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 % 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.

Keywords: Hookworm, *Ancylostoma ceylanicum*, *Heligmosomoides bakeri, Heligmosomoides polygyrus*, chemotherapy, combination chemotherapy, tribendimidine, *in vitro*, *in vivo*

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 promising broad-spectrum activity, rapid onset of action and good tolerability were documented (Keiser et al., 2007; Keiser et al., 2008; Steinmann et al., 2008; Xiao et al., 2005). In humans, tribendimidine showed excellent efficacy against hookworms (*Necator americanus* and

Ancylostoma 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; Xue et al., 2010). Tribendimidine and dADT exhibited high efficacies against *Necator 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; Xue et al., 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 *H. bakeri* (formerly known as *H. polygyrus*) and *A. 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)ethylideneaminophenyl)-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

Ancylostoma 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., in press-b). Briefly, male Syrian gold 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 larvae. 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 hours 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 hours microscopically (inverted microscope, Carl Zeiss, Germany, magnification 20x) 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 20x) 72 hours following drug exposure (final concentrations of 100 to 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. (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 RMPI 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 80-160x) for embryonation, and 40 hours 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., in press-a). Briefly, 30 L3 per well (96-well plates) were incubated for 72 hours 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 20x) 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 4x IC_{50} to 0.25x IC_{50} for each drug.

Drug susceptibilities of adult worms (obtained from hamster's 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 hours. The motility was determined microscopically (magnification 20x) 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 deionised water containing 10 µg/ml of the test drug. After 24 hours, 20

eggs per well were examined microscopically (magnification 80-160x) for embryonation, and after 48 hours, 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 (2x 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]*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., in press-b). 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 ($0.5ED_{50}$:$05ED_{50}$) 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 hours following treatment were collected from each hamster and soaked in 0.9 % NaCl. The entire sample was then carefully examined under a binocular (magnification 10-40x) 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)*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). 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*) were 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* third-stage larvae (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 >1000, 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 hours, 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 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* third-stage larvae (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 tribendimidine-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: IC50s >

< Table 2: ovicidal activity >

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.

< Table 3: *in vivo H. bakeri* >

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}. Figure 1 illustrates this finding using an isobologram.

< Table 4: *in vivo A. ceylanicum* >

< Figure 1: isobologram >

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 *Necator americanus*, *Nippostrongylus braziliensis, Strongyloides ratti*, and *Toxocara canis* and the trematodes *Clonorchis sinensis* and *Opisthorchis viverrini* (Keiser et al., 2007; Keiser et al., 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, 2008; Yuan et al., 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 ≤ 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 ≥ 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. ceylanicum* in context with findings from previous work shows that tribendimidine and dADT displayed a higher 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., in press-b), 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., in press-b), 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

365 different drugs, a phenomenon well-studied for drug effects against *Ancylostoma* spp. and *N.*
366 *americanus* (Behnke et al., 1993; Xue et al., 2005).
367 Tribendimidine combinations with albendazole, levamisole and ivermectin interacted
368 antagonistically *in vitro* against *H. bakeri* L3 larvae. These findings were confirmed in our *in*
369 *vivo* studies. Interestingly, increased tribendimidine doses resulted in a decreased treatment
370 effect, particularly notable for albendazole- or ivermectin-tribendimidine combinations. On the
371 other hand, against *A. ceylanicum*, additive to synergistic effects were observed with a
372 combination of tribendimidine-levamisole *in vitro* and *in vivo.* This finding suggests that the
373 two drugs might act via an at least partially different mechanism, perhaps at different nAChR
374 subunits. Initially, it had been proposed that tribendimidine also belongs to the L-type nAChR
375 agonists group and shares the same mode of action as levamisole. On the other hand, it was
376 noticed that tribendimidine does not behave like a typical nAChR agonist (levamisole or
377 pyrantel), acting more rapidly, and paralyzing the worms starting from the head (Hu et al.,
378 2009). It might be important to note that levamisole was shown to have a broad range of
379 immunomodulatory effects in rodents and humans. In anti-cancer chemotherapy, levamisole
380 is thought to potentiate the combined drug 5-fluorouracil (Mitchell, 2003; Stevenson et al.,
381 1991). Possibly, these immunomodulatory properties play a role in the additive to synergistic
382 interaction with tribendimidine. Though not tested here we would have expected a similar
383 nature of interaction combining dADT with levamisole, since these drugs have comparable
384 efficacies and are structurally very close.
385 In conclusion, tribendimidine and dADT show potent anti-hookworm properties, when
386 administered alone, in single oral doses. We have confirmed that the *H. bakeri* mouse model
387 is an excellent laboratory model to study drug effects on hookworms. Since *H. bakeri* can be
388 maintained in mice and worms have matured already 14 days post-infection this model is fast
389 and cost-effective. Promisingly, the combination tribendimidine-levamisole revealed excellent
390 efficacy (synergism at the ED_{90}), against *A. ceylanicum*. This combination should therefore
391 be investigated in further detail, i.e. in drug interaction studies and against other soil-
392 transmitted helminths.

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

	***H. bakeri* IC50s (r)**		***A. ceylanicum* IC50s (r)**	
Drug	**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[#]	> 100 (n.d.)	32.4 (0.87)[§]	> 100 (0.77)[§]
Levamisole-HCl	0.02[#]	0.56 (0.70)	1.6 (0.95)[§]	> 100 (0.79)[§]
Ivermectin	6.92[#]	> 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 [#](Nwosu et al., 2011) [§] (Tritten et al., in press-b)

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 ≥ 85 indicates a satisfactory fit).

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

	H. bakeri		*A. ceylanicum*	
Group	**% 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, * P<0.001

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 [#]	10	–	52.6	NA
Levamisole-HCl [#]	1.25	–	61.5	NA
Ivermectin [#]	0.125	–	53.7	NA
Combination Tribendimidine-Albendazole	0.5 + 10[2]	36.0 (32.2)	67.1	0.486[α]
Combination Tribendimidine-Albendazole	1 + 10[3]	70.0 (40.3)	36.1	0.057[β]
Combination Tribendimidine-Levamisole	0.5 + 1.25[2]	34.25 (25.7)	68.7	0.686[α]
Combination Tribendimidine-Levamisole	1 + 1.25[3]	41.5 (65.2)	62.1	0.543[β]
Combination Tribendimidine-Ivermectin	0.5 + 0.125[2]	15.5 (17.1)	85.8	0.229[α]
Combination Tribendimidine-Ivermectin	1 + 0.125[3]	64.5 (55.1)	41.1	> 0.05[β]

SD= standard deviation. NA= Not assessed.

The numbers in superscript refer to the corresponding control group. [α] versus tribendimidine 0.5 mg/kg, [β] versus tribendimidine 1 mg/kg. [#] For comparison, worm burden reductions obtained with the subcurative doses of the partner drugs (albendazole, levamisole, ivermectin) are also listed (Nwosu et al., 2011)

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-HCl [§]	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 CI at IC_{90}= 0.19
	5+5[2]	13.3 (11.8)	9.3 (11.2)	70.0	70.1	
	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. [§] The worm expulsion rate and the worm burden reduction obtained with 10 mg/kg levamisole are given (Tritten et al., in press-b).

Figure legend

Figure 1: Isobologram for the combined effect of tribendimidine and levamisole.

Fa= effect level.