

1 **Activity of OZ78 analogues against *Fasciola hepatica* and *Echinostoma caproni***

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23 Running title: Activity of OZ78 derivatives against *Fasciola hepatica*

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26 The rapid spread of triclabendazole resistance in veterinary medicine is an important
27 motivation for fasciocidal drug discovery and development. The aim of this study was to
28 characterize the fasciocidal properties of 1,2,4,5-tetraoxane (MT04 and MT14) and
29 1,2,4-trioxane (ST16 and ST28) analogues of the fasciocidal drug candidate OZ78, an
30 1,2,4-trioxolane. Dose response relationships were determined against juvenile and
31 adult *Fasciola hepatica* in rats and *Echinostoma caproni* in mice. The temporal effects of
32 MT04, MT14, ST16, and ST28 compared to OZ78 on the viability of *F. hepatica* were
33 tested *in vitro*. The heat flow of OZ78 and MT04 treated flukes was studied with
34 isothermal microcalorimetry. Finally, surface changes to adult flukes were monitored by
35 scanning electron microscopy (SEM) 18, 24, and 48 h post-treatment of rats with 50
36 mg/kg MT04. Administration of 50-100 mg/kg of the synthetic peroxides resulted in
37 complete elimination of adult *F. hepatica* from rats. SEM pictures revealed sloughing and
38 blebbing already 18 h post-treatment with MT04. MT04 (100 mg/kg) cured infections with
39 juvenile *F. hepatica*, whereas MT14, ST16, and ST28 showed only low to moderate
40 worm burden reductions. At 300 mg/kg, MT14 was the only compound to completely
41 eliminate worms from *E. caproni* infected mice. MT14 showed the highest activity against
42 juvenile *F. hepatica in vitro*. MT04 was very active against adult *F. hepatica in vitro*,
43 which was confirmed by heat flow measurements. In conclusion, we have identified
44 MT04 as another lead compound with potential against *F. hepatica*, hence further
45 preclinical studies are necessary to determine if MT04 can be considered a drug
46 development candidate.

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50 **Keywords:** *Fasciola hepatica*, *Echinostoma caproni*, synthetic peroxides, *in vivo* studies,
51 *in vitro* studies, microcalorimetry

52 1. Introduction

53 *Fasciola hepatica* and *F. gigantica* are hepatic plant-borne trematodes causing
54 fascioliasis (Keiser and Utzinger, 2009; Robinson and Dalton, 2009). Fascioliasis is an
55 important public health problem in many countries on different continents (Bolivia, Chile,
56 Cuba, Ecuador, Egypt, France, Peru, Portugal and Spain) (Mas-Coma et al., 2007). It
57 has been estimated that more than 91 million people are at risk of infection, with 2.4-17
58 million infections (Keiser and Utzinger, 2009). In the veterinary field, the economic loss
59 due to fascioliasis of cattle and sheep is enormous (Schweizer et al., 2005).

60 Today's first line therapy of infections with *Fasciola* spp. is triclabendazole
61 (Fasinex®, Egaten®), a benzimidazole anthelmintic, which is highly effective against
62 immature and mature flukes. The drug is widely available in veterinary medicine but
63 registered in only four countries for the treatment of human fascioliasis (Fairweather,
64 2009; Fairweather and Boray, 1999). Triclabendazole-resistant *F. hepatica* populations,
65 which have emerged on different continents in sheep and cattle, are of major concern
66 such as Australia and North Europe (Moll et al., 2000), but poorly studied and
67 documented in some parts of the world, such as the Andean Region, where
68 triclabendazole is widely used in cattle (Espinoza et al., 2010).

69 The rapid spread of triclabendazole resistance is an important motivation for
70 fasciocidal drug discovery. Recent studies have shown that the artemisinins and the
71 synthetic 1,2,4-trioxolane (ozonide) OZ78 have potent flukicidal activity (Halferty et al.,
72 2009; Keiser et al., 2006; Vennerstrom et al., 2004). In an effort to identify more effective
73 trematocidal synthetic peroxides, a structurally diverse library of OZ78 analogues was
74 recently studied. It was found that a peroxide group, a spiroadamantane substructure
75 and acidic functional group (or ester prodrug) were required for fasciocidal activity (Zhao
76 et al., 2010). We also observed that 1,2,4-trioxane and 1,2,4,5-tetraoxane isosteres are

77 usually more effective than the corresponding 1,2,4-trioxolanes (unpublished
78 observation).

79 The aim of the present work was to study and compare the fasciocidal activity of
80 synthetic peroxides MT04, MT14, ST16, and ST28 (Figure 1). We determined dose-
81 response relationships against juvenile and adult *F. hepatica in vitro* and *in vivo*. We
82 studied the *in vivo* effect of the compounds against the intestinal fluke *Echinostoma*
83 *caproni*, a non-haematophagous feeder to determine the contribution of haemoglobin
84 digestion to the activity of these peroxides. Finally, scanning electron microscopy (SEM)
85 and isothermal microcalorimetry was used to characterize the fasciocidal properties of
86 MT04 in greater detail.

87

88 **2. Materials and methods**

89 **2.1. Ethical clearance, parasites and host-parasite model**

90 All animal studies were carried out at the Swiss Tropical and Public Health Institute
91 (Basel, Switzerland) and were approved by Swiss and cantonal authorities (permission:
92 2070). Female Wistar rats (n=124, age: 4-5 weeks, weight: ~ 150 g) and female NMRI
93 mice (n=36, age: 3-4 weeks, weight: ~ 20 g) were purchased from RCC (Horst, The
94 Netherlands). Animals were kept in groups of 5 (rats) and 10 (mice) in macrolon cages in
95 environmentally-controlled conditions (temperature: ~ 25°C; humidity: ~ 70%; 12 h
96 light/dark cycle) and acclimatized for one week. They had free contact to water and
97 rodent diet.

98 Metacercariae (Pacific Northwest Wild Strain) of *F. hepatica* were purchased from
99 Baldwin Aquatics (Monmouth, OR, USA). Metacercarial cysts of *E. caproni* were
100 obtained from infected *Biomphalaria glabrata* snails kept in our laboratories.

101

102 **2.2. Test compounds**

103 OZ78, MT04, MT14, ST16, and ST28 were synthesized following literature
104 methods (Tang et al., 2005). The chemical structures of MT04, MT14, ST16, ST28, and
105 OZ78 are depicted in Figure 1.

106 **<Figure 1 near here>**

107 For the *in vivo* studies MT04, MT14, ST16, and ST28 were suspended in 7% (v/v)
108 Tween-80 and 3% (v/v) ethanol. Stock solutions of MT04, MT14, ST16, ST28, and OZ78
109 were prepared in 60% in DMSO (v/v) for *in vitro* studies.

110

111 **2.3. *In vivo* studies**

112 **2.3.1. *Fasciola hepatica* infection**

113 Approximately 20 metacercarial cysts of *F. hepatica* were orally administered to
114 each rat using the gavage technique. Three (juvenile infection) and eight (adult infection)
115 weeks post-infection, groups of 3 to 4 rats were treated orally with MT04, MT14, ST16,
116 and ST28 at single doses of 25-100 mg/kg. Untreated rats served as controls. One week
117 after treatment, rats were killed using CO₂. The livers of rats harbouring juvenile flukes
118 were flattened and examined for the presence of worms. Adult *F. hepatica* flukes were
119 harvested from the livers and excised bile ducts and placed in Petri dishes. The worm
120 count and the viabilities of all flukes recovered were recorded.

121

122 **2.3.2. *Echinostoma caproni* infection**

123 Approximately 35 metacercarial cysts of *E. caproni* were applied to each mouse
124 using the gavage technique. Two weeks post-infection, 5 groups of 3 to 5 mice were
125 treated orally with MT04, MT14, ST16, and ST28 at single 150-300 mg/kg oral doses.
126 Untreated mice served as control. Seven days after treatment, mice were euthanized by

127 CO₂. At necropsy, all *E. caproni* were removed from the pylorus to the ileocaecal valve of
128 the mice and counted.

129

130 **2.4. SEM observations**

131 Three rats were infected orally with 20 *F. hepatica* metacercariae each. Eight
132 weeks post-infection, the rats were treated with MT04 (50 mg/kg). At 18, 24, and 48 h
133 post-treatment, respectively one rat was killed by CO₂. Flukes were collected from the
134 livers and bile ducts and fixed for 24 h in 2.5% glutaraldehyde in PBS buffer at room
135 temperature. The specimens were then thoroughly washed with buffer, dehydrated with
136 ethanol and critically point dried (Bomar SPC-900; Tacoma, USA). Flukes were mounted
137 on aluminum stubs, sputter-coated with gold of 20 nm (Baltec Med 020, Tucson, USA)
138 and observed in a high-resolution SEM (Philips XL30 ESEM; Eindhoven, the
139 Netherlands) at an accelerating voltage of 5 kV.

140

141 **2.5. *In vitro* studies**

142 Adult and juvenile *F. hepatica* flukes were recovered from livers and bile ducts of
143 infected rats. In addition, adult *F. hepatica* collected from infected bovine livers obtained
144 from the local slaughterhouse (Basel, Switzerland) were used. The worms were quickly
145 washed with 0.9% (w/v) NaCl and placed in 6 or 12-well plates (Costar).

146 Culture medium in each well contained RPMI 1640 (Gibco) at 37°C, which was
147 supplemented with antibiotics (50 µg/ml streptomycin and 50 IU/ml penicillin; Gibco) and
148 80 µg/ml of a haemin solution. The haemin solution was prepared as follows: 5 mg
149 haemin was dissolved in 1 ml of 0.1 M aqueous solution of NaOH, and 3.95 ml of PBS
150 (pH = 7.4) and 0.05 ml of 1 M HCl were added to adjust the pH to 7.1 – 7.4 (Keiser and
151 Morson, 2008). Cultures were kept at 37°C in an atmosphere of 5% CO₂.

152 To monitor the temporal drug effect of MT04, MT14, ST16, ST28, and OZ78 *in*
153 *vitro*, 3-6 flukes were incubated for 72 h in the presence of 50 µg/ml of the test drugs. At
154 24, 48, and 72 h, worms were examined using a dissecting microscope. For the adult
155 worms, a viability scale ranging from 4 (normal movements) to 1 (death; no movement
156 observed for two min using a microscope) was used. The experiment was repeated 2-4
157 times. For the juvenile worms, we applied a viability scale from 3 (normal movements
158 observed using a microscope) to 1 (death; no movement observed for two min using a
159 microscope).

160

161 **2.6. Microcalorimetry**

162 A multi-channel isothermal multi-calorimeter (Model “TAM III”, TA instruments,
163 New Castle, DE) was used to monitor the heat-production of *F. hepatica* over time as a
164 result of their metabolic activity. The calorimeter was set at 37°C two days before the
165 start of the experiment. All materials used were sterilized and drug solutions were sterile
166 filtered (0.2 µm).

167 Worms recovered from the bile ducts of infected rats were washed and placed in
168 20 ml glass ampoules containing 3 ml culture medium supplemented with antibiotics,
169 haemin-solution, and drug solution as described above. The heat-flow of 4 flukes
170 (incubated in 50 µg/ml OZ78), 5 flukes (incubated in 50 µg/ml MT04) and 5 controls was
171 recorded every 10 min over 96 h. Drug effects were analysed by comparing the heat-
172 flow curves of medium containing dead worms or medium only, worms alive with no
173 treatment, and worms incubated in drug solution. Inhibition of activity of adult *F. hepatica*
174 was calculated by comparing (random) oscillation amplitudes, which were derived from
175 the worm motor activities (Fig. 2) of untreated and treated worms (Manneck et al., 2011).

176

<Figure 2 near here>

177 **2.7. Statistical analysis**

178 Statistical analyses were performed with version 2.4.5 of StatsDirect statistical
179 software (StatsDirect Ltd; Cheshire, UK). Average worm burdens were expressed as
180 arithmetic means. The Kruskal-Wallis (KW) test was applied to compare the medians of
181 the responses between the treatment and control groups. A difference in median was
182 considered to be significant at a level of 5%. Analyses of noise amplitudes for
183 calorimetric measurements were performed using R software and Microsoft Excel® (R
184 Development Core Team, 2008).

185

186 **3. Results**

187 **3.1. Effect of MT04, MT14, ST16, and ST28 against adult and juvenile *F. hepatica*** 188 **harboured in rats**

189 OZ78 analogues MT04, MT14, ST16, and ST28 were first administered as single
190 100 mg/kg oral doses to rats infected with adult *F. hepatica*. This dose was chosen
191 based on previous findings documented for OZ78 (ED₅₀ of 23 mg/kg and ED₉₉ of 99
192 mg/kg) (Duthaler et al., 2010). In a next step, doses were titrated down to 50 and 25
193 mg/kg. Compound efficacies from these experiments are summarized in Table 1. At 100
194 mg/kg, all compounds were completely curative. At 50 mg/kg, MT04, ST16, and ST28
195 resulted in worm burden reductions of 100%, respectively, whereas MT14 produced only
196 a 61% worm burden reduction. At the lowest dose administered (25 mg/kg), MT04,
197 ST16, and ST28 effected worm burden reductions of 71, 88, and 0%, respectively.

198

199 **<Table 1 near here>**

200 Since a fasciocidal drug development candidate should have a broad spectrum of
201 activity, activities against juvenile *F. hepatica* were studied. Compound efficacies of
202 MT04, MT14, ST16, and ST28 administered at 50 and 100 mg/kg oral doses to rats
203 infected with juvenile *F. hepatica* are presented in Table 2. Administration of 100 and 50

204 mg/kg MT04 resulted in worm burden reductions of 100 and 61%, respectively, an
205 outcome almost identical to that previously observed for OZ78, which at the same doses,
206 decreased worm burden by 100 and 67% (Keiser et al., 2006). A significant difference
207 was observed between MT04 treated and untreated rats in the juvenile infection model
208 (KW = 13.26; P = 0.0003). On the other hand, low to moderate worm burden reductions
209 (0-46%) were observed for ST16, ST28, and MT14.

210 <Table 2 near here>

211

212 **3.2. Effect of OZ78 analogues against *E. caproni***

213 We assessed the efficacies of the 4 OZ78 derivatives against the non-blood
214 feeder *E. caproni* to obtain further insight into the mechanism of action of these
215 compounds. In more detail, our goal was to determine whether trematocidal activity
216 entirely depends on haeme iron-mediated reactivity or whether also other targets are
217 involved. In addition, as juvenile *F. hepatica* show a preference for hepatic cells rather
218 than blood (Dawes, 1961) we were wondering whether there would be a relationship
219 between an echinostomocidal activity and activity against juvenile *F. hepatica*. At 300
220 mg/kg, MT04, ST16, and ST28 showed no activity against *E. caproni* in mice. In
221 comparison, 1000 mg/kg OZ78 was required for good echinostomocidal activity (Keiser et
222 al., 2006). On the other hand, a worm burden reduction of 100% was observed with
223 MT14 at 300 mg/kg (Table 3).

224 <Table 3 near here>

225

226 **3.3. *In vitro* activity against juvenile and adult *F. hepatica***

227 The temporal effects of MT04, MT14, ST16, ST28, and OZ78 (50 µg/ml) on adult
228 *F. hepatica in vitro* collected from rats and bovine are presented in Figure 3A and 3B.
229 Control *Fasciola* showed normal movements at all examination time points. Flukes

230 obtained from rats incubated in the presence of MT04 showed reduced activities at the
231 24 h time point (mean viability: 2.3). Twenty-four h later, only minimal viability was
232 observed (mean viability: 1.6). 72 h post-incubation with MT04, all flukes were dead.
233 Bovine flukes incubated with 50 µg/ml MT04 showed reduced viabilities 72 h post-
234 incubation (mean viability: 1.6). Flukes incubated with ST28, and OZ78 showed reduced
235 movements 72 h post exposure (mean viabilities rat flukes: 2.1 and 2.0 and mean
236 viabilities bovine flukes: 1.9 and 1.8). Slightly contradictory results were observed with
237 ST16: while flukes obtained from rats were affected by the drug 72 h post-incubation
238 (mean viability: 1.3) a less pronounced effect on *Fasciola* obtained from bovines was
239 observed at this examination time point (mean viability: 2.1). Finally, the majority of
240 worms incubated with MT14 had died 72 h post-exposure (mean viability: 1.2; rat flukes
241 and mean viability: 1.1 bovine flukes).

242 **<Figure 3A and 3B near here>**

243 The fasciocidal activities of the test drugs against juvenile *F. hepatica in vitro* are
244 presented in Figure 3C. Control flukes were alive for 72 h. Incubation with MT14 (50
245 µg/ml) resulted in death of all *F. hepatica* 48 h post-incubation. MT04, ST16, and OZ78
246 showed no effect against juvenile flukes *in vitro* (mean viability after 72 h: 2.7, 2.8 and
247 2.4, respectively). *F. hepatica* incubated in ST28 showed reduced movements after 72 h
248 (mean viability: 1.5).

249 **<Figure 3C near here>**

250

251 **3.4. Microcalorimetry of adult *F. hepatica***

252 Thermogenic noise value curves of control adult *F. hepatica* and worms
253 incubated with 50 µg/ml MT04 and OZ78 are depicted in Fig. 4. Consistently low signals
254 of 1.46 µW were measured for dead worms or medium only (data not shown). The
255 intersection of the sample amplitude curve (following exponential decay) with the

256 background signal noise of dead worms (1.46 μ W) was set as an endpoint of worm
257 motility. Worms incubated with MT04 and OZ78 were dead after 29.6 h and 43.4 h,
258 respectively. Control worms were viable for 69.3 h.

259 **<Figure 4 near here>**

260

261 **3.5. *In vivo* SEM observations**

262 SEM studies were only performed with MT04 since it was the most efficacious
263 analogue of OZ78. At 18 h post-treatment with 50 mg/kg of MT04, 6 flukes were
264 collected from a rat and processed for SEM. Disruption of the tegument was visible, in
265 particular on the anterior region of *F. hepatica* where blebbing and sloughing were
266 observed (Figures 5A and B). Twenty-four h post-treatment, we collected 2 dead
267 specimens and 1 *F. hepatica* that showed minor activity. Similar abnormalities such as
268 blebbing and furrowing, which had not progressed further in severity, were observed on
269 these worms (Figure 5C and 5D). Forty-eight h post-treatment only dead *F. hepatica*
270 were recovered and since flukes were broken they were not processed for SEM
271 analyses.

272 **<Figure 5 near here>**

273

274 **4. Discussion**

275 Triclabendazole is an ideal fasciocidal drug as it is orally active against both
276 juvenile and adult *F. hepatica* (Fairweather and Boray, 1999). However, since drug
277 resistance is spreading it is imperative that novel fasciocidal drugs are discovered and
278 developed. The synthetic ozonides seem to offer an excellent starting point as recent
279 studies showed that OZ78 is active against adult and juvenile *F. hepatica in vitro* and *in*
280 *vivo*, including resistant isolates (Keiser and Utzinger, 2007). In the present work, the
281 fasciocidal activities of 4 OZ78 analogues were studied in greater detail.

282 MT04 had the highest activities against both juvenile and adult *F. hepatica in vivo*.
283 MT04 was superior to OZ78, in particular against adult *F. hepatica*. A single 50 mg/kg
284 oral dose of MT04 achieved complete worm burden reductions against adult *F. hepatica*
285 in rats, while 100 mg/kg doses of OZ78 were required to cure *F. hepatica* infected rats
286 (Keiser et al., 2006). Forty-eight h after treatment with 50 mg/kg MT04, only dead flukes
287 were recovered from a rat. Flukes collected at earlier time points showed disrupted
288 teguments including sloughing and blebbing and some flukes had already died.
289 Comparable tegumental alterations (blebs, sloughing, and furrows) were also seen 24-
290 72 h after treatment with 100 mg/kg OZ78 (Keiser and Morson, 2008). The main
291 difference observed between the two drugs was the onset of action. Eighteen-24 h after
292 treatment with MT04, *F. hepatica* showed reduced viabilities or had already died,
293 whereas dead worms were collected from OZ78-treated rats 72 h post-treatment (Keiser
294 and Morson, 2008). Whether differences in *in vivo* efficacy and the onset of action
295 between the two compounds derive from pharmacodynamic or pharmacokinetic
296 parameters is not clear, but it is evidently a function of their two different peroxide
297 heterocycles. In this respect, O'Neill et al. have recently shown that the red blood cell
298 stability of tetraoxanes is higher than that of the corresponding trioxolanes (ozonides)
299 (O'Neill et al., 2010). The mechanism of action of the secondary ozonides against
300 *Fasciola* spp. has not yet been elucidated. However, a formation of carbon-centered
301 radicals, similar to the antimalarial mechanism of action might play a role (Dong et al.,
302 2010).

303 Our *in vitro* studies on adult *F. hepatica* confirmed the excellent flukicidal activity of
304 MT04. After 72 h, the majority of adult worms incubated in presence of 50 µg/ml MT04
305 were dead. It is interesting to note that OZ78 and MT04 did not show any effects against
306 juvenile *F. hepatica in vitro* in line with results obtained with OZ78 in a recent study
307 (Duthaler et al., 2010). Why juveniles are affected *in vivo*, but not *in vitro* is not known,

308 but drug metabolism may account for these differences. A good relationship with regard
309 to compound sensitivity was observed between the *F. hepatica* Pacific Northwest wild
310 strain harboured in rats and bovine slaughterhouse isolates, although flukes obtained
311 from infected bovine livers were slightly less susceptible to the test drugs.

312 We speculated that a drug effect against *E. caproni* might point to an activity
313 against juvenile *F. hepatica*, since both parasites do not feed on large quantities of blood
314 (Dawes, 1961; Keiser and Utzinger, 2007). However, no relationship was observed
315 between drug sensitivities on echinostomes and juvenile *F. hepatica*. Though ST16 and
316 ST28 lacked activity against both parasite stages, MT14 had activity against *E. caproni*,
317 while lacking activity against juvenile *F. hepatica in vivo*. On the other hand, MT04
318 revealed no activity against echinostomes but cured infections with juvenile *F. hepatica*.

319 We have shown for the first time that heat flow measurements are an excellent
320 tool to study the effects of fasciocidal drugs. The usefulness of this method to study drug
321 effects on helminths has recently been demonstrated for another trematode, namely
322 *Schistosoma mansoni* (Manneck et al., 2011). In the present work, heat flow
323 measurements confirmed data obtained by morphological *in vitro* testing.
324 Microcalorimetry showed that worms incubated in MT04 died earlier than worms
325 incubated with OZ78. Compared to our standard *in vitro* assays, untreated worms died
326 earlier (69 hours) which might be due to a lack of oxygen in the calorimetry vials. Further
327 studies are currently ongoing in our laboratories, including experiments with juvenile
328 flukes and the reference drug triclabendazole in order to validate and standardize the
329 use of microcalorimetry to study drug effects on *Fasciola* spp..

330 In conclusion, this assessment of 4 promising synthetic peroxide derivatives of
331 OZ78 has identified MT04 as another lead compound with potential against *F. hepatica*
332 and perhaps other haemoglobin-degrading flukes. We anticipate that ongoing

333 pharmacokinetic and mechanism of action studies with MT04 should provide the
334 necessary data to determine if MT04 can be considered a drug development candidate.

335

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342

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433 **Figure legend:**

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435 **Figure 1:** Chemical structures of MT04, MT14, ST16, ST28, and OZ78.

436 **Figure 2:** Noise analysis of adult *F. hepatica* (A): heat-flow curve of a sample containing
437 1 adult worm, showing the occurrence of noise/oscillations over time (black curve).

438 Oscillations amplitude values follow exponential decay (grey curve) (B): magnification of
439 oscillations derived from A, (C): Maximum values of the amplitude over a window of 20
440 min during the entire course of the experiment. The intersection of the background noise
441 of the calorimetric system (grey dotted line) with the smoothed sample curve (grey
442 curve) is the endpoint and corresponds to the calculated death of the worm.

443 **Figure 3A:** *In vitro* activity of MT04, MT14, ST16, and ST28 at a concentration of 50
444 µg/ml against adult *F. hepatica* (obtained from rats) compared to control worms and
445 worms incubated with OZ78 (50 µg/ml). Black line with white diamond: control; black
446 dotted line with black circle: OZ78; black line with black square: MT04; grey line with
447 white circle: MT14; dotted and dashed black line with black diamond: ST16; grey line
448 with black triangle: ST28. The limits of the whiskers correspond to the standard error of
449 the mean values per time point.

450 **Figure 3B:** *In vitro* activity of MT04, MT14, ST16, and ST28 at a concentration of 50
451 µg/ml against adult *F. hepatica* (obtained from bovine livers) compared to control worms.
452 Black line with white diamond: control; black dotted line with black circle: OZ78; black
453 line with black square: MT04; grey line with white circle: MT14; dotted and dashed black
454 line with black diamond: ST16; grey line with black triangle: ST28. The limits of the
455 whiskers correspond to the standard error of the mean values per time point.

456 **Figure 3C:** *In vitro* activity of 50 µg/ml MT04, MT14, ST16, and ST28 against juvenile *F.*
457 *hepatica* compared to control worms and worms incubated with OZ78 50 µg/ml. Black

458 line with white diamond: control; black dotted line with black circle: OZ78; black line with
459 black square: MT04; grey line with white circle: MT14; dotted and dashed black line with
460 black diamond: ST16; grey line with black triangle: ST28. The limits of the whiskers
461 correspond to the standard error of the mean values per time point.

462 **Figure 4:** Absolute noise values of untreated and treated worms (OZ78 50 µg/ml and
463 MT04 50 µg/ml). Dotted black line: background; black line: MT04, dark-grey shaded line:
464 OZ78, light-grey line: control.

465 **Figure 5A-D:** Fig. 5A, B: SEM observation of adult *F. hepatica* 18 h post treatment with
466 50 mg/kg MT04. (A) Disruption and sloughing (s) of the tegument near the oral sucker
467 (OS). (B) Blebbing (b) observed on the tegument. Fig. 5C, D: SEM observation of adult
468 *F. hepatica* 24 h post treatment with 50 mg/kg MT04. (C) Blebs in the OS region, (D)
469 furrows (f) visible in the mid body region.

Table 1: Worm burden reductions achieved against adult *F. hepatica* harboured in rats following the administration of MT04, MT14, ST16, and ST28 at different doses.

Treatment	Dose (mg/kg)	No. of rats investigated	No. of rats cured	Mean worm burden	Total flukes recovered		Total worm burden reduction (%)	KW	P
					Live	Dead			
Control	- ¹	7	0	7.7	54	0	-		
	- ²	7	0	7	49	0	-		
	- ³	5	0	2	10	0	-		
	- ⁴	5	0	7.2	36	0	-		
	- ⁵	5	0	4	20	0	-		
	- ⁶	7	0	7.6	53	0	-		
MT04	25 ¹	4	0	2.25	9	0	70.8		
	50 ⁴	4	4	0	0	3	100	17.06	<0.0001
	100 ⁴	4	4	0	0	7	100		
MT14	50 ²	4	1	2.75	11	3	60.7		
	100 ⁶	3	3	0	0	8	100	11.96	0.0005
ST16	25 ³	4	3	0.25	1	0	87.5		
	50 ²	4	4	0	0	1	100	17.48	<0.0001
	100 ²	3	3	0	0	1	100		
ST28	25 ⁵	4	1	5	20	0	0		
	50 ⁵	3	3	0	0	7	100	2.625	0.1052
	100 ⁵	3	3	0	0	0	100		

KW Kruskal Wallis; Superscript number matches control group with the corresponding treatment group

Table 2: Worm burden reductions achieved against juvenile *F. hepatica* harboured in rats following the administration of MT04, MT14, ST16, and ST28 at two different doses.

Treatment	Dose (mg/kg)	No. of rats investigated	No. of rats cured	Mean worm burden	Total flukes recovered		Total worm burden reduction (%)	KW	P
					Live	Dead			
Control	- ¹	7	0	7	49	0	-		
	- ²	4	0	6.75	27	0	-		
	- ³	3	0	8.33	25	0	-		
MT04	50 ¹	4	0	2.75	11	0	60.7	13.26	0.0003
	100 ²	6	6	0	0	2	100		
MT14	50 ³	4	0	7.75	31	0	7.0	0.52	0.4688
	100 ³	4	0	6.75	27	0	19.0		
ST16	50 ²	4	0	5.75	23	0	14.8	1.44	0.2304
	100 ²	4	0	3.75	15	0	44.4		
ST28	50 ³	4	0	10.5	42	0	0	0.17	0.6789
	100 ³	4	0	4.5	18	0	46.0		

KW Kruskal Wallis

Superscript number matches control group with the corresponding treatment group

Table 3: Worm burden reductions achieved against adult *E. caproni* harboured in mice following the administration of MT04, MT14, ST16, and ST28 at different doses.

Treatment	Dose (mg/kg)	No. of mice investigated	No. of mice Cured	Mean worm burden	Total flukes recovered		Total worm burden reduction (%)	KW	P
					Live	Dead			
Control	- ¹	7	0	19.9	139	0	-		
	- ²	5	0	24.2	121	0	-		
	- ³	5	0	29.6	148	0	-		
MT04	300 ³	4	1	13.5	54	0	54.4	5.46	0.0195
MT14	150 ²	5	1	15	75	0	38.0	5.59	0.0180
	300 ¹	3	3	0	0	0	100		
ST16	300 ²	3	0	25.7	77	0	0	0.02	0.8815
ST28	300 ²	4	0	20	80	0	17.4	1.54	0.2148

KW Kruskal Wallis

Superscript number matches control group with the corresponding treatment group