Activity of OZ78 analogues against Fasciola hepatica and Echinostoma caproni

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

**Keywords:** Fasciola hepatica, Echinostoma caproni, synthetic peroxides, in vivo studies, in vitro studies, microcalorimetry
1. Introduction

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

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

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

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

2. Materials and methods

2.1. Ethical clearance, parasites and host-parasite model

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

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

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

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

2.3. In vivo studies

2.3.1. Fasciola hepatica infection

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

2.3.2. Echinostoma caproni infection

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

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

2.5. In vitro studies

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

Culture medium in each well contained RPMI 1640 (Gibco) at 37°C, which was supplemented with antibiotics (50 µg/ml streptomycin and 50 IU/ml penicillin; Gibco) and 80 µg/ml of a haemin solution. The haemin solution was prepared as follows: 5 mg haemin was dissolved in 1 ml of 0.1 M aqueous solution of NaOH, and 3.95 ml of PBS (pH = 7.4) and 0.05 ml of 1 M HCl were added to adjust the pH to 7.1 – 7.4 (Keiser and Morson, 2008). Cultures were kept at 37°C in an atmosphere of 5% CO₂.
To monitor the temporal drug effect of MT04, MT14, ST16, ST28, and OZ78 in vitro, 3-6 flukes were incubated for 72 h in the presence of 50 µg/ml of the test drugs. At 24, 48, and 72 h, worms were examined using a dissecting microscope. For the adult worms, a viability scale ranging from 4 (normal movements) to 1 (death; no movement observed for two min using a microscope) was used. The experiment was repeated 2-4 times. For the juvenile worms, we applied a viability scale from 3 (normal movements observed using a microscope) to 1 (death; no movement observed for two min using a microscope).

2.6. Microcalorimetry

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

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

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

3. Results

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

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

Since a fasciocidal drug development candidate should have a broad spectrum of activity, activities against juvenile F. hepatica were studied. Compound efficacies of MT04, MT14, ST16, and ST28 administered at 50 and 100 mg/kg oral doses to rats infected with juvenile F. hepatica are presented in Table 2. Administration of 100 and 50
mg/kg MT04 resulted in worm burden reductions of 100 and 61%, respectively, an outcome almost identical to that previously observed for OZ78, which at the same doses, decreased worm burden by 100 and 67% (Keiser et al., 2006). A significant difference was observed between MT04 treated and untreated rats in the juvenile infection model (KW = 13.26; P = 0.0003). On the other hand, low to moderate worm burden reductions (0-46%) were observed for ST16, ST28, and MT14.

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

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

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

The temporal effects of MT04, MT14, ST16, ST28, and OZ78 (50 µg/ml) on adult *F. hepatica in vitro* collected from rats and bovine are presented in Figure 3A and 3B. Control *Fasciola* showed normal movements at all examination time points. Flukes
obtained from rats incubated in the presence of MT04 showed reduced activities at the 24 h time point (mean viability: 2.3). Twenty-four h later, only minimal viability was observed (mean viability: 1.6). 72 h post-incubation with MT04, all flukes were dead.

Bovine flukes incubated with 50 µg/ml MT04 showed reduced viabilities 72 h post-incubation (mean viability: 1.6). Flukes incubated with ST28, and OZ78 showed reduced movements 72 h post exposure (mean viabilities rat flukes: 2.1 and 2.0 and mean viabilities bovine flukes: 1.9 and 1.8). Slightly contradictory results were observed with ST16: while flukes obtained from rats were affected by the drug 72 h post-incubation (mean viability: 1.3) a less pronounced effect on Fasciola obtained from bovines was observed at this examination time point (mean viability: 2.1). Finally, the majority of worms incubated with MT14 had died 72 h post-exposure (mean viability: 1.2; rat flukes and mean viability: 1.1 bovine flukes).

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

3.4. Microcalorimetry of adult F. hepatica

Thermogenic noise value curves of control adult F. hepatica and worms incubated with 50 µg/ml MT04 and OZ78 are depicted in Fig. 4. Consistently low signals of 1.46 µW were measured for dead worms or medium only (data not shown). The intersection of the sample amplitude curve (following exponential decay) with the
background signal noise of dead worms (1.46 μW) was set as an endpoint of worm
motility. Worms incubated with MT04 and OZ78 were dead after 29.6 h and 43.4 h,
respectively. Control worms were viable for 69.3 h.

<Figure 4 near here>

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

<Figure 5 near here>

4. Discussion
Triclabendazole is an ideal fasciocidal drug as it is orally active against both
juvenile and adult F. hepatica (Fairweather and Boray, 1999). However, since drug
resistance is spreading it is imperative that novel fasciocidal drugs are discovered and
developed. The synthetic ozonides seem to offer an excellent starting point as recent
studies showed that OZ78 is active against adult and juvenile F. hepatica in vitro and in
vivo, including resistant isolates (Keiser and Utzinger, 2007). In the present work, the
fasciocidal activities of 4 OZ78 analogues were studied in greater detail.
MT04 had the highest activities against both juvenile and adult *F. hepatica* *in vivo*. MT04 was superior to OZ78, in particular against adult *F. hepatica*. A single 50 mg/kg oral dose of MT04 achieved complete worm burden reductions against adult *F. hepatica* in rats, while 100 mg/kg doses of OZ78 were required to cure *F. hepatica* infected rats (Keiser et al., 2006). Forty-eight h after treatment with 50 mg/kg MT04, only dead flukes were recovered from a rat. Flukes collected at earlier time points showed disrupted tegumental including sloughing and blebbing and some flukes had already died. Comparable tegumental alterations (blebs, sloughing, and furrows) were also seen 24-72 h after treatment with 100 mg/kg OZ78 (Keiser and Morson, 2008). The main difference observed between the two drugs was the onset of action. Eighteen-24 h after treatment with MT04, *F. hepatica* showed reduced viabilities or had already died, whereas dead worms were collected from OZ78-treated rats 72 h post-treatment (Keiser and Morson, 2008). Whether differences in *in vivo* efficacy and the onset of action between the two compounds derive from pharmacodynamic or pharmacokinetic parameters is not clear, but it is evidently a function of their two different peroxide heterocycles. In this respect, O'Neill et al. have recently shown that the red blood cell stability of tetraoxanes is higher than that of the corresponding trioxolanes (ozonides) (O'Neill et al., 2010). The mechanism of action of the secondary ozonides against *Fasciola* spp. has not yet been elucidated. However, a formation of carbon-centered radicals, similar to the antimalarial mechanism of action might play a role (Dong et al., 2010).

Our *in vitro* studies on adult *F. hepatica* confirmed the excellent flukicidal activity of MT04. After 72 h, the majority of adult worms incubated in presence of 50 µg/ml MT04 were dead. It is interesting to note that OZ78 and MT04 did not show any effects against juvenile *F. hepatica* *in vitro* in line with results obtained with OZ78 in a recent study (Duthaler et al., 2010). Why juveniles are affected *in vivo*, but not *in vitro* is not known,
but drug metabolism may account for these differences. A good relationship with regard to compound sensitivity was observed between the *F. hepatica* Pacific Northwest wild strain harboured in rats and bovine slaughterhouse isolates, although flukes obtained from infected bovine livers were slightly less susceptible to the test drugs.

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

We have shown for the first time that heat flow measurements are an excellent tool to study the effects of fasciocidal drugs. The usefulness of this method to study drug effects on helminths has recently been demonstrated for another trematode, namely *Schistosoma mansoni* (Manneck et al., 2011). In the present work, heat flow measurements confirmed data obtained by morphological *in vitro* testing.

Microcalorimetry showed that worms incubated in MT04 died earlier than worms incubated with OZ78. Compared to our standard *in vitro* assays, untreated worms died earlier (69 hours) which might be due to a lack of oxygen in the calorimetry vials. Further studies are currently ongoing in our laboratories, including experiments with juvenile flukes and the reference drug triclabendazole in order to validate and standardize the use of microcalorimetry to study drug effects on *Fasciola* spp..

In conclusion, this assessment of 4 promising synthetic peroxide derivatives of OZ78 has identified MT04 as another lead compound with potential against *F. hepatica* and perhaps other haemoglobin-degrading flukes. We anticipate that ongoing
pharmacokinetic and mechanism of action studies with MT04 should provide the necessary data to determine if MT04 can be considered a drug development candidate.

Acknowledgements

This investigation received financial support from the Swiss National Science Foundation (project no. PPOOA--114941) and the NIH (Grant R21AI076783).

We are thankful Mrs Evi Bieler at the Center for Microscopy of University Basel for expert help with the SEM pictures. Thanks are also addressed to Urs Duthaler for his laboratory support and scientific help.

References


Figure legend:

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

Figure 2: Noise analysis of adult *F. hepatica* (A): heat-flow curve of a sample containing 1 adult worm, showing the occurrence of noise/oscillations over time (black curve). Oscillations amplitude values follow exponential decay (grey curve) (B): magnification of oscillations derived from A, (C): Maximum values of the amplitude over a window of 20 min during the entire course of the experiment. The intersection of the background noise of the calorimetric system (grey dotted line) with the smoothed sample curve (grey curve) is the endpoint and corresponds to the calculated death of the worm.

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

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

Figure 3C: *In vitro* activity of 50 µg/ml MT04, MT14, ST16, and ST28 against juvenile *F. hepatica* compared to control worms and worms incubated with OZ78 50 µg/ml. Black
line with white diamond: control; black dotted line with black circle: OZ78; black line with black square: MT04; grey line with white circle: MT14; dotted and dashed black line with black diamond: ST16; grey line with black triangle: ST28. The limits of the whiskers correspond to the standard error of the mean values per time point.

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

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

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</tr>
<tr>
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</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No. of rats investigated</th>
<th>No. of rats cured</th>
<th>Mean worm burden</th>
<th>Total flukes recovered</th>
<th>Total worm burden reduction (%)</th>
<th>KW</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Live</td>
<td>Dead</td>
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<tr>
<td>Control</td>
<td>-1</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>49</td>
<td>0</td>
<td>-</td>
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<tr>
<td></td>
<td>-2</td>
<td>4</td>
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<td>6.75</td>
<td>27</td>
<td>0</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>-3</td>
<td>3</td>
<td>0</td>
<td>8.33</td>
<td>25</td>
<td>0</td>
<td>-</td>
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<tr>
<td>MT04</td>
<td>50&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4</td>
<td>0</td>
<td>2.75</td>
<td>11</td>
<td>0</td>
<td>60.7</td>
<td>13.26</td>
</tr>
<tr>
<td></td>
<td>100&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>6</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>MT14</td>
<td>50&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4</td>
<td>0</td>
<td>7.75</td>
<td>31</td>
<td>0</td>
<td>7.0</td>
<td>0.52</td>
</tr>
<tr>
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<td>100&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4</td>
<td>0</td>
<td>6.75</td>
<td>27</td>
<td>0</td>
<td>19.0</td>
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</tr>
<tr>
<td>ST16</td>
<td>50&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4</td>
<td>0</td>
<td>5.75</td>
<td>23</td>
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<td>14.8</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>100&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4</td>
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<td>3.75</td>
<td>15</td>
<td>0</td>
<td>44.4</td>
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</tr>
<tr>
<td>ST28</td>
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<td>10.5</td>
<td>42</td>
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<tr>
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<td>4.5</td>
<td>18</td>
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<td>46.0</td>
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</table>

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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No. of mice investigated</th>
<th>No. of mice Cured</th>
<th>Mean worm burden</th>
<th>Total flukes recovered</th>
<th>Total worm burden reduction (%)</th>
<th>KW</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>-³</td>
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<td>0</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>MT04</td>
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<td>4</td>
<td>1</td>
<td>13.5</td>
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<td>0</td>
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<td>5.46</td>
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<tr>
<td>MT14</td>
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<td>15</td>
<td>75</td>
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<td>38.0</td>
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</tr>
<tr>
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<td>ST28</td>
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<td>20</td>
<td>80</td>
<td>0</td>
<td>17.4</td>
<td>1.54</td>
</tr>
</tbody>
</table>

KW Kruskal Wallis

Superscript number matches control group with the corresponding treatment group