

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

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16

17 **Abstract**

18 Worldwide, 3 billion people are at risk of hookworm infection, particularly in resource-poor
19 countries. While control of soil-transmitted helminthiases relies mostly on chemotherapy, only
20 few drugs are available and concern about potential emergence of drug resistance is rising.
21 In the present study, tribendimidine, a derivative of amidantel, and its metabolites deacylated
22 amidantel (dADT) and acetylated deacylated amidantel (AdADT) were tested *in vitro* and *in*
23 *vivo* against *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*, two hookworm rodent
24 models, alone or in combination with standard drugs.
25 Tribendimidine achieved $IC_{50}s \leq 5 \mu\text{g/ml}$ against both *H. bakeri* third-stage larvae and adults
26 *in vitro* and a single 2 mg/kg oral dose resulted in complete worm elimination *in vivo*.
27 Comparable results were obtained with dADT, whereas AdADT displayed no effect *in vitro*
28 and gave a moderate worm burden reduction of 42 % in *H. bakeri*-infected mice.
29 Tribendimidine combined with albendazole, levamisole or ivermectin revealed antagonistic
30 interactions against *H. bakeri in vitro* and no significant killing effect *in vivo*. Tribendimidine
31 and dADT exerted high efficacies against *A. ceylanicum* third-stage larvae ($IC_{50}s < 0.5 \mu\text{g/ml}$)
32 whereas adults were moderately affected *in vitro* ($IC_{50}s > 88 \mu\text{g/ml}$). *In vivo* at single oral
33 doses of 10 mg/kg, dADT showed a slightly higher efficacy than tribendimidine, achieving
34 worm burden reductions of 87.4 % and 74.8 %, respectively. At the same dose, AdADT
35 reduced the worm burden by 57.9 %. Synergistic interactions were observed with
36 tribendimidine-levamisole combinations against *A. ceylanicum in vitro* (combination index at
37 $IC_{50} = 0.5$), and *in vivo* (combination index at $ED_{90} = 0.19$). In conclusion, tribendimidine and
38 dADT show potent anti-hookworm properties. The potential of the promising tribendimidine-
39 levamisole combination should be investigated in greater detail.

40

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

44

45 **1. Introduction**

46 Hookworms are intestinal parasitic nematodes of great public health significance.
47 *Necator americanus* and *Ancylostoma duodenale* are the two most important species
48 infecting humans. They occur mainly in Sub-Saharan Africa, South-East Asia, China and in
49 the Pacific Islands. More than 3 billion people are at risk of acquiring hookworm infections
50 (de Silva et al., 2003). Hookworm infection is one of the so-called neglected diseases,
51 affecting particularly people living in resource-poor settings (Hotez et al., 2007). Disease
52 morbidities depend directly on the infection intensity, ranging from asymptomatic cases to
53 anaemia, nutrient loss and profound physical and mental deficits (Hotez et al., 2004; Roche
54 and Layrisse, 1966; Stoltzfus et al., 1997). The current mainstay to control hookworm
55 infections is chemotherapy administered in the framework of periodic mass drug
56 administration campaigns, targeting high-risk groups (Harhay et al., 2010; Hotez, 2008). Only
57 4 drugs are currently recommended by the World Health Organisation (WHO) against soil-
58 transmitted helminthiases: albendazole, mebendazole, levamisole and pyrantel pamoate
59 (Keiser and Utzinger, 2010), all of them in use for decades (Holden-Dye and Walker, 2007;
60 Hotez et al., 2006; Utzinger and Keiser, 2004).

61 Since resistance to these drugs has spread widely in livestock (Kaplan, 2004), there is rising
62 concern about potential emergence of resistance among human nematode populations
63 (Albonico et al., 2003; De Clercq et al., 1997; Geerts and Gryseels, 2001; Reynoldson et al.,
64 1997). New drugs are therefore urgently needed, and combinations of existing drugs have to
65 be thoroughly explored to prevent emergence of drug resistance (Barnes et al., 1995; Nyunt
66 and Plowe, 2007; van den Enden, 2009).

67 Tribendimidine, a successor of a drug developed by Bayer, amidantel (Bay d 8815), was
68 discovered in the 1980s by the Institute of Parasitic Diseases in Shanghai, China (Xiao et al.,
69 2005). Tested against a range of helminths in the laboratory and in humans, a promising
70 broad-spectrum activity, rapid onset of action and good tolerability were documented (Keiser
71 et al., 2007; Keiser et al., 2008; Steinmann et al., 2008; Xiao et al., 2005). In humans,
72 tribendimidine showed excellent efficacy against hookworms (*Necator americanus* and

73 *Ancylostoma duodenale*), performing even better than albendazole, the current drug of
74 choice (Xiao et al., 2005). The Chinese authorities approved tribendimidine for human use in
75 2004 (Sun, 1999; Utzinger and Keiser, 2004).

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

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

89 **2. Materials and methods**

90 2.1. Drugs

91 Tribendimidine (*N,N'*-bis(4-(1-dimethylamino)ethylideneaminophenyl)-1,4-phenylene
92 dimethylidyneamine), MW: 452.594 g/mol, and the metabolites deacylated amidantel (dADT),
93 MW: 173.214 g/mol, and acetylated deacylated amidantel (AdADT), MW: 215.251 g/mol,
94 were donated by Shandong Xinhua Pharmaceutical Company (China) and stored at 4 °C.
95 Albendazole, levamisole and ivermectin were purchased from Sigma-Aldrich (Buchs,
96 Switzerland). For the *in vitro* studies, 10 mg/ml (*H. bakeri*) or 5 mg/ml (*A. ceylanicum*) stock
97 solutions were prepared for all drugs in 100 % DMSO (Fluka, Buchs, Switzerland) and stored
98 at 4 °C. For the *in vivo* studies, drugs were suspended in 7 % (v/v) Tween 80 % and 3 %
99 (v/v) ethanol shortly before treatment.

100

101 2.2. Animals

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

111

112 2.3. Parasites and infections

113 *Ancylostoma ceylanicum* third-stage larvae (L3) were kindly provided by Prof. Jerzy
114 Behnke (University of Nottingham). The *A. ceylanicum* life cycle has been maintained at the
115 Swiss TPH as described earlier (Garside and Behnke, 1989; Ray and Bhopale, 1972; Tritten
116 et al., in press-b). Briefly, male Syrian gold hamsters were immunosuppressed with

117 hydrocortisone (3 mg/kg, twice weekly) or dexamethasone (1 mg/l continuously in the
118 drinking water) and infected orally at 4 weeks, with 150 L3 larvae. For *in vivo* studies,
119 hamsters were not immunosuppressed and infected orally with 300 L3. Details on the life
120 cycle of *H. bakeri*, maintained at the institute since 2009 have recently been described
121 (Nwosu et al., 2011). Briefly, 4-week old NMRI mice were infected orally with 150 *H. bakeri*
122 L3 (for *in vitro* studies), or only with 80 L3 (for *in vivo* work).

123

124 2.4. In vitro studies

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

128

129 2.4.1. H. bakeri

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

141 Similarly, 4 adult worms (males and females, gained upon dissection of infected mice
142 guts) were incubated in 48-well plates in 500 µl phenol-red free RPMI 1640 supplemented
143 with HEPES and antibiotics, at 37 °C, 5 % CO₂. The motility was assessed microscopically

144 (magnification 20x) 72 hours following drug exposure (final concentrations of 100 to 0.1
145 µg/ml) and the viability determined. Assays were conducted at least twice.

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

155

156 2.4.2. *A. ceylanicum*

157 Experiments were conducted as described recently (Tritten et al., in press-a). Briefly,
158 30 L3 per well (96-well plates) were incubated for 72 hours at room-temperature in the
159 presence of 200 µl HBSS medium (containing 25 µg/ml amphotericin B (Sigma-Aldrich) and
160 1% (v/v) penicillin-streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin,
161 Sigma-Aldrich) and drug dilutions (ranging from 100 to 0.01 µg/ml). The larval motility was
162 investigated using microscopy (magnification 20x) following addition of hot water (~80 °C)
163 and exposure to microscope light. For combination chemotherapy experiments, 50 µl of the
164 individual drug solutions were added to each well and serially diluted, in order to have final
165 concentrations ranging from 4x IC₅₀ to 0.25x IC₅₀ for each drug.

166 Drug susceptibilities of adult worms (obtained from hamster's guts upon dissection)
167 were tested in 48-well plates (Costar) with 3-4 worms per well containing 1 ml medium and
168 drugs at 37 °C, 5 % CO₂ for 72 hours. The motility was determined microscopically
169 (magnification 20x) using a viability scale ranging from 2 (worms healthy, fit) and 0 (death).

170 The ovicidal activity was evaluated using 50 freshly isolated eggs per well which were
171 incubated in 1 ml deionised water containing 10 µg/ml of the test drug. After 24 hours, 20

172 eggs per well were examined microscopically (magnification 80-160x) for embryonation, and
173 after 48 hours, 20 eggs were examined for hatching. In all assays (L3, adults and eggs)
174 control wells contained the highest DMSO concentration used in the tests (max. 2 % v/v).

175

176 2.5. In vivo studies

177 2.5.1. *H. bakeri*

178 Six groups (n=4) of mice were treated orally with 0.5, 1 or 2 mg/kg single doses of
179 tribendimidine, or 1 or 2 mg/kg single doses of dADT or AdADT, 21-28 days post-infection
180 (p.i.). Untreated mice (2x n=4) served as controls. To assess the effect of drug combinations,
181 0.5 mg/kg tribendimidine was combined with 10 mg/kg albendazole, 1.25 mg/kg levamisole
182 or 0.125 mg/kg ivermectin, each dose being the estimated ED₅₀ value for the single drug.
183 Worms remaining in the gut on day 8 post-treatment were counted after killing the mice with
184 the CO₂ method. Worm burden reductions were calculated as following: $[(a-b)/a]*100$, where
185 a = average worm count in the control group upon dissection and b = average worm count in
186 a treated group upon dissection (Bartley et al., 2008; Xue et al., 2005).

187

188 2.5.2. *A. ceylanicum*

189 The experimental procedure was carried out as described recently (Tritten et al., in
190 press-b). Briefly, the fecal egg burden was established on average from days 21 and 22 p.i.
191 On the basis of the fecal egg burden, hamsters were assigned to equally balanced treatment
192 groups (single oral doses of 10 or 5 mg/kg tribendimidine, 10 mg/kg dADT or AdADT) or
193 control groups (4 animals each). For combination chemotherapy studies, we first calculated
194 approximative ED₅₀ values of both drugs (tribendimidine 10 mg/kg and levamisole 10 mg/kg).
195 Both drugs were then combined at a constant ED₅₀ ratio (10 mg/kg:10 mg/kg (1ED₅₀:1ED₅₀);
196 5 mg/kg:5 mg/kg (0.5ED₅₀:0.5ED₅₀) and 2.5 mg/kg:2.5 mg/kg (0.25ED₅₀:0.25ED₅₀)).

197 Hamsters were treated on day 23 p.i. The complete stools over a period of 48 hours
198 following treatment were collected from each hamster and soaked in 0.9 % NaCl. The entire
199 sample was then carefully examined under a binocular (magnification 10-40x) and all worms

200 counted. Worms remaining in the gut 7 days post-treatment were collected and counted after
201 killing the hamsters with the CO₂ method. Worm burden reductions (see *H. bakeri*) and worm
202 expulsion rates were calculated. The worm expulsion rates were calculated as follows:
203 [(c/d)*100], where c is the total number of expelled worms in a treated group and d, the total
204 worm count (expelled worms as well as worms present in gut counted following dissection) of
205 the same group.

206

207 2.6. Statistical analysis

208 IC₅₀s were calculated based on the median effect principle using CompuSyn (version
209 1.0). The r value is the linear correlation coefficient of the median-effect plot, indicating the
210 goodness of fit, hence how accurate the IC₅₀ is (Chou, 1976). Variance in ovicidal activities
211 was analyzed using the Fisher's exact test (StatsDirect, version 2.4.5; StatsDirect Ltd;
212 Cheshire, UK). Worm burden reductions and worm expulsion rates were calculated in
213 Microsoft[®] Excel. The statistical significance of the worm burden reduction was evaluated
214 with the Kruskal-Wallis test (multiple doses against control), or the Mann-Whitney U test
215 (single dose against control), using StatsDirect. Combination indices (CI) at IC₅₀ and IC₉₀
216 were calculated for *in vitro* combination studies using CompuSyn. For *in vivo* studies, either
217 the CI value (*A. ceylanicum*) or the statistical significance (Mann-Whitney test, *H. bakeri*)
218 were determined.

219

220 **3. Results**

221 3.1. *In vitro* studies against *H. bakeri*

222 3.1.1. Single drug assays against third-stage larvae and adults

223 Tribendimidine was highly active against both *H. bakeri* third-stage larvae (IC₅₀: 0.32 µg/ml, r
224 = 0.85) and adult worms *in vitro* (IC₅₀: 5.09 µg/ml, r = 0.95) (Table 1). dADT showed a slightly
225 lower efficacy than tribendimidine against L3 (IC₅₀: 0.62 µg/ml, r = 0.94) but higher activity
226 against adults (IC₅₀: 3.52 µg/ml, r = 0.93). Only a very low activity was observed with AdADT
227 (both IC₅₀s >100 µg/ml, r = 0.91 and 0.84 respectively).

228

229 3.1.2. Drug combination studies against third-stage larvae

230 Third-stage larvae were exposed simultaneously to tribendimidine and albendazole,
231 levamisole or ivermectin (Table 1) using a fixed dose ratio based on the IC₅₀s and 2-fold
232 dilutions up and down. The calculated CIs for the tribendimidine-albendazole, tribendimidine-
233 levamisole and tribendimidine-ivermectin combinations were >1000, 1.20, and 9.76,
234 respectively. Hence, all combinations tested showed antagonistic interactions (CI >1) at the
235 fixed dose ratio.

236

237 3.1.3. Ovicidal activity

238 In the presence of tribendimidine, 99.6 % of the eggs were fully embryonated after 24 hours,
239 while hatching was moderately reduced by 13.4 % (P=0.116), compared to the controls
240 (Table 2). dADT achieved similar low reductions in embryonation (2 %) and hatching (12.8
241 %) (P=0.106). AdADT had no effect on embryonation and hatching.

242

243 3.2. *In vitro* studies against *A. ceylanicum*

244 3.2.1. Single drug assays against third-stage larvae and adults

245 Tribendimidine strongly affected *A. ceylanicum* third-stage larvae (IC₅₀: 0.32 µg/ml, r = 0.89),
246 while adult worms' viability was only moderately reduced (IC₅₀: 88.44 µg/ml, r = 0.83) (Table
247 1). A similar effect was observed for dADT, which exhibited excellent activity against L3 but

248 not against the adults (IC₅₀s: 0.14 µg/ml, r = 0.95 and >100 µg/ml, r = 0.50, respectively).
249 AdADT showed a moderate effect against both stages (IC₅₀s: 70.0 µg/ml, r = 0.66 and 21.93
250 µg/ml, r = 0.88, respectively).

251

252 3.2.2. Drug combination studies against third-stage larvae

253 Third-stage larvae were incubated with tribendimidine combined with albendazole,
254 levamisole or ivermectin (Table 1) based on their respective IC₅₀ values and 2-fold dilutions
255 were carried up and down. The combination index at the IC₅₀ value indicated synergism for
256 the tribendimidine-levamisole combination (CI: 0.50), whereas the combinations of
257 tribendimidine with albendazole and ivermectin were antagonistic (CIs: 2.53 and 4.21,
258 respectively).

259

260 3.2.3. Ovicidal activity

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

264

< Table 1: IC₅₀s >

265

< Table 2: ovicidal activity >

266

267 3.3. In vivo studies against *H. bakeri*

268 3.3.1. Monotherapy

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

274

275 3.3.2. Combination chemotherapy

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

286

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

288

289 3.4. *In vivo* studies against *A. ceylanicum*

290 3.4.1. Monotherapy

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

297

298 3.4.2. Combination chemotherapy

299 Based on our *in vitro* findings, only the combination of tribendimidine and levamisole
300 was further studied *in vivo*, using a fixed dose ratio based on the approximate ED_{50} doses of
301 both drugs and diluted twice (10 mg/kg : 10 mg/kg, 5 mg/kg : 5 mg/kg, 2.5 mg/kg : 2.5
302 mg/kg). These combination treatments achieved worm burden reductions of 92.7 %, 70.1 %
303 and 3.6 %, respectively (Table 4). The calculated CI revealed an additive effect tending

304 towards synergism, with a CI of 1.02 at the ED₅₀ and a CI of 0.19 at the ED₉₀. Figure 1
305 illustrates this finding using an isobologram.

306

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

308 < Figure 1: isobologram >

309

310 **Discussion**

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

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

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

328 *In vitro*, tribendimidine was highly active against both studied stages of *H. bakeri* ($IC_{50}s \leq 5$
329 $\mu\text{g/ml}$), and a similar result was observed for dADT. For comparison, tribendimidine and
330 dADT showed a higher activity against *H. bakeri* third-stage larvae and adults than the
331 standard drugs albendazole and ivermectin but was less active than levamisole (Nwosu et
332 al., 2011).

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

338 al., 2009). Putting the findings obtained with tribendimidine against *A. ceylanicum* in context
339 with findings from previous work shows that tribendimidine and dADT displayed a higher
340 activity against L3 and adults than albendazole and levamisole. Adult worms were more
341 affected by ivermectin compared to tribendimidine and its metabolites, while the effect of
342 ivermectin on L3 was slightly lower.

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

346 *In vivo*, tribendimidine cleared *H. bakeri* infections in mice at a dose of 2 mg/kg and reduced
347 the worm burden by more than 50 % at 0.5 mg/kg. A comparable result was obtained with
348 dADT, as the worm burden reduction following a 2 mg/kg treatment was of 97 %. As
349 expected from the *in vitro* data, AdADT had a moderate impact on the *H. bakeri* worm burden
350 (2 mg/kg dose, 42 % worm burden reduction).

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

356 It is interesting to note that in a recent study worm burden reductions observed after
357 tribendimidine oral treatments against *N. americanus* were significantly higher than those
358 achieved by the metabolite dADT (Xue et al., 2010), which contradicts the findings observed
359 in the *A. ceylanicum*- model and stability issues highlighted above. The efficacy of AdADT
360 against *N. americanus* was moderate, in line with our results (Xue et al., 2010). Albendazole,
361 which showed a superior efficacy over tribendimidine against *A. ceylanicum in vivo* (Tritten et
362 al., in press-b), produced a less pronounced effect against *N. americanus* (Xue et al., 2005).
363 Similarly, *H. bakeri* was less affected by albendazole (Nwosu et al., 2011). Overall, our
364 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 ED₉₀), 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.

393

394 **Acknowledgements**

395 J. Keiser is grateful to the Swiss National Science Foundation (project no. PPOOA-
396 114941) for financial support. This study was supported by the Institute of One World Health
397 with funds from the Bill and Melinda Gates Foundation.

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520 **Table 1. Activity of tribendimidine, dADT and AdADT and tribendimidine combinations**
 521 **against *H. bakeri* and *A. ceylanicum* in vitro 72 hours post-incubation.**
 522

Drug	<i>H. bakeri</i> IC50s (r)		<i>A. ceylanicum</i> IC50s (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 [#]	> 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.

523
 524 IC₅₀: Fifty-percent inhibitory concentrations. For comparison, IC₅₀ values of albendazole,
 525 levamisole and ivermectin are shown [#](Nwosu et al., 2011) [§] (Tritten et al., in press-b)
 526 CI at IC₅₀ = combination index at IC₅₀. CI <1: synergism; CI =1: additive effect; CI >1:
 527 antagonism. n.d. = not determined (no fitting possible).
 528 r = linear correlation coefficient of the median-effect plot, indicating the goodness of fit. (r ≥
 529 85 indicates a satisfactory fit).

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

532

533

534

Group	<i>H. bakeri</i>		<i>A. ceylanicum</i>	
	% 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)

535

536 SD = standard deviation, * P<0.001

537

538

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

541

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

542
 543

544 SD= standard deviation. NA= Not assessed.

545 The numbers in superscript refer to the corresponding control group. ^α versus tribendimidine

546 0.5 mg/kg, ^β versus tribendimidine 1 mg/kg. # For comparison, worm burden reductions

547 obtained with the subcurative doses of the partner drugs (albendazole, levamisole,

548 ivermectin) are also listed (Nwosu et al., 2011)

549

550 **Table 4: Dose-response relationships of tribendimidine, dADT and AdADT**
 551 **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	

552

553

554 SD= standard deviation. NA=Not assessed. The numbers in superscript refer to the
 555 corresponding control group. P-value determined for worm burden reductions. CI at IC₅₀ =
 556 combination index at IC₅₀, CI at IC₉₀ = combination index at IC₉₀. CI <1: synergism; CI =1:
 557 additive effect; CI >1: antagonism. [§] The worm expulsion rate and the worm burden reduction
 558 obtained with 10 mg/kg levamisole are given (Tritten et al., in press-b).

559

560 **Figure legend**

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

562 Fa= effect level.