed a strongly reduced viability and/or were only able to move on one end. Worms which showed less viability than the controls were recorded as category 2 (low motility).

2.3.2. Alamar Blue assay

Following incubation of 4–6 L4 larvae with drugs for 72 h, larvae were transferred into 200 µl fresh RPMI medium. The motility of worms was examined as described in Section 2.3.1. Ten microliter resazurin was added and the plates were incubated for another 4 h. The fluorescent emission of the reduced dye was measured at 588 nm using a spectrofluorometer (SpectraMax, Gemini XS, Molecular Devices, UK).

2.3.3. MTT assay

Four L4 larvae were placed into 100 µl medium within a 96-well plate. One hundred microliters of the drug solution to be tested was added and the plate was incubated for 72 h. The motilities of worms were documented. The worms were then carefully transferred into 100 µl fresh medium. In a next step, 100 µl MTT reagent (6 mg/ml in deionized water) was added per well and the plate was incubated for 1 h at 37 °C and 5% CO_2. To solubilize the formazan crystals, the larvae were transferred into a new 96-well plate containing 200 µl 100% DMSO and incubated for another hour. Then the worms were removed and the plate agitated gently to homogenize the solution.

The absorbance of formazan was measured at 490 nm with a microplate reader (VersaMax, Molecular Device, UK).

2.3.4. Acid phosphatase activity assay

Three adult worms were placed into one well of a 48-well plate, containing 500 µl pre-warmed HBSS medium supplemented with antibiotics and 10% FCS. Five hundred µl of the drug solutions were added and the plate was incubated for 72 h. The motilities of the worms were recorded using a motility scale as described above.

Seventy-two hours after incubation, 100 µl of the medium was transferred from each well into 1.5 ml Eppendorf tubes. Then 50 µl of the substrate pNPP (15 mM in 50 mM sodium acetate buffer, pH 4) was added and the mixture was incubated for 2–3 h. The reaction was stopped with 70 µl 0.3 M NaOH (Fluka). Eighty microliters of each solution was transferred (in duplicate) into each well of a 96-well plate and the optical density was measured at 405 nm using a microplate reader (VersaMax, Molecular Devices, UK).

Since nitazoxanide has the same color (yellow) as the reaction product of the acid phosphatase enzyme reaction, additional controls had to be used for the nitazoxanide experiments. Solutions with 200, 100 and 50 µg/ml nitazoxanide in HBSS medium (with antibiotics and 10% FCS) were therefore prepared and the absorbances of these controls measured (nitazoxanide controls). In order to calculate the absorbance of nitazoxanide-treated worms, the absorbance of the respective nitazoxanide control was substracted from the measured absorbance of nitazoxanide-treated worms.

2.3.5. xCELLigence System

Four L4 larvae and adult worms were distributed to 4 different wells of an E-plate (Roche Diagnostics) containing RPMI medium. The plate was placed into the xCELLigence RTCA SP instrument and scanned for two minutes at 37 °C and 5% CO_2. The plates were incubated for 3 h to obtain a stable noise signal. Following exposure to 50 µg/ml of the drugs, the noise amplitudes of the worms, obtained from Cell Index curves, were measured every minute for at least 72 h. The Cell Index is a dimensionless parameter that measures electrical impedance. Samples containing untreated live and dead worms served as controls.

2.3.6. Isothermal microcalorimetry

For the measurement, a multi-channel isothermal microcalorimeter (Model "TAM 48", TA Instruments, New Castle, DE, USA) was used. One to six adult worms were placed into glass ampoules containing 2.5 ml pre-warmed RPMI medium. Five hundred microliters of the drug solutions (50 µg/ml) were prepared and filled into insulin syringes. The glass ampoules were placed into the microcalorimeter together with an injection system (Manneck et al., 2010) and incubated at 37 °C to allow an adjustment of the temperature. After a 20 hour equilibration period, the drug solution was injected into the medium and the heat flow of the worms was measured continuously for at least 72 h. The motor activity of the worms was reflected by random oscillations occurring in heat flow curves. Live and dead worms served as controls.

2.4. Statistical analyses

All data obtained were analyzed by Excel (Microsoft Office, 2007). Means (+/− standard deviations) of motilities, emissions and absorbances were calculated from repeated tests. For microcalorimetry the reduction in heat flow (HFR) from the starting point (after a 20 hour equilibration period) as well as the amplitude length was calculated for each drug 24 and 72 h after drug injection. The amplitude length of drugs analyzed with xCELLigence was calculated the same way after 24 and 72 h.

The Pearson correlation coefficient (r) was calculated with Excel to characterize the relationship between microscopic (motility assay) and measured data from the Alamar Blue, MTT and acid phosphatase assay.

The software R (R 2.12.0, R Development Core Team) with the packages zoo, stats and waveslim was used to analyze the heat flow and motor activity data from microcalorimetry as well as noise amplitude data from the xCELLigence System. For both assays the exponential decay of noise amplitude values was described through the following equation: $H_0e^{-\lambda t}+3e\text{-}07$, where λ described the decay rate of the noise amplitudes, H_0 the starting point and t the time in hours (Manneck et al., 2010). In both test systems the noise amplitude of dead control worms was used to set the background noise.

For the motility assays the Fisher's exact test was used to identify whether the drug effect was significant, compared to untreated conditions. The p-value was calculated, based on degree of freedom and the significance level of 5% ($\alpha = 0.05$). For the Alamar Blue, the MTT and the acid phosphatase assays the unpaired *t*-test was used to determine whether or not the drug effect of treated worms differs significantly from untreated ones. The critical t-value, based on degree of freedom and the significance level of 5% ($\alpha = 0.05$), was read from the t-distribution table and compared with the calculated t-value. For microcalorimetry and xCELLigence the paired *t*-test was used (significance level = 5%). In the case of the motility assay, the null hypothesis was rejected when the calculated p-value was smaller than the 5% significance level. For the Alamar Blue, MTT and acid phosphatase activity assays, the null hypothesis was rejected when the calculated t-value was smaller than the critical t-value, for xCELLigence and microcalorimetry when the calculated t-value led to a significant p-value ($p<0.05$).

3. Results

3.1. Motility assay

The mean viabilities of L4 larvae examined after 72 h incubation following exposure to nitazoxanide, ivermectin and levamisole are presented in Fig. 1. Control worms showed a normal viability (motility = 2.94 +/− 0.13) Worms treated with nitazoxanide were dead (motility = 0), regardless of the concentration used (200, 100 and 50 µg/ml) ($p<0.005$). Ivermectin-treated worms (100 and 50 µg/ml) showed nearly normal movements (mean motilities = 2.71 +/− 0.21 and 2.61 +/− 0.31, respectively), which did not significantly differ from the controls ($p>0.1$). Only the highest concentration of ivermectin tested (200 µg/ml) slightly reduced the motility of *T. muris* larvae (motility = 2.0 +/− 0.6) ($p=0.001$). All concentrations of levamisole (200, 100 and 50 µg/ml) reduced the viability of the worms by more than 50% (all $p<0.005$).

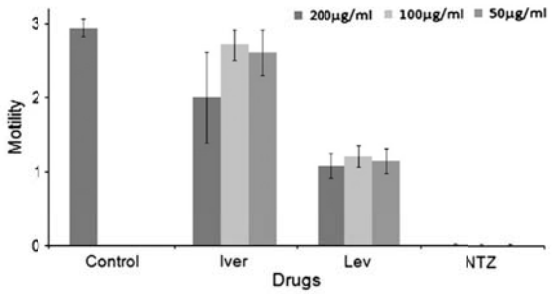

Fig. 1. In vitro motility assay with *T. muris* L4 larvae. Worms were incubated in the presence of 200, 100 and 50µg/ml of the drugs. Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide. Motility scale: 0 = dead, 1 = very low motility, 2 = low motility, 3 = normal motility.

3.2. Alamar Blue assay

Preliminary experiments showed that metabolic activity of *T. muris* could readily be detected following incubation of worms with Alamar Blue. Experiments using 4 worms/well and a volume of 10 µl resazurin resulted in the highest emission signal in comparison to the background noise of the medium. There was no enhanced effect using 6 worms instead of 4 and increased resazurin doses (20, 30, 40 and 50 µl) resulted only in a shift in the signal-to-noise ratio. The emission was measured every hour after the experiment onset but, with increasing time, the signal-to-noise ratio increased simultaneously. An incubation period of 4 h (signal-to-noise ratio = 2.9) was identified as ideal and selected for our experiments. Ivermectin-treated worms (200, 100 and 50 µg/ml) showed high fluorescent emissions of 1924.5 +/− 238.5, 1795.0 +/− 249.3 and 2302.0 +/− 102.0, respectively, which were even higher than emissions obtained following incubation of control worms. Control worms and levamisole-treated worms (200 µg/ml) showed nearly identical emissions (1400.1 +/− 45.8 and 1374.0 +/− 371.6) as depicted in Fig. 2A. Significantly lower emissions of 987.1 +/− 446.3, 908.4 +/− 196.4 and 820.8 +/− 151.0 (all $p<0.05$) were observed with levamisole (100 µg/ml and 50 µg/ml) and nitazoxanide (50 µg/ml) ($p<0.05$), respectively. Nitazoxanide at the two highest doses (200 and 100 µg/ml) had a highly significant impact on the emission with low values of 109.0 +/− 119.5 ($p<0.05$) and 390.5 +/− 154.7 ($p<0.05$) observed.

The microscopic data observed with the motility assay (Fig. 2B) and the Alamar Blue data showed correlation ($r = 0.84$).

3.3. MTT assay

The MTT assay indicated changes in the health status of the worms. A color change from yellow to blue was observed after transferring

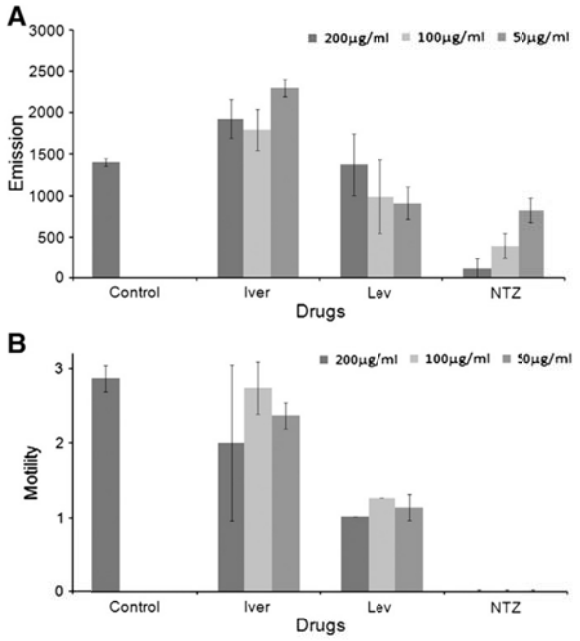

Fig. 2. A: In vitro Alamar Blue assay with *T. muris* L4 larvae. worms were incubated in the presence of 200, 100 and 50 µg/ml of the drugs. Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide. B: In vitro motility assay with *T. muris* L4 larvae. Data correspond to the Alamar Blue assay depicted in Fig. 2A. Worms were incubated in the presence of 200, 100 and 50 µg/ml of the drugs; Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide; Motility scale: 0 = dead, 1 = very low motility, 2 = low motility, 3 = normal motility.

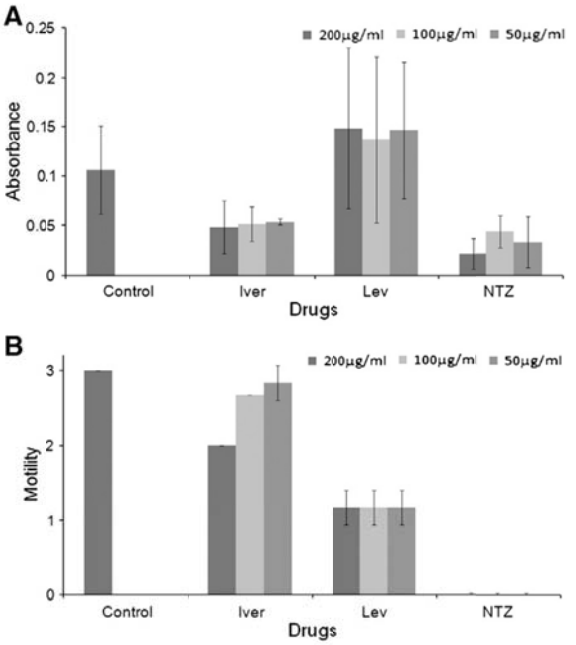

Fig. 3. A: In vitro MTT assay with *T. muris* L4 larvae. Worms were incubated in the presence of 200, 100 and 50 µg/ml of the drugs; Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide. B: In vitro motility assay with *T. muris* L4 larvae. Data correspond to the MTT assay depicted in Fig. 3A. Worms were incubated in the presence of 200, 100 and 50 µg/ml of the drugs. Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide. Motility scale: 0 = dead, 1 = very low motility, 2 = low motility, 3 = normal motility.

untreated worms into 100% DMSO. Results of the MTT assay are depicted in Fig. 3A. The signal-to-noise ratio between controls and medium blanks was 3.4. For nitazoxanice and ivermectin-treated worms (200, 100 and 50 µg/ml) low absorbances of 0.022 +/− 0.016, 0.044 +/− 0.016 and 0.034 +/− 0.026 as well as 0.048 +/− 0.026, 0.052 +/− 0.017 and 0.054 +/−0.003, respectively, were measured (all $p<0.05$). A significant difference in absorbance could be also documented for levamisole-treated worms (200 and 100 µg/ml) (all $p<0.05$).

No correlation could be observed between motility assay (Fig. 3B) and MTT assay data ($r = 0.2$).

3.4. Acid phosphatase activity assay

Signals obtained measuring activity of acid phosphatase secreted by adult *T. muris* incubated for 72 h in nitazoxanide, levamisole and ivermectin are shown in Fig. 4A. Control worms yielded an absorbance of 0.31 +/− 013. Significantly decreased absorbances (all $p<0.05$) were documented for worms incubated with 200, 100 and 50 µg/ml ivermectin and nitazoxanide. Levamisole-treated worms (200, 100 and 50 µg/ml) showed absorbances of 0.36 +/− 0.13, 0.17 +/− 0.02 and 0.13 +/− 0.03, respectively ($p<0.05$). The signal-to-noise ratio of control wells to medium blanks was 3.1.

Corresponding microscopic data is presented in Fig. 4B. A positive correlation was calculated between the acid phosphatase assay and microscopic data ($r = 0.67$).

3.5. xCELLigence System

L4 larvae and adults were incubated in the presence of levamisole, nitazoxanide and ivermectin and the drug effect was analyzed using the xCELLigence System, which indirectly detects the viability of the worms. The mean noise amplitude length of dead worms ($1.3x10^{-3}$)

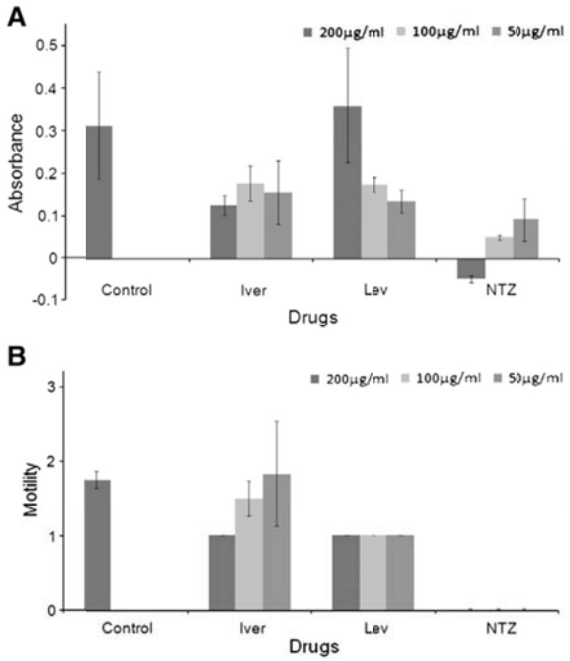

Fig. 4. A: In vitro acid phosphatase activity assay with *T. muris* adult worms. Worms were incubated in the presence of 200, 100 and 50 µg/ml of the drugs. Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide. B: In vitro motility assay with *T. muris* adult worms. Data correspond to the acid phosphatase assay presented in Fig. 4A. Worms were incubated in the presence of 200, 100 and 50 µg/ml of the drugs. Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide. Motility scale: 0 = dead, 1 = very low motility, 2 = low motility, 3 = normal motility.

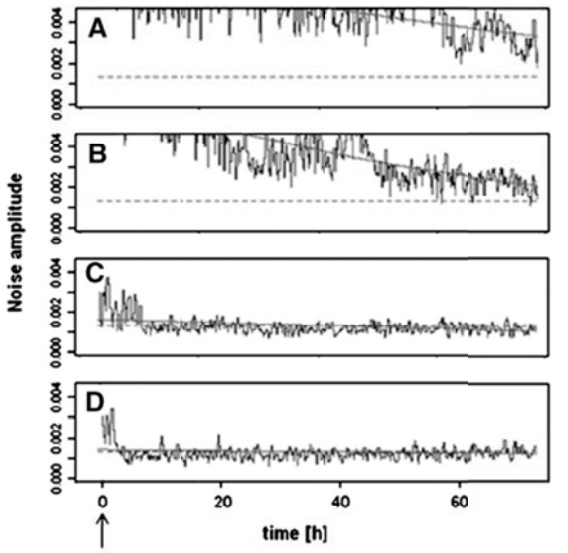

Fig. 5. xCELLigence System measurements of *T. muris* adult worms incubated with 50 µg/ml ivermectin, levamisole and nitazoxanide at 37 °C for 72 h. A) DMSO control, B) ivermectin (50 µg/ml), C) levamisole (50 µg/ml), D) nitazoxanide (50 µg/ml). Continuous line = absolute values of the noise amplitudes obtained for 20 min over the whole experiment, undulated line = noise amplitude values following an exponential decay according to the equation: $H_0e^{-\lambda t} + 3e\text{-}07$, where λ described the decay rate of the noise amplitudes, H_0 the starting point and t the time in hours, dotted line = background noise of the system (1.3×10^{-3}), arrow = medium/drug injection after an equilibration time of 3 h, the time point of death of worms is defined as the intersection between the background and the drug curve (continuous line).

was set as the background noise of the system. After 3 h of equilibration time of the system, medium or drug solutions were added (arrow Fig. 5). The intersection between the noise exponential decay curve of treated worms (undulated line) and the background noise line (dotted line) indicated the time point of death of the worms. Control worms (Fig. 5A) were active, showing amplitude lengths (a.l.) of 5.5×10^{-3} after 24 h of incubation, while slightly decreased amplitudes were observed 72 h after medium injection (a.l. = 3.4×10^{-3}). Ivermectin-treated worms were not affected by the drug (50 µg/ml) as shown in Fig. 5B (a.l. ranging from 4.7×10^{-3} (24 h) to 2.2×10^{-3} (72 h)). The viability of these worms was comparable to the controls. Worms incubated in 50 µg/ml levamisole (Fig. 5C) showed a low activity (a.l. of 1.4×10^{-3} at the 24 hour time point, which further decreased to 1.1×10^{-3} 72 h post-incubation) during the entire experiment, almost in the range of dead worms (1.3×10^{-3}). The time point of death for levamisole treated worms was determined as approximately 35 h after drug injection. Similarly, nitazoxanide-treated worms (50 µg/ml) revealed a low activity with amplitude lengths of 1.3×10^{-3} and 1.2×10^{-3} after 24 and 72 h of incubation, respectively as shown in Fig. 5D. Nitazoxanide treated worms died after an incubation period of around 25 h. All drug-treated worms showed significant reductions in viability (amplitudes) (all $p<0.005$).

Several tests using the xCELLigence System were also done with L4 larvae incubated in the presence of different drugs and without drug exposure. However, with these worms, noise amplitudes were not greater than the baseline noise (data not shown).

3.6. Isothermal microcalorimetry

In a preliminary assay the heat productions of 1, 2 and 4 adult worms per ampoule were measured over a period of 300 h. Calorimetric measurements with 1 and 2 worms did not yield strong signals. The heat flow and noise amplitude curves obtained from 4 worms were acceptable but in order to get a better and more stable signal, we increased the worm number to 6 per ampoule.

Heat flow curves of ivermectin, levamisole and nitazoxanide-treated worms as well as controls are depicted in Fig. 6. Each curve represents the heat production of 6 worms per ampoule. The mean heat flow signal obtained from the measurements of dead worms was 10^{-7} µW, set as the background signal of the system. The system was equilibrated for 20 h followed by medium and drug injections (arrow Fig. 6).

3.6.1. Heat flow

The levels of heat production of treated and untreated worms (Fig. 6) were compared at 24 and 72 h post-incubation and heat flow reductions (HFR) were calculated. Control worms showed constant heat flow (HF) values of 1.7×10^{-5} µW and 1.0×10^{-5} µW 24

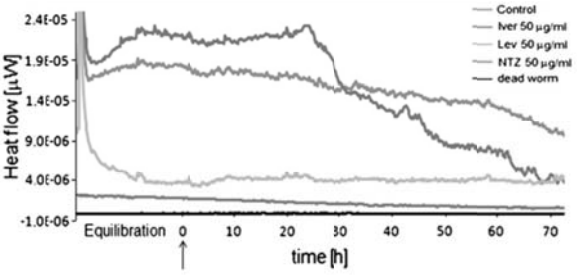

Fig. 6. Heat flow curves of *T. muris* adult worms incubated with 50 µg/ml ivermectin, levamisole and nitazoxanide at 37 °C for 72 h. Arrow = medium/drug injection after an equilibration time of 20 h. Iver = ivermectin, Lev = levamisole, NTZ = nitazoxanide.

and 72 h, respectively after experiment onset. A higher heat flow value of $2.1x10^{-5}$ μW (24 h) was observed with ivermectin-treated worms (50 μg/ml), which decreased to $4.3x10^{-6}$ μW 72 h after starting the experiment (HFR of 81%). The heat flow of levamisole-treated worms was reduced by 28% (HF = $4.7x10^{-6}$ μW) 24 h following incubation start and remained at this reduced level over the next 48 h. Nitazoxanide-treated worms showed a reduced heat flow of $1.2x10^{-6}$ μW 24 h after starting the experiment (HFR of 32%) decreasing to $6.1x10^{-7}$ μW 72 h post-incubation (HFR of 66%). All treated worms showed significant differences in heat production compared to the controls (all $p<0.005$).

3.6.2. Motor activity (noise amplitudes)

Control worms showed amplitude lengths of $6.8x10^{-7}$ μW and $6.4x10^{-7}$ μW 24 and 72 h following the start of incubation, respectively, hence motor activities remained constant (Fig. 6). Ivermectin-treated worms showed an amplitude length of $9.8x10^{-7}$ μW (24 h post-exposure), which even increased to $1.4x10^{-6}$ μW at the 72 h examination time point. Levamisole-treated worms (50 μg/ml) had stable noise amplitudes during the entire observation period (a.l. = $4.4x10^{-7}$ μW (24 h) and $4.6x10^{-7}$ μW (72 h)). Strongly reduced noise amplitudes were observed with worms treated with 50 μg/ml nitazoxanide. Amplitude lengths were in the range of dead worms (10^{-7} μW) already shortly after drug exposure (e.g. a.l. = $1.1x10^{-7}$ μW (24 h post-incubation)).

4. Discussion

T. trichiura is one of the most neglected parasites worldwide and treatment options are not satisfactory. To date the motility assay is the most widely used assay to evaluate drug candidates on nematodes in vitro. Hence, our objective was to evaluate, characterize and compare several different in vitro drug sensitivity assays, namely the motility assay, the Alamar Blue assay, the MTT assay, the acid phosphatase activity assay, the xCELLigence System and isothermal microcalorimetry, to identify the best methods for analyzing possible drug effects on *Trichuris* in vitro. We used two standard anthelminthic drugs, levamisole and ivermectin, to analyze these test systems. We did not use albendazole, which is the currently most widely used drug to treat infections with *T. trichiura* since albendazole is very insoluble in water and therefore tends to precipitate. We additionally included nitazoxanide in this work, a human antiprotozoal drug, blocking the anaerobic energy metabolism of parasites (Zhang et al., 2010), since clinical trials have documented some trichuricidal properties (Juan et al., 2002). Our own in vitro studies showed that nitazoxanide revealed surprisingly an even higher activity against *T. muris* than the standard drugs (Tritten et al., manuscript submitted for publication).

The motility assay, the MTT assay, the acid phosphatase activity assay and the xCELLigence System were tested already in previous studies on other gastrointestinal nematodes as for example *Ancylostoma caninum*, *Necator americanus*, *Strongyloides stercoralis*, *Strongyloides ratti* or *Heligmosomoides polygyrus* (Maki and Yanagisawa 1980a, 1980b, 1980c; Moulay and Robert-Gero 1995; Fetterer and Rhoads 2000; Kotze et al., 2004, 2005; Hordegen et al., 2006; Smout et al., 2010). In addition, the Alamar Blue assay is commonly used to study drug effects against trypanosomes (O'Brien et al., 2000). Finally, microcalorimetry was found to be a useful tool to study drug effects on schistosomes and has been used to study the effect of synthetic peroxides on *Fasciola* spp. (Manneck et al., 2010; Kirchhofer et al., 2011).

An overview of the different assays, highlighting assets and drawbacks (processing time, reagents, necessary equipment, technology and costs), is provided in Table 1.

Comparing the 6 in vitro assays, we found that the Alamar Blue assay is an excellent alternative to the standard motility assay. First of all, the Alamar Blue test was very easy to perform. After 72 h of incubation, resazurin was added to the wells and the plates were incubated for another 4 h. Second, the costs for the assay are low and no high-tech equipment is necessary since a spectrofluorometer often belongs to the basic equipment of a scientific institute. Furthermore, the test provides exact values (i.e. the fluorescence emission) hence data are more precise than the subjective data obtained from visual analysis of worms done by microscopy. For example, the Alamar Blue assay revealed that worms treated with 50 or 100 μg/ml nitazoxanide were still alive 72 h post-incubation although they had been classified as dead using microscopy. This finding might be due to a paralysis of worms while the worms were metabolically still alive. Similarly, L4 larvae incubated with the highest concentration of levamisole (200 μg/ml) revealed high absorbances though all levamisole concentrations showed reduced viabilities using the motility assay (Figs. 2a and b). A possible explanation might be that the drug precipitated at the high concentration of 200 μg/ml, resulting in a lower effect on the worms.

The MTT assay and the acid phosphatase activity assay also both generated accurate data and the procedure was comparable to the

Table 1
Comparison of 6 different in vitro assays to study drugs against *Trichuris* spp.; Processing time (pt) = preparation time for each assay (e.g. addition of reagents, transfer of parasites, setting of parameters, measurements); it = incubation time; MTT = Thiazolyl Blue Tetrazolium Bromide, DMSO = dimethylsulfoxid, FCS = Fetal Calf Serum, pNPP = p-nitrophenyl phosphate, NaAc = sodium acetate anhydrous, NaOH = sodium hydroxide RTCA SP = xCELLigence system.

Assay	Technology	Duration of assay (estimation)	Reagents	Equipment	Costs	Comment
Motility assay	Motility scale used to determine worm's viability (visual)	pt = 3x ~15 min it = ~72 h	–	Light microscope	Low	Subjective nature due to visual characterization of worms
Alamar Blue assay	Enzymatic reaction within parasites, reduction process of substrate	pt = ~25 min it = ~76 h	Alamar Blue	Spectrofluorometer	Medium	Possible worm damage due to transfer
MTT assay	Enzymatic reaction within parasites, reduction process of substrate	pt = ~35 min it = ~74 h	MTT, DMSO	Spectrophotometer	Medium	Several worm transfer steps, possible worm damage due to transfer
Acid phosphatase (AP) assay	Enzymatic reaction of AP within gastro intestinal tract of worms	pt = ~20 min it = ~74–75 h	FCS, pNPP, NaAc, NaOH	Spectrophotometer	Medium	Optimal medium (RPMI) could not be used, additional controls to remove background noise (color) of nitazoxanide
Isothermal microcalorimetry	Analysis of the heat flow of biological processes. Analysis of noise amplitudes due to viability of worms	pt = ~30 min it = ~72 h	–	Isothermal microcalorimeter	High	Time extensive for data analysis
xCELLigence	Analysis of noise amplitudes due to viability of worms	pt = ~30 min it = ~72 h	–	RTCA SP	High	Time extensive for data analysis

Alamar Blue assay. However, both assays had decisive drawbacks. Most importantly, the acid phosphatase assay could not be performed in the medium of choice (RPMI) because the assay seems to be highly sensitive to normal bacterial growth. The acid phosphatase experiment presented here was performed using HBSS medium supplemented with fetal calf serum. Since RPMI and not HBSS was identified as the optimal medium for *T. muris* in vitro tests, the experiment was therefore not done under optimal conditions. The health status of all worms was not ideal, as the control worms showed a low motility (see Fig. 4b, motility = 1.75 +/−0.12). Hence, this assay would need further improvements before it could be used as an in vitro assay to characterize drug effects on *T. muris*. In addition, of note, additional nitazoxanide controls had to be used to remove the disturbing color signal of nitazoxanide (yellow color) in the acid phosphatase assay. The MTT assay showed no good correlation with the motility assay. For example, though worms exposed to ivermectin showed high motilities (and high absorbances with the Alamar Blue assay), only low absorbances were seen with the MTT assay. Finally, it is interesting to note that in both MTT and acid phosphatase activity assays, levamisole-treated worms showed strong absorbances, even higher than the controls. This effect disturbed a correct analysis of the effect of levamisole on *T. muris* in these assays. This finding can not be explained at the moment but might have been caused by stress of worms, triggering ingestion of larger amounts of medium.

The xCELLigence System and isothermal microcalorimetry were both excellent methods to measure the activity of adult *T. muris* worms and drug effects on the worms continuously and highly accurately. The time point of death of worms following drug exposure could precisely be determined with xCELLigence. For example, heat flow random oscillation analyses from both test systems revealed an immediate parasite immobility following exposure of *T. muris* to nitazoxanide. Microcalorimetry measured a low heat flow of nitazoxanide over 72 h. These observations correlate nicely with findings observed using Alamar Blue as described above. The fact that levamisole-treated worms were defined as dead with xCELLigence (Fig. 5) after around 35 h whereas motility (Fig. 1) was still observed under the microscope cannot be explained. It might be possible that the worms used for xCELLigence were not very healthy because worms treated with 200 and 100 μg/ml levamisole and analyzed with xCELLigence showed reduced activity but were still alive during the whole experiment (data not shown). A disadvantage of both systems is the high costs for the equipment, such as the xCELLigence 96-well E-plates and the machines. Hence, if the xCELLigence System and isothermal microcalorimetry could be used in collaboration with other institutes to reduce the costs, both assays would be a good choice to generate drug-effect data on *Trichuris* worms of high quality with a continuous and very precise chronology.

5. Conclusion

In conclusion, the Alamar Blue assay is an excellent in vitro assay to study drug effects on *Trichuris* spp and might be a good alternative to microscopic evaluation. The Alamar Blue assay is characterized by low costs, uncomplicated procedure and handling as well as precise data outputs.

Acknowledgments

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Chapter 3

In vitro and *in vivo* efficacy of monepantel (AAD 1566) against laboratory models of human intestinal nematode infections

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In Vitro and *In Vivo* Efficacy of Monepantel (AAD 1566) against Laboratory Models of Human Intestinal Nematode Infections

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Abstract

Background: Few effective drugs are available for soil-transmitted helminthiases and drug resistance is of concern. In the present work, we tested the efficacy of the veterinary drug monepantel, a potential drug development candidate compared to standard drugs *in vitro* and in parasite-rodent models of relevance to human soil-transmitted helminthiases.

Methodology: A motility assay was used to assess the efficacy of monepantel, albendazole, levamisole, and pyrantel pamoate *in vitro* on third-stage larvae (L3) and adult worms of *Ancylostoma ceylanicum*, *Necator americanus* and *Trichuris muris*. *Ancylostoma ceylanicum*- or *N. americanus*-infected hamsters, *T. muris*- or *Ascaris suum*-infected mice, and *Strongyloides ratti*-infected rats were treated with single oral doses of monepantel or with one of the reference drugs.

Principal Findings: Monepantel showed excellent activity on *A. ceylanicum* adults (IC_{50} = 1.7 µg/ml), a moderate effect on *T. muris* L3 (IC_{50} = 78.7 µg/ml), whereas no effect was observed on *A. ceylanicum* L3, *T. muris* adults, and both stages of *N. americanus*. Of the standard drugs, levamisole showed the highest potency *in vitro* (IC_{50} = 1.6 and 33.1 µg/ml on *A. ceylanicum* and *T. muris* L3, respectively). Complete elimination of worms was observed with monepantel (10 mg/kg) and albendazole (2.5 mg/kg) in *A. ceylanicum*-infected hamsters. In the *N. americanus* hamster model single 10 mg/kg oral doses of monepantel and albendazole resulted in worm burden reductions of 58.3% and 100%, respectively. *Trichuris muris*, *S. ratti and A. suum* were not affected by treatment with monepantel *in vivo* (following doses of 600 mg/kg, 32 mg/kg and 600 mg/kg, respectively). In contrast, worm burden reductions of 95.9% and 76.6% were observed following treatment of *T. muris*- and *A. suum* infected mice with levamisole (200 mg/kg) and albendazole (600 mg/kg), respectively.

Conclusions/Significance: Monepantel reveals low or no activities against *N. americanus*, *T. muris*, *S. ratti and A. suum* *in vivo*, hence does not qualify as drug development candidate for human soil-transmitted helminthiases.

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Introduction

The hookworm species *Ancylostoma duodenale* and *Necator americanus*, the whipworm *Trichuris trichiura*, the threadworm *Strongyloides stercoralis*, and the roundworm *Ascaris lumbricoides* are soil-transmitted helminths (STH) of great public health importance. Cumulatively, these parasites affect more than one billion people globally, particularly in developing regions of Asia, Africa, and Latin America [1,2]. If untreated, infections with STH are present for years and patients suffer from moderate to severe intestinal disturbances, anemia, nutrient loss and profound physical and mental deficiencies [3,4].

Helminth control relies primarily on the regular administration of anthelmintics, typically carried out within the framework of school-based deworming programs, once or twice a year [5–7]. Five drugs are currently available for the treatment of infections with STH (albendazole, mebendazole, pyrantel pamoate, levamisole, and ivermectin), all of which have been registered for human use before or during the 1980's [8,9]. No new anthelmintic drug for human use has reached the market since then. Moreover, none of these drugs are efficacious using single doses on all STH species, with particularly low efficacy observed on *T. trichiura* [10]. Relying on only a handful of drugs is a precarious situation, in the light of a possible emergence of drug resistance [10].

Since drug resistance to nematodes of veterinary importance is widely spread and increasing in frequency, most of the anthelmintic drug research and development efforts are motivated by veterinary needs [11]. For example, albendazole, mebendazole, and pyrantel pamoate were originally developed for livestock and pets [12].

Monepantel (AAD1566) belongs to a new class of veterinary anthelmintics, the amino-acetonitrile derivatives. It has been proposed that monepantel interferes with nematode-specific acetylcholine receptor subunits, leading to body wall muscle

Author Summary

Soil-transmitted helminthiases affect more than one billion people among the most vulnerable populations in developing countries. Currently, control of these infections primarily relies on chemotherapy. Only five drugs are available, all of which have been in use for decades. None of the drugs are efficacious using single doses against all soil-transmitted helminths (STH) species and show low efficacy observed against *Trichuris trichiura*. In addition, the limited availability of current drug treatments poses a precarious situation should drug resistance occur. Therefore, there is great interest to develop novel drugs against infections with STH. Monepantel, which belongs to a new class of veterinary anthelmintics, the amino-acetonitrile derivatives, might be a potential drug candidate in humans. It has been extensively tested against livestock nematodes, and was found highly efficacious and safe for animals. Here we describe the *in vitro* and *in vivo* effect of monepantel, on *Ancylostoma ceylanicum*, *Necator americanus*, *Trichuris muris*, *Strongyloides ratti*, and *Ascaris suum*, five parasite-rodent models of relevance to human STH. Since we observed that monepantel showed only high activity on one of the hookworm species and lacked activity on the other parasites tested we cannot recommend the drug as a development candidate for human soil-transmitted helminthiases.

paralysis and subsequent death of worms. Due to its unique mode of action, the drug has proven efficacy against nematodes infecting livestock which are resistant to current anthelmintic drugs [13]. Monepantel has been extensively tested on different nematode isolates. Administered at a single oral dose of 2.5 mg/kg, it was found to be safe, well-tolerated by ruminant hosts, and showed high cure rates on fourth stage larvae and adult worms of 15 nematode species [13–16]. Due to its high and broad nematocidal activity, monepantel was considered to be a candidate for a human health directed program.

The aim of the present investigation was to study the activity of monepantel, compared with the reference drugs, albendazole, levamisole, and pyrantel pamoate, in five parasite-rodent models, that correspond to important human STH and *in vitro*. *Ancylostoma ceylanicum* and *Necator americanus* were both adapted using eggs from infected dogs or humans to an unnatural host, the golden hamster, and represent robust rodent models for hookworm infections [17,18]. The *Trichuris muris* mouse model is an excellent model for trichuriasis [19,20]. *Strongyloides ratti* in rats is a commonly used murine model for strongyloidiasis [21]. Finally, murine infection with *Ascaris suum* is a model which mimics the early infection of *A. lumbricoides*. The survival of different larval stages and adult worms of *A. ceylanicum*, *N. americanus*, and *T. muris* was evaluated *in vitro*, following monepantel incubation using a motility assay. The *in vitro* activity of monepantel on *S. ratti* has been described recently [22]. *In vivo*, we studied worm burden reductions and for *A. ceylaniam*, *N. americanus*, and *T. muris*, worm expulsion rates were also measured.

Methods

Drugs

Monepantel was kindly provided by Novartis Animal Health, St-Aubin, Switzerland. Albendazole and pyrantel pamoate were purchased from Sigma-Aldrich (Buchs, Switzerland), and levamisole-hydrochloride from Fluka (Buchs, Switzerland).

For the *in vitro* studies, stock solutions of the drugs were prepared in 100% DMSO (Fluka, Buchs, Switzerland) and stored at 4°C. For the *in vivo* studies, drugs were suspended in 7% (v/v) Tween 80% and 3% (v/v) ethanol or DMSO/PEG shortly before treatment.

Animals and Parasites

Three-week-old male Syrian Golden hamsters were purchased from Charles River (Sulzfeld, Germany). Four-week-old female NMRI mice and 3-week-old female C57Bl/6J mice were purchased from Harlan (Horst, The Netherlands). Three-week-old female Wistar rats were purchased from Harlan (Horst, The Netherlands).

All animals were kept in macrolon cages under environmentally-controlled conditions (temperature: 25°C, humidity: 70%, light/dark cycle 12 h/12 h) and had free access to water and rodent food (Rodent Blox from Eberle NAFAG, Gossau, Switzerland). They were allowed to acclimatize in the animal facility of the Swiss Tropical and Public Health Institute (Swiss TPH) for 1 week before infection. The current study was approved by the local veterinary agency based on Swiss cantonal and national regulations (permission no. 2070).

Parasites and Infections

Ancylostoma ceylanicum third-stage larvae (L3) were kindly provided by Prof. J. M. Behnke (University of Nottingham). The *A. ceylanicum* life cycle [23] had been maintained at the Swiss TPH since June 2009 [17]. To maintain the life cycle, hamsters were treated orally 1 day before infection and then twice weekly with 3 mg/kg hydrocortisone (Hydrocortone®, MSD) or with 1 mg/l dexamethasone (dexamethasone water-soluble, Sigma-Aldrich) in the drinking water. They were orally infected with 150 *A. ceylanicum* L3, which had been harvested less than 1 month before infection and had been assessed microscopically for viability. Animals assigned to *in vivo* studies were not treated with hydrocortisone and were infected with 300 L3.

Infective *N. americanus* L3 were the gift of Prof. S. H. Xiao (National Institute for Parasitic Diseases, Shanghai). Hamsters were immunosuppressed with dexamethasone as described above and were infected subcutaneously with 250 viable *N. americanus* L3.

Embryonated *T. muris* eggs were kindly obtained from Prof. J. M. Behnke and Prof. H. Mehlhorn. The life cycle had been maintained at the Swiss TPH since January 2010 as described elsewhere [19]. Briefly, *T. muris* eggs were evaluated for embryonation under the microscope (magnification 80–160×, Carl Zeiss, Germany). NMRI mice were orally infected with 400 embryonated eggs. Mice were treated either subcutaneously (s.c.) 1 day before infection and then every second day between days 5 and 15 with 15 mg hydrocortisone (Hydrocortisone 21-hemisuccinate sodium salt, Sigma-Aldrich) in 0.9% NaCl solution, or with 8 mg/l dexamethasone in the drinking water until the end of the experiment.

The *S. ratti* life cycle had been maintained over decades at the Swiss TPH, by serial passage through rats. Rats were infected subcutaneously with 735 freshly harvested *S. ratti* L3.

Infective *A. suum* eggs were obtained from Prof. S. M. Thamsborg, University of Copenhagen and Prof. G. Cringoli, University of Naples. Briefly, C57Bl/6J mice were orally infected with 500 embryonated eggs, according to a procedure described elsewhere [24].

In vitro Studies

The larval or adult motility assay is currently the method of choice to evaluate drug sensitivity of different nematode species [25–27]. Non-motile worms were considered as dead and the percent viability or survival in each well was calculated.

A. ceylanicum and *N. americanus*

Activity against L3. Thirty L3 in 100 µl deionized water, supplemented with antibiotics, were placed in each well of a 96-well plate (Costar). The L3 had been harvested less than 1 month before the studies and were stored in deionized water containing 25 µg/ml amphotericin B (Sigma-Aldrich) and 1% (v/v) penicillin-streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin, Sigma-Aldrich). Drugs were serially-diluted in HBSS (Gibco) supplemented with 25 µg/ml amphotericin B (Sigma Aldrich) and 1% (v/v) penicillin-streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin, Sigma-Aldrich). Diluted drug (100 µl; 100–0.01 µg/ml, final concentration) were added to the wells and the plate was incubated for 72 h at room-temperature in a dark and humid box. Larval motility was evaluated under the microscope (magnification 20×) following addition of 100 µl hot water (~90°C) and exposure to microscope light. A minimum of 2 wells served as controls, which were L3 incubated with the highest concentration of DMSO used in the test (2% v/v).

Activity against Adults. Drug susceptibility on adults was tested in 48-well plates (Costar). Three to 4 worms were added to wells containing 500 µl supplemented HBSS medium, containing 10% v/v fetal calf serum (Gibco). Drug dilutions (500 µl) were added and the plate was incubated for 72 h at 37°C and 5% CO_2. The motility of adult worms was evaluated under the microscope (magnification 20×), after adding 500 µl hot water (~90°C), using a viability scale (2: good motility, 1: lowered motility, and 0: no motility, death). A minimum of 3 worms served as controls which were incubated in the presence of the highest concentration of DMSO used in the test (2% v/v).

Ovicidal Activity. The drug effects on eggs were observed using a modified protocol of the egg hatch test [28]. Stools of infected hamsters were collected overnight, filtered and subjected to flotation by mixing the stool with a 2 M $NaNO_3$ solution and centrifuged at 2000 rpm. The upper quarter of the solution (containing the eggs) was kept and washed twice in deionized water. The test was carried out in quadruplicate for each drug concentration. Fifty eggs in 500 µl deionized water were distributed to each well and 500 µl drug diluted in deionized water were added (10 µg/ml, final concentration). After 24 and 48 h, 20 eggs per well were examined under the microscope (magnification 80–160×) for embryonation and hatching, respectively.

T. muris

Activity against L3 and Adults. L3 or adults were collected from the intestines of infected mice and 3–4 worms were distributed to each well of 48 or 96-well plates (Costar), containing 100 µl (L3) or 500 µl (adults) RPMI medium supplemented with 5% (v/v) amphotericin B (Sigma Aldrich) and 1% (v/v) penicillin-streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin, Sigma-Aldrich). Drug dilutions (100 µl for testing on L3 or 500 µl for adults) ranging from 200–50 µg/ml, final concentration, were added and the plate was incubated for 72 h at 37°C and 5% CO_2. The larval and adult motilities were evaluated under the microscope (magnification 20–80×) using a viability scale (3: good motility, 2: low motility, 1: very low motility, and 0: death), as described by Stepek and colleagues [27]. A minimum of 3 worms served as controls, which were incubated in the highest concentration of DMSO used in the test (2% v/v).

In vivo Studies

***A. ceylanicum* and *N. americanus*.** The immunosuppressive treatment of hamsters was stopped at least 2 days before treatment. Hamsters were housed individually from day 20 post-infection (p.i.) onwards. On days 21 and 22 (*A. ceylanicum*) or 46 and 47 (*N. americanus*), a fecal sample was collected from each hamster and processed using an in-house sedimentation method. Briefly, stools were collected overnight, soaked in 0.9% NaCl solution, filtered and washed with 150 ml saline through 8 gauze layers. The filtrate was allowed to sediment for 1–2 h and the solution adjusted to 0.1 g filtrate per ml. To calculate the number of eggs per gram (epg), the average of 4 egg counts of 20 µl of fecal solution was determined microscopically. On the basis of the fecal egg burden, *A. ceylanicum*-infected hamsters were assigned to the following treatment groups - monepantel 10 mg/kg or 5 mg/kg, albendazole 5 mg/kg, 2.5 mg/kg or 1.25 mg/kg, levamisole 10 mg/kg or pyrantel pamoate 10 mg/kg- or control groups, with 4 animals per group. Similarly, groups of 3–4 *N. americanus*-infected animals received 20 or 10 mg/kg monepantel, 10 or 5 mg/kg albendazole, or served as controls.

Hamsters were treated on day 23 p.i (*A. ceylanicum*) or 48 p.i. (*N. americanus*) with single oral doses of the test drugs. Expelled worms were counted from the collected stools 24 h and 48 h after treatment. On day 7 post-treatment, the hamsters were killed by the CO_2 method and the remaining worms in the gut counted [29–31].

***T. muris*.** Treatment with dexamethasone was stopped at least 2 days before treatment. Mice were housed individually from day 40 onwards. A fecal sample was examined from each mouse and egg negative animals were excluded from the study. Groups of 4 infected animals were assigned to treatment (monepantel 600 mg/kg, albendazole 600 mg/kg, levamisole 200 mg/kg, or pyrantel pamoate 300 mg/kg) or control groups. Expelled worms were counted from the collected stools 24 h and 48 h after treatment. On day 7 post-treatment, mice were killed by the CO_2 method and the remaining worms in the gut counted [30].

***S. ratti*.** Five days p.i, 1 group of 4 rats was treated orally with 32 mg/kg monepantel. Four rats were left untreated and served as controls. Seven days post-treatment, the rats were dissected. The intestine was removed, opened and incubated for 3 h at 37°C in PBS, as described before [32]. The liquid and the intestines were screened under a binocular, and the larvae counted (magnification 10–40×).

***A. suum*.** One hour p.i, 2 groups of 4 mice were treated with single oral doses of 600 mg/kg monepantel or 600 mg/kg albendazole. A third group of 4 mice was left untreated and served as control. On day 7 post-treatment, the mice were killed by the CO_2 method. The lungs and livers were removed, cut with fine scissors, and incubated in 0.9% NaCl for 24 h at 37°C to allow larvae to migrate out of the organs into the saline [24]. Larvae in solution were counted using a microscope (magnification 20×).

Statistical Analyses

The average of motility scores for one drug was calculated for each concentration and normalized into percentage, relative to control. IC_{50} values were expressed based on the median effect principle using CompuSyn (version 1.0). The r value represents the linear correlation coefficient of the median-effect plot, indicating the goodness of fit, hence the accuracy of the IC_{50} [33]. Variance analysis in the ovicidal activity studies was performed with the Fisher's exact test, using StatsDirect (version 2.4.5; StatsDirect Ltd; Cheshire, UK). The worm burden reductions were determined by comparing the mean number of adult worms in the intestine of a treated group with the mean numbers of worms in the control group. Means and standard deviations were calculated using Microsoft® Excel 2003. The worm expulsion rates were calculated by dividing the number of expelled worms of a treatment group by the group's total worm burden. The

Table 1. 50% inhibitory concentrations of monepantel, albendazole, levamisole, and pyrantel pamoate on *A. ceylanicum*, *N. americanus*, and *T. muris*.

Drugs	*A. ceylanicum*		*N. americanus*		*T. muris*	
	IC_{50} in µg/ml (r)		IC_{50} in µg/ml (r)		IC_{50} in µg/ml (r)	
	L3	Adults	L3	Adults	L3	Adults
Monepantel	>100 (0.70)	1.7 (0.93)	>100 (n.d.)	>100 (0.32)	78.7 (1.0)	>200 (n.d.)
Albendazole	32.4 (0.87)	>100 (0.77)	>100 (n.d.)	>100 (0.57)	>200 (0.39)	>200 (0.40)
Levamisole-HCl	1.6 (0.95)	>100 (0.79)	0.5 (0.97)	13.4 (0.99)	33.1 (1.0)	16.5 (1.0)
Pyrantel pamoate	90.9 (0.84)	>100 (0.79)	2.0 (0.94)	7.6 (0.98)	95.5 (0.99)	34.1 (0.99)

IC_{50}s (µg/ml) were calculated for monepantel, albendazole, levamisole, and pyrantel pamoate after 72 h on L3 and adult stages of *A. ceylanicum*, *N. americanus*, and *T. muris*. r = linear correlation coefficient of the median-effect plot, indicating the goodness of fit. r≥0.85 indicates a satisfactory fit. n.d.: not determined, fitting not possible.

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Kruskal-Wallis test and the Mann-Whitney U test were used to assess the statistical significance of the worm burden reduction, using StatsDirect.

Results

In vitro Findings

The effects of monepantel and reference drugs on L3 and adult worms of *A. ceylanicum* and *T. muris* after 72 h of exposure *in vitro* are presented in Table 1.

A. ceylanicum. The sensitivity of *A. ceylanicum* L3 to monepantel was low at the highest concentration tested (100 µg/ml), with over 70% of the larvae still showing activity after 72 h following stimulation with hot water and exposure to light (IC_{50}>100 µg/ml). Pyrantel pamoate affected the larvae only moderately (IC_{50} = 90.9 µg/ml, r = 0.84), whereas an IC_{50} of 32.4 µg/ml was calculated for albendazole (r = 0.87). Levamisole showed superior efficacy: at a concentration of 10 µg/ml a survival rate of 26.7% was determined. An IC_{50} value of 1.6 µg/ml (r = 0.95) was calculated for levamisole (Table 1).

Ancylostoma ceylanicum adult worms were highly sensitive to monepantel at concentrations of 1 µg/ml and above (IC_{50} = 1.7 µg/ml, r = 0.93). Lower activities against adult *A. ceylanicum* were observed for albendazole, levamisole, and pyrantel pamoate, which showed survival rates of 50–95% at 100 µg/ml (all IC_{50}s>100 µg/ml) (Table 1).

As shown in Table 2, albendazole was the only compound that inhibited both egg embryonation and hatching *in vitro* (90.5% and 98.5% inhibition of embryonation and hatching, respectively).

Table 2. Ovicidal activity of monepantel, albendazole, levamisole, and pyrantel pamoate on *A. ceylanicum* eggs.

Group	% Embryonation at 24 h (SD)	% Hatching at 48 h (SD)
Control	100 (3.5)	100 (9.8)
Monepantel	99.5 (9.3)	68.6 (9.2)*
Albendazole	9.5 (5.2)*	1.5 (3.0)*
Levamisole-HCl	88.6 (8.2)*	58.5 (3.5)*
Pyrantel pamoate	95.4 (7.0)	95.3 (12.5)

SD = standard deviation.

*P-value <0.001 (Fisher's exact test).

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Levamisole, pyrantel pamoate, and monepantel showed no effect on embryonation and only a moderate inhibition on hatching (reduction of 4.7–41.5%).

N. americanus. Neither monepantel nor albendazole had an effect on the viability of larvae or adult *N. americanus* (Table 1). Levamisole was found the most efficacious drug at both stages, with calculated IC_{50}s of 0.5 µg/ml (r = 0.97) against larvae and 13.4 µg/ml (r = 0.99) against adults. Both stages were found to be sensitive to pyrantel pamoate (IC_{50}s = 2.0 µg/ml, r = 0.94 and 7.6 µg/ml, r = 0.98, respectively).

T. muris. A reduction in viability of *T. muris* L3 was observed following exposure to monepantel (IC_{50} = 78.7 µg/ml, r = 1.0), whereas adult worms were not affected (IC_{50}>200 µg/ml). Albendazole showed no activity against *T. muris* L3 or adults *in vitro* (IC_{50}>200 µg/ml). The highest activity on *T. muris* L3 and adults *in vitro* was observed with levamisole (IC_{50}s = 33.1 µg/ml, r = 1.0, and 16.5 µg/ml, r = 1.0, respectively) followed by pyrantel pamoate (IC_{50}s = 95.5 µg/ml, r = 0.99 and 34.1 µg/ml, r = 0.99, respectively) (Table 1).

In vivo Findings

A. ceylanicum. The worm expulsion rates and worm burden reductions determined for monepantel, albendazole, levamisole, and pyrantel pamoate administered to *A. ceylanicum*-infected hamsters at single oral doses are shown in Table 3. Treatment with monepantel at 10 mg/kg resulted in complete elimination of the worms. At a dose of 5 mg/kg a worm expulsion rate of 42.7% and a worm burden reduction of 56.8% were observed. The worm burden reductions in monepantel-treated hamsters were statistically significant (P = 0.046). Treatment with albendazole at 5 and 2.5 mg/kg cured all *A. ceylanicum*-infected hamsters. At a dose of 1.25 mg/kg the worm expulsion rate and worm burden reduction were 70.5% and 87.8%, respectively. There was a highly significant difference between the worm burden of albendazole-treated hamsters (1.25–5 mg/kg) and control hamsters (P<0.001). Moderate activities were observed for levamisole (worm burden reduction of 60.2%, P = 0.057) and pyrantel pamoate (worm burden reduction of 87.2%, P = 0.057 and worm expulsion rate of 63.4%) administered at 10 mg/kg.

N. americanus. As presented in Table 4, no dose-response relationship could be observed following administration of monepantel to *N. americanus*-infected hamsters. Both worm expulsion rate and worm burden reduction were 58.3% after treatment with a single dose of 10 mg/kg monepantel, whereas a worm expulsion rate and worm burden reduction of 38.6% and 0%, respectively were obtained following treatment with 20 mg/

Table 3. Dose response relationships of monepantel, albendazole, levamisole, and pyrantel pamoate on *A. ceylanicum in vivo*.

Group	Dose (mg/kg)	Mean number of worms (SD)	Mean number of expelled worms (SD)	Worm expulsion rate (%)	Worm burden reduction (%)	*P*-value
Control 1	–	29.5 (21.2)	0	0	–	–
Control 2	–	24.5 (8.8)	0	0	–	–
Control 3	–	29.7 (4.2)	0.3 (0.6)	1.1	–	–
Control 4	–	27.0 (4.0)	0.3 (0.6)	1.2	–	–
Monepantel	5[1]	22.3 (10.1)	9.5 (4.4)	42.7	55.8	0.046*
	10[1]	16.0 (9.7)	16.0 (9.7)	100	100	
Albendazole	1.25[4]	11.0 (6.9)	7.8 (3.8)	70.5	87.8	<0.001*
	2.5[3]	6.3 (7.1)	6.3 (7.1)	100	100	
	5[2]	6.3 (7.1)	6.3 (7.1)	100	100	
Levamisole-HCl	10[2]	17.5 (7.2)	7.8 (2.5)	44.3	60.2	0.057[§]
Pyrantel pamoate	10[3]	10.3 (9.3)	6.5 (6.2)	63.4	87.2	0.057[§]

SD = standard deviation. The numbers in superscript refer to the corresponding control group.
*Kruskal Wallis test comparing the median of the worm burdens of control and treated hamsters (all doses versus control),
[§]Mann-Whitney U-test comparing the median of the worm burdens of control and treated hamsters (one dose versus control).
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kg. The worm burden reductions in monepantel-treated hamsters were not statistically significant ($P = 0.830$). In comparison, albendazole administered at 5 mg/kg achieved a worm expulsion rate of 69.6% and a worm burden reduction of 70.8%. A complete elimination of worms was observed at 10 mg/kg albendazole ($P = 0.028$).

T. muris. The worm expulsion rates and worm burden reductions determined for monepantel, albendazole, levamisole, and pyrantel pamoate administered to *T. muris*-infected mice at single oral doses are shown in Table 5. A single dose of 200 mg/kg levamisole resulted in a worm expulsion rate of 90.5% and a worm burden reduction of 95.9% ($P = 0.036$). Albendazole (600 mg/kg) achieved a worm expulsion rate of 49.4% and a worm burden reduction of 20.2%. No effect was observed with pyrantel pamoate (300 mg/kg) and monepantel (600 mg/kg) (worm expulsion rate and worm burden reduction <10%).

S. ratti. *S. ratti*-infected rats treated with 32 mg/kg monepantel showed a worm burden reduction of 0% compared to the untreated control group ($P = 0.77$).

A. suum. Monepantel displayed negligible effect on the worm burden in the mice's lungs and liver, 7 days after treatment with 600 mg/kg (worm burden reduction = 3.3%, $P = 0.886$, Table 6), compared to the control animals. Albendazole administered at the same dose achieved a worm burden reduction of 76.6% ($P = 0.171$).

Discussion

To date, only five drugs are included in the WHO model list of essential medicines to treat infections with human STH. Most of these anthelmintics were discovered before the 1980s. Though there is no evidence yet for emerging resistance to any of these drugs in human helminth populations, there are worrying signs that anthelminthic efficacy may be declining [34,35]. In addition, the increased frequency of reported low cure rates, in particular against *T. trichiura* and hookworm infections, highlight the need to find alternative drugs [10].

A. ceylanicum, *N. americanus*, *T. muris*, *S. ratti*, and *A. suum* are five well-established laboratory parasite-rodent models of relevance to human STH. The aim of the present study was to determine their sensitivities to monepantel, a broad spectrum and safe drug used for livestock which recently entered the market for veterinary use. It is one of the few available drug candidates eligible for rapid transitioning into development for human STH infections [36].

Monepantel activates signaling *via* nematode-specific DEG-3 subtype nicotinic acetylcholine receptors (nAChRs), causing a hypercontraction of the body wall muscles leading to paralysis and hence, death of the worm [13]. ACR-23 protein, a member of the DEG-3 group in *Caenorhabtitis elegans*, and its homolog MPTL-1 in *Haemonchus contortus*, another model for gastrointestinal nematodes, are major targets of monepantel. The absence of MPTL-1,

Table 4. Effects of monepantel and albendazole on *N. americanus in vivo*.

Group	Dose (mg/kg)	Mean number of worms (SD)	Mean number of expelled worms (SD)	Worm expulsion rate (%)	Worm burden reduction (%)	*P*-value*
Control	–	8.0 (7.5)	0 (0)	0	–	–
Monepantel	20	19.0 (12.0)	7.3 (4.5)	38.6	0	0.330
	10	8.0 (4.4)	4.7 (3.1)	58.3	58.3	
Albendazole	10	6.7 (2.5)	6.7 (2.5)	100	100	0.028
	5	7.7 (3.1)	5.3 (3.8)	69.6	70.8	

SD = standard deviation.
*Kruskal Wallis test comparing the median of the worm burdens of control and treated hamsters (all doses versus control).
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In conclusion, to our knowledge, we have for the first time analyzed the efficacy of monepantel in animal models corresponding to human intestinal helminthiases. A recently developed target product profile suggested that a drug development candidate for the treatment of infections with STH should ideally target all stages (at least adult and ova) and species of the major geohelminths such as *Ascaris*, *Trichuris*, both hookworm species and *Enterobius* [36]. Hence, based on our results, established in nematode-rodent models, monepantel does not fulfill the required minimal product characteristics for a new intestinal anthelmintic.

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Author Contributions

Conceived and designed the experiments: LT AS JK. Performed the experiments: LT AS. Analyzed the data: LT AS JK. Wrote the paper: LT JK.

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Chapter 4

In vitro and *in vivo* efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*

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ABSTRACT

Worldwide, 3 billion people are at risk of hookworm infection, particularly in resource-poor countries. While control of soil-transmitted helminthiases relies mostly on chemotherapy, only few drugs are available and concern about potential emergence of drug resistance is rising. In the present study, tribendimidine, a derivative of amidantel, and its metabolites deacylated amidantel (dADT) and acetylated deacylated amidantel (AdADT) were tested in vitro and in vivo against *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*, two hookworm rodent models, alone or in combination with standard drugs.

Tribendimidine achieved IC_{50}s ≤ 5 μg/ml against both *H. bakeri* third-stage larvae and adults in vitro and a single 2 mg/kg oral dose resulted in complete worm elimination in vivo. Comparable results were obtained with dADT, whereas AdADT displayed no effect in vitro and gave a moderate worm burden reduction of 42.9% in *H. bakeri*-infected mice. Tribendimidine combined with albendazole, levamisole or ivermectin revealed antagonistic interactions against *H. bakeri* in vitro and no significant killing effect in vivo. Tribendimidine and dADT exerted high efficacies against *A. ceylanicum* third-stage larvae (IC_{50}s < 0.5 μg/ml) whereas adults were moderately affected in vitro (IC_{50}s > 88 μg/ml). In vivo at single oral doses of 10 mg/kg, dADT showed a slightly higher efficacy than tribendimidine, achieving worm burden reductions of 87.4% and 74.8%, respectively. At the same dose, AdADT reduced the worm burden by 57.9%. Synergistic interactions were observed with tribendimidine–levamisole combinations against *A. ceylanicum* in vitro (combination index at IC_{50} = 0.5), and in vivo (combination index at ED_{90} = 0.19). In conclusion, tribendimidine and dADT show potent anti-hookworm properties. The potential of the promising tribendimidine–levamisole combination should be investigated in greater detail.

1. Introduction

Hookworms are intestinal parasitic nematodes of great public health significance. *Necator americanus* and *Ancylostoma duodenale* are the two most important species infecting humans. They occur mainly in Sub-Saharan Africa, South-East Asia, China and in the Pacific Islands. More than 3 billion people are at risk of acquiring hookworm infections (de Silva et al., 2003). Hookworm infection is one of the so-called neglected diseases, affecting particularly people living in resource-poor settings (Hotez et al., 2007). Disease morbidities depend directly on the infection intensity, ranging from asymptomatic cases to anaemia, nutrient loss and profound physical and mental deficits (Hotez et al., 2004; Roche and Layrisse, 1966; Stoltzfus et al., 1997). The current mainstay to control hookworm infections is chemotherapy administered in the framework of periodic mass drug administration campaigns, targeting high-risk groups (Harhay et al., 2010; Hotez, 2008). Only 4 drugs are currently recommended by the World Health Organisation (WHO) against soil-transmitted helminthiases: albendazole, mebendazole, levamisole and pyrantel pamoate (Keiser and Utzinger, 2010), all of them in use for decades (Holden-Dye and Walker, 2007; Hotez et al., 2006; Utzinger and Keiser, 2004).

Since resistance to these drugs has spread widely in livestock (Kaplan, 2004), there is rising concern about potential emergence of resistance among human nematode populations (Albonico et al., 2003; De Clercq et al., 1997; Geerts and Gryseels, 2001; Reynoldson et al., 1997). New drugs are therefore urgently needed, and combinations of existing drugs have to be thoroughly explored to prevent emergence of drug resistance (Barnes et al., 1995; Nyunt and Plowe, 2007; van den Enden, 2009).

Tribendimidine, a successor of a drug developed by Bayer, amidantel (Bay d 8815), was discovered in the 1980s by the Institute of Parasitic Diseases in Shanghai, China (Xiao et al., 2005). Tested against a range of helminths in the laboratory and in humans, a

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promising broad-spectrum activity, rapid onset of action and good tolerability were documented (Keiser et al., 2007, 2008; Steinmann et al., 2008; Xiao et al., 2005). In humans, tribendimidine showed excellent efficacy against hookworms (*N. americanus* and *A. duodenale*), performing even better than albendazole, the current drug of choice (Xiao et al., 2005). The Chinese authorities approved tribendimidine for human use in 2004 (Sun, 1999; Utzinger and Keiser, 2004).

Like levamisole and pyrantel, tribendimidine belongs to the L-subtype nAChR (nicotinic acetylcholine receptor) agonists family (Hu et al., 2009). When administered in vivo, tribendimidine is rapidly metabolised into deacylated amidantel (dADT), which undergoes acetylation, resulting in acetylated deacylated amidantel (AdADT) (Xue et al., 2005, 2010). Tribendimidine and dADT exhibited high efficacies against *N. americanus* in a hamster model, whereas AdADT showed only moderate effects, suggesting that dADT is the key metabolite for nematocidal activity (Xue et al., 2005, 2010). Tribendimidine was also found to be very potent against *Ancylostoma caninum* (Xiao et al., 2005).

The aim of the present investigation was to study the in vitro and in vivo activities of tribendimidine and its metabolites dADT and AdADT against *Heligmosomoides bakeri* (formerly known as *Heligmosomoides polygyrus*) and *Ancylostoma ceylanicum*, two hookworm laboratory animal models. In addition, we tested the combination dose effects of tribendimidine with albendazole, levamisole or ivermectin and evaluated them using combination indices.

2. Materials and methods

2.1. Drugs

Tribendimidine (*N,N′*-bis(4-(1-dimethylamino)ethylidene-aminophenyl)-1,4-phenylene dimethylidyneamine), MW: 452.594 g/mol, and the metabolites deacylated amidantel (dADT), MW: 173.214 g/mol, and acetylated deacylated amidantel (AdADT), MW: 215.251 g/mol, were donated by Shandong Xinhua Pharmaceutical Company (China) and stored at 4 °C.

Albendazole, levamisole and ivermectin were purchased from Sigma–Aldrich (Buchs, Switzerland). For the in vitro studies, 10 mg/ml (*H. bakeri*) or 5 mg/ml (*A. ceylanicum*) stock solutions were prepared for all drugs in 100% DMSO (Fluka, Buchs, Switzerland) and stored at 4 °C. For the in vivo studies, drugs were suspended in 7% (v/v) Tween 80% and 3% (v/v) ethanol shortly before treatment.

2.2. Animals

Three week-old male Syrian Golden hamsters were purchased from Charles River (Sulzfeld, Germany). Four week-old female NMRI mice were purchased from Harlan (Horst, the Netherlands) or Charles River (Sulzfeld, Germany). All animals were kept in macrolon cages under environmentally controlled conditions (temperature: 25 °C; humidity: 70%; light/dark cycle: 12/12 h) and had free access to water and rodent food (Rodent Blox from Eberle NAFAG, Gossau, Switzerland). They were allowed to acclimatize in the animal facility of the Swiss Tropical and Public Health Institute (Swiss TPH) for one week before infection. The current study was approved by the local veterinary agency based on Swiss cantonal and national regulations (permission no. 2070).

2.3. Parasites and infections

A. ceylanicum third-stage larvae (L3) were kindly provided by Prof. Jerzy Behnke (University of Nottingham). The *A. ceylanicum* life cycle has been maintained at the Swiss TPH as described earlier (Garside and Behnke, 1989; Ray and Bhopale, 1972; Tritten et al., 2011). Briefly, male Syrian Golden hamsters were immunosuppressed with hydrocortisone (3 mg/kg, twice weekly) or dexamethasone (1 mg/l continuously in the drinking water) and infected orally at 4 weeks, with 150 L3. For in vivo studies, hamsters were not immunosuppressed and infected orally with 300 L3. Details on the life cycle of *H. bakeri*, maintained at the institute since 2009 have recently been described (Nwosu et al., 2011). Briefly, 4-week old NMRI mice were infected orally with 150 *H. bakeri* L3 (for in vitro studies), or only with 80 L3 (for in vivo work).

2.4. In vitro studies

The motility assay was used to evaluate drug susceptibilities of L3 and adult worms of *A. ceylanicum* and *H. bakeri* (Kopp et al., 2008a; Stepek et al., 2005). Drug effects on egg development were assessed observing embryonation and hatching.

2.4.1. H. bakeri

In vitro assays with *H. bakeri* L3 were performed following procedures presented elsewhere (Nwosu et al., 2011). Briefly, in 24-well plates (Costar), 20 µl of a larval solution containing 30 freshly isolated *H. bakeri* L3 were added to 470 µl RPMI 1640 supplemented with 25 mM HEPES, 500 U/ml penicillin, 500 µg/ml streptomycin and 0.6 µg/ml amphotericin B. Ten µl drug solutions with appropriate drug concentrations were added, to reach final concentrations ranging from 100 to 0.1 µg/ml. The assays were incubated for 72 h at room-temperature. For drug combination studies, the assay was performed in a total volume of 1 ml, with final drug concentrations of 1 or 0.1 µg/ml. Larval motility was assessed at 72 h microscopically (inverted microscope, Carl Zeiss, Germany, magnification 20×) following addition of hot water (~80 °C) and exposure to microscope light, and the percentage survival determined. Assays were conducted three times in duplicate.

Similarly, 4 adult worms (males and females, gained upon dissection of infected mice guts) were incubated in 48-well plates in 500 µl phenol-red free RPMI 1640 supplemented with HEPES and antibiotics, at 37 °C, 5% CO_2. The motility was assessed microscopically (magnification 20×) 72 h following drug exposure (final concentrations of 100–0.1 µg/ml) and the viability determined. Assays were conducted at least twice.

The ovicidal activity of drugs was assessed following a slightly modified protocol by Fonseca-Salamanca et al. (2003). Briefly, in 48-well plates (Costar), 20 µl egg solution containing 30 freshly isolated unembryonated eggs were added to 470 µl RPMI 1640, supplemented with HEPES and antibiotics. The test drugs (10 µl) at concentrations ranging from 100 to 1 µg/ml were added. The plates were incubated at room-temperature. Twenty-four hours post-exposure, the eggs were examined microscopically (magnification 80–160×) for embryonation, and 40 h post-incubation hatching was assessed. In all assays (L3, adults and eggs) control wells contained the highest DMSO concentration used in the tests (1%, v/v).

2.4.2. A. ceylanicum

Experiments were conducted as described recently (Tritten et al., 2012). Briefly, 30 L3 per well (96-well plates) were incubated for 72 h at room-temperature in the presence of 200 µl HBSS medium containing 25 µg/ml amphotericin B (Sigma–Aldrich) and 1% (v/v) penicillin–streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin, Sigma–Aldrich) and drug dilutions (ranging from 100 to 0.01 µg/ml). The larval motility was investigated using microscopy (magnification 20×) following addition of hot water (~80 °C) and exposure to microscope light. For combination chemotherapy experiments, 50 µl of the individual drug solutions were added to each well and serially diluted, in order to

have final concentrations ranging from 4× IC_{50} to 0.25× IC_{50} for each drug.

Drug susceptibilities of adult worms (obtained from hamsters' guts upon dissection) were tested in 48-well plates (Costar) with 3–4 worms per well containing 1 ml medium and drugs at 37°C, 5% CO_2 for 72 h. The motility was determined microscopically (magnification 20×) using a viability scale ranging from 2 (worms healthy, fit) and 0 (death).

The ovicidal activity was evaluated using 50 freshly isolated eggs per well which were incubated in 1 ml deionized water containing 10 μg/ml of the test drug. After 24 h, 20 eggs per well were examined microscopically (magnification 80–160×) for embryonation, and after 48 h, 20 eggs were examined for hatching. In all assays (L3, adults and eggs) control wells contained the highest DMSO concentration used in the tests (max. 2%, v/v).

2.5. In vivo studies

2.5.1. H. bakeri

Six groups ($n = 4$) of mice were treated orally with 0.5, 1 or 2 mg/kg single doses of tribendimidine, or 1 or 2 mg/kg single doses of dADT or AdADT, 21–28 days post-infection (p.i.). Untreated mice (2× $n = 4$) served as controls. To assess the effect of drug combinations, 0.5 mg/kg tribendimidine was combined with 10 mg/kg albendazole, 1.25 mg/kg levamisole or 0.125 mg/kg ivermectin, each dose being the estimated ED_{50} value for the single drug.

Worms remaining in the gut on day 8 post-treatment were counted after killing the mice with the CO_2 method. Worm burden reductions were calculated as following: $[[(a - b)/a] \times 100]$, where a = average worm count in the control group upon dissection and b = average worm count in a treated group upon dissection (Bartley et al., 2008; Xue et al., 2005).

2.5.2. A. ceylanicum

The experimental procedure was carried out as described recently (Tritten et al., 2011). Briefly, the fecal egg burden was established on average from days 21 and 22 p.i. On the basis of the fecal egg burden, hamsters were assigned to equally balanced treatment groups (single oral doses of 10 or 5 mg/kg tribendimidine, 10 mg/kg dADT or AdADT) or control groups (4 animals each). For combination chemotherapy studies, we first calculated approximative ED_{50} values of both drugs (tribendimidine 10 mg/kg and levamisole 10 mg/kg). Both drugs were then combined at a constant ED_{50} ratio (10 mg/kg:10 mg/kg ($1ED_{50}$:$1ED_{50}$); 5 mg/kg:5 mg/kg ($0.5ED_{50}$:$0.5ED_{50}$) and 2.5 mg/kg:2.5 mg/kg ($0.25ED_{50}$:$0.25ED_{50}$)).

Hamsters were treated on day 23 p.i. The complete stools over a period of 48 h following treatment were collected from each hamster and soaked in 0.9% NaCl. The entire sample was then carefully examined under a binocular (magnification 10–40×) and all worms counted. Worms remaining in the gut 7 days post-treatment were collected and counted after killing the hamsters with the CO_2 method. Worm burden reductions (see *H. bakeri*) and worm expulsion rates were calculated. The worm expulsion rates were calculated as follows: $[(c/d) \times 100]$, where c is the total number of expelled worms in a treated group and d, the total worm count (expelled worms as well as worms present in gut counted following dissection) of the same group.

2.6. Statistical analysis

IC_{50}s were calculated based on the median effect principle using CompuSyn (version 1.0) (Tritten et al., 2011). The r value is the linear correlation coefficient of the median-effect plot, indicating the goodness of fit, hence how accurate the IC_{50} is (Chou, 1976). Variance in ovicidal activities was analyzed using the Fisher's exact test (StatsDirect, version 2.4.5; StatsDirect Ltd., Cheshire, UK). Worm burden reductions and worm expulsion rates were calculated in Microsoft® Excel. The statistical significance of the worm burden reduction was evaluated with the Kruskal–Wallis test (multiple doses against control), or the Mann–Whitney U test (single dose against control), using StatsDirect. Combination indices (CI) at IC_{50} and IC_{90} were calculated for in vitro combination studies using CompuSyn. For in vivo studies, either the CI value (*A. ceylanicum*) or the statistical significance (Mann–Whitney test, *H. bakeri*) was determined.

3. Results

3.1. In vitro studies against H. bakeri

3.1.1. Single drug assays against third-stage larvae and adults

Tribendimidine was highly active against both *H. bakeri* L3 (IC_{50}: 0.32 μg/ml, $r = 0.85$) and adult worms in vitro (IC_{50}: 5.09 μg/ml, $r = 0.95$) (Table 1). dADT showed a slightly lower efficacy than tribendimidine against L3 (IC_{50}: 0.62 μg/ml, $r = 0.94$) but higher activity against adults (IC_{50}: 3.52 μg/ml, $r = 0.93$). Only a very low activity was observed with AdADT (both IC_{50}s >100 μg/ml, $r = 0.91$ and 0.84, respectively).

3.1.2. Drug combination studies against third-stage larvae

Third-stage larvae were exposed simultaneously to tribendimidine and albendazole, levamisole or ivermectin (Table 1) using a fixed dose ratio based on the IC_{50}s and 2-fold dilutions up and down. The calculated CIs for the tribendimidine–albendazole, tribendimidine–levamisole and tribendimidine–ivermectin combinations were >100, 1.20, and 9.76, respectively. Hence, all combinations tested showed antagonistic interactions (CI > 1) at the fixed dose ratio.

3.1.3. Ovicidal activity

In the presence of tribendimidine, 99.6% of the eggs were fully embryonated after 24 h, while hatching was moderately reduced by 13.4% ($P = 0.116$), compared to the controls (Table 2). dADT achieved similar low reductions in embryonation (2%) and hatching (12.8%) ($P = 0.106$). AdADT had no effect on embryonation and a very low effect on hatching.

3.2. In vitro studies against A. ceylanicum

3.2.1. Single drug assays against third-stage larvae and adults

Tribendimidine strongly affected *A. ceylanicum* L3 (IC_{50}: 0.32 μg/ml, $r = 0.89$), while adult worms' viability was only moderately reduced (IC_{50}: 88.44 μg/ml, $r = 0.83$) (Table 1). A similar effect was observed for dADT, which exhibited excellent activity against L3 but not against the adults (IC_{50}s: 0.14 μg/ml, $r = 0.95$ and >100 μg/ml, $r = 0.50$, respectively). AdADT showed a moderate effect against both stages (IC_{50}s: 70.0 μg/ml, $r = 0.66$ and 21.93 μg/ml, $r = 0.88$, respectively).

3.2.2. Drug combination studies against third-stage larvae

Third-stage larvae were incubated with tribendimidine combined with albendazole, levamisole or ivermectin (Table 1) based on their respective IC_{50} values and 2-fold dilutions were carried up and down. The combination index at the IC_{50} value indicated synergism for the tribencimidine–levamisole combination (CI: 0.50), whereas the combinations of tribendimidine with albendazole and ivermectin were antagonistic (CIs: 2.53 and 4.21, respectively).

3.2.3. Ovicidal activity

Tribendimidine exerted minor, not significant, reductions of egg embryonation (7.3%) and hatching (12.3%) (Table 2). dADT and AdADT did not affect either egg embryonation or hatching (all $P > 0.05$).

Table 1
Activity of tribendimidine, dADT and AdADT and tribendimidine combinations against *H. bakeri* and *A. ceylanicum* in vitro 72 h post-incubation.

Drug	*H. bakeri* IC_{50}s (*r*)		*A. ceylanicum* IC_{50}s (*r*)	
	L3	Adults	L3	Adults
Tribendimidine	0.32 (0.85)	5.09 (0.95)	0.32 (0.89)	88.44 (0.83)
dADT	0.62 (0.94)	3.52 (0.93)	0.14 (0.95)	>100 (0.50)
AdADT	>100 (0.91)	>100 (0.84)	70.0 (0.66)	21.93 (0.88)
Albendazole	9.05[a]	>100 (n.d.)	32.4 (0.87)[b]	>100 (0.77)[b]
Levamisole	0.02[a]	0.56 (0.70)	1.6 (0.95)[b]	>100 (0.79)[b]
Ivermectin	6.92[a]	>100 (n.d.)	1.15 (0.88)	1.22 (0.89)
CI at IC_{50} tribendimidine–albendazole	>1000	n.d.	2.53	n.d.
CI at IC_{50} tribendimidine–levamisole	1.20	n.d.	0.50	n.d.
CI at IC_{50} tribendimidine–ivermectin	9.76	n.d.	4.21	n.d.

IC_{50}: Fifty-percent inhibitory concentrations. For comparison, IC_{50} values of albendazole, levamisole and ivermectin are shown. CI at IC_{50} = combination index at IC_{50}; CI < 1: synergism; CI = 1: additive effect; CI > 1: antagonism; n.d. = not determined (no fitting possible); *r* = linear correlation coefficient of the median-effect plot, indicating the goodness of fit ($r \geq 0.85$ indicates a satisfactory fit).
[a] Nwosu et al. (2011).
[b] Tritten et al. (2011).

Table 2
Ovicidal activity (embryonation and hatching) of tribendimidine, dADT and AdADT at a concentration of 10 μg/ml.

Group	*H. bakeri*		*A. ceylanicum*	
	% embryonation (SD)	% hatching (SD)	% embryonation (SD)	% hatching (SD)
Control	100 (1.3)	100 (2.9)	100 (3.5)	100 (9.8)
Tribendimidine	99.6 (2.1)	86.6 (10.6)	92.7 (10.0)	87.7 (22.4)
dADT	98.0 (3.6)	87.2 (0.8)	100 (2.7)	100 (4.4)
AdADT	100 (0)	98.4 (5.6)	100 (5.8)	100 (3.0)

SD, standard deviation.

3.3. In vivo studies against H. bakeri

3.3.1. Monotherapy

Tribendimidine achieved statistically significant worm burden reductions of 53.9% at a treatment dose of 0.5 mg/kg, 68.6% at 1 mg/kg, and complete elimination of worms at 2 mg/kg, compared to the controls (Table 3). Comparably, 1 mg/kg and 2 mg/kg dADT resulted in significant worm burden reductions of 76.2% and 97.1%, respectively. A moderate worm burden reduction of 42.9% was observed following AdADT at 2 mg/kg ($P = 0.343$).

3.3.2. Combination chemotherapy

Results obtained in combination chemotherapy experiments are shown in Table 3. Tribendimidine (0.5 and 1 mg/kg) combined with albendazole (10 mg/kg) revealed worm burden reductions of 67.1% and 36.1%, which was comparable to the effect produced by tribendimidine alone, or significantly weaker ($P > 0.05$). Similarly, no increased dose response effect was observed for combinations of levamisole (1.25 mg/kg) and tribendimidine (68.7% and 62.1%). The highest activity of 85.8% was observed with ivermectin (0.125 mg/kg) combined with 0.5 mg/kg tribendimidine ($P = 0.229$). Doubling of the tribendimidine dose (1 mg/kg), in combination with ivermectin resulted in a lower worm burden reduction of 41.1% ($P > 0.05$). Further dose effect studies with the three combinations were not done since these data showed that neither additive nor synergistic effects were present.

3.4. In vivo studies against A. ceylanicum

3.4.1. Monotherapy

The worm expulsion rate and the worm burden reduction achieved administering a 10 mg/kg dose of tribendimidine to *A. ceylanicum*-infected hamsters were 63.3% and 74.8%, respectively ($P = 0.436$) (Table 4). Following dADT treatment at the same dose, a worm expulsion rate of 88.5% and a significant worm burden reduction of 87.4% were measured, whereas AdADT had low to moderate effects against *A. ceylanicum* in vivo (worm expulsion rate: 6.3%, worm burden reduction: 57.9% ($P > 0.999$)).

3.4.2. Combination chemotherapy

Based on our in vitro findings, only the combination of tribendimidine and levamisole was further studied in vivo, using a fixed dose ratio based on the approximate ED_{50} doses of both drugs and diluted twice (10 mg/kg:10 mg/kg, 5 mg/kg:5 mg/kg, 2.5 mg/kg:2.5 mg/kg). These combination treatments achieved worm burden reductions of 92.7%, 70.1% and 3.6%, respectively (Table 4). The calculated CI revealed an additive effect tending towards synergism, with a CI of 1.02 at the ED_{50} and a CI of 0.19 at the ED_{90}. Fig. 1 illustrates this finding using an isobologram.

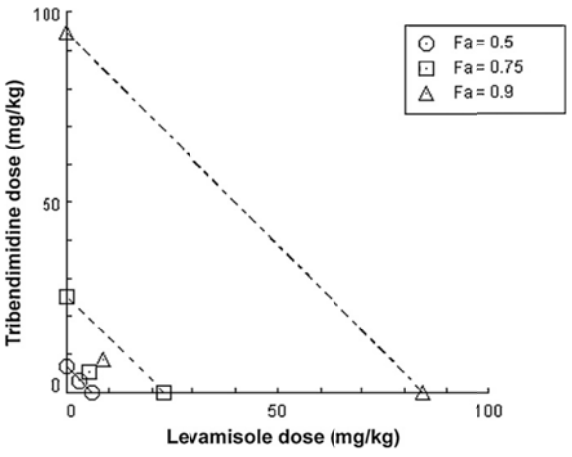

Fig. 1. Isobologram for the combined effect of tribendimidine and levamisole. Fa = effect level.

Table 3
Dose-response relationships of tribendimidine, dADT and AdADT administered to *H. bakeri*-infected mice.

Group	Dose (mg/kg)	Mean number of worms after 7 days (SD)	Worm burden reduction (%)	*P*-value
Control 1	–	26.25 (9.9)	–	–
Control 2	–	109.5 (74.1)	–	–
Control 3	–	32.75 (14.7)	–	–
Tribendimidine	0.5[2]	50.5 (36.5)	53.9	0.006
	1[1]	8.25 (3.6)	68.6	
	2[1]	0.0 (0.0)	100	
dADT	1[1]	6.25 (8.8)	76.2	0.010
	2[1]	0.75 (1.0)	97.1	
AdADT	2[1]	15.0 (12.8)	42.9	0.343
Albendazole[a]	10	–	52.6	NA
Levamisole[a]	1.25	–	61.5	NA
Ivermectin[a]	0.125	–	53.7	NA
Combination tribendimidine–albendazole	0.5 + 10[2]	36.0 (32.2)	67.1	0.486[b]
Combination tribendimidine–albendazole	1 + 10[3]	70.0 (40.3)	36.1	0.057[c]
Combination tribendimidine–levamisole	0.5 + 1.25[2]	34.25 (25.7)	68.7	0.686[b]
Combination tribendimidine–levamisole	1 + 1.25[3]	41.5 (65.2)	62.1	0.543[c]
Combination tribendimidine–ivermectin	0.5 + 0.125[2]	15.5 (17.1)	85.8	0.229[b]
Combination tribendimidine–ivermectin	1 + 0.125[3]	64.5 (55.1)	41.1	>0.05[c]

SD, standard deviation; NA, not assessed. The numbers in superscript refer to the corresponding control group.
[a] For comparison, worm burden reductions obtained with the subcurative doses of the partner drugs (albendazole, levamisole, and ivermectin) are also listed (Nwosu et al., 2011).
[b] Significance calculated versus tribendimidine 0.5 mg/kg.
[c] Significance calculated versus tribendimidine 1 mg/kg.

4. Discussion

Since chemotherapy is the current mainstay for treatment of soil-transmitted helminthiases, there is a pressing need for increased efforts on drug research. Since the drug discovery and development process is extremely long and expensive (Dickson and Gagnon, 2004), combining existing marketed drugs is a powerful strategy for avoiding the spread of drug resistance and for obtaining efficacy against a broader range of parasites (Smith, 1990a,b; Smith et al., 1999).

Tribendimidine, a Chinese amidantel derivative, has shown activity against a wide range of helminths maintained in rodents such as the nematodes *N. americanus*, *Nippostrongylus braziliensis*, *Strongyloides ratti*, and *Toxocara canis* and the trematodes *Clonorchis sinensis* and *Opisthorchis viverrini* (Keiser et al., 2007, 2008; Xiao et al., 2005). In the present work, we aimed to generate comprehensive efficacy data on tribendimidine and its metabolites dADT and AdADT against *A. ceylanicum* and *H. bakeri*, two laboratory hookworm models, in vitro and in vivo. In addition, drug combinations of tribendimidine plus standard drugs were tested.

Tribendimidine is unstable in aqueous solution, and within minutes, dADT is the only active compound present (Yuan et al., 2008, 2010). Hence, similar efficacies are expected for both compounds.

In vitro, tribendimidine was highly active against both studied stages of *H. bakeri* ($IC_{50}s \leq 5$ μg/ml), and a similar result was observed for dADT. For comparison, tribendimidine and dADT showed a higher activity against *H. bakeri* third-stage larvae and adults than the standard drugs albendazole and ivermectin but was less active than levamisole (Nwosu et al., 2011).

However, while tribendimidine and dADT were highly active against *A. ceylanicum* L3 the drugs were only moderately active against adult *A. ceylanicum* ($IC_{50}s \geq 88.5$ μg/ml), suggesting some stage-specificity. It has been proposed that the presence of different nAChR subunit populations varies during the different *A. caninum* life-cycle stages, resulting in altered degrees of susceptibility to drugs of the nAChR group (Kopp et al., 2008b; Kotze et al., 2009). Putting the findings obtained with tribendimidine against *A.*

Table 4
Dose-response relationships of tribendimidine, dADT and AdADT administered to *A. ceylanicum*-infected hamsters.

Group	Dose (mg/kg)	Mean number of worms (SD)	Mean number of expelled worms (SD)	Worm expulsion rate (%)	Worm burden reduction (%)	*P*-value
Control 1	–	18.0 (18.2)	0.5 (0.8)	2.8	–	–
Control 2	–	13.8 (8.0)	0	0	–	–
Tribendimidine	5[1]	17 (16.7)	3.8 (4.9)	22.1	25.7	0.436
	10[1]	12.3 (6.5)	7.8 (5.3)	63.3	74.8	
dADT	5[1]	13.8 (7.3)	7.5 (8.7)	54.5	64.9	0.023
	10[1]	19.5 (9.3)	17.3 (8.6)	88.5	87.4	
AdADT	5[1]	19.3 (11.3)	0.5 (1)	2.6	0	>0.999
	10[1]	8.0 (3.4)	0.5 (0.6)	6.3	57.9	
Levamisole[a]	10	–	–	44.3	60.2	NA
Combination tribendimidine–levamisole	10 + 10[2]	8.7 (5.7)	7.7 (6.0)	88.5	92.7	CI at IC_{50} = 1.02
	5 + 5[2]	13.3 (11.8)	9.3 (11.2)	70.0	70.1	CI at IC_{90} = 0.19
	2.5 + 2.5[2]	19.3 (15.1)	6.0 (3.3)	31.2	3.6	

SD, standard deviation; NA, not assessed. The numbers in superscript refer to the corresponding control group. *P*-value determined for worm burden reductions. CI at IC_{50} = combination index at IC_{50}, CI at IC_{90} = combination index at IC_{90}. CI < 1: synergism; CI = 1: additive effect; CI > 1: antagonism.
[a] The worm expulsion rate and the worm burden reduction obtained with 10 mg/kg levamisole are given (Tritten et al., 2011).

ceylanicum in context with findings from previous work shows that tribendimidine and dADT displayed a higher in vitro activity against L3 and adults than albendazole and levamisole. Adult worms were more affected by ivermectin compared to tribendimidine and its metabolites, while the effect of ivermectin on L3 was slightly lower.

In both models, in contrast to albendazole which strongly inhibited embryonation and hatching (Nwosu et al., 2011; Tritten et al., 2011), tribendimidine and its metabolites displayed no overt ovicidal activity at the tested drug concentrations.

In vivo, tribendimidine cleared *H. bakeri* infections in mice at a dose of 2 mg/kg and reduced the worm burden by more than 50% at 0.5 mg/kg. A comparable result was obtained with dADT, as the worm burden reduction following a 2 mg/kg treatment was of 97%. As expected from the in vitro data, AdADT had a moderate impact on the *H. bakeri* worm burden (2 mg/kg dose, 42% worm burden reduction).

Roughly 75% of *A. ceylanicum* worms were expelled following a 10 mg/kg treatment with tribendimidine, while 87% were cleared by dADT at the same dose, showing the slightly better efficacy of the latter. AdADT reduced the worm burden by approximately 6% (WER). Hence, in both models, tribendimidine and dADT displayed similar potent efficacies, both in vitro and in vivo, whereas AdADT showed only moderate activities.

It is interesting to note that in a recent study worm burden reductions observed after tribendimidine oral treatments against *N. americanus* were significantly higher than those achieved by the metabolite dADT (Xue et al., 2010), which contradicts the findings observed in the *A. ceylanicum*-model and stability issues highlighted above. The efficacy of AdADT against *N. americanus* was moderate, in line with our results (Xue et al., 2010). Albendazole, which showed a superior efficacy over tribendimidine against *A. ceylanicum* in vivo (Tritten et al., 2011), produced a less pronounced effect against *N. americanus* (Xue et al., 2005). Similarly, *H. bakeri* was less affected by albendazole (Nwosu et al., 2011). Overall, our results re-emphasize that all hookworm species show varying degrees of sensitivity to different drugs, a phenomenon well-studied for drug effects against *Ancylostoma* spp. and *N. americanus* (Behnke et al., 1993; Xue et al., 2005).

Tribendimidine combinations with albendazole, levamisole and ivermectin interacted antagonistically in vitro against *H. bakeri* L3. These findings were confirmed in our in vivo studies. Interestingly, increased tribendimidine doses resulted in a decreased treatment effect, particularly notable for albendazole-tribendimidine or ivermectin–tribendimidine combinations. On the other hand, against *A. ceylanicum*, additive to synergistic effects were observed with a combination of tribendimidine–levamisole in vitro and in vivo. This finding suggests that the two drugs might act via an at least partially different mechanism, perhaps at different nAChR subunits. Initially, it had been proposed that tribendimidine also belongs to the L-type nAChR agonists group and shares the same mode of action as levamisole. On the other hand, it was noticed that tribendimidine does not behave like a typical nAChR agonist (levamisole or pyrantel), acting more rapidly, and paralyzing the worms starting from the head (Hu et al., 2009). It might be important to note that levamisole was shown to have a broad range of immunomodulatory effects in rodents and humans. In anti-cancer chemotherapy, levamisole is thought to potentiate the combined drug 5-fluorouracil (Mitchell, 2003; Stevenson et al., 1991). Possibly, these immunomodulatory properties play a role in the additive to synergistic interaction with tribendimidine. Though not tested here we would have expected a similar nature of interaction combining dADT with levamisole, since these drugs have comparable efficacies and are structurally very close.

In conclusion, tribendimidine and dADT show potent anti-hookworm properties, when administered alone, in single oral doses. We have confirmed that the *H. bakeri* mouse model is an excellent laboratory model to study drug effects on hookworms. Since *H. bakeri* can be maintained in mice and worms have matured already 14 days post-infection this model is fast and cost-effective. Promisingly, the combination tribendimidine–levamisole revealed excellent efficacy (synergism at the ED_{90}), against *A. ceylanicum*. This combination should therefore be investigated in further detail, i.e. in drug interaction studies and against other soil-transmitted helminths.

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Chapter 5

Nitazoxanide: *In vitro* and *in vivo* drug effects against *Trichuris muris* and *Ancylostoma ceylanicum*, alone or in combination

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Nitazoxanide: *In vitro* and *in vivo* drug effects against *Trichuris muris* and *Ancylostoma ceylanicum*, alone or in combination

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ABSTRACT

Soil-transmitted helminths cause more than 1 billion human infections globally, mostly in the poorest regions of the world. Control relies essentially on a limited panel of four drugs, and drug resistance might be inescapable. Nitazoxanide, an anti-infective drug, has been shown to exert anthelmintic activity in human clinical trials. In the present work, nitazoxanide was tested alone or combined with commercialized anthelmintics on *Trichuris muris*, a whipworm mouse model, and *Ancylostoma ceylanicum*, a hookworm hamster model, *in vitro* and *in vivo*. IC_{50}s of ⩽1 and 12.87 μg/ml were achieved with nitazoxanide on *T. muris* third-stage larvae (L3) and adult worms *in vitro*, respectively. An IC_{50} of ⩽1 μg/ml was obtained exposing *A. ceylanicum* adults worms to nitazoxanide, whereas *A. ceylanicum* L3 were not affected. Using scanning electron microscopy, the tegument of adult *T. muris* appeared unchanged following nitazoxanide treatment, whereas swellings were seen on the tegument of the anterior region of half of the *A. ceylanicum* specimen analyzed. Synergism was observed *in vitro* when nitazoxanide was combined with levamisole or ivermectin on *T. muris* adult worms, and when combined with levamisole, pyrantel pamoate, or ivermectin on *A. ceylanicum* adult worms. In *T. muris*-infected mice, oral nitazoxanide achieved worm burden reductions of 56.09% and 17.37% following a single dose of 100 mg/kg and three doses of 50 mg/kg, respectively. None of the tested drug combinations displayed activity on *T. muris in vivo*. In *A. ceylanicum*-infected hamsters, no effect was observed for oral nitazoxanide alone, and none of the tested combinations reached the threshold for additive effect. In conclusion, nitazoxanide failed to demonstrate promising activity against *T. muris* and *A. ceylanicum in vivo*, regardless whether tested as monotherapy or combined with standard drugs. Reasons for the discrepancy of these findings compared to results obtained in clinical trials remain to be elucidated.

1. Introduction

Soil-transmitted helminths (STH) are parasitic intestinal nematodes affecting more than one billion people worldwide (de Silva et al., 2003; Bethony et al., 2006; Hotez et al., 2008). The diseases caused by STHs belong to the so-called neglected tropical diseases, because they receive only a small percentage (<1%) of global research funding (de Silva et al., 2003; Bethony et al., 2006; Hotez et al., 2007). Pre-school and school-aged children, pregnant women and fetuses are most vulnerable and may suffer profound morbidities, including anemia, growth impairment and mental retardation (Bethony et al., 2006; Hotez et al., 2007). The current global approach to control helminth infections is based on chemotherapy, mainly administered in the frame of school deworming campaigns (Bethony et al., 2005; Mascarini-Serra, 2011). The arsenal of available drugs for the treatment of STH infections is limited: albendazole, mebendazole, levamisole, and pyrantel pamoate are recommended by the World Health Organization (WHO, 2011). Almost all human anthelmintics were originally developed for veterinary needs and have not been optimized for human use (Geary et al. 2010). This is reflected in low cure rates, in particular when single oral doses are administered against *Trichuris trichiura* infections (Keiser and Utzinger, 2008, 2010).

In addition, anthelmintic resistance is widespread among nematodes of livestock as a consequence of frequent drug administration of the same class of compounds over long periods (Geerts et al., 1997; Wolstenholme et al., 2004). Though there is no conclusive evidence of anthelmintic resistance in humans, there is an urgent need to develop backup drugs. However, despite the large global burden of human STH (Bethony et al., 2006), few efforts have been made to discover and develop novel nematocidal drug candidates (Keiser and Utzinger, 2010; Olliaro et al., 2011). In addition to the discovery and development of novel antiparasitic drugs, combination chemotherapy is a powerful strategy to slow emergence of drug resistance (Barnes et al., 1995; Nyunt and Plowe,

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2007). Therefore, combinations with both new and marketed drugs have to be explored.

Nitazoxanide, first described in the 1980s, is a broad-spectrum thiazolide compound with anthelmintic, antiprotozoal and antiviral properties (Rossignol and Maisonneuve, 1984; Rossignol and Stachulski, 1999; Hemphill et al., 2006). More recently, it has been demonstrated that nitazoxanide exhibits also activity when administered in multiple doses against a number of geohelminths, including *Ascaris lumbricoides* and *T. trichiura* (Fox and Saravolatz, 2005; Anderson and Curran, 2007; van den Enden, 2009). Nitazoxanide was therefore listed as a potential drug development candidate eligible for rapid transitioning into development for human STH infections (Olliaro et al., 2011).

Hence, it was our aim to further characterize the potential of nitazoxanide as nematocidal drug using the parasite-rodent models *Trichuris muris* and the hookworm *Ancylostoma ceylanicum*. *In vitro*, the worms' viability of larvae and adult worms was determined following incubation with nitazoxanide using the motility assay (Stepek et al., 2006; Kopp et al., 2008; Silbereisen et al., 2011). Scanning-electron microscopy pictures are presented to underscore our results. *In vivo*, *T. muris*-infected mice and *A. ceylanicum*-infected hamsters were treated with single or multiple doses of nitazoxanide. In addition, the drug was combined *in vitro* with each standard drug (albendazole, mebendazole, levamisole, pyrantel pamoate) as well as ivermectin and the interaction of each combination was assessed. Synergistic combinations were tested *in vivo*.

2. Materials and methods

2.1. Drugs

Nitazoxanide was kindly provided from Laboratoria Wolfs (Zwijndrecht, Belgium) and for *in vitro* assays a drug stock (5–10 mg/ml) was prepared in 100% DMSO (Sigma–Aldrich, Buchs, Switzerland) and stored at 4 °C. For *in vivo* assays, a nitazoxanide suspension in 10% [Tween 80, 80% EtOH (70:30 v/v, Fluka)] and 90% dH_2O was prepared before treatment. Albendazole, mebendazole, pyrantel pamoate and ivermectin were purchased from Sigma–Aldrich, and levamisole hydrochloride, from Fluka (Buchs, Switzerland), and prepared in the same way.

2.2. Animals and parasites

Three to five week-old female C57BL/10 mice and 3 week-old male Syrian golden hamsters were purchased from Charles River (Blackthorn, UK and Sulzfeld, Germany, respectively). Before infection, animals were allowed to acclimatize for 1 week, kept in groups of 10 (mice) or 5 (hamsters) in macrolon cages with free access to water and rodent food pellets (Rodent Blox from Ebetle NAFAG, Gossau, Switzerland) and with a 12 h light/dark cycle according to Swiss cantonal and national regulations (Permission No. 2070). *T. muris* was obtained from Prof. J.M. Behnke (University of Nottingham) and Prof. H. Mehlhorn (University of Düsseldorf) in 2010 and *A. ceylanicum* was provided by Prof. J.M. Behnke (University of Nottingham), in 2009.

2.3. Parasites and infections

The *T. muris* and *A. ceylanicum* rodent models have been described elsewhere (Tritten et al., 2011). Mice and hamsters were immunosuppressed with dexamethasone in the drinking water (4 mg/l and 1 mg/ml, respectively, dexamethasone-water soluble, Sigma–Aldrich) from 2 days before infection until 2 days before treatment (Campbell, 1968; Wakelin, 1970). Mice received an oral dose of 200 embryonated *T. muris* eggs (Wakelin, 1970; Stepek et al., 2006). Hamsters were infected orally with 150 *A. ceylanicum* third-stage larvae (L3) (Ray and Bhopale, 1972; Garside and Behnke, 1989). Hamsters assigned to *in vivo* studies were not immunosuppressed and were infected with 300 L3.

2.4. In vitro studies

2.4.1. T. muris

2.4.1.1. Activity of nitazoxanide on L3 and adults. In vitro studies with *T. muris* were performed as described previously (Stepek et al., 2006; Silbereisen et al., 2011). Briefly, L3 (on day 20 p.i.) and adult worms (from day 35 p.i. onwards) were isolated from the intestines of mice (Stepek et al., 2006). Three to four parasites were incubated for 72 h at 37 °C, 5% CO_2 in 100 µl (96-well) or 500 µl (48-well) RPMI medium [10.44 g RPMI 1640 (Gibco, Basel, Switzerland), 5 g albumax H (Gibco), 5.94 g HEPES (Sigma–Aldrich), 2.1 g sodium bicarbonate (Sigma–Aldrich), in 1 l dH_2O supplemented with 5% v/v amphotericin B (250 µg/ml, Sigma–Aldrich) and 1% v/v penicillin–streptomycin (10,000 U/ml penicillin + 10 mg/ml streptomycin, Sigma–Aldrich), in the presence of 12.5, 25, 50, 100 and 200 µg/ml (adults) and 50, 100 and 200 µg/ml (L3) nitazoxanide (Fonseca-Salamanca et al., 2003). Parasite motility was evaluated microscopically (Carl Zeiss, Germany, magnification 20–80×) 24, 48 and 72 h after start of incubation, using a motility scale from 3 (normal motility, full viability) to 0 (no movement, death). As controls, 3 worms incubated in the highest DMSO concentration used in the assays and in RPMI medium alone were used (2% v/v).

2.4.1.2. Combination chemotherapy studies. Nitazoxanide was combined with alberdazole, mebendazole, levamisole, pyrantel pamoate or ivermectin using a constant dose ratio (DR) based on estimated IC_{50} values: nitazoxanide = 25 µg/ml, albendazole = 200 µg/ml, mebendazole and ivermectin = 100 µg/ml, levamisole and pyrantel pamoate = 50 µg/ml (Tritten et al., 2011 and unpublished work). Three to four adult *T. muris* were incubated in four different concentrations of each drug combination ($2xIC_{50}$: $2xIC_{50}$, IC_{50} : IC_{50}, $0.5xIC_{50}$: $0.5xIC_{50}$ and $0.25xIC_{50}$: $0.25xIC_{50}$).

2.4.2. A. ceylanicum

2.4.2.1. Activity of nitazoxanide on L3 and adults. In vitro studies with *A. ceylanicum* were conducted as described recently (Tritten et al., 2011, 2012). Briefly, 30 L3 per well (96-well plates, Costar) were incubated for 72 h at room-temperature in 200 µl HBSS medium (Gibco) supplemented with 10% v/v amphotericin B (250 µg/ml, Sigma–Aldrich) and 1% v/v penicillin–streptomycin (10,000 U/ml penicillin + 10 mg/ml streptomycin, Sigma–Aldrich) containing drug concentrations ranging from 0.01 to 100 µg/ml. The larval survival (ability to move, alive; no movement, death) was investigated microscopically (magnification 20×) following addition of hot water (~80 °C) and exposure to microscope light. Drug susceptibilities of adult worms (obtained by dissection of hamster intestines) were tested in 48-well plates (Costar) with 2–3 worms per well and 1 ml HBSS medium supplemented with antibiotics and 10% v/v fetal calf serum (Connectorate AG, Switzerland) in the presence of 0.01–100 µg/ml of the drugs at 37 °C, 5% CO_2 for 72 h. The motility was determined microscopically (magnification 20×) using a viability scale ranging from 2 (normal motility, full viability) to 0 (no movement, death). Based on the motility values obtained with L3 and adults IC_{50}s were calculated. Control worms were incubated in the highest DMSO concentration used in the test (2% v/v). Assays were conducted in duplicate at least twice.

2.4.2.2. Combination chemotherapy studies. L3 and adult worms were incubated with nitazoxanide combined with albendazole,

mebendazole, levamisole, pyrantel pamoate or ivermectin, as described recently (Tritten et al., 2012). Briefly, 50 µl of the individual drug solutions were added to each well and serially diluted, in order to have final concentrations ranging from 2×IC_{50} to 0.25×IC_{50} for each drug. IC_{50} values of the partner drugs have been presented recently (Tritten et al., 2011, 2012). The IC_{50} values of mebendazole were determined in the present work. The assay was conducted at least twice in duplicate and the interaction of each combination was assessed.

2.4.2.3. Ovicidal activity of nitazoxanide. The ovicidal activity of nitazoxanide was assessed following a modified protocol based on Fonseca-Salamanca et al. (2003) and Tritten et al. (2011). Briefly, in 48-well plates (Costar), 50 eggs were incubated in dH_2O containing 10 µg/ml nitazoxanide. After 24 h exposure at room-temperature, 20 eggs per well were examined microscopically (magnification 80–160×) for embryonation, and after 48 h, for hatching. Control wells contained the same amount of DMSO used as in the drug assay (0.2% v/v). The assay was conducted in quadruplicate.

2.4.3. Scanning electron microscopy studies

The effect of nitazoxanide on the tegument of adult *T. muris* and *A. ceylanicum* was observed using scanning electron microscopy (SEM). After 24 h of incubation with the drug, both parasites were fixed in 2.5% glutaraldehyde (Alfa Aesar GmbH, Germany) in PBS (pH 7.4) for approximately 24 h at room-temperature. Each worm was washed three times in PBS and stored in 500 µl PBS at 4 °C until use. Before SEM examination, the samples were dehydrated stepwise for 10 min in 500 µl ascending ethanol concentrations (30%, 50%, 70% and >96% (ethanol absolute, Merck)), at room-temperature and kept in 500 µl 96% ethanol at 4 °C (Manneck et al., 2010). Finally, the worms were dried to critical point (Bal-tec CPD 030), fixed on aluminum stubs and sputter coated with 20 nm gold particles. SEM pictures were acquired with a high-resolution scanning electron microscope (Phillips XL30 ESEM and Nova™ Nano SEM 230) at an accelerating voltage of 5 kV.

2.5. In vivo studies

2.5.1. T. muris

2.5.1.1. Nitazoxanide monotherapy. *In vivo* studies with *T. muris*-infected mice were conducted as described recently (Tritten et al., 2011). Briefly, each mouse was checked for presence of infection (presence of eggs in the stools) on day 41 p.i. Groups of 3–4 infected animals were assigned to treatment (100, 300, 600 mg/kg single dose or 50 mg/kg nitazoxanide per day over 3 days (Fonseca-Salamanca et al., 2003) or control groups (4 animals each). Expelled worms present in the mouse stools (collected up to 72 h after treatment) were counted. On day 7 post-treatment, the mice were sacrificed and the remaining worms in the gut counted. The worm burden reduction (WBR) as well as the worm expulsion rate (WER) were calculated for each treatment (Rajasekariah et al., 1991).

2.5.1.2. Combination chemotherapy studies. Combinations found synergistic at IC_{50} *in vitro* were tested *in vivo*. Drugs were combined using a constant ratio based on the approximate ED_{50} values of the partner drugs (unpublished data). Nitazoxanide (100 mg/kg, best-performing dose) was combined with levamisole (46 mg/kg) or ivermectin (4 mg/kg). Drug effects were analyzed as described above.

2.5.2. A. ceylanicum

2.5.2.1. Nitazoxanide monotherapy. The *A. ceylanicum* experiments were carried out as summarized recently (Tritten et al., 2011). Briefly, the fecal egg burden was established on days 21 and 22 p.i. and hamsters were assigned to equally balanced treatment groups accordingly (3–4 animals each). Hamsters were treated with a single oral dose of 10 mg/kg nitazoxanide on day 23 p.i. Four animals were left untreated and served as controls. Stools were collected from each hamster over a period of 48 h after treatment and carefully searched for worms. Hamsters were sacrificed on day 7 post-treatment and the remaining worms counted. WBR and WER were calculated (Rajasekariah et al., 1986; Xue et al., 2005; Tritten et al., 2011).

2.5.2.2. Combination chemotherapy studies. Combinations behaving synergistically at IC_{50} *in vitro* were tested *in vivo*. Nitazoxanide (10 mg/kg) was combined with levamisole, pyrantel pamoate or ivermectin. Approximate ED_{50} values were calculated for the partner drugs (levamisole: 10 mg/kg (Tritten et al., 2012), pyrantel pamoate: 10 mg/kg, ivermectin: 0.04 mg/kg (unpublished data)).

2.6. Statistical analyses

The average of motility scores was calculated for each concentration, converted into a percentage value and normalized to the control, using Microsoft® Excel 2010. Differences in the motility of drug-treated and control worms and ovicidal activity were examined using the Fisher's exact test, with a significance level of 0.05, using Microsoft Excel 2010 or StatsDirect (version 2.4.5; StatsDirect Ltd., Cheshire, UK). IC_{50} values were expressed based on the median effect principle using the CompuSyn software (version 1.0), where a fitted curve is modeled and r is the linear correlation coefficient of the median-effect plot, indicating the goodness of fit (Chou, 1976). Combination indices (CI) were calculated with CompuSyn. Briefly, the behavior of a combination was categorized according to the following scale: CI > 1: antagonism; CI = 1: additive effect; CI < 1: synergism (Chou, 1976). Worm burden reductions were determined by comparing the mean number of adult worms in the intestine of a treated group with the mean number of the control group, in Microsoft® Excel 2010. Worm expulsion rates were calculated by dividing worm output of a treatment group by the total worm burden, using Microsoft® Excel 2010. ED_{50}s were calculated based on the WBRs, using CompuSyn. The Kruskal–Wallis test (multiple doses against control) or the Mann–Whitney U test (single dose against control) was used to assess the statistical significance of the WBRs, using StatsDirect.

3. Results

3.1. In vitro studies

3.1.1. T. muris

3.1.1.1. Activity of nitazoxanide on L3 and adults. Nitazoxanide had a marked effect on both *T. muris* L3 and adult worms. L3 were either dead (200, 50 µg/ml) or showed a large reduction in viability (reduction >85%, all $P < 0.001$) following incubation with 100 µg/ml for 72 h (data not shown). The temporal effect of five different concentrations of nitazoxanide on the viability of adult *T. muris* is presented in Fig. 1. Adults incubated in nitazoxanide (200, 100, 50 µg/ml) were markedly affected (>65% viability reduction) 24 h post-incubation (all $P < 0.001$). At 200 µg/ml adult *T. muris* were dead after 48 h ($P < 0.001$). After 72 h, adult *T. muris* were still alive when exposed to 100 and 50 µg/ml nitazoxanide, but showed little movement (motility <1, all $P < 0.001$). Nitazoxanide concentrations of 25 µg/ml reduced viability by 44% ($P < 0.03$), whereas a concentration of 12.5 µg/ml of the drug had no effect on adult worms ($P > 0.05$) 72 h post-incubation. All control worms showed normal movement (motility = 3) during the entire incubation period

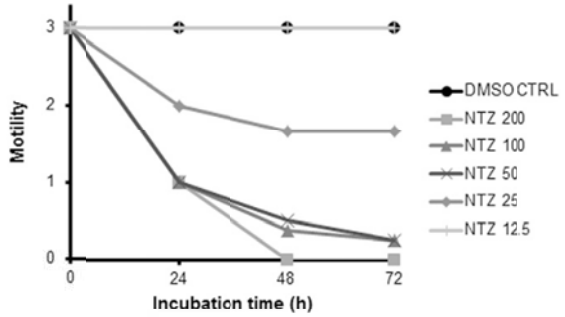

Fig. 1. *In vitro* effect of nitazoxanide on adult *T. muris* worms after 24, 48 and 72 h of incubation with 12.5, 25, 50, 100 and 200 μg/ml. NTZ = nitazoxanide. Motility scale: 3 = normal motility; 2 = low motility; 1 = very low motility; 0 = dead.

(72 h). IC_{50}s of 0.27 μg/ml ($r = 0.92$) and 12.87 μg/ml ($r = 0.70$) were calculated for L3 and adults, respectively. IC_{50}s are summarized in Table 1.

3.1.1.2. Combination chemotherapy studies. Five different drug combinations were tested against adult *T. muris*. A synergistic effect was observed with the combination of nitazoxanide and ivermectin (CI = 0.63, DR = 1:4). A nearly additive effect was calculated for nitazoxanide combined with levamisole (CI = 0.99, DR = 1:2). For example, even the lowest concentrations of nitazoxanide (6.25 μg/ml) and levamisole (25 μg/ml) tested reduced the viability of the worms to less than 50% (motility: 1.29 ± 0.49, $P < 0.001$). The combinations of nitazoxanide–pyrantel pamoate (DR = 1:2), nitazoxanide–albendazole (DR = 1:8), and nitazoxanide–mebendazole (DR = 1:4) revealed antagonistic effects (CI = 4.94, 2.64 and 2.28, respectively). Normal viability was observed with control worms (motility = 3) until the end of the experiment (72 h).

3.1.2. A. ceylanicum

3.1.2.1. Activity of nitazoxanide on L3, adults and eggs. No effect on L3 motility was observed when *A. ceylanicum* were incubated with 100 μg/ml nitazoxanide ($P = 0.50$) for 72 h. As many as 74.2% of larvae survived in the presence of 10 μg/ml ($P < 0.001$), 81.1% in 1 μg/ml ($P < 0.001$) and 93.3% in 0.1 μg/ml ($P = 0.059$) (Fig. 2). An IC_{50} of >100 μg/ml (r not determined) was calculated for L3 (Table 1).

Adult worms were very sensitive to nitazoxanide. A concentration of 100 μg/ml killed all adult worms ($P < 0.001$). At 10 μg/ml, the worms showed strongly reduced viability (motility of 0.8; $P = 0.028$). The lower nitazoxanide concentrations of 1 and 0.1 μg/ml only slightly decreased adult hookworm motility (motility values of 1.6 and 1.75, respectively (both $P > 0.05$)). An IC_{50} of 0.74 μg/ml ($r = 0.93$) was determined (Table 1).

For comparison, control worms incubated with DMSO showed an average motility of 1.93. No reduction in egg embryonation and hatching was observed in nitazoxanide-treated wells, compared to the controls (both $P = 0.50$).

3.1.2.2. Combination chemotherapy studies. Nitazoxanide combined with albendazole resulted in a CI of 1.90, suggesting an antagonistic behavior against adult worms *in vitro* (Table 1). An additive effect (CI = 1.0) was observed with the nitazoxanide–mebendazole combination. Nitazoxanide combined with levamisole, pyrantel pamoate or ivermectin showed synergism, with CIs of 0.32, 0.14 and 0.69, respectively

3.1.3. Scanning electron microscopy studies

3.1.3.1. T. muris. Nitazoxanide treated *T. muris* (200 μg/ml) and control worms were examined after incubation for 24 h (Fig. 3A–D). According to our *in vitro* studies worms are already strongly affected at this examination point.

No tegumental changes were observed with treated worms (Fig. 3A and C) compared to controls (Fig. 3B and D) at the anterior as well as the posterior tegument.

3.1.3.2. A. ceylanicum. In 2 out of 4 specimen, SEM revealed many areas of swelling (knobs) in the anterior part of adult *A. ceylanicum* 24 h post-incubation in the presence of a lethal concentration of 100 μg/ml nitazoxanide (all worms were dead, Fig. 4A). These structures were not observed on the tegument of control worms that had been incubated with DMSO (Fig. 4B).

3.2. In vivo studies

3.2.1. T. muris

3.2.1.1. Nitazoxanide monotherapy. Mice were treated with single 100, 300 or 600 mg/kg nitazoxanide, or 3 doses of 50 mg/kg given over 3 consecutive days (Table 2). The highest WBR of 56.09% was achieved following administration of 100 mg/kg nitazoxanide. However, a low WER of 16.23% was observed for this dose. Higher dosages did not result in increased activity (WERs of 2.0% and 0% and WBRs of 0% and 0%, at 300 and 600 mg/kg, respectively). Worm burden reductions in nitazoxanide-treated mice (all single dosages) were not significant ($P = 0.910$). Fifty mg/kg given in multiple doses reduced the worm burden by 17.37% ($P = 0.543$) and resulted in a WER of 1.84%.

3.2.1.2. Combination chemotherapy studies. Nitazoxanide (100 mg/kg) combined with levamisole (ED_{50}) resulted in a moderate WER of 33.9% and a very low WBR (0.27%) ($P = 0.629$). Nitazoxanide

Table 1
Median effect doses (IC_{50}s) of nitazoxanide, mebendazole and ivermectin and combination indices of selected drug combinations on *T. muris* and *A. ceylanicum*.

Drug	IC_{50}s (r) (μg/ml)			
	T. muris		*A. ceylanicum*	
	L3	Adults	L3	Adults
Nitazoxanide	0.27 (0.92)	12.87 (0.70)	>100 (n.d.)	0.74 (0.93)
Mebendazole	n.d.	n.d.	11.55 (0.87)	>100 (0.87)
CI at IC_{50} nitazoxanide–albendazole	n.d.	2.64	n.d.	1.90
CI at IC_{50} nitazoxanide–mebendazole	n.d.	2.28	n.d.	1.00
CI at IC_{50} nitazoxanide–levamisole	n.d.	0.99	n.d.	0.32
CI at IC_{50} nitazoxanide–pyrantel pamoate	n.d.	4.94	n.d.	0.14
CI at IC_{50} nitazoxanide–ivermectin	n.d.	0.63	n.d.	0.69

IC_{50}s were calculated 72 h post incubation; r = linear correlation coefficient of the median-effect plot, indicating the goodness of fit. $r \geq 0.85$ indicates a satisfactory fit. n.d.: not determined.

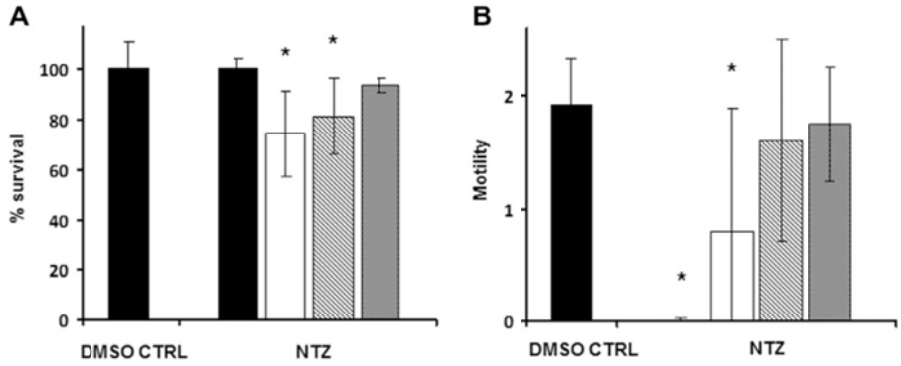

Fig. 2. Comparison of the *in vitro* effect of nitazoxanide on different parasite stages of *A. ceylanicum*. (A) L3; (B) adults. NTZ = nitazoxanide. Black bars represent incubation with 100 µg/ml, white bars incubation with 10 µg/ml striped bars incubation with 1 µg/ml and grey bars incubation with 0.1 µg/ml. Motility scale for adults: 2 = normal motility; 1 = low motility; 0 = dead.

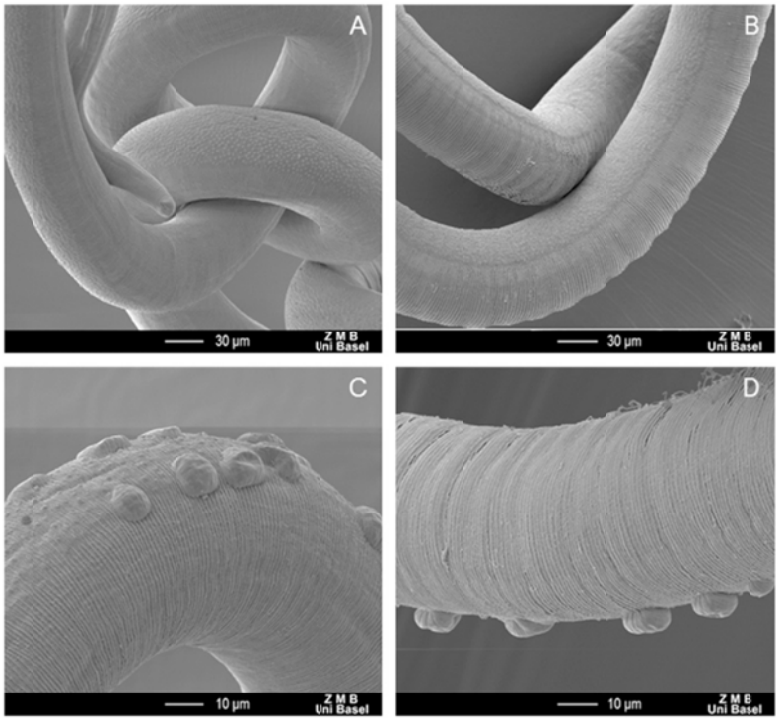

Fig. 3. SEM pictures of *T. muris* adults incubated for 24 h with nitazoxanide (A + C) or medium without drug (B + D). (A) Tegument of a nitazoxanide-treated worm (200 µg/ml) and the corresponding control (B), (both magnification 1000×). (C) anterior part with glands of nitazoxanide-treated worms and control (D) both at higher magnifications (3000×).

combined with ivermectin at the same ratio achieved a WER of 25% and had no effect on the WBR ($P = 0.629$).

3.2.2. A. ceylanicum

3.2.2.1. Nitazoxanide monotherapy. Treatment of *A. ceylanicum*-infected hamsters with a single 10 mg/kg oral dose had no effect on the worm burden (WER and WBR = 0%) ($P = 0.857$), as shown in Table 3.

3.2.2.2. Combination chemotherapy studies. Levamisole (10 mg/kg) administered simultaneously with nitazoxanide (10 mg/kg) produced a WER of 64.29% and a low WBR of 18.02% ($P = 0.80$) (Table 3). The combination nitazoxanide–pyrantel pamoate (both 10 mg/kg) moderately reduced the infection, with an observed WER of 27.78% and a WBR of 70.27% ($P = 0.60$). Ivermectin administered together with nitazoxanide (0.04 mg/kg + 10 mg/kg, respectively) resulted in a WER of 56.34% and a WBR of 70.19%

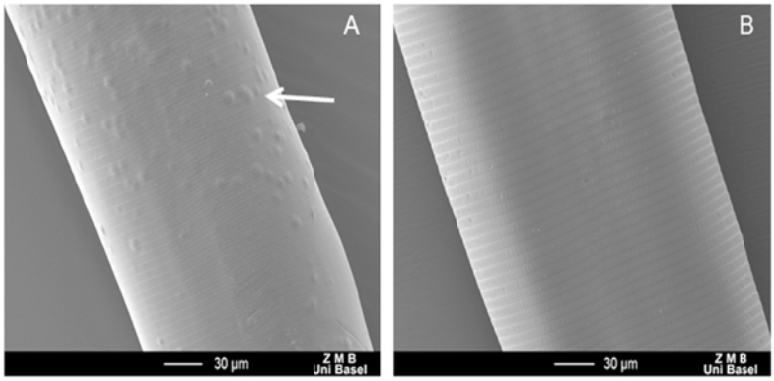

Fig. 4. SEM pictures of *A. ceylanicum* adult worms. (A) Treated with nitazoxanide, anterior part showing tegumental knobs (arrow) (magnification 1000×), (B) anterior part of a control worm (magnification 1000×).

Table 2
In vivo effect of nitazoxanide on *T. muris*, alone and in combination.

Group	Dose (mg/kg)	Mean number of worms (SD)	Mean number of expelled worms (SD)	Worm expulsion rate (%)	Worm burden reduction (%)	*P*-value
Control 1	–	109.5 (32.91)	0.68 (0.50)	0	–	–
Control 2	–	157.5 (62.57)	0.2 (045)	0	–	–
Control 3	–	98.33 (28.04)	0.33 (0.58)	0.34	–	–
Control 4	–	56.0 (55.32)	0 (0)	0	–	–
Nitazoxanide	100[1]	57.0 (60.55)	9.25 (13.20)	16.23	56.09	0.910[a]
	300[3]	182.5.0 (114.54)	3.66 (6.35)	2.0	0	
	600[4]	87.0 (74.48)	0 (0)	0	0	
	50 × 3[2]	122.0 (32.84)	2.25 (3.30)	1.84	17.37	0.543[b]
Control 5	–	91.25 (23.73)	0 (0)	0	–	–
Nitazoxanide–levamisole	100 + 46[5]	137.66 (99.80)	46.67 (37.16)	33.90	0.27	0.629[b]
Nitazoxanide–ivermectin	100 + 4[5]	185.33 (150.51)	46.33 (21.96)	25.0	0	0.629[b]

Numbers in superscript refer to the corresponding control group.
[a] Kruskal–Wallis test comparing the median of the worm burdens of control and treated mice.
[b] Mann–Whitney *U* test comparing the median of the worm burdens of control and treated mice.

Table 3
In vivo effect of nitazoxanide on *A. ceylanicum*, alone and in combination.

Group	Dose (mg/kg)	Mean number of worms (SD)	Mean number of expelled worms (SD)	Worm expulsion rate (%)	Worm burden reduction (%)	*P*-value[a]
Control 1	–	13.8 (8.0)	0 (0)	0	–	–
Nitazoxanide	10[1]	17.8 (2.10)	0 (0)	0	0	0.857
Control 2	–	18.5 (23.33)	0 (0)	0	–	–
Control 3	–	26.0 (4.24)	0 (0)	0	–	–
Nitazoxanide–levamisole	10 + 10[2]	9.33 (8.10)	6.0 (4.60)	64.29	18.02	0.80
Nitazoxanide–pyrantel pamoate	10 + 10[2]	18.0 (20.10)	5.0 (2.70)	27.78	70.27	0.60
Nitazoxanide–ivermectin	10 + 0.04[3]	17.75 (15.80)	10 (5.89)	56.34	70.19	0.80

Numbers in superscript refer to the corresponding control group.
[a] Mann–Whitney *U* test comparing the median of the worm burdens of control and treated hamsters.

($P = 0.80$). Hence, none of the drug combinations tested achieved a higher efficacy than levamisole, pyrantel pamoate or ivermectin alone (Tritten et al., 2011).

4. Discussion

Worldwide, about 3 billion people are estimated to be at risk of STH infections (de Silva et al., 2003). Since we rely on only four drugs for the treatment of STH infections, many of which are suboptimal, new therapies and/or drug combinations are urgently needed.

Nitazoxanide was first described for its cestocidal properties, later for anti-protozoal and anti-viral activities and recently for its potential against different nematodes and trematodes (Rossignol and Maisonneuve, 1984; Anderson and Curran, 2007; van den Enden, 2009). Among the helminths, *Fasciola hepatica* (Favennec et al., 2003), *A. lumbricoides*, *T. trichiura*, *Ancylostoma duodenale*, *Enterobius vermicularis* and *Strongyloides stercoralis* have been shown in clinical trials to be affected by treatment with nitazoxanide (Romero Cabello et al., 1997; Abaza et al., 1998; Ortiz et al., 2002).

In the present investigation, we evaluated the activity of nitazoxanide against *T. muris* and *A. ceylanicum*, two well-established

parasite-rodent models that mimic human soil-transmitted helminthiases, *in vitro* and *in vivo*. With minor differences in sensitivity, both *T. muris* stages were markedly affected by nitazoxanide *in vitro*. Adult worms incubated with 50, 100 and 200 µg/ml nitazoxanide showed significantly decreased viabilities and at the highest concentration (200 µg/ml) tested, all *T. muris* died. Additionally, nitazoxanide significantly decreased the motility of L3. On the other hand, differing stage susceptibilities were observed for hookworms; *A. ceylanicum* L3 were only moderately affected by nitazoxanide *in vitro*, while adult worms were killed only at the highest tested nitazoxanide dose. The hookworm third stage larva is a developmentally arrested non-feeding stage, where physiological processes and exchanges with the environment are greatly reduced (Cassada and Russell, 1975; O'Riordan and Burnell, 1989, 1990; Burnell et al., 2005). Hence, if nitazoxanide requires ingestion by the worm to exert its anthelmintic activity, or if the target enzymes are missing at this stage, it would not be surprising that *A. ceylanicum* L3 would remain unaffected by the drug. Similarly, monepantel only affected adult *A. ceylanicum* and showed a low activity on L3 (Tritten et al., 2011).

To our knowledge, we have investigated the effect of nitazoxanide on *A. ceylanicum* and *T. muris* using SEM for the first time. SEM did not identify any marked effect of nitazoxanide (200 µg/ml) on the *T. muris* adult worm tegument compared to controls. However, SEM revealed small tegumental knobs in the anterior region of half of the treated *A. ceylanicum* specimen, possibly associated with exposure to the drug.

Note that, in mice, nitazoxanide is rapidly transformed and only tizoxanide, the active metabolite, is measurable in plasma. However, *in vitro*, nitazoxanide and tizoxanide have shown similar activities against a wide range of protozoa and bacteria (Stettler et al., 2004; Anderson and Curran, 2007). Surprisingly, however, nitazoxanide administered *in vivo* had no significant impact on the helminths infections studied, regardless of the dose and treatment schedule. It is striking that the greatest activity against *T. muris* (WER and WBR of 16% and 56%, respectively) was observed with the lowest dose (100 mg/kg) of nitazoxanide tested. It is also worth mentioning that again a pronounced inter-individual variability was noted treating mice at this dose, as the worm burden reduction was 0% in half of the mice and in others high WBRs and WERs were observed.

An interaction between host immunity and the effect of nitazoxanide has been reported by Cabada and White (2010), who found that the drug lacked efficacy against cryptosporidiosis in immunocompromised patients. Since the *T. muris*-infected mice were immunosuppressed until 2 days before treatment (and the mice immune status had therefore not reached normality yet at the time treatment was administered), this could have induced the observed unresponsiveness to the drug. However, this hypothesis is not supported by the fact that also in *A. ceylanicum*-infected hamsters which had not been immunosuppressed, no drug effect was observed. In addition, nitazoxanide achieved consistent oocyst shedding inhibition in an immunosuppressed rat model of cryptosporidiasis (Li et al., 2003).

The low activity of nitazoxanide observed in our study *in vivo* is in contrast to findings from human clinical trials. High cure and egg reduction rates were observed against *A. lumbricoides*, *A. duodenale* and *T. trichiura*, comparing favorably to albendazole, the current drug of choice (Romero Cabello et al., 1997; Abaza et al., 1998; Ortiz et al., 2002).

The striking differences between our *in vivo* studies and human clinical trial results cannot be explained at the moment. In protozoa and anaerobic bacteria, nitazoxanide was shown to be a non-competitive inhibitor of the pyruvate oxydoreductases (Hoffman et al., 2007). However, evidence for this is lacking and alternative mechanisms have been proposed (Hemphill et al., 2006; Müller and Hemphill, 2011). It has been suggested that the activity of nitazoxanide is quenched in the stomach by gastric acids (pH 3), by transforming the drug into a biologically inactive protonated form which might be restored to the active anion by the more alkaline pH in the small intestine. In the same report, the presence of the active anion form of nitazoxanide was revealed from pH 6 and above (Hoffman et al., 2007). In mice, the mean intestinal pH is <5.2, much lower than values observed in humans (pH 7.5), with some consistent inter-individual variations (Evans et al., 1988; McConnell et al., 2008). If the proposed mode of action for nitazoxanide also applies to helminths, the substantial intestinal pH differences between humans and rodents might explain the unexpectedly low *in vivo* activity, and would make rodent models inappropriate to test drugs strongly relying on narrow intestinal pH ranges.

To our knowledge, we have for the first time evaluated the effect of nitazoxanide drug combinations. Though synergistic activity was observed using combinations of nitazoxanide and standard anthelminthic drugs *in vitro*, these effects could not be confirmed *in vivo*, possibly also because of intestinal pH incompatibilities.

In conclusion, nitazoxanide was found highly potent against both *T. muris* stages tested, whereas only *A. ceylanicum* adult worms were found sensitive to the drug *in vitro*. In both parasite-rodent models, the drug exhibited poor efficacy. Further studies in non-rodent systems are necessary to explain the differences between our results and findings obtained in clinical trials.

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Chapter 6

Activity of oxantel pamoate monotherapy and combination chemotherapy against *Trichuris muris* and hookworms: revival of an old drug

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Activity of oxantel pamoate monotherapy and combination chemotherapy against *Trichuris muris* and hookworms: revival of an old drug

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Short Title: Oxantel against *Trichuris muris* and hookworms

Abstract

***Background*:** It is widely recognized that only a handful of drugs are available against soil-transmitted helminthiases, which are characterized by a low efficacy against *Trichuris trichiura*, when administered as single doses. The re-evaluation of old, forgotten drugs is a promising strategy to identify alternative anthelminthic drug candidates or drug combinations.

Methodology: We studied the activity of the veterinary drug oxantel pamoate against *Trichuris muris*, *Ancylostoma ceylanicum* and *Necator americanus in vitro* and *in vivo*. In addition, the dose-effect of oxantel pamoate combined with albendazole, mebendazole, levamisole, pyrantel pamoate and ivermectin was studied against *T. muris in vitro* and combinations behaving additive or synergistic were followed up *in vivo*.

Principal Findings: We calculated an ED_{50} of 4.7 mg/kg for oxantel pamoate against *T. muris* in mice. Combinations of oxantel pamoate with pyrantel pamoate behaved antagonistically *in vitro* (combination index (CI)=2.53). Oxantel pamoate combined with levamisole, albendazole and ivermectin using ratios based on their ED_{50}s revealed antagonistic effects *in vivo* (CI=1.27, 1.90 and 1.27, respectively). A highly synergistic effect (CI=0.15) was observed when oxantel pamoate-mebendazole was administered to *T. muris*-infected mice. Oxantel pamoate (10 mg/kg) lacked activity against *Ancylostoma ceylanicum* and *Necator americanus in vivo*.

Conclusion/Significance: Our study confirms the excellent trichuricidal properties of oxantel pamoate. Since the drug lacks activity against hookworms it is necessary to combine oxantel pamoate with a partner drug with anti-hookworm properties. Synergistic effects were observed for oxantel pamoate-mebendazole, hence this combination should be studied in more detail. Since of the standard drugs albendazole has the highest efficacy against hookworms additional investigations on the combination effect of oxantel pamoate-albendazole should be launched.

Author Summary

The roundworm *Ascaris lumbricoides,* the whipworm *Trichuris trichiura* and the hookworms are responsible for the most common infections worldwide and place more than 5 billion people at risk. To control these infections, at risk populations are treated regularly with anthelminthic drugs, mostly albendazole and mebendazole. Since both drugs have a low therapeutic effect against *T. trichiura* alternative drugs should be discovered and developed. Possible strategies are to re-evaluate forgotten compounds and to thoroughly study drug combinations. We evaluated the activity of the “old”, veterinary drug oxantel pamoate against *T. muris*, *Ancylostoma ceylanicum* and *Necator americanus in vitro* and *in vivo*. In addition, we studied the activity of combinations of oxantel pamoate with the 4 standard treatments for soil-transmitted helminthiases. Our results confirm that oxantel pamoate has excellent trichuricidal properties. We show that the drug lacks activity against hookworms. It is therefore necessary to combine oxantel pamoate with an anti-hookworm drug. Synergistic effects were observed with oxantel pamoate-mebendazole in our study. Additional preclinical studies should be launched.

1. Introduction

Infections with the three major soil-transmitted helminths (STH), *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma duodenale* are among the most common parasitic diseases in areas of rural poverty in developing countries [1]. In countries where soil-transmitted helminthiases are endemic, preventive chemotherapy, i.e. regular anthelminthic drug administration to all people at risk of morbidity, is one of the key strategies [2]. In 2009 the number of school-aged children treated for soil-transmitted helminthiases was estimated at 204 million [3]. Albendazole and mebendazole, 2 drugs belonging to the benzimidazole group are the most widely used drugs in preventive chemotherapy programs. At present, 2 alternative drugs, pyrantel pamoate and levamisole are available but currently have a less prominent role since they require weight-based dosing [4]. Despite their excellent safety profile the drugs have serious limitations with regard to their efficacy. When delivered as a single dose, as in preventive chemotherapy programs, all 4 compounds have a limited effect against infections with *T. trichiura* as calculated by a recent meta-analysis [5]. In addition, drug resistance is a concern. Efforts are therefore ongoing to discover and develop the next generation of anthelminthic drugs [6]. Promising strategies to identify potential anthelminthic drug candidates are to assess compounds derived from animal health, to re-evaluate forgotten compounds and to thoroughly study drug combinations [6].

Oxantel is the meta-oxyphenol analog of pyrantel. It was discovered in the early 1970s by Pfizer and showed high activity in *T. muris*-infected mice and *T. vulpis*-infected dogs [7, 8]. Subsequent clinical trials demonstrated that the drug was safe and effective in the treatment of trichuriasis [9-12]. For example, complete cure was observed in 10 *T. trichiura*-infected patients treated with 20 mg/kg oxantel pamoate [9]. In veterinary medicine oxantel pamoate was later combined with pyrantel pamoate, which has with the exception of activity against *Trichuris* spp. a broad spectrum of activity against different nematodes [13]. Today oxantel-pyrantel is widely available as a dewormer for dogs and cats. The combination of oxantel-

pyrantel was also evaluated in a few clinical trials against human STH infections [11, 14-16]. For example, a decade ago oxantel-pyrantel (10 mg/kg) was tested in school aged children in Pemba. The combination achieved cure rates of 38.2% and 12.7% against infections with *T. trichiura* and hookworms, respectively [17]. To our knowledge, despite the interesting trichuricidal properties of oxantel, combinations of this drug with other recommended anthelminthic drugs have not been evaluated to date.

The aim of the present study was to study the trichuricidal potential of oxantel pamoate combined with the 4 WHO recommended anthelminthic drugs for the treatment of hookworm, *T. trichiura* and *A. lumbricoides* infections (albendazole, mebendazole, levamisole or pyrantel pamoate) as well as combinations of ivermectin and oxantel pamoate. Ivermectin, the first line drug for strongyloidiasis, is known to have trichuricidal properties and combinations of albendazole-ivermectin and mebendazole-ivermectin have been tested clinically [18]. In a first step the EC_{50} (ED_{50}) values of oxantel pamoate against *T. muris* were determined *in vitro* and *in vivo*. Next to oral administration we also tested the activity of intraperitoneal oxantel pamoate in mice. We then elucidated whether oxantel pamoate combined with albendazole, mebendazole, ivermectin, levamisole or pyrantel interacts in an additive, antagonistic or synergistic manner *in vitro* using the combination index equation [19]. Additive and synergistic combinations were followed up *in vivo*. In addition, the activity of oxantel pamoate was studied against *A. ceylanicum* and *N. americanus in vitro* and *in vivo*.

2. Materials and Methods

2.1. Drugs

Albendazole and levamisole were purchased from Fluka (Buchs, Switzerland), oxantel pamoate, mebendazole, ivermectin and pyrantel pamoate were obtained from Sigma-Aldrich

(Buchs, Switzerland). Note that, the pamoate salts of oxantel and pyrantel contain only 35.8% and 34.7% of the active ingredients, oxantel and pyrantel base, respectively.

For *in vitro* studies, drug stocks (5-10 mg/ml) were prepared in 100% DMSO (Sigma-Aldrich, Buchs, Switzerland) and stored at 4°C pending usage. For *in vivo* studies, the drugs were suspended in 10% Tween 80 [80% EtOH (70:30 v/v)] (Buchs, Switzerland) and 90% dH_2O shortly before treatment.

2.2. Animals

Four week-old female C57BL/10 mice and 3 week-old male Syrian golden hamsters were purchased from Charles River (Blackthorm, UK and Sulzfeld, Germany, respectively). Before infection, animals were allowed to acclimatize for one week in our animal facility. They were kept in groups of maximum ten (mice) or three (hamsters) in macrolon cages with free access to water and rodent food pellets (Rodent Blox from Eberle NAFAG, Gossau, Switzerland).

2.3. Ethics statement

Experiments were performed in an attempt to comply with the 3R rules for animal experiments. The current study was approved by the cantonal veterinary office Basel-Stadt (Switzerland) based on Swiss cantonal and national regulations (permission no. 2070).

2.4. Parasites and infections

2.4.1. *Trichuris muris*

The life cycle of *T. muris* has been maintained at the Swiss TPH since January 2010, and described in detail in previous publications [20-23]. Mice were treated with dexamethasone (1

mg/l, dexamethasone-water soluble, Sigma-Aldrich) supplied with the drinking water 2 days before infection onwards and were infected orally with 200 embryonated *T. muris* eggs.

2.4.2. *Ancylostoma ceylanicum* and *Necator americanus*

The *A. ceylanicum* and *N. americanus* life cycles have been maintained at the Swiss TPH since June 2009 and April 2011, respectively, and have been described previously [23, 24, 25, 26]. Hamsters were treated with 0.5 mg/l dexamethasone in the drinking water, 2 days before infection onwards. They were infected orally with 150 L3 (*A. ceylanicum*) or subcutaneously with 250 L3 (*N. americanus*). Hamsters assigned to *in vivo* studies were not immunosuppressed and were infected with 300 L3.

2.5. *In vitro* studies with *Trichuris muris*

2.5.1. Oxantel monotherapy

Three to 4 fourth-stage larvae (L4) (days 26-28 p.i.) were collected from the intestines (binocular, magnification 16x) and transferred into each well of a 96-well plate containing 100 µl pre-warmed RPMI medium [10.44 g RPMI 1640 (Gibco, Basel, Switzerland), 5 g albumax H (Gibco), 5.94 g HEPES (Sigma-Aldrich) and 2.1 g sodium bicarbonate (Sigma-Aldrich) in 1 l dH_2O] supplemented with 5% v/v amphotericin B (250 µg/ml, Sigma-Aldrich) and 1% v/v penicillin-streptomycin (10'000 U/ml penicillin + 10 mg/ml streptomycin, Sigma-Aldrich). One hundred µl of an oxantel pamoate solution were added to obtain 0.15-600 µg/ml (final concentrations) and the plate was incubated at 37°C and 5% CO_2 for 72 hours. Control worms were incubated in medium with the highest DMSO concentration used in the test (1% v/v). After 24, 48 and 72 hours of incubation the viability of the worms was evaluated according to a motility scale from 3 to 0 (3=normal, 100% motility, 0=dead). Assays were conducted in duplicate.

2.5.2. Combination chemotherapy studies

Drug combination assays were carried out as described for single drug assays, with slight alterations. Three to 4 adult worms were transferred into each well of a 48-well plate containing 500 µl pre-warmed supplemented RPMI medium. Then, 250 µl of the drug solution #1 and 250 µl of the drug solution #2 were added at a constant dose ratio based on the calculated IC_{50} values (inhibitory concentration 50%) and 2-fold dilutions were carried out up and down. In more detail, the following combinations were tested: $2IC_{50}$:$2IC_{50}$ (for oxantel pamoate-pyrantel pamoate and oxantel pamoate-levamisole only), IC_{50}:IC_{50}, $0.5IC_{50}$:$0.5IC_{50}$ and $0.25IC_{50}$:$0.25IC_{50}$. Since for albendazole, mebendazole and ivermectin no IC_{50} value could be calculated (IC_{50}s > 200 µg/ml) [27] a concentration of 400 µg/ml was selected as IC_{50} value. A combination index (CI) was calculated to characterize the interaction of each combination: synergism (CI<1), antagonism (CI>1) and additive effect (CI=1) [19].

2.6. *In vitro* studies with *Ancylostoma ceylanicum* and *Necator americanus*

2.6.1. Oxantel monotherapy

In vitro studies with *A. ceylanicum* and *N. americanus* third-stage larvae (L3) and adult worms were conducted as described recently [23]. Briefly, in a 96-well plate (Costar), 30 L3 per well were incubated for 72 hours at room-temperature in 200 µl HBSS medium supplemented with 10% v/v amphotericin B (250 µg/ml, Sigma-Aldrich), 1% v/v penicillin-streptomycin (10'000 U/ml penicillin + 10 mg/ml streptomycin, Sigma-Aldrich) containing oxantel pamoate dilutions ((0.1), 1, 10 and 100 µg/ml, final concentrations). The larval survival was determined microscopically (magnification 20x) following addition of hot water (~80°C) and exposure to microscope light. Two to 3 adult worms, collected from the hamsters intestines (binocular, magnification 16x) were incubated per well in 48-well plates for 72 hours in 1 ml supplemented HBSS medium and 10% v/v fetal calf serum containing oxantel pamoate dilutions (ranging from 0.1 to 100 µg/ml) at 37°C, 5% CO_2. The motility was

determined microscopically (magnification 20x) using a viability scale ranging from 2 (normal viability, 100% motility) to 0 (death). Control worms were incubated with the highest DMSO concentration used in the test (2% v/v). Assays were conducted in triplicate.

2.7. *In vivo* studies

2.7.1. *Trichuris muris*

Each animal was checked for the presence of eggs in the stools on day 40 p.i. and assigned to treatment or control groups (n=4 mice per group) and treated with a single oral drug dose on the following day. Oxantel pamoate was administered at 10 mg/kg, 5 mg/kg, 2.5 mg/kg and 1 mg/kg. Two groups of mice were treated intraperitoneally with 10 mg/kg oxantel pamoate and 10 mg/kg ivermectin. Expelled worms, recovered from stools collected for up to 72 hours after treatment, were counted. At dissection, worms remaining in the gut 7 days post-treatment were collected and counted. Worm burden arithmetic means were calculated for each treatment and control group. Worm burden reductions (WBRs) and worm expulsion rates (WERs) were calculated as described previously [23, 27]. Drug combinations revealing synergism *in vitro* (CI<1) (oxantel pamoate-albendazole, oxantel pamoate-levamisole, oxantel pamoate-mebendazole and oxantel pamoate-ivermectin), were tested *in vivo* using a constant dose ratio. The ratio of the ED_{50}s (effective dose 50%) of each drug was chosen as starting dose (ED_{50}:ED_{50}). If the treatment reduced the worm burden by more than 75% (threshold for additivity when the dose effect curves for both drugs are hyperbolic [19]), the drug doses were divided in half. ED_{50} values of the partner drugs were 345 mg/kg for albendazole, 79 mg/kg for mebendazole (both values determined in the frame of the present work), 4 mg/kg for ivermectin, and 46 mg/kg levamisole [22, 23].

2.7.2. *Ancylostoma ceylanicum* and *Necator americanus*

The experiments were carried out as described recently [27]. Briefly, the fecal egg burden was established on days 21 and 22 p.i. (*A. ceylanicum*) and 46 and 47 (*N. americanus*) and treatment and control groups formed on the basis of arithmetic mean fecal egg burden. Hamsters were treated with a single oral dose of 10 mg/kg oxantel pamoate on the following day. Animals left untreated served as controls. Forty-eight hours after treatment, the complete stools were collected from each hamster and searched for expelled worms (binocular, magnification 16x). Worms remaining in the gut 7 days post-treatment were collected and counted after sacrificing the hamsters. The WERs were calculated.

2.8. Statistical analyses

All data obtained were analyzed by Excel (Microsoft Office, 2007). *In vitro* data obtained from the individual data with the motility assay were averaged and normalized to the controls. IC_{50}s (median-effect dose), defined as the concentration of a drug required to decrease the mean worm's motility to 50% at the 72 hour time point, were calculated with the CompuSyn software (CompuSyn, version 3.0.1). The combination index (CI) was calculated for the combination chemotherapy data with the CompuSyn software. To test the significance of the worm burden reductions *in vivo*, the Kruskal-Wallis (several treatment doses vs. controls) or the Mann-Whitney U-test (one treatment dose vs. control) was applied, using StatsDirect (version 2.4.5; StatsDirect Ltd; Cheshire, UK).

3. Results

3.1. *In vitro* studies with *T. muris*

3.1.1. Oxantel monotherapy

Temporal drug effects of different oxantel pamoate concentrations over the incubation period of 72 hours are depicted in Figure 1. Exposure of *T. muris* to 0.15 and 0.3 µg/ml oxantel pamoate achieved only a negligible effect (mean motilities of 76.7% (±31.3%) and 83.3% (±23.3%) respectively) on the worms 24-72 hours post-treatment. Incubation of *T. muris* L4 for 24-72 hours with 0.6-600 µg/ml oxantel pamoate resulted in strongly reduced viabilities within 24 hours but did not kill the worms. Control worms showed normal movements over the entire incubation period. We calculated an IC_{50} of 2.35 µg/ml for oxantel pamoate (corresponding to 0.78 µg/ml for the free base oxantel) on *T. muris* L4 (Table 1).

3.1.2. *Trichuris muris* combination chemotherapy

Oxantel pamoate was combined with albendazole, mebendazole, pyrantel pamoate, ivermectin or levamisole using ratios based on their IC_{50}s and *T. muris* adults were exposed simultaneously to one of these combinations. The results are presented in Table 1 and dose response relationships of the combinations depicted in Figure 2. Synergistic effects were observed for four of the combinations, namely oxantel pamoate-mebendazole (CI=0.06), oxantel pamoate-ivermectin (CI=0.27), oxantel pamoate-albendazole (CI=0.37) and oxantel-pamoate levamisole (CI=0.46). An antagonistic interaction was found when oxantel pamoate was combined with pyrantel pamoate (CI=2.53). Worms exposed to this combination were only affected at the 2 highest concentration ratios ($2IC_{50}$:$2IC_{50}$ and IC_{50}:IC_{50}) and showed normal viabilities at the 2 lowest concentration ratios examined ($0.5IC_{50}$:$0.5IC_{50}$ and $0.25IC_{50}$:$0.25IC_{50}$).

3.2. *In vitro* studies with *Ancylostoma ceylanicum*

A. ceylanicum L3 incubated with oxantel pamoate revealed high survival rates (92.9% ± 0.01% at 1 µg/ml, 100% ± 0.0% at 10 µg/ml and 95.3% ± 0.07% at 100 µg/ml), compared to controls. Similarly, adult worms were only weakly affected by the drug, showing an average motility of 100% (±0.0%) at 0.1 and 1 µg/ml and 83.5% (±29.0%) at 10 and 100 µg/ml compared to controls (motility of 100% (±0.0%)).

3.3. *In vitro* studies with *Necator americanus*

N. americanus L3 incubated with oxantel pamoate revealed high survival rates (100% ± 0.05% at 0.1 µg/ml, 97.7% ± 0.04% at 1 µg/ml, 97.1% ± 0.003% at 10 µg/ml and 96.6% ± 0.0% at 100 µg/ml), compared to controls. In contrast, adult worms were markedly affected by the drug, resulting in an average motility of 100% (±0.0%) at 0.1 µg/ml, 50% (±25.0%) at 1 µg/ml and 62.5% (±40.5%) at 10 µg/ml and only 12.5% (±25.0%) at 100 µg/ml compared to controls (motility of 100% (±0.0%)). An IC_{50} of 11.80 (r=0.89) was calculated for *N. americanus* adult worms (Table 1).

3.4. *In vivo* studies with *Trichuris muris*

3.4.1. Oxantel monotherapy

Oxantel pamoate displayed a high activity against *T. muris in vivo*, with an ED_{50} of 4.71 mg/kg. In more detail, a worm burden reduction of 92.5% and worm expulsion rate of 88.4% was achieved after administration of 10 mg/kg (Table 2). Administration of 5 mg/kg resulted in a WBR of 81.1% and a WER of 78.2%. A moderate activity was observed with oxantel pamoate at 2.5 mg/kg (WER=24.1%, WBR=13.5%) and no effect was observed when mice were treated with 1 mg/kg (WER=1.5%, WBR=0%). The worm burden in orally oxantel pamoate treated mice was significantly different from untreated mice (*P*=0.041). An

intraperitoneal treatment of 10 mg/kg lacked activity against *T. muris* (both WER and WBR=0%). For comparison, 10 mg/kg ivermectin given intraperitoneally resulted in a worm burden reduction of 93.5%.

3.4.2. Combination chemotherapy

The 4 drug combinations that displayed synergistic effects *in vitro* were followed up *in vivo* (Table 2). Simultaneous treatment of *T. muris*-infected mice with a combination of oxantel pamoate and albendazole using the approximate ED_{50} doses resulted in a WBR of 76.6%, while combining $0.5ED_{50}$s was inefficacious (WBR=0%). The combination was modeled as antagonistic (CI=1.90). A synergistic interaction was found for the combination oxantel pamoate-mebendazole, as illustrated by a combination index of 0.15. A WBR of 88.8% was achieved combining both drugs using the ED_{50} doses and a still moderate WBR of 58.2% was observed when doses of 0.63 mg/kg oxantel pamoate and 10 mg/kg mebendazole (1/8 ED_{50}s) were administered. Oxantel pamoate combined with ivermectin achieved a WBR of 84.8% at the highest dose tested (ED_{50}:ED_{50} (5 and 4 mg/kg) of oxantel pamoate and ivermectin, respectively), but the combination at $0.5ED_{50}$: $0.5ED_{50}$ revealed a worm burden reduction of 37.5% only. The combination dose-effect analysis yielded antagonistic properties for the oxantel pamoate-ivermectin combination (CI=1.27). Similarly although the combination of oxantel pamoate and levamisole at the ED_{50}:ED_{50} removed most of the worms (WBR=82.0%, WER=71.7%), using half of the dosage reduced the worm burden by less than 50% (WBR=34.3%, WER=31.1%). The overall behavior of the combination of oxantel pamoate and levamisole was found to be antagonistic (CI=1.27).

3.5. *In vivo* studies with *A. ceylanicum* and *N. americanus*

Oxantel pamoate exerted no effect on *A. ceylanicum in vivo* following a single dose treatment of 10 mg/kg, illustrated by a WER of 0% (data not shown). The same oral treatment (10 mg/kg) in the *N. americanus* model resulted in a very low WER of 10.3% (data not shown).

4. Discussion

Since the introduction of albendazole, mebendazole, levamisole, and pyrantel pamoate in the human armamentarium to treat STH infections 3-4 decades ago, efforts to discover and develop a novel nematocidal drug have been scarce. The danger of resistance development therefore raises concern for the availability of therapy options in the future. Furthermore, all four above-mentioned drugs have a limited activity against *Trichuris* spp when administered as single oral doses. To spur the discovery of novel anthelminthic treatments potential drug candidates have recently been examined *in vitro*, *in vivo* and in clinical trials. Disappointingly, nitazoxanide, a potential drug candidate identified in systematic literature search [6] as well as a combination of albendazole and nitazoxanide revealed low trichuricidal activity in a randomized placebo controlled trial on Pemba [28]. Furthermore, monepantel, a safe nematocidal drug recently marketed in veterinary medicine showed a very low activity against *Ascaris suum* and *T. muris in vitro* and *in vivo* [23]. Hence, neither nitazoxanide nor monepantel can be recommended for the treatment of infections with STH.

In the present work, another potential candidate, oxantel pamoate, widely used in veterinary medicine was evaluated against *T. muris* and hookworms *in vitro* and *in vivo*. Oxantel pamoate revealed an excellent trichuricidal activity in mice. We calculated an ED_{50} of 4.7 mg/kg in *T. muris*-infected mice. A similarly low ED_{50} of 1.7 mg/kg was reported previously in this model [8]. For comparison, the WHO recommended drugs for the treatment of STH infections are characterized by much higher ED_{50} values in this model, namely 345 mg/kg for

albendazole, 79 mg/kg for mebendazole, 46 mg/kg for levamisole and > 300 mg/kg for pyrantel pamoate [23, 27]. Ivermectin, used in the treatment of strongyloidiasis and filarial infections, displayed a comparable ED_{50} value of 4 mg/kg in our *T. muris* model [29].

Interestingly, a dose of 10 mg/kg oxantel pamoate administered intraperitoneally lacked activity in *T. muris*-infected mice. For comparison, the same i.p. dose of ivermectin resulted in a high reduction of the worm load (>93%). This demonstrates that in contrast to ivermectin oxantel pamoate does not kill the worm via the blood stream, which might also be explained by the poor absorption of the drug in the gastrointestinal tract [13].

Oxantel pamoate lacked *in vivo* activity against both hookworm species *A. ceylanicum* and *N. americanus*. This finding is in line with a previous study in *A. caninum*-infected mice [30]. Interestingly, *N. americanus* adults were affected by the drug *in vitro* while no activity was observed on *A. ceylanicum*. To our knowledge, the activity of oxantel pamoate against hookworms has not been studied in humans.

Oxantel pamoate showed also no effect against the third major soil-transmitted helminth species, *A. lumbricoides* in humans (all 53 patients treated with oxantel revealed *Ascaris* eggs in the stools collected post-treatment regardless of the dose administered) [9]. It is therefore necessary to combine oxantel pamoate with a partner drug, which therapeutic profile covers roundworms and hookworms. In the present work we have for the first time thoroughly evaluated the potential of oxantel pamoate in drug combinations. Interestingly, antagonistic effects were observed with oxantel pamoate-pyrantel pamoate against *T. muris in vitro*, hence this combination was not pursued further. However, we cannot exclude a better trichuricidal effect *in vivo* for this combination, in particular as a pharmacodynamic interference at the target is unlikely. Oxantel is classified as an N-subtype AChR agonist, while pyrantel is considered an L-subtype suggesting differences in drug action [31]. The combination of oxantel pamoate-pyrantel pamoate is widely used in veterinary medicine and has also been studied in several human clinical trials. For example, in Korea oxantel pamoate-pyrantel pamoate at 20 mg/kg achieved a cure rate of 75% and egg reduction rate

of 97% against *T. trichiura* infections and cleared *A. lumbricoides* infections [11]. A high egg reduction rate against *T. trichiura* following oxantel pamoate-pyrantel pamoate at 20 mg/kg was also reported in a Malaysian study [16]. A lower effect of this combination administered at 10 mg/kg was observed on Pemba with cure rates of 96.3, 38.2 and 12.7% against *A. lumbricoides*, *T. trichiura* and hookworm, respectively [17]. In 2 Korean trials both oxantel monotherapy as well as an oxantel-pyrantel combination were used, however since different formulations were used (syrup versus tablets), different dosages applied and sample sizes were small no conclusion can be drawn whether the combination was superior to oxantel monotherapy [11,12].

Antagonistic effects were observed *in vivo* using combinations of oxantel pamoate-albendazole, oxantel pamoate-levamisole and oxantel pamoate-ivermectin. Since the molecular basis of actions of these drugs is not yet fully elucidated it is impossible to explain the antagonistic interaction profile observed for these combinations. In addition, drug scheduling, host behavior, environmental factors and genetic variations might also influence the level of activity [32]. Furthermore, note that these findings are based on a single ratio of the combined agents (ED_{50} values) and it might be worthwhile to assess other ratios of the drug dosages in particular for the combination of oxantel pamoate-albendazole, since of the standard drugs albendazole has the highest efficacy against hookworms [5]. On the other hand, the oxantel pamoate-mebendazole combination revealed highly synergistic effects against *T. muris in vivo*. Why the two benzimidazole derivates behave so differently when administered as partner drugs in oxantel pamoate combinations cannot be explained at the moment, but differences in their pharmacokinetic properties [33] might play a role.

In conclusion, our studies confirm that oxantel pamoate has excellent trichuricidal properties. In the *T. muris* mouse model oxantel pamoate showed a higher activity than the standard drugs albendazole, mebendazole, levamisole and pyrantel pamoate. Since the drug has no activity against hookworms it is necessary to combine oxantel pamoate with a partner drug revealing anti-hookworm properties. Synergistic effects were observed for oxantel pamoate-

mebendazole. Despite of our results pointing to an antagonistic behavior of oxantel pamoate-albendazole additional investigations on the combination effect of these agents should be launched (e.g. evaluation of a different dosing ratio or schedule) since of the standard drugs albendazole has the highest efficacy against hookworms. Systemic drug interactions between oxantel pamoate and partner drugs are most likely not to be expected given that, as mentioned, the absorption of oxantel pamoate is very poor. Nonetheless, preclinical studies should carefully elucidate metabolic and pharmacokinetic interactions of oxantel pamoate and the benzimidazoles.

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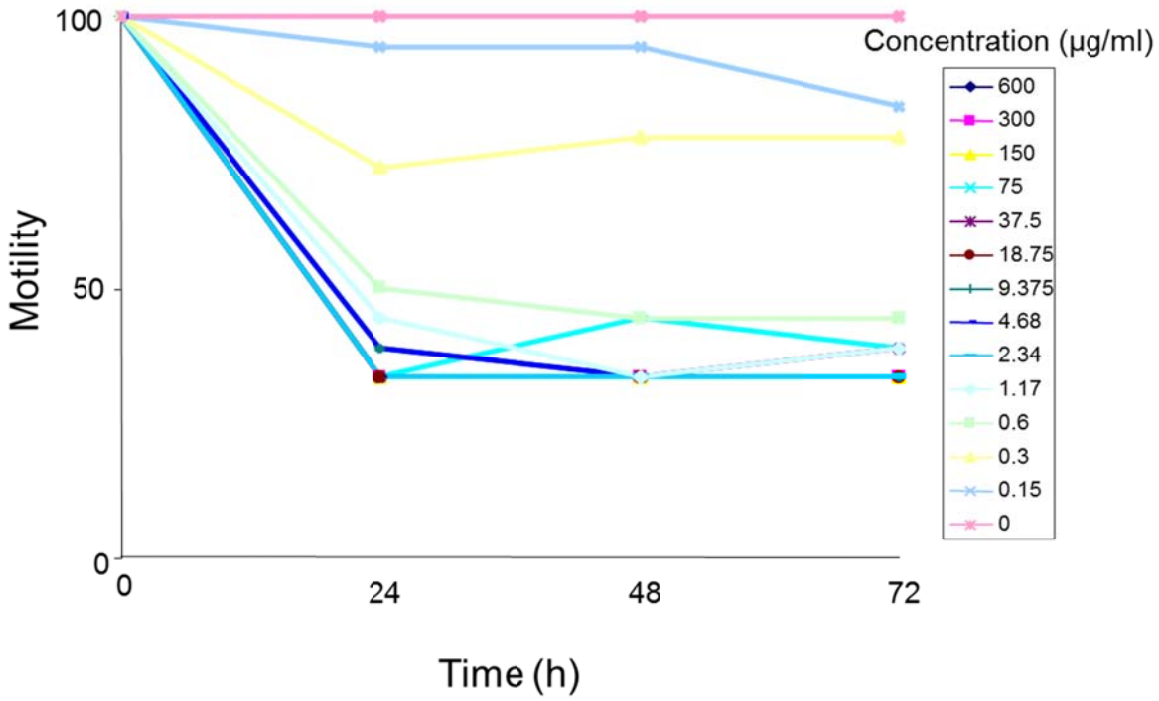
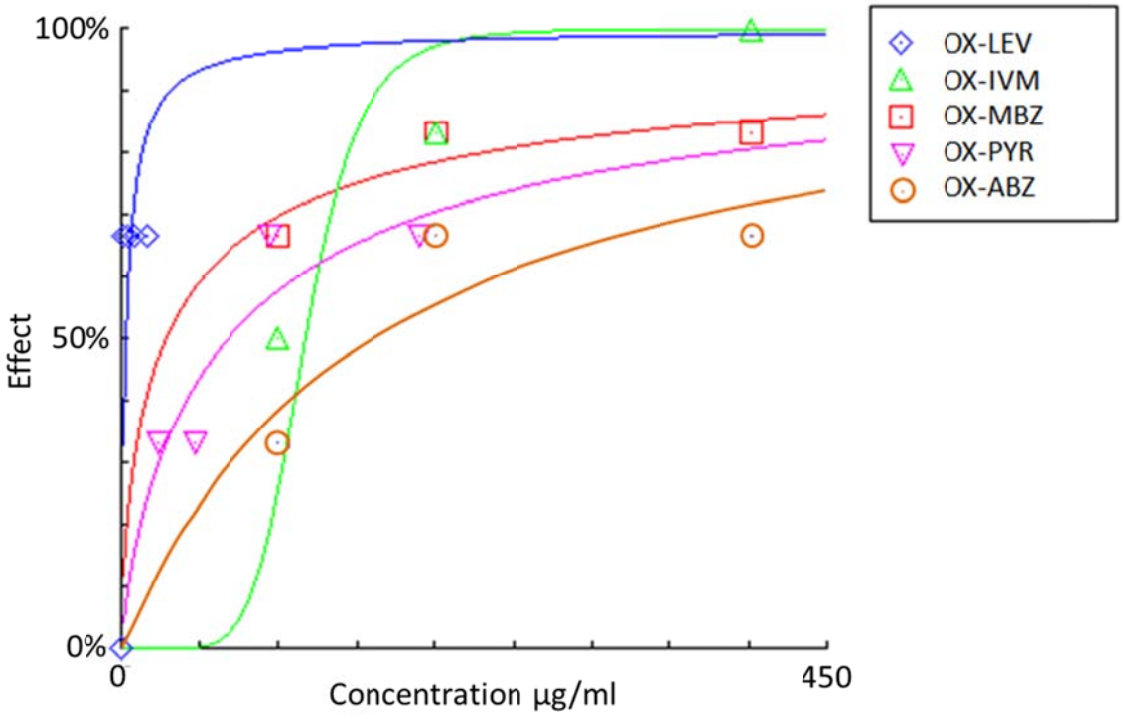
Figures and figure legends

Figure 1: Temporal effect of different concentrations of oxantel pamoate on the viability of *T. muris*.



T. muris were exposed concentrations of 0.15-600 µg/ml oxantel pamoate and examined 24, 48 and 72 hours post-incubation. Data derived from two independent experiments.

Figure 2: Dose response relationship of oxantel pamoate combinations against *T. muris in vitro*.



Oxantel pamoate-levamisole (blue line), oxantel pamoate-ivermectin (green line), oxantel pamoate-mebendazole (red line), oxantel pamoate-pyrantel pamoate (pink line) and oxantel pamoate-albendazole (orange line) were combined using ratios based on their IC_{50}s.

Table 1: *In vitro* activity of oxantel pamoate against *T. muris*, *A. ceylanicum* and *N. americanus.*

Drugs			**IC_{50} (r)**			
	***T. muris* L4**		***A. ceylanicum***		***N. americanus***	
	IC_{50} (r)	**Combination index (CI) at IC_{50}**	**L3**	**Adults**	**L3**	**Adults**
Oxantel	2.35 (0.68)	-	> 100 (n.d.)	> 100 (n.d.)	> 100 (n.d.)	11.80 (0.89)
Oxantel-Albendazole	159.61 (0.87)	0.37	-	-	-	-
Oxantel-Mebendazole	27.95 (0.87)	0.06	-	-	-	-
Oxantel-Levamisole	2.93 (0.99)	0.46	-	-	-	-
Oxantel-pyrantel pamoate	67.13 (0.90)	2.53	-	-	-	-
Oxantel-Ivermectin	116.86 (0.94)	0.27	-	-	-	-

IC_{50} median effect dose. r = linear correlation coefficient of the median-effect plot, indicating the goodness of fit. r ≥ 0.85 indicates a satisfactory fit. IC_{50}s of albendazole, mebendazole, levamisole, pyrantel pamoate, and ivermectin have been published elsewhere [23]. n.d. = not determined.

Table 2: Activity of oxantel pamoate monotherapy or combination chemotherapy against *T. muris in vivo*.

Group	Dose (mg/kg)	Mean number of worms (SD)	Mean number of expelled worms (SD)	Worm expulsion rate (%)	Worm burden reduction (%)	*P*-value	Combination index (CI)
Control 1	–	157.5 (62.6)	0.2 (0.5)	0.1	–	–	–
Control 2	–	93.3 (9.5)	0.8 (1.0)	0.8	–	–	–
Control 3	–	109.5 (32.9)	0.8 (0.5)	0.7	–	–	–
Control 8	–	56.5 (22.1)	0 (0)	0	–	–	–
Oxantel pamoate	10[1]	91.7 (46.1)	81.0 (44.0)	88.4	92.5	0.041[a]	–
	5[2]	80.3 (40.0)	62.8 (37.5)	78.2	81.1		
	2.5[2]	105.7 (58.5)	25.7 (16.0)	24.3	13.51		
	1[2]	148.3 (94.1)	2.3 (2.1)	1.5	0		
Albendazole	300[3]	69.0 (63.7)	5.8 (4.5)	8.3	41.8	0.293[a]	–
	75[1]	139.0 (54.9)	0.5 (1.0)	0.4	2.6		
Mebendazole	150[2]	99.8 (47.8)	70.3 (36.1)	70.4	68.1	0.006[a]	–
	75[1]	115.8 (36.5)	42.7 (18.6)	36.5	48.3		
Oxantel pamoate	10[8] i.p.	44.0 (18.7)	0 (0)	0	0	0.857[b]	–
Ivermectin	10[8] i.p.	80.7 (59.5)	77 (62.0)	95.5	93.5	0.057[b]	–
Control 4	–	123.3 (35.1)	0.3 (0.5)	0.2	–	–	–
Control 5	–	91.3 (23.7)	0 (0)	0	–	–	–
Control 6	–	78.3 (20.8)	0 (0)	0	–	–	–
Control 7	–	94.4 (39.2)	0 (0)	0	–	–	–
Control 8	–	56.5 (22.1)	0 (0)	0	–	–	–
Oxantel pamoate-albendazole	5+345[4]	105.0 (80.1)	76.3 (80.5)	72.6	76.6	0.529[a]	1.90
	2.5+172.5[8]	191.0 (123.4)	44.5 (45.4)	23.3	0		
Oxantel pamoate-mebendazole	5+79[4]	101.3 (39.8)	87.5 (27.6)	86.4	88.8	<0.001[a]	0.15
	2.5+39.5[4]	53.0 (27.8)	41.3 (28.6)	77.8	90.5		
	1.25+19.75[5]	128.8 (68.8)	107.5 (69.3)	83.5	76.7		
	0.63+10[6]	106.8 (49.9)	74.0 (42.7)	69.3	58.2		
Oxantel pamoate-ivermectin	5+4[4]	79.0 (20.8)	60.3 (14.5)	76.3	84.7	0.008[a]	1.27
	2.5+2[5]	116.7 (28.3)	59.7 (15.9)	51.1	37.5		
Oxantel pamoate-levamisole	5+46[7]	60.0 (40.2)	43.0 (30.5)	71.7	82.0	0.028[a]	1.27
	2.5+23[7]	90.0 (45.6)	28.0 (19.4)	31.1	34.3		

Numbers in superscript refer to the corresponding control group. [a]*P*-values were obtained from the Kruskal-Wallis test (several treatment doses vs. controls), [b]*P*-values were obtained from the Mann-Whitney U test (one treatment dose vs. controls). The CI at IC_{50} are based on WBR.

Chapter 7

Validation of candidate metabolic biomarkers across co-infection in murine models for malaria and hookworm disease

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Working manuscript

Validation of candidate metabolic biomarkers across co-infection in murine models for malaria and hookworm disease

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Abstract

Background: The prevalence of hookworm disease caused by *Ancylostoma duodenale* and *Necator americanus* and *Plasmodium falciparum*-induced malaria shows a large geographic overlap, in particular in sub-Saharan Africa. Robust, accurate, and highly sensitive diagnostic tools are required to detect even low parasitic burdens in both diseases, especially in low-transmission settings.

Methods: Murine hookworm (*Heligmosomoides bakeri*) and malaria (*Plasmodium berghei*) single and co-infection models (delayed and simultaneous) were established in NMRI outbred mice to study infection-related metabolic responses. Urine and plasma samples were collected from every mouse at 6 key time points over the course of the infection. The samples were analyzed using ^{1}H NMR spectroscopy and subsequent multi- and univariate statistical approaches, in order to reveal and identify differences between the infection groups, and to establish time trajectories within each infection. Identified candidate biomarkers were documented and related to the parasitological and physiological measures of the rodent models.

Findings and conclusion: A characteristic metabolic fingerprint has been found for each of the five infection scenarios assessed. Pipecolic acid, which has been identified previously as biomarker for *P. berghei* single infection has shown here to be not only specific to all *P. berghei* exposed groups but the compound remained to be present in significantly higher levels than in *P. berghei* unexposed groups, even in co-infection with a nematode worm. Pipecolic acid may hence represent a promising candidate for human malaria diagnostics and will be further validated.

1. Introduction

In 2010, *Plasmodium*-induced malaria accounted globally for about 216 million cases and more than 600,000 deaths mostly amongst children in sub-Saharan Africa. *P. falciparum* is the most deadly species, inflicting the greatest annual mortality burden of all parasitic diseases (WHO 2011). The two nematode worms *Necator americanus* and *Ancylostoma duodenale*, on the other hand, are the causative agents of hookworm disease, which represents the most frequent chronic tropical infection affecting an estimated 576-740 million people, whereby about 5 billion people are at risk worldwide (de Silva, Brooker et al. 2003; Bethony, Brooker et al. 2006; Pullan and Brooker 2012). One quarter of all school-aged children on the African continent (~45 million) are likely to harbor concomitant infections with *Plasmodium falciparum* malaria and a hookworm species at any given time (Brooker, Clements et al. 2006) due to the large geographical overlap of the parasite incidence. By affecting mostly the poor, each disease contributes to an entrenched cycle of ill-health, missing education in children, and declined work performance of affected adults. The burden of a co- or poly-infection, which represents the more common scenario in resource-poor settings, is often even more debilitating. Besides a frequently observed increase in anaemia caused by the co-existence of both parasites in the same host (Brooker, Akhwale et al. 2007), controversial reports exist on the interactions between *Plasmodium* and the nematodes in humans. Whereas hookworm infections were associated with *P. falciparum*-induced malaria in some field studies (Hillier, Booth et al. 2008; Midzi, Sangweme et al. 2008; Nacher 2011), no association was discovered by Shapiro and colleagues (Shapiro, Tukahebwa et al. 2005). Degarege and colleagues recently studied the nature of interaction between hookworms and *P. falciparum* malaria and found that light hookworm infections tend to decrease malaria parasitaemia, but a trend for heavy hookworm infections to increase the malaria parasitaemia (Degarege, Animut et al. 2009). Despite sometimes inconsistent findings, the overall trend shows a worsened malaria pathogenesis if a concomitant hookworm infection is present (Adegnika and Kremsner 2012). However, so far, little attention has been given to the hookworm-malaria interactions in the human host.

At present, the gold standard to diagnose any *Plasmodium* infection relies on microscopy. This is the only method to accurately quantify malaria parasite density, but the approach is time-consuming, prone to individual subjectivity and relies on blood samples obtained by finger prick (O'Meara, McKenzie et al. 2005; malERA 2011). Hookworm infections are detected by copromicroscopic methods, based on microscopical detection of helminth eggs in faecal material, whereby sensitivity is sub-optimal, and does not relate to the intensity of infection. Light and early infections may easily be missed, and multiple stool samples are often required (Knopp, Mgeni et al. 2008; Glinz, Silue et al. 2010). PCR-based techniques and antigen-based assays are often presented as alternative means for detection of many parasitic diseases (Knopp, Mohammed et al. 2010; Taniuchi, Verweij et al. 2011). However, although they may offer improved sensitivity and/or specificity, they present major drawbacks, including potential cross-reactivity, cost, high detection thresholds, the necessity of a laboratory off field, technology and substantial expertise (Neppert 1974; Bergquist, Johansen et al. 2009; malERA 2011).

Diagnostic means for detection of multiple infections at the same time (multiplexing) and application for large scale screenings in the field are strongly desirable. Ideally, diagnostic would be based on a sample matrix that can be obtained in a non-invasive way (e.g. urine or saliva). Such a tool would indeed greatly facilitate integrated control programs for multiple parasitic species.

Metabolic profiling has broadly been used to gain knowledge about host-parasite interactions, and to discover infection-related diagnostic markers. However, the approach has been substantially developed since the initial screening of urine samples for *Schistosoma mansoni*-infected mice and their uninfected counterparts. Eleven single infection rodent models have been reported so far, whereby each of them showed an infection-specific fingerprint in urine and/or plasma, our most commonly characterized biological matrices in the rodent (Li, Wang et al. 2008; Saric, Li et al. 2009; Wang, Xiao et al. 2009). Although efforts have been made recently for describing metabolic effects in humans

(Denery, Nunes et al. 2010; Balog, Meissner et al. 2011), the biggest challenge in human biomarker discovery remains the high prevalence of co- and multiple infections in low income countries. In order to predict the metabolic fingerprint of a co- or poly-infection it is necessary to gain more understanding on the effect of parasite co-existence to the host metabolism.

The goal of the present work was to investigate the metabolic effects of a malaria-hookworm co-infection in the murine host in a laboratory-controlled experiment, and to identify candidate biomarkers that overlap between single and each co-infection. Mice underwent either single-infection with *P. berghei* or the mouse hookworm *Heligmosomoides bakeri,* co-infection with both parasites in a simultaneous or delayed manner, or they remained uninfected and served as control group. Plasma and urine samples were collected one day pre-infection and five times during the course of infection in order to capture acute and chronic effects and evaluate the consistency of the candidate metabolic markers over both phases. Samples were acquired by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and analyzed using multivariate and univariate statistical approaches.

2. Materials and methods

2.1. Animals and parasites

Forty 3-week-old female NMRI mice were purchased from Charles River (Sulzfeld, Germany) and kept in groups of 8 in macrolon cages under standard environmentally-controlled conditions (temperature: 25°C, humidity: 70%, light/dark cycle 12/12 h). Mice had access to water and rodent food *ad libitum* (Rodent Blox from Eberle NAFAG, Gossau, Switzerland) and were allowed to acclimatize in the animal facility of the Swiss Tropical and Public Health Institute (Swiss TPH) for one week before the first sampling was conducted. The current work was approved by the local and national regulations of laboratory animal welfare (permission no. 2081). Mice were tagged 2 days prior to infection.

2.2. Study design

Each group of 8 mice was allocated to a different infection schedule (See Figure 1). On day 0, groups H and CD received 80 infective *H. bakeri* L3 larvae which were administered orally in 150 µl water. Details on the life cycle of *H. bakeri,* maintained at the institute since 2009 have recently been described (Nwosu, Vargas et al. 2011).

Three groups of mice received 2 x 10^7 erythrocytes, parasitized with the GFP-transfected *P. berghei* ANKA strain in 0.2 ml intravenously (Franke-Fayard, Trueman et al. 2004), on day 15, resulting in *P. berghei* single infection (Group P), delayed co-infection (Group CD) and simultaneous co-infection (Group CS), whereby the latter received additionally *H. bakeri* at the same time. Group C remained uninfected and served as control. All mice were sacrificed by spinal dislocation on day 19.

2.3. Biofluid collection, weight and PCV determination

Urine and blood were collected one day before infection and 1, 8, 14, 16 and 19 days after experiment start (day 0), always between 8 and 11 AM (Figure 1).

Mice were individually placed into empty cages and monitored until they released a minimum of 40 µl urine which was immediately collected into 1.5 ml Eppendorf tubes and frozen over dry ice. All samples were stored at -80°C prior to ^{1}H NMR acquisition.

Approximately 50 µl tail blood was sampled from each mouse into a Na-heparinized hematocrit tube (1.55 mm Ø, BRAND GMBH + CO KG; Wertheim, Germany) and centrifuged at 11,000 x rpm for 5 minutes (microcentrifuge Sigma 1-15). The ratio of plasma to red blood cells was determined by measuring packed cell volume (PCV) and the whole blood volume (in mm). The plasma fraction was transferred into 1.5 ml Eppendorf tubes and stored at -80°C. On day 19, a heart puncture was performed after euthanasia of the animals by spinal dislocation. On each sampling day, mice were monitored for weight, to the nearest 0.05 g.

For weight and PCV, intergroup median variation was analyzed using the Mann-Whitney U test with Bonferroni correction in StatsDirect (version 2.4.5; StatsDirect Ltd; Cheshire, UK), with a significance level of 5%.

2.4. Worm burden and parasitemia

The worm burden was determined upon dissection of the intestine and manual counting of adult worms, whereas the parasitemia in the *P. berghei* infections was evaluated by FACS analysis (FACScan, Becton Dickinson, Basel, Switzerland) on day 19, by counting 100,000 erythrocytes (Franke-Fayard, Trueman et al. 2004). The worm burden in groups H and CD was compared using the Mann-Whitney U test (StatsDirect). Eventual intergroup variation in median blood parasitemia was evaluated using the Mann-Whitney U test with Bonferroni correction.

2.5. Sample preparation and ^{1}H NMR spectroscopic analysis

All samples were sent to Imperial College London on dry ice for ^{1}H NMR-based analysis. Urine samples were prepared by mixing 30 µl phosphate buffer (43.8 mM NaH_2PO_4 and ≈ 0.2 M Na_2HPO_4, 70% D_2O v/v, 0.1% sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 pH = 7.4) with 30 µl urine and centrifuged 5 minutes at 13,000 rpm (microcentrifuge Sigma 1-14). Fifty-five µl of the sample were transferred into NMR microtubes (Bruker, 1.7 mm Ø), and stored at 4°C prior to data acquisition.

Plasma samples were prepared by mixing 25 µl plasma with 30 µl NaCl buffer (0.9 % NaCl, 10% D_2O v/v, pH = 7.4) in Eppendorf tubes and centrifuged 5 minutes at 13,000 rpm. A volume of 50 µl was transferred into NMR microtubes (Bruker, 1.7 mm Ø) shortly before measurement, and stored at 4°C prior to spectral acquisition.

A standard ^{1}H NMR spectrum was acquired from each individual sample on a Bruker 600 DRX MHz spectrometer (Rheinstetten, Germany), in a one-dimensional (1D) NOESY (nuclear overhauser effect spectroscopy) experiment, using the standard solvent suppression pulse delay [recycle delay (RD)-90°-t_1-90°-t_m-90°-acquire free induction decay (FID)] (Nicholson, Foxall et al. 1995). A constant temperature of 300 K was maintained during spectral acquisition. For plasma samples, a second set of data was acquired, using a 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse [RD-90°-(τ-180°-τ)$_n$] sequence (Meiboom 1958). The samples were scanned 256 times in each experiment.

2.6. Data reduction, multivariate analysis and metabolic trajectories

All ^{1}H NMR spectra were manually phased and baseline-corrected in Topspin (Version 3.1, Bruker). Plasma spectra were referenced to lactate at δ 1.33, whereas urine samples were adjusted to the TSP peak at δ 0.00. The complete spectra from δ 0.5-9 were imported into MATLAB (Version 7.12.0, R2011a) and the water peak region was removed in all spectra (δ 4.18-5.77). Since signals from ethanol and methanol were detected in plasma which may represent potential contaminations, the corresponding spectral regions (δ 1.15-1.22, 3.64-3.69 and 3.355-3.375, in plasma NOESY spectra) were additionally removed to not bias the data modeling process. Spectral pre-processing further included median-fold normalization and peak alignment using in-house developed scripts (Veselkov, Vingara et al. 2011).

In plasma spectra, only the aliphatic region was included in the multivariate modeling in order to minimize the impact of the water related baseline distortion and to facilitate preprocessing of the whole data set, in particular the process of phasing and baseline alignment (δ 0.5-4.6). However, the group discriminatory power of formate, which was the only peak found between the water peak region and δ 9 was tested with the Mann-Whitney U test (StatsDirect).

Orthogonal projection to latent structure discriminant analysis (O-PLS-DA) (Trygg and Wold 2002; Cloarec, Dumas et al. 2005) was applied to compare ^{1}H NMR spectral data between

the different murine infection groups and identify the discriminating compounds (biomarkers). Metabolic trajectories based on principal components analysis (PCA) were constructed for urine as well as for both plasma data sets (NOESY and CPMG). The mean position in the principal component (PC) scores plot was therefore taken for each infection group and at each time point separately, as described before (Saric, Li et al. 2008). In a next step, the coordinates were connected chronologically to establish any systematic change in the global metabolic composition over time, expressed by the 6 sampling days (Figure 4).

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2.7. Metabolite Identification

Metabolite identity was determined using the literature (Bell, Brown et al. 1987; Nicholson, Foxall et al. 1995; Liu, Nicholson et al. 1997; Coen, Lenz et al. 2003; Tang, Wang et al. 2004; Li, Wang et al. 2008), statistical total correlation spectroscopy (STOCSY) (Cloarec, Dumas et al. 2005), and the software Chenomx Profiler (Chenomx NMR Suite, 7.1, evaluation version). Assignments in urine were additionally confirmed with two-dimensional (2D) ^{1}H-^{1}H correlation spectroscopy (COSY), with the parameters described previously (Bax and Davis 1985; Hurd 1990).

One sample with high relative signals between 2.0-2.1 ppm was selected for conducting an enzymatic digestion with β-galactosidase (Jack Bean, Sigma) in order to confirm the nature of the glycoprotein. A baseline sample was therefore acquired and compared to the sample immediately after addition of 0.5 U of the enzyme (Bell, Brown et al. 1987) and during the next 20 h window. The assay was run on a 400 MHz spectrometer at an ambient temperature of 300 K and at a pH of ~ 7.4. The enzyme addition resulted in cleaving off of the acetyl group resulting in a marked increase of acetate and a subsequent decrease of the 2.04 signal proving signal contribution from an *N*-acetyl glycoprotein, whereby α_1-acid glycoprotein is likely to be the main contributor.

2.8. Validation of the candidate biomarkers

Candidate biomarkers identified in O-PLS-DA ($p < 0.05$) were further validated by univariate analysis. The area under the curve (AUC) was extracted from the clearest signal for each metabolite found significant *via* O-PLS-DA analysis. Variance analysis between the mean values of the groups was performed using a Mann-Whitney U test with Bonferroni correction, with a significance level of 5%. Peaks that were found to be statistically insignificant according to the univariate analysis (Mann-Whitney U test) were removed unless they belonged to top 5 correlation coefficients extracted by multivariate (O-PLS-DA) analysis. All identified peaks that showed significance in both analysis sets were documented (Tables 1 and 2).

3. Results

3.1. Parasitology

Average worm counts of 42.38 (+/- 17.69) and 61.88 (+/- 18.37) were found in groups H and CD, respectively, but the difference was not statistically significant ($p = 0.0531$). The worm burden could not be established in the simultaneous co-infection, since larvae were too small and embedded in the intestinal mucosa.

Blood parasitemia of 39.5%, 35.3% and 37.1% were determined for the groups P, CD and CS, respectively. They did not significantly differ in any of the pairwise comparisons between the 3 *P. berghei*-infected groups.

3.2. Weight and PCV

No significant weight variation could be identified between groups at any of the time points assessed. PCV values, on the other hand, were found significantly lower in group P

compared to group H (p = 0.012), and group CS respectively (p = 0.019) one day before infection. However, this was no longer the case on day 1 post infection. On day 16 only, the mean PCV was found lower in group P than in group CD (p = 0.04). On day 19, mice with a *P. berghei* single infection presented a significantly lower average PCV value (37.68%) compared to animals harboring a *H. bakeri* single infection or control mice (52.60% and 51.90%, p = 0.003 and 0.006, respectively). Delayed and simultaneously co-infected mice presented the same feature over helminth-infected or control mice, with average PCV values of 28.46% and 34.29% (all $p \leq 0.003$). Mean PCV values did not vary significantly between both co-infection groups, nor between *P. berghei*-infected mice and both co-infection groups. Infection with *H. bakeri* alone did not lead to a decreased PCV compared to the uninfected group.

3.3. Urine and plasma ^{1}H NMR spectral profiles of *H. bakeri*, *P. berghei* single and co-infected mice

Figure 2 depicts representative 600 MHz ^{1}H NMR mouse urine spectra from *P. berghei* single infection (A), the delayed co-infection (B), and a *H. bakeri* single infection (C) on day 19. Urine spectra were characterized by the presence of 3-oxoisovalerate, 2-oxoisocaproate, creatine, hippurate, and formate, and a variety of amino acids, such as leucine and alanine. Two unknown metabolites (UK 1: d 1.21; d 1.24 and UK 2: t 6.05; d 6.3; d 7.4) were present in both the *P. berghei* infection and in the delayed co-infection (number 16 and 17, Table 1), but not the single *H. bakeri* infection. *P. berghei*-infected groups presented also elevated levels of 2-oxoisocaproate, trimethylamine and pipecolic acid. Infection with *H. bakeri* (single and delayed co-infection) was mostly characterized by elevated hippurate levels. Figure 3 shows the typical spectral profile of a delayed co-infection plasma sample (NOESY, day 19). Plasma spectra showed the presence of several lipids, amino acids (leucine, isoleucine, valine, alanine and glycine), lactate, choline species as well as glucose. In *P. berghei*

infections (both single and delayed co-infections), an accumulation of lactate was typically observed, whereas increased glucose levels were noticed in *H. bakeri*-infected groups.

3.4. Multivariate data analysis

All infection groups were pairwise compared with each other, at all time points separately using O-PLS-DA within each urine and plasma data set. Candidate metabolic markers that have been found to be discriminatory between two given infection groups are presented in detail in Tables 1 and 2 (days 16 and 19 post infection, respectively). In brief, on day 16, urine samples indicated few metabolic changes (Table 1) including decreased hippurate levels in the *H. bakeri* single infection compared to uninfected controls and the delayed co-infection, and increase in acetate in the simultaneous co-infection compared to controls. However, on day 19, a significant increase in pipecolic acid was detected in *P. berghei*-infected animals compared to the *H. bakeri* single infection and the control group, whereby the delayed co-infection additionally revealed relatively higher levels than the *P. berghei* mono-infection. An accumulation in the unknown metabolites UK 1 and UK 2 (d 1.21; d 1.24 and t 6.05; d 6.3; d 7.4; d 7.56) was furthermore observed in all *P. berghei*-infected groups on days 16 and 19. Succinate on the other hand showed depletion upon *P. berghei* exposure as revealed by comparison between all *P. berghei* positive groups and the uninfected control group. On day 19, an increase in 2-oxoisocaproate appeared in the *P. berghei* mono-infection group and the simultaneous co-infected animals compared to all other groups. Increases in creatine and 2-oxoisovalerate characterized the simultaneous co-infection, however, not significantly when compared to the delayed co-infection. An increase in acetate was observed in the delayed co-infection compared to the *P. berghei* single infection.

Plasma spectra obtained by CPMG and NOESY pulse programs revealed a greater amount of information whereby the trends were more pronounced on day 19 (Table 2). On day 16,

glucose was found decreased in *P. berghei* mono-infected animals compared to all other groups. Interestingly, this association became insignificant on day 19. In the simultaneous co-infection model the same trend was confirmed, i.e. lower glucose levels in presence of *Plasmodium*, on day 19 only. Consequently on day 19, a significant increase in lactate was observed in every *P. berghei*-infected group, without significant difference between them.

Observed on day 16 only, a decreased level in the amino acid lysine characterized the *P. berghei* infections in single and delayed co-infection, whereas relatively increased levels were found in the *H. bakeri* mono-infection and the simultaneous co-infection, when compared to uninfected controls. Other amino acids such as leucine, valine and alanine were found augmented in *P. berghei* single and co-infections when compared to uninfected controls. On day 16, a decrease in glycerophosphocholine (GPC) was observed in *P. berghei*-infected animals and in the *H. bakeri* single infection group compared to uninfected controls. The same trend was still present but appeared less pronounced on day 19. The lipid metabolism was markedly affected on day 19, with a trend of accumulation of several lipid fractions in *P. berghei* single and co-infections. Pyruvate (tentatively assessed) characterized the simultaneous co-infection versus the *H. bakeri*-infected group and uninfected animals.

3.5. Time trajectory analysis

Metabolic time trajectories of each infection group are represented in Figure 4, based on spectra derived from urine (A), plasma acquired *via* NOESY (B) and plasma acquired applying a CPMG pulse program (C). Stars of the same color indicate significant differences between time points. The plasma CPMG data set revealed a general fundamental difference of day 14 compared to other time points in the uninfected control (C5) and simultaneous co-infection (C4) groups (where no infection was induced before day 15). In plasma samples, a general metabolic impact inflicted by *P. berghei* infections was observed between day 14 or 16 and day 19, reflecting the quick evolution of the infection, especially in the mono-infection and simultaneous co-infections. In both urine and plasma, *H. bakeri* infection was

characterized by significant movements between the early time points (day -1 and 1) and late time points (day 14 onwards). No significant movement was noted between all time points for *P. berghei* single infection in urine (A1) for the uninfected controls in plasma using the NOESY pulse program (B5) and for the *H. bakeri* single infection in plasma using the CPMG pulse program (C2). The uninfected control group showed the largest amount of significant changes in urine (A5), as well as the *P. berghei* single infection in plasma CPMG (A1).

4. Discussion

We have, for the first time, established a co-infection model of murine malaria and hookworm disease to study the impact of concurrent infections on the host metabolism. The 6 sampling time points were strategically set one day before and after each infection day. The *H. bakeri* infection is considered established after 14 days, when adult worms are present in the gut, whereas the evolution of *P. berghei* infections in NMRI mice is very quick (mice start dying from 5 days post-infection). The experiment was therefore terminated 4 days after exposure to the malaria parasite i.e. on day 19 of the experiment. Days 16 and 19 revealed the most information regarding metabolic changes. Pairwise comparison of each infection scenario with the uninfected control group revealed that the *P. berghei*-infected mice (single as well as co-infected groups) induced most metabolic changes in the rodent host. As shown by the time trajectories (Figure 4), most of the overall metabolic changes in the *H. bakeri* single infection group occurred on day 19, compared to the first 2 sampling time points, which may be reflective of the slow and chronic progress of the disease. In plasma from *P. berghei*-infected animals perturbed metabolic profiles were observed after infection (on day 15) compared to the early sampling time points, but also between day 16 and day 19, reflecting the rapid development of the malaria infection. More biomarkers were found in urine on day 19 than on day 16, whereby on day 19, elevated levels of pipecolic acid were detected in all *P. berghei*-infected groups, as reported earlier (Li, Wang et al. 2008). The compound can

accumulate due to a number of consequences of malaria infection such as liver dysfunction, disturbance of the gut microbiota, and neurological damage (Li, Wang et al. 2008). Pipecolic acid has also been described in human *P. vivax* infections and may represent a unique biomarker for *Plasmodium* (Sengupta, Ghosh et al. 2011). Pipecolic acid is therefore of great interest regarding diagnostics of *Plasmodium* spp.

The depletion of plasma glucose was one of the main findings in the *P. berghei* single infection group on day 16, while this effect appeared with delay in the simultaneous co-infection group, on day 19. As already discussed in detail by Li and colleagues (Li, Wang et al. 2008), *Plasmodium*-infected erythrocytes consume higher amounts of glucose than normal, as the parasites rely on anaerobic glycolysis (combined to reductive and oxidative Krebs cycle pathways) to obtain energy. Consequently, prominent lactate peaks appeared on day 19, in all *P. berghei*-infected groups. This is consistent with the exacerbated glucose consumption observed, since lactate is a waste product of the anaerobic glycolysis. In severe *P. falciparum* malaria, lactate is a death predictor, which accumulation can lead to cardiac impairment (Agbenyega, Angus et al. 2000).

In a previous *N. americanus* hamster model, blood sugars tended to be higher than in uninfected controls (Kaul, Talwalker et al. 1982). Malabsorption of sugars has been demonstrated in hookworm-infected patients (Falaiye, Oladapo et al. 1974). However, the concentration of blood glucose was found decreased in a *N. americanus* hamster model for metabolic profiling (Wang, Xiao et al. 2009). Here, we used an NMRI mouse background and another hookworm species. Our results suggest that an established *H. bakeri*-infection compensates at least in part the *P. berghei*-induced plasma glucose depletion. In the simultaneous co-infection, the presence of hookworm larvae delayed glucose depletion. A significant increase in glucose level was observed in *H. bakeri*-infected animals compared to uninfected controls at the experimental day 8.

Plasma GPC decreased in all *P. berghei* single and co-infections compared to the mouse groups absent of the protozoan parasite, a finding that is in accordance with the results of Li

and colleagues (Li, Wang et al. 2008). This effect was in the current experiment more pronounced on day 16 than on day 19. The breakdown of GPC into choline has been shown necessary for uptake by the infected erythrocyte and transformation to phosphatidylcholine, the most abundant membrane phospholipid in *P. falciparum* (Ancelin and Vial 1986; Lehane, Saliba et al. 2004; Dechamps, Maynadier et al. 2010).

BOX 1

- *Specificity to infection*
- *Stability over time*
- *Reproducibility*
- *Stability across co- and multiple infections*
- *Transferability across host species, human particularly*
- *Stability across infection intensity*

Since the delayed co-infection is reflective of the usual "real-life" scenario, i.e. underlying chronic helminth infection and occasional episodes of malaria, the main goal here was to identify valuable discriminators between each single infection and this particular co-infection scenario. The consequent overlap between metabolic profiles of these two diseases allowed isolating 3 metabolites from urine, namely pipecolic acid and the 2 unknowns UK 1 and UK 2 which were related to the presence of *P. berghei*. For the hookworm single infection, a decrease in hippurate could be identified in urine on day 16. In plasma, increases in lactate and decreases in GPC were the most consistent changes following *P. berghei* infection. However, an accumulation of GPC was also observed in relation to the *H. bakeri* infection and therefore, should not be selected as candidate diagnostic biomarker. In the same way, the glucose metabolism was perturbed by both infections, disease-specificity is lacking. Ideally, a combination of infection-specific biomarkers should be selected as candidates for diagnostics. A set of validation criteria for candidate diagnostic biomarkers has been defined (Box 1), whereby deeper investigations on infection specificity, temporal stability, co-infection, and human transferability have already been conducted.

In the time trajectories (Figure 2), an overall aging effect at the small molecular weight level but not on lipidic larger molecular weight molecules has been observed in urine and plasma

(CPMG pulse program) as indicated by significant changes in the uninfected control group (Figure 2, A5 and C5, respectively). Since plasma analysis with the NOESY pulse program includes more pronounced signals from larger molecular weight compounds compared to CPMG, this suggests that the aging effect is mainly due to changes in small molecules. Both plasma data sets from *P. berghei*-infected mice were in agreement, whereby the main differences were noticed between day 1 and day 16 and between day 16 and day 19, correlating with the course of the infection and rapid health decline, whereas urine did not reflect any infection-related metabolic effect at all. The differences between day 14 and day 19 observed in the delayed co-infection group (Figure 2, A3, B3 and C3) are consistent with the fact that the superinfection with the malaria parasite occurred on day 15.

Altogether, our results largely agree with previous work on metabolic profiling of a *P. berghei* infection in NMRI mice, however, a few trends were not reproduced in the present investigation. For instance, we found no urinary creatinine and not the same unknown metabolites as did Li and colleagues (Li, Wang et al. 2008). In our study, the contribution of 2-oxoisocaproate and 2-oxoisovalerate appeared later than 1 day post infection with *P. berghei*. Interestingly, in our results, only few traits were recognized in the urinary and plasma metabolic profiles of previous *N. americanus* hamster models, whereby both the host and the infectious agent differ. A hookworm-driven decrease in urinary hippurate levels had already been observed in correlation with *N. americanus* infection (Wang, Xiao et al. 2009; Wu, Holmes et al. 2010) as well as in trematodiases models (Saric, Li et al. 2008; Li, Holmes et al. 2009). Hippurate is a gut microbiotal-related metabolite (Nicholls, Mortishire-Smith et al. 2003; Wu, Holmes et al. 2010). The metabolite 2-aminoadipate had been the most discriminant element in urine (Wang, Xiao et al. 2009), while it was absent in our samples. *N. americanus* induces anemia (Xue, Hui-Qing et al. 2005). Whether *H. bakeri* can cause anemia was not known (Knowles 2011), but our findings suggest that it is not the case (no significant difference in PCV between *H. bakeri* and uninfected groups) and would constitute a major difference between the two models. No general pattern for hookworms could be

identified, emphasizing that the metabolic response is strongly specific, with regard to host- and infective agent-specificity.

Worm counts did not significantly vary in the presence of the *P. berghei* superinfection. In other co-infection murine models (C57Bl/6 background) between *P. berghei* and *H. bakeri*, no interaction was found between the pathogens and the development of cerebral malaria was normal (de Souza and Helmby 2008), or the *Plasmodium* parasitemia was exacerbated without changing the immunopathogenesis in cerebral malaria development (Tetsutani, Ishiwata et al. 2008). A meta-analysis of murine malaria-helminthiases co-infection models revealed a large heterogeneity of reported interactions (Knowles 2011). The authors suggested a dual role for helminth infections in malaria disease: in models of resolving malaria, the presence of the worm leads to increased mortality as the host fails to clear *Plasmodium*, and in lethal models, helminths tend to reduce host mortality (Knowles 2011).

In the present work, no interaction has been found. We suggest that a hookworm model where no anemia is induced is not representative of the actual hookworm symptoms in humans, and therefore not representative of the stress imposed (Roche and Layrisse 1966; Stoltzfus, Dreyfuss et al. 1997).

In conclusion, we found characteristic metabolic changes in the NMRI mouse urinary and plasma metabolic profiles due to single or co-infection with *P. berghei* and *H. bakeri*, reflecting the complex metabolic host-parasites cross-talk. In addition, we showed the capacity of ^{1}H NMR-spectroscopy coupled with uni- and multivariate statistics to trace concurrent parasitic infections and highlight an inherent potential as diagnostic tool. Two unknown urinary metabolites have been found in correlation with *P. berghei* infection. In addition, we confirmed the presence of urinary pipecolic acid in *P. berghei* single and co-infections, hence this metabolite might become a candidate diagnostic biomarker. The established helminth infection compensated for the plasma glucose depletion due to the malaria infection. No specific candidate biomarker was found for *H. bakeri*, most likely since this infection does not induce much physiological stress (i.e. due to anemia). Although no

interaction has been found between *P. berghei* and *H. bakeri* regarding the parasitemia and worm counts, this work highlights that the complexity and specificity of host-parasite interactions can still be reflected on the metabolic level.

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Figure 1. Study design

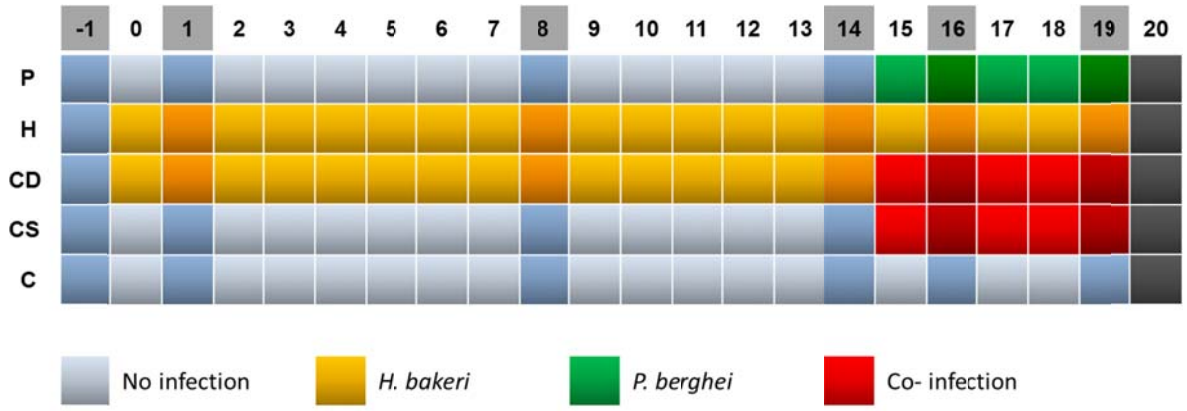

Figure 1: Urine and plasma were collected from all mice (5 groups, n = 8) one day before and 1, 8, 14, 16 and 19 days after experiment start (day 0). The darker cells represent sampling days. P: *P. berghei* only; H: *H. bakeri* only; CD: delayed co-infection (superinfection); CS: simultaneous co-infection; C: uninfected control.

Figure 2: Representative urine spectra.

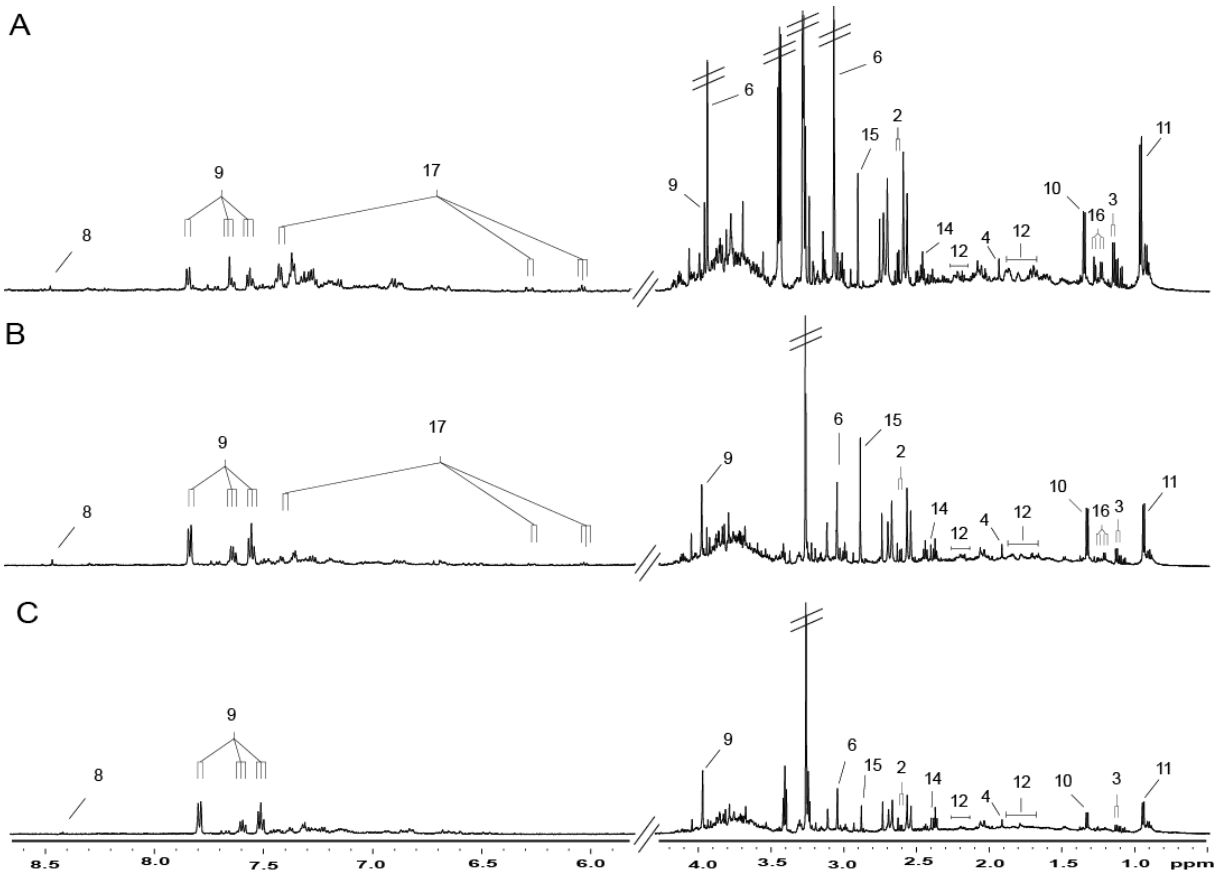

Figure 2: A = *P. berghei* single infection; B = delayed co-infection; C = *H. bakeri* single infection. The key to metabolite identity is the one indicated in Table 1. The water peak region and high peaks were cut out and indicated by double bars.

Figure 3: Representative plasma spectra.

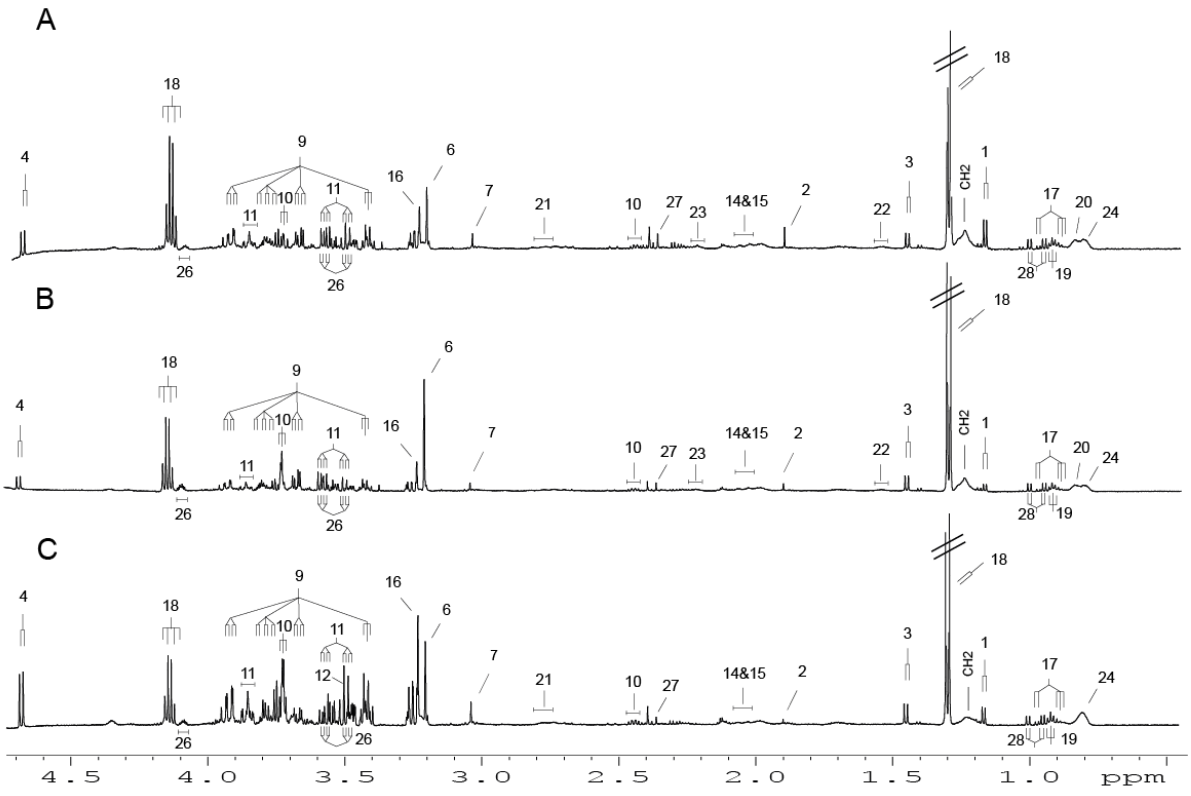

Figure 3: A = *P. berghei* single infection; B = delayed co-infection; C = *H. bakeri* single infection. The key to metabolite identity is the one indicated in Table 2. The aromatic region (δ 4.7-9), little informative, was removed. High peaks were cut out and indicated by double bars.

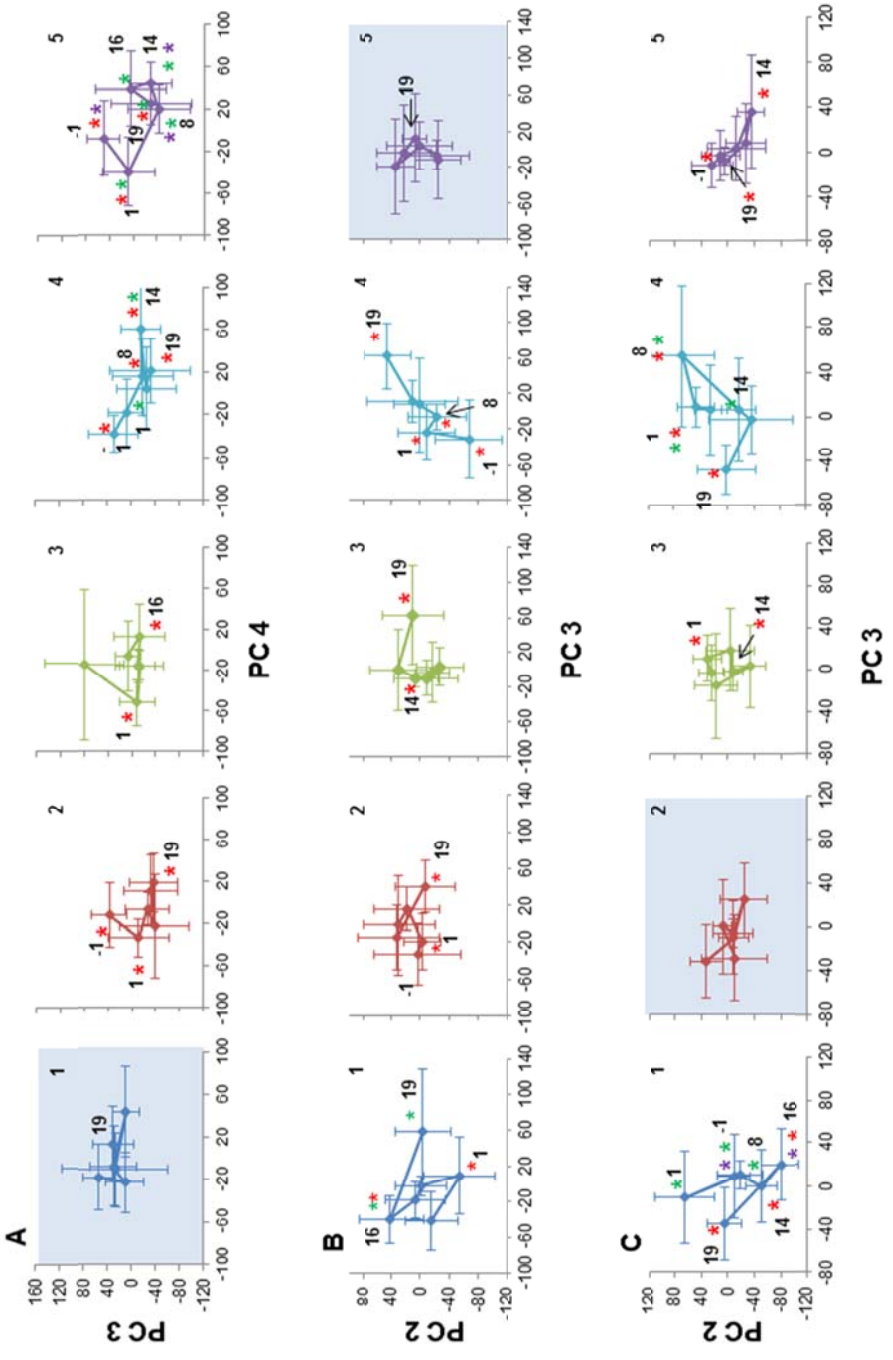
A
B
C
PC 3
PC 2
PC 4
PC 3
PC 2
PC 3

Table 1. List of metabolites found in urine.

URINE

No.	Metabolite	δ ^{1}H (multiplicity)	Day 16										Day 19									
			P/H	P/CD	P/C	P/CS	H/CD	H/C	H/CS	CD/C	CD/CS*	C/CS	P/H	P/CD	P/C	P/CS	H/CD	H/C*	H/CS	CD/C	CD/CS	C/CS
1	1-methylnicotinamide TA	t 8.19/d 8.9/d 8.97			↗																	
2	2-oxoisocaproate	d 2.62											↗	↗					↘			↘
3	2-oxoisovalerate TA	d 1.13																	↘			↘
4	acetate	s 1.91										↘		↘								
5	cholesterol TA	m 0.66/m 0.79/m 0.83	↘														↗					
6	creatine	s 3.05/s 3.93																	↘			↘
7	dimethylurea TA	s 2.69										↗										
8	formate	s 8.46																		↗	↗	
9	hippurate	s 3.97/t 7.55/t 7.64/d 7.84					↘	↘														
10	lactate	d 1.33/q 4.11															↘					
11	leucine	t 0.96																				
12	pipecolic acid	m 1.68/m 1.85/ m 2.22/m 3.03											↗		↗	↘			↘	↗		↘
13	scyllo-inositol	s 3.36												↗								
14	succinate	s 2.41													↘					↘		↗
15	trimethylamine	s 2.88																		↗		
16	unknown 1	d 1.21/d 1.24	↗		↗		↘		↘	↗		↘	↗		↗		↘			↗		↘
17	unknown 2	t 6.05/d 6.3/d 7.4/d 7.56	↗		↗		↘		↘	↗		↘					↘					

Table 1: Arrows indicate changes in the first group cited compared to the second (i.e. P in P/H). P: *P. berghei* only; H: *H. bakeri* only; CD: delayed co-infection (superinfection); CS: simultaneous co-infection; C: uninfected control. s = singlet; d = doublet; t = triplet; q = quadruplet; m = multiplet. TA: tentatively assigned. * Bad model.

Table 2. List of metabolites found in plasma

PLASMA																					
No.	**Metabolite**	**δ ^{1}H (multiplicity)**	**Day 16**										**Day 19**									
			P/H	P/CD	P/C	P/CS	H/CD	H/C	H/CS	CD/C	CD/CS	C/CS	P/H	P/CD*	P/C	P/CS	H/CD	H/C	H/CS	CD/C	CD/CS	C/CS
1	3-hydroxybutyrate	d 1.21/m 2.32						↘					↘		↘		↗			↘		
2	acetate	s 1.91																				
3	alanine	d 1.48	↘		↘										↗					↗		↘
4	beta-glucose	d 4.65	↘	↘	↘	↘																↗
5	cholesterol TA	m 0.91		↘									↘									
6	choline	s 3.20/m 3.52	↘		↘	↘									↗	↗						
7	creatine	s 3.05/s 3.93																				
8	formate	s 8.46			↘					↘												
9	glucose	t 3.42/dd 3.54/t 3.71/dd 3.72/ddd 3.83/m 3.84/dd 3.91	↘	↘	↘	↘													↗		↗	↗
10	glutamine	m 2.09/m 2.45/t 3.78																		↗		↗
11	glycerol	dd 3.56/dd3.64/m 3.87						↘	↘		↘				↗			↗				↘
12	glycine	s 3.56				↘									↗			↗		↗		
13	glyceryl of lipids TA	m 4.26		↗															↘			↘
14	glycoprotein	m 2.08	↗		↗																	
15	glycoprotein	m 2.05																	↘			↘
16	GPC	s 3.23/m 4.3	↘		↘	↘	↗	↘	↗	↘	↘	↗	↘		↘				↗	↘		↗
17	isoleucine	t 0.93/d 1.01	↘				↘											↗				
18	lactate	d 1.33/q 4.11		↘									↗		↗		↘		↘	↗		↘
19	leucine	t 0.96													↗					↗		↘
20	lipid (CH3)	m 0.88	↗					↘														
21	lipid (CH2**CH2**CH)	m 2.79														↘	↘		↘		↘	
22	lipid (**CH2**CH2CO)	m 1.57															↘		↘			↘
23	lipid (CH2**CH2**CO)	m 2.23		↗	↗								↘		↗		↘		↘	↗		↘

24	lipid VLDL (**CH3**CH2CH2C=)	t 0.87	↗					↘														
25	lysine	t 3.03	↘		↘		↗	↗				↘										
26	myo-inositol	dd 3.56/dd 3.63/t 4.06	↘																			
27	pyruvate (TA)	s 2.38																	↘			↘
28	valine	d 0.99/d 1.04	↘												↗					↗		↘

Table 2: Arrows indicate changes in the first group cited compared to the second (i.e. P in P/H). P: *P. berghei* only; H: *H. bakeri* only; CD: delayed co-infection (superinfection); CS: simultaneous co-infection; C: uninfected control. s = singlet; d = doublet; t = triplet; q = quadruplet; m = multiplet. TA: tentatively assigned. * Bad model. GCP = glycerophosphocholine.

Chapter 8

Discussion

8. Discussion

8.1. Background, objectives and key findings of the thesis

Infections with soil-transmitted helminths belong to the 13 most neglected tropical diseases (NTDs) [1]. An infectious disease is considered “neglected” when effective, affordable and user-friendly drugs are lacking. Since these diseases affect the poorest people in developing countries, who cannot afford drugs, the pharmaceutical companies have largely ignored these diseases, putting an emphasis on profitable drugs [2]. Note that malaria, HIV and tuberculosis, all presenting market opportunities in Western countries, were no longer considered most neglected after the formation of public-private partnerships (PPPs) and substantial investments in drug research and development. However, most regrettably, the 13 most neglected bacterial and parasitic diseases represent an insufficient incentive for the pharmaceutical industry to invest in drug research and development (R&D), as they have absolutely no rewards potential [2]. Fortunately, for some of these diseases a few PPPs came into play, combining industry and the public sector thus giving rise to several new drug R&D projects for NTDs [3].
Chemotherapy is still the most significant tool to control STHs. Given the restrained panel of “unsatisfactory” drugs available, and the potential emergence of resistance, the situation is considered precarious and there is an urgent need for concerted efforts to seek and develop new treatment options. Drug discovery and development “from scratch” is a complex, expensive, long and risky process and is strongly regulated. The clinical development phase is the most expensive step and requires solid preclinical data acting as filters before pursuing the endeavor [4,5].

Preceding this project, a number of compounds were approved for human or veterinary medicine, showing anthelmintic activity and favorable prerequisites (safety, broad-spectrum, affordable and possible administration in single dose) [5,6,7]. As these selected drugs had already undergone extensive testing, they are potentially amenable to rapid development, to be available on the market in a few years.
In the present work, once I had set up these nematode models at the Swiss TPH a series of alternative *in vitro* drug sensitivity assays in use for other organisms have been tested in an attempt to facilitate drug research against parasitic nematodes. Furthermore, the efficacies of four compounds have been investigated *in vitro* and *in vivo* using human STHs rodent

models, with special emphasis on drug combinations. This part of the drug pipeline is depicted in Figure 1. Finally, metabolic profiling was applied to a malaria-hookworm co-infection murine model, with the aim of discovering infection-related metabolic signatures and of examining the potential of ^{1}H NMR-based metabonomics as a diagnostic tool.

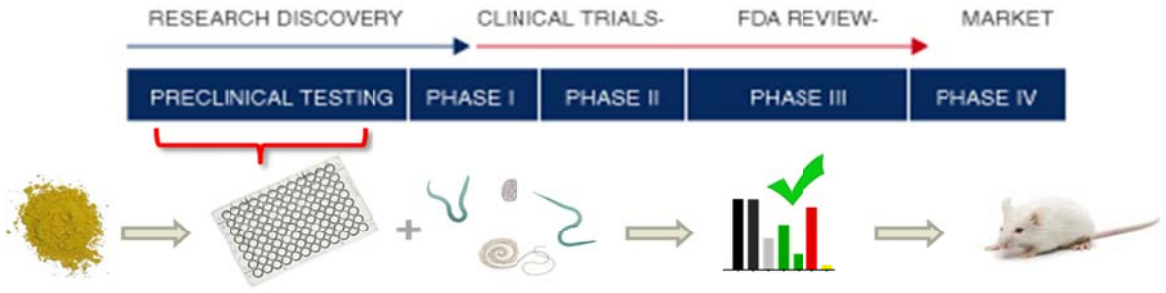

Figure 1. Drug development process. In the early preclinical steps, *in vitro* drug efficacy testing is the first filter before pursuing the investigations *in vivo*.

The key findings of this thesis are listed below:

Alternative *in vitro* assays:

- No alternative test was found superior to the assay of choice, the motility assay, while using *A. ceylanicum* third-stage larvae (L_3). Impedance measurement by the xCELLigence System performed at least as well as the comparative motility assay, when testing on adult worms (Chapter 2a).

- The Alamar Blue assay, the xCELLigence System and isothermal microcalorimetry compared favorably to the motility assay for drug sensitivity testing on *T. muris* fourth-stage larvae (L_4) and adult worms (Chapter 2b). The Alamar Blue assay is the preferred assay of these three due to the simplicity of equipment required.

Potential drug development candidates:

- Monepantel (AAD 1566) showed excellent activity against *A. ceylanicum in vivo* (a single oral dose of 10 mg/kg resulted in a worm burden reduction of 100%), a moderate effect against *N. americanus* (a single oral dose of 10 mg/kg resulted in a worm burden reduction of 58.3%), but failed to show any effect in *T. muris*-, *A. suum* and *S. ratti*-infected animals (Chapter 3). Because of its narrow activity profile, monepantel does not qualify as a drug development candidate for human STHs.

- In the hookworm models *A. ceylanicum* and *H. bakeri*, both tribendimidine and its active metabolite dADT displayed potent activities *in vitro* and *in vivo*. A single oral dose of 10 mg/kg tribendimidine reduced the *A. ceylanicum* worm burden by 74.8%, while dADT killed 87.4% of the worms at the same dose. In *H. bakeri*-infected mice, single oral doses of 2 mg/kg tribendimidine and dADT achieved worm burden reductions of 100% and 97.1%, respectively. In the *A. ceylanicum* model, the combination tribendimidine-levamisole displayed a promising additive to synergistic interaction (Chapter 4).

- Nitazoxanide exhibited strong nematocidal activity against *T. muris* L_3 and adult worms (IC_{50}s = 0.27 and 12.87 µg/ml) and *A. ceylanicum* adult worms *in vitro* (IC_{50} = 0.74 µg/ml). However, in both models, nitazoxanide failed to show any anthelmintic effect *in vivo.* Also, all combinations found synergistic *in vitro* did not decrease the worm burdens significantly compared to control animals *in vivo* (Chapter 5).

- An ED_{50} of 4.7 mg/kg was determined for oxantel pamoate in *T. muris*-infected mice. Combined with mebendazole, a synergistic combination index (CI = 0.158) was calculated. Oxantel pamoate was found inefficacious against *A. ceylanicum* and *N. americanus in vivo*. (Chapter 6). Because the currently used drugs are not satisfactory in terms of efficacy against *T. trichiura*, oxantel pamoate and the promising combination with mebendazole should be further investigated.

Metabolic profiling:

- A characteristic metabolic fingerprint has been found for each of the 5 infection groups (*P. berghei* only, *H. bakeri* only, delayed co-infection, simultaneous co-infection and uninfected control group).

- The presence of increased levels of urinary pipecolic acid in *P. berghei*-infected mice (in single and co-infections) confirmed findings from a previous study. Two unknown urinary metabolites were consistently detected in all *P. berghei*-infected mice (in single and co-infections). Pipecolic acid and possibly the two unknown biomarkers may represent candidate diagnostic biomarkers for human malaria.

Objective-specific issues have been discussed in individual chapters. The following three aspects were chosen for a detailed discussion here:

1) A few considerations on drug screening against soil-transmitted helminths and implications for drug discovery (section 8.2)

2) Further thoughts on the current drug research situation and the means to control STHs (section 8.3)

3) Comments on metabolic profiling as a tool for diagnosing infectious diseases (section 8.4)

8.2. Considerations on drug screening for soil-transmitted helminthiases

The use of *in vitro* assays to identify efficacious agents has been pivotal for anthelmintic drug discovery [8,9]. At present, drug screening for new compounds or assessment of drug resistance in field strains (at least in veterinary medicine) relies on whole-organism *in vitro* development assays, motility assays and egg hatch assays [10,11]. The motility assay consists of a microscopic assessment of the worms' ability to move as a viability criterion [10,12]. Briefly, each adult worm is individually scored according to an arbitrary scale, whereas the proportion of moving infective larvae (L_3, i.e. hookworm larvae), is used to establish the survival rate for each drug concentration. In both cases, a dose-response curve is plotted and a measure of efficacy (i.e. IC_{50}) is calculated, which facilitates comparison between compounds [10,13].

Since cultivation of parasitic nematodes throughout their life cycles is not possible, the test organisms depend substantially upon sacrifice of the rodent host. Although thousands of hookworm and *S. ratti* L_3 larvae can be obtained by culturing eggs released with stools of infected animals, additional tests on adult worms are required since both stages can present discrepancies in drug sensitivity. An ideal drug should show activity against both the infective and adult stages [5]. So far, testing on *T. muris* necessitates killing of the host to recover any stage of interest [12]. However, it might be worth mentioning that an alternative *T. muris* assay is currently being developed at the Swiss TPH. *A. suum* cannot be maintained in mice to the adult stage, and therefore testing is possible on larval stages only [14].

In vivo data are the most meaningful. Besides technical limitations of *in vitro* systems, such as drug solubility in a culture medium, key factors from the host's biology determine the outcome of a drug treatment. These are among others absorption, distribution and extensive

metabolism of the drug [15]. I would like to emphasize that the way in which soil-transmitted helminths take up drugs is not known (absorption through the cuticle versus ingestion), for all of the routinely used drugs. Especially for blood-feeders, this could have a major impact on a given treatment outcome, and open room for optimization of treatment and administration route.

In some cases, *in vivo* results did not correspond to the effect observed *in vitro*. For example, nitazoxanide was found highly efficacious against *A. ceylanicum* adult worms and both stages of *T. muris in vitro,* but the effect could not be reproduced *in vivo* (Chapter 5). Oxantel pamoate exhibited anthelmintic activity against *N. americanus* which was not reproduced *in vivo* (Chapter 6). The contrary was observed with monepantel, where moderate efficacy against *N. americanus in vivo* would have been overlooked from the preceding negative *in vitro* data (Chapter 3). Since *in vitro* activity cannot always be translated into effect *in vivo*, questioning whether *in vitro* filters prior to *in vivo* testing are useful is justified.

For ethical reasons and with the implementation of the 3R rules (reduce, replace, refine), it became important to set criteria in order to limit the use of animals to compounds presenting *in vitro* activity only. Today, the strategy of choice is still *in vitro* compound testing, against larval and adult stages (and sometimes against ova). Although it necessitates little laboratory equipment, the larval/adult motility assay is time-consuming, prone to the reader's subjectivity and does not support high-throughput screening. This assay has proven useful in many cases but does not differentiate death from paralysis, the latter being reversible after treatment with some paralytic agents like levamisole [16,17]. The egg hatch test, commonly used to detect benzimidazole resistance [18], describes ovicidal activity (embryonation and hatching), which does not necessarily relate to anthelmintic activity against larval and adult stages [16,19].

Because most of these methods present drawbacks [20], *in vitro* testing should be optimized to increase the quality of data and enable at least medium-throughput reading. In the present work, we have compared 6 different assays to the current assay of choice, the larval and adult motility assays for drug susceptibility testing on *A. ceylanicum* and *T. muris* (Chapter 2). An ideal assay should be precise, fast, affordable, highly reproducible, and require uncomplicated equipment with a simple (and preferably automated) readout. Such an assay would be a huge step forward in the field of drug discovery for parasitic nematodes. Overall, the xCELLigence System exhibited some of these aimed characteristics, offering a medium-throughput automated readout, precision, and relative user-friendliness, but is suitable for *A. ceylanicum* and *T. muris* intestinal stages only, hence requiring dissection of the animal host.

For screening against *T. muris*, the Alamar Blue test was preferred over the xCELLigence System, because of its higher cost-effectiveness and extreme simplicity of the procedure. No examined assay was found suitable to measure the larvicidal effect on *A. ceylanicum* L_3. Regrettably, the production of an instrument developed to measure the response of larval and adult nematodes to anthelmintic drugs, called the micromotility meter [21], has been stopped [22]. It was based on the angle of light refraction, which was altered by the worm's movement and subsequently computer-processed. Although presenting some of the limitations shown by the motility assay discussed here, the readout was automated and was a suitable platform for medium-throughput screening [23,24]. Hopefully, additional studies planned in our laboratory using a microcalorimetry chip will be a step forward and will allow testing drug sensitivity on *A. ceylanicum* and *T. muris* precisely, quickly and simply. An advantage of the microcalorimetry chip is that it will enable a low consumption of biological test material.

With the rising costs of drug development, the pharmaceutical industry experienced a paradigm-shift in how to conduct drug discovery and reoriented their screening strategies from random-compounds collections towards mechanism-based screening a few decades ago, a strategy that was expected to be more quickly rewarding [9,25]. Mechanism-based screening looks for compounds that selectively act on a well-defined receptor, using recombinant systems in a next step [25]. Hence, an essential prerequisite to this approach is a strong knowledge of the parasites' genomes in order to identify, characterize and prioritize drug targets after target validation. Few genomes of parasitic nematode strains have been fully sequenced (only *A. suum* among STHs of present interest), and understanding the function of every gene remains as a venture. At present, this bioinformatics-based approach does not represent a realistic alternative to drug discovery for helminths, as much of the nematode biology remains to be understood and the potential targets are not sufficiently characterized. Indeed, the youngest class of compounds studied in the frame of this thesis, the AADs and its representative monepantel, discovered in the 2000s, have been identified by extensive *in vitro* whole-organism screening and monepantel exhibited the best properties of the 600 compounds synthesized subsequently [26].

Besides random-compound collection screening, testing marketed drugs with suggested anthelmintic activities via literature search or extending the spectrum of drugs in use in animal health appears to be a powerful strategy. In the same direction, screening large compound databases (i.e. DrugBank) using *in silico* methods will perhaps reveal to be utmost helpful. In this way, the support vector machines (SVM) approach predicted recently 6

potential anthelmintic leads targeted towards parasitic nematodes and was claimed to be suitable where little three-dimensional information is available about a given target [27].

In conclusion, *in vitro* anthelmintic activity screening is so far the only valuable strategy to filter compounds that deserve testing in animals, although some candidates might be missed. Testing of potential candidates should still be carried out on both infective larvae (if relevant) and adult stages. Since complementary information is gained from the different stages, one assay cannot replace the other. Because several compounds can be tested in different concentrations with the amount of worms recovered from one infected animal, *in vitro* testing on adult worms is ethical. I would recommend the use of the Alamar Blue assay for drug discovery against *T. muris* adult stages and would encourage more basic research to enable mechanism-based screening, a more ethical approach. If extensive whole-organism *in vitro* screening against nematodes was destined to remain, a renewed manufacture of the motility-meter would be of great advantage. In order to identify inherent anthelmintic activity of existing drugs, bioinformatic methods could be an excellent start for the screening procedure but obviously animal models will not be easily replaceable.

8.3. Drugs and control of soil-transmitted helminthiases

At present, we rely on preventive chemotherapy for morbidity control in STHs endemic regions. In 2001, the WHO endorsed annual to biannual large-scale drug administration as the strategy of choice with the aim to cover at least 75% of school-aged children at risk of infection [28,29]. The currently restrained pharmacopeia is not satisfactory in terms of cure rates. Here, I would like to discuss potential novel drug candidates, the gaps hindering control of STHs, and the complementary strategies that can help.

Although the demand is not lacking, no new drug for human use has entered the market since the 1970s. All of them are derived from veterinary medicine, have been translated without prior adjustments regarding the dosage, and relatively little is known about their clinical pharmacology [30]. Even if drug resistance represents no serious threat yet, an empty pipeline is certainly no bright strategy. The process of drug discovery and development is a long procedure (lasting up to 20 years [4]) and drug resistance might emerge faster than that. Ironically, the veterinary pharmaceutical industry is more active and has brought a few new drugs to the market since the 1970s [31]. Worth mentioning, criteria for animal health are much more stringent than actual human requirements, requiring a minimal efficacy of 90%

[30]. In humans, an efficacy of ≈75% (2x100 mg/kg mebendazole over 3 days, against *T. trichiura*) is accepted and there are no efficacy standards for drugs to be included in a mass drug administration (MDA) program [30,32].

It has been proposed that the best hope of treating the world's most neglected diseases is for the public sector to take responsibility for drug development, moving away from market-driven forces [2]. On the other hand, research already suggests that the long-standing beliefs on NTDs drug development (nearly non-existing) activity are no longer true, and that these inaccurate ideas have led to a number of poorly designed government policies and motivations [3]. In the post-2000 era, the landscape of drug discovery for NTDs has changed dramatically. Indeed, the increasing formation of drug development PPPs (defined as public health-driven not-for-profit organizations that drive NTD drug development in conjunction with industry groups [3]), has allowed increasing research activity combining the manufacturing skills of pharmaceutical companies with the public health expertise of academia and the public sector, and catalyzed activity in this domain. Moreover, in the case of multinational drug companies (GlaxoSmithKline, Novartis, AstraZeneca and Sanofi-Aventis), money is no longer the driving motivation to get involved into NTDs research and development. Rather, it comes more from a concern for their image stemming from high pressure from the public sector regarding the paucity of activity for the poorest world's needs, social responsibility and ethical concerns, and strategic considerations [3]. Because the PPPs usually subsidize further development costs (i.e. clinical trials) "no profit-no loss" partnerships are created. Smaller companies are active in dozens of projects, motivated by more modest, but profitable, commercial returns [3].
However, so far, PPPs exist for several neglected diseases, but no PPP is dedicated to drugs for treating infections with soil-transmitted helminths. Only very recently, the first activities have started in our laboratories in collaboration with the institute for One World Health (mainly focused on malaria, diarrheal diseases and visceral leishmaniasis) and DND*i* (Drugs for Neglected Diseases initiative, mainly focused on Chagas disease and human African trypanosomiasis). Since STHs are not their main focus, there is no guarantee that these new areas of interest will persist in their portfolios. Although the pipeline for STH is still empty, there is (cautious) reason for hope.

Some of the drugs examined within the frame of this thesis were derived from veterinary medicine. Since, as mentioned, only scarce drug discovery efforts are ongoing for human helminthiases, aiming to extend the spectrum of veterinary anthelmintics is a logical development, encouraged by a favorably short transitioning time (3 years on average, when solid pre-clinical data was available [5]). Monepantel, oxantel pamoate and the

cyclooctadepsipeptides were three of these “low-hanging fruits” examined in our laboratories that showed a promising broad spectrum of activity.
Besides discovery of novel drugs, drug combinations should be more often envisioned. Combining commercialized drugs represents not only a potent strategy to delay emergence of drug resistance, but may also eventually increase the spectra of action of the respective drugs on additional helminth species. In the present work, we identified two promising synergistic drug combinations: tribendimidine-levamisole against *A. ceylanicum*, and oxantel pamoate-mebendazole against *T. muris*. Additional pre-clinical trials are necessary with these 2 combinations.

In addition to suboptimal drugs, we have suboptimal diagnostics. The pivotal role of accurate diagnostics at every stage of a disease control program is often neglected [33] and monitoring subtle changes after repeated treatments is very difficult. This lack of robust diagnostic tools has significant consequences, impairing the collection of precise information on the ecological distribution of soil-transmitted helminths, the definition of at risk populations and the implementation of spatially-targeted control programs [29]. In mass drug administration campaigns, no diagnosis is undertaken prior treatment, a decision based on cost-effectiveness considerations [29,34]. However, if in some places, the prevalence decreased sufficiently, morbidity control should be replaced by infection/transmission control, and integrated diagnostics would help to better refine the scope of the intervention. The preferred diagnostic methods rely on helminth eggs detection. Since the amount of eggs recovered from a stool sample is not directly related to the intensity of infection (a phenomenon also observed in our animal models) these diagnostic methods based on the demonstration of eggs in stools show poor sensitivity in low-intensity infections [35]. Only recently, the available methods to detect hookworm eggs were compared. For instance for hookworms, the Koga agar plate method performed better than Kato-Katz [36], and in another study, FLOTAC outperformed both methods in terms of sensitivity, but the actual egg counts were not equal between methods [37].
New robust and accurate diagnostics are needed. In 2007, only approximately US$ 58,000 were spent on diagnostic research for STHs [38].

Preventive chemotherapy does not prevent from rapid re-infection and therefore, complementary interventions are required to limit the frequency of infections. Improved access to sanitation is a rewarding key factor of integrated control programs [39,40]. In a recent meta-analysis, the availability and use of water supply and appropriate waste management revealed a protective effect on STHs, resulting in reduced transmission [39].

Overall reduction of extreme poverty, but also means such as health education and hygienic behavior are intimately linked with decreased STH transmission [41,42].

In conclusion, control of STHs should rely on a multifactorial approach, using drugs as a backbone, but keeping in mind that sanitation and health education play a decisive role. Without proper knowledge of the drugs we use, without highly active novel drugs and without efficient diagnostic tools to optimize treatment strategies and monitor emergence of drug resistance, we are seriously ill-equipped to understand, monitor and react in case of emergence of drug resistance. However, I am convinced that we do have the tools to do a lot better, by concerting MDA efforts with sanitation improvements and strong health education and within reasonable expenses, by possibly combining the five standard drugs plus oxantel pamoate, the cyclooctadepsipeptides and tribendimidine.

8.4. The use of metabolic profiling to diagnose tropical infectious diseases

In this thesis, we aimed to acquire knowledge on metabolic profiling as a diagnostic tool in a mouse hookworm-malaria co-infection model. This work is one of the multiple pieces of a long term investigation with the aim to critically evaluate the scope and limits of ^{1}H NMR-based metabolic profiling as a new tool to extend our understanding of host-parasite interactions in general and with particular emphasis on diagnostic potential [43]. Blood plasma and urine samples were collected from 5 groups of mice, a *P. berghei* single infection, a *H. bakeri* single infection, a delayed and a simultaneous co-infection, as well as an uninfected control group. Our study revealed a characteristic fingerprint for each different group. Urinary pipecolic acid, found in a previous study in *P. berghei*-infected mice, may represent a promising candidate for human malaria diagnostics. Although further analysis will be required for confirmation, two potential new malaria biomarkers have been identified from *P. berghei*-infected mouse urine. These major findings and their implications are already discussed in Chapter 7 and here, I would like to reflect more on the potential of metabolic profiling as a diagnostic tool for tropical infectious diseases.

The science of metabonomics has established itself as an important contributor to personalized healthcare which will remain a luxury of the world's richest people [44]. For tropical infectious diseases, a diagnosis should be rapid and inexpensive. As mentioned before, development of reliable, sensitive and reproducible diagnostic tests for understanding

the epidemiology of tropical diseases is an integral aspect of the outcome of worldwide control programs and fundament to all subsequent implemented case-management or surveillance strategies [45]. Especially when speaking of elimination, when a disease's transmission level and prevalence are very low, accurate diagnostic tools are of utmost need for effective active case detection [35,41,46].

Diagnostic issues for STHs have been discussed in the section 8.3. Diagnostic approaches for malaria all present drawbacks. Particularly, suboptimal or inconstant detection levels are criticized [47,48], or the required equipment is not easy to adapt to field settings [47]. The malERA (Malaria Eradication Research Agenda) Consultative Group on Diagnoses and Diagnostics proposed a list of priorities for research and development regarding diagnostics [46]. The high priority-rated case-management as well as surveillance tools should be simple, field-applicable, minimally invasive and sensitive. Because malaria occurs in resource-poor settings, the cost of diagnostics should be low as well. The capacity to detect other diseases simultaneously, or severity markers (multiplexing) was mentioned under "medium priority".

^{1}H NMR-based metabolic profiling offers several favorable characteristics. While as little as 20 µl blood plasma is required, and obtained in humans by a simple finger prick, urine recovery is absolutely non-invasive [49]. Both matrices are metabolically rich and well-characterized across several species [50,51,52]. In addition, the method undoubtedly holds promise to detect disease severity markers and multiple species at one time, with reasonable sensitivity. The major disadvantages of this technique are on the one hand the risk of spectral overlaps, compromising the identification of the multiple species at play, and on the other hand the cost of equipment, the required infrastructure and the high level of skills necessitated for interpretation of the spectra. To solve the latter problem to some extent, there may be scope for implementation of analytic platforms that are less expensive than NMR (like HPLC-MS) in some chosen laboratories in endemic countries [53].

The quality and quantity of biomarkers are important for the choice of a suitable diagnostic template but do not conclusively elucidate how well a disease is reflected by them [43]. The specificity of a biomarker to any disease is the most difficult point to uncover and extensive evaluation across several species is required [43]. Although identification of a single specific biomarker would be ideal, the use of multiple biomarkers would improve diagnostic accuracy [54].

How useful rodent metabolic biomarkers are transposable from laboratory-controlled models to humans still needs to be better defined. In human populations, the inter- and intra-individual variability is more pronounced. The age and gender, the intensity of infection as well as nutritional status etc. impact the individual metabolic profile and might render the interpretation difficult. In a study with *Schistosoma mansoni*-infected human subjects in

Uganda [55], a good overlap with metabolic fingerprints in previous animal studies was observed, suggesting the existence of a specific metabolic response to this infection [56]. In a study based on *Onchocerca volvulus*-infected patients, 14 discriminating candidate biomarkers (showing sensitivity and specificity of >99%) have been identified using LC-MS-based metabolic profiling [57]. The sensitivity of the method was such that the authors mentioned the eventual possibility to monitor worms' viability in the patient following treatment. A *P. vivax* metabonomic investigation using urine from infected patients revealed the potential of differentiation between malarial versus non-malarial fevers, due to discriminant biomarkers [58]. Overall, the authors proposed additional human studies to confirm this specificity and to validate the test against the widest genetic backgrounds possible.
Ultimately, the optimized biomarkers can be transposed into field-ready portable diagnostic tests such as immunochromatographic, microfluidic-based tests or lab-on-chip technologies [57,59].

In conclusion, the use of metabolic profiling is a powerful approach to identify disease-related metabolic fingerprints, delivering a holistic read-out on one individual's health. With the growing knowledge about biomarkers from animal models but also studies in the field, the use of metabolic-based diagnostics may be on the horizon. However, given the high price of the necessary equipment and the complexity of interpretation of the spectra, a mandatory prerequisite to apply this technology to diagnostics for tropical infectious diseases would be the transformation into a simple biochemical assay: ideally a dipstick or lab-on-chip technology. This is in itself a new endeavor.

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Chapter 9

Conclusion and Outlook

9. Conclusion and Outlook

In this thesis, several aspects in research on soil-transmitted helminths have been addressed: the optimization of *in vitro* drug screening assays, the characterization of the efficacy of potential novel drug candidates, and the investigation of a potential diagnostic tool.
For *T. muris*, one alternative *in vitro* assay, the Alamar Blue assay, compared favorably to the standard motility assay.
Fortunately, out of four examined compounds, one drug has been positively evaluated for its trichuricidal properties (oxantel pamoate), one for promising anti-hookworm effects (tribendimidine) and two potent drug combinations have been uncovered (tribendimidine-levamisole and oxantel pamoate-mebendazole). Two drugs failed to show sufficient activity (monepantel and nitazoxanide).
^{1}H NMR spectroscopy was found to be powerful for detecting metabolic changes in a co-infection model, but the identified biomarkers would necessitate validation across other species (in particular transferability to humans) and transposition into a field-applicable format (such as a dipstick) in order to present a realistic option for diagnostics.

I am convinced that drug combinations are an accessible and extremely powerful tool to control STHs. Unfortunately, they are still scandalously overlooked. Policy-makers need to review their positions not only regarding the few but strong efforts to discover and develop new drugs, but also by recognizing the “low-hanging fruits” represented by combined chemotherapy (first between approved drugs, including novel drugs then).
It is largely accepted that long-term control will stem from several sides and that missing knowledge from precise epidemiological data, drug pharmacology and worm physiology are impeding implemented strategies. Further research is needed to fill all these gaps.

In some bullet points, I would like to list a few study directions which may constitute logical next steps to this work:

- Further pre-clinical studies should be carried out with the additive/synergistic drug combinations identified here. For example, drug interaction studies are desirable.
- Investigations on drug uptake by the worms (ongoing in our laboratory).
- Identification of the urinary unknown metabolites found consistently in all *P. berghei*-infected mouse groups (ongoing) and evaluation of transferability through species (e.g. *P. falciparum*) and of stability through infection intensities.

Curriculum Vitae

Curriculum Vitae

Personal data

Address	Lucienne Tritten Oberwilerstrasse 72 4054 Basel Switzerland
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Date of birth	09/19/1984
Nationality	Swiss

Education

2006 - 2008	**MSc in Infection Biology and Epidemiology** **University of Basel - Swiss Tropical and Public Health Institute** Major in Infection Biology Thesis: 'Development of a new double-antibody sandwich ELISA to evaluate antimalarial drug sensitivity' (including 9 months at F. Hoffmann-La Roche, Basel)
2003 - 2006	**BSc in Biology** **University of Neuchâtel**
2000 - 2003	**Highschool in La Chaux-de-Fonds - Lycée Blaise Cendrars** Major in Biology and Chemistry
Others	**Introductory Course in Laboratory Animal Science** Berne, with certificate, 2008 **Metabonomics Shortcourse** Imperial College London, 2009

Work experience

06/2009 - today	**PhD student at the Swiss Tropical and Public Health Institute** **University of Basel** Departement of Medical Parasitology & Infection Biology, supervisor: Prof. Dr. Jennifer Keiser

Activities:

- Establishment of the *Ancylostoma ceylanicum*, *Necator americanus*, and *Trichuris muris* life cycles in rodents at the institute.

- Searching for alternative in vitro assays for assessing drug sensitivity of *T. muris* and *A. ceylanicum* larvae and adult worms (colorimetric and fluorescent assays, microcalorimetry and measurement of impedance).

- Testing of standard drugs, monepantel, nitazoxanide, tribendimidine and oxantel pamoate as well as drug combinations in diverse STH animal models, in vitro and in vivo.

- Establishment of a mouse hookworm-malaria co-infection model, sample generation for a systemic metabolic profiling study using ^{1}H NMR spectroscopy in collaboration with Imperial College London (4 months spent in London) and data analysis with PCA and O-PLS-DA.

- Supervision of master students.

03/2008 - 04/2009 **Scientific collaborator in immunology at the Theodor Kocher Institute**
University of Berne
(PhD program)
Project: 'The individual contribution of α4β1- and α4β7-integrins in the multi-step recruitment of different leukocyte subpopulations across the blood-brain barrier during health and ongoing experimental autoimmune encephalomyelitis in vivo'

2003 - 2007 **Attendant at the Natural History Museum of La Chaux-de-Fonds**

Languages

French (native language)
German (full professional proficiency)
English (full professional proficiency)

Computer skills

MS Office (Word, Excel, PowerPoint)
EndNote
Stata, StatsDirect
MATLAB, R

Conferences

2011 **British Society for Parasitology, Spring Meeting 2011, Nottingham (UK)**
Oral presentation: 'Alternative in vitro assays and potential drug candidates against *Ancylostoma ceylanicum*'

2010 **PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology, Spiez (CH)**
Oral presentation: 'In vitro assays for drug susceptibility testing on hookworm and the example of monepantel'

2008 **Swiss Tropical and Public Health Institute research seminar, Basel (CH)**
Oral presentation: 'Development of a new double antibody sandwich ELISA to evaluate antimalarial drug sensitivity'

Publications

2012 Tritten L, Silbereisen A, Keiser J (2012). Nitazoxanide: *In vitro* and *in vivo* drug effects against *Trichuris muris* and *Ancylostoma ceylanicum*, alone or in combination. International Journal for Parasitology: Drugs and Drug Resistance, 2:98-105.

Tritten L, Braissant O, Keiser J (2012). Comparison of novel and existing tools for studying drug sensitivity against the hookworm *Ancylostoma ceylanicum in vitro*. Parasitology, 139(3):348-57

Tritten L, Nwosu U, Vargas M, Keiser J (2012). *In vitro* and *in vivo* efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*. Acta Tropica, 122(1):101-7

2011 Tritten L, Silbereisen A, Keiser J (2011). *In vitro* and *in vivo* efficacy of monepantel (AAD 1566) against laboratory models of human intestinal nematode infections. PLoS Neglected Tropical Diseases 5(12), e 1457

Silbereisen A, Tritten L, Keiser J (2011). Exploration of novel *in vitro* assays to study drugs against *Trichuris* spp. Journal of Microbiological Methods 87(2):169-75

2009 Tritten L, Matile H, Brun R, Wittlin S (2009). A new double-antibody sandwich ELISA targeting *Plasmodium falciparum* aldolase to evaluate anti-malarial drug sensitivity. Malaria Journal 8:226

Obtained grants

2011	2800 CHF, Mathieu-Stiftung
2012	4000 CHF, Freiwillige Akademische Gesellschaft

Additional information

Memberships	Swiss Society of Tropical Medicine and Parasitology British Society for Parasitology

Personal statements

I am passionate about understanding neglected tropical diseases and finding tools to combat them. During the time spent in different institutes, universities or in industry, I had the chance to acquire a growing expertise in this research area. My goal is to continue to employ and develop my research skills in a challenging position and in a competitive environment.

As a student and PhD student, I attended lectures by

B. Betschart, G. Süss-Fink, T. Ward, R. Deschenaux, M. Burkhard, J. Faist, M. Zuber, P. Küpfer, M. Aragno, M. Brossard, K. Föllmi, O. Maggioni, E. Verrecchia, R. Bshari, B. Engelhardt, U. Jenal, R. Brun, I. Felger, H.-P. Beck, G. Pluschke, C. Daubenberger, P. Vounatsou, C. Lengeler, C. Burri, J. Keiser, J. Utzinger, M. Tanner.

Cover picture: Stoma of an adult *Ancylostoma ceylanicum* showing the lateral cutting plates. The picture has been taken in collaboration with ZMB (Zentrum für Mikroskopie), University of Basel.