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The synthetic peroxide OZ78 is effective against *Echinostoma caproni* and *Fasciola hepatica*

Jennifer Keiser[1]*, Jürg Utzinger[1], Marcel Tanner[1], Yuxiang Dong[2] and Jonathan L. Vennerstrom[2]

[1]Swiss Tropical Institute, PO Box, CH-4002 Basel, Switzerland; [2]College of Pharmacy, University of Nebraska Medical Center, Nebraska, NE 68198-6025, USA

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***Objectives*: The trematocidal properties of a synthetic peroxide, 1,2,4-trioxolane (OZ78) were determined both *in vivo* and *in vitro*.**

***Methods*: Two weeks post-infection *Echinostoma caproni*-infected mice were administered single oral doses of 400–1000 mg/kg OZ78. *Fasciola hepatica*-infected rats were treated orally with 50–400 mg/kg OZ78 3 and 8–9 weeks post-infection. Worm burden reductions were assessed against untreated control animals. Adult *F. hepatica* were observed by scanning electron microscopy (SEM) after recovery from the bile duct of a rat 3 days after administration of a single oral dose of 100 mg/kg OZ78 and after *in vitro* exposure to concentrations of 1, 10 and 100 μg/mL OZ78.**

***Results*: In the *E. caproni*–mouse model 100% worm burden reductions were achieved with a single oral dose of 1000 mg/kg OZ78. A single dose of 100 mg/kg OZ78 resulted in worm burden reductions of 100% against juvenile and adult *F. hepatica*. *F. hepatica* recovered from rats 3 days post-treatment displayed feeble activity and some flukes had died. Typical features revealed by SEM included extensive blebbing and sloughing. Exposure of *F. hepatica* to 10–100 μg/mL OZ78 *in vitro* resulted in the death of all trematodes. *F. hepatica* showed focal blebbing and sloughing of the tegument at all concentrations investigated.**

***Conclusions*: Our data indicate that OZ78 is highly efficacious against *F. hepatica* and *E. caproni* and provide a sound platform for identification of a synthetic peroxide drug development candidate against major trematode infections.**

Keywords: food-borne trematodiasis, 1,2,4-trioxolanes, *in vivo* studies, *in vitro* studies, scanning electron microscopy

Introduction

Fascioliasis is a zoonotic disease of considerable public health and great veterinary significance. The causative agents of fascioliasis are liver flukes, i.e. *Fasciola hepatica* and *Fasciola gigantica.* Human fascioliasis occurs worldwide, with the highest number of infected people reported in South America, Cuba, Western Europe, Egypt and the Islamic Republic of Iran.[1] An estimated 91 million people are at risk and as many as 17 million people might be infected with either *F. hepatica* or *F. gigantica*.[2,3] Typical symptoms of the disease include fever, urticaria, pain in the right hypochondrium, hepatomegaly, hypergammaglobulinaemia and marked eosinophilia, cholangitis and cholestasis.[4] Today, treatment of human fascioliasis relies on a single drug, i.e. triclabendazole. However, since this drug is currently registered in only four countries,[5] novel treatment options are of high priority. There is considerable concern about the development of resistance to triclabendazole, which already hampers use of this drug in veterinary medicine.[6]

We have recently reported fasciocidal properties of the artemisinins, which are best known for their antimalarial[7] and to a lesser extent their antischistosomal properties.[8] In the *F. hepatica*-rat model we found that single oral doses of 200 mg/kg artemether and 400 mg/kg artesunate resulted in worm burden reductions of 100%.[9] However, the artemisinins have chemical, economic and biopharmaceutical liabilities.[10] Hence, we were motivated to evaluate the fasciocidal properties of other peroxides, particularly the antimalarial synthetic 1,2,4-trioxolanes (secondary ozonides, OZs). The OZs exhibit structural simplicity, ease of synthesis and improved pharmacokinetic

*Corresponding author. Tel: +41-61-284-8218; Fax: +41-61-284-8105; E-mail: jennifer.keiser@unibas.ch

parameters when compared with the artemisinins.[10] The antimalarial OZ277 has recently entered Phase II clinical trials. For the present study we selected OZ78, which has weak antimalarial but promising antischistosomal properties (S. H. Xiao, J. Keiser, J. Chollet, Y. Dong, J. Utzinger, M. Tanner and J. L. Vennerstrom, unpublished data). Previous research revealed that OZ78 is not mutagenic and has a toxicological profile similar to artesunate in a multiple dose 5 day exploratory toxicity experiment in male Wistar rats.[10]

We firstly present our approach to screen for trematocidal activity employing an *Echinostoma caproni*–mouse model. Emphasis is then placed on the evaluation of the fasciocidal properties of OZ78 after single oral doses at various concentrations administered to rats infected with either juvenile or adult *F. hepatica*. Finally, we document drug-induced alterations on *F. hepatica* following exposure to OZ78 *in vitro* and after administration of OZ78 *in vivo* by means of scanning electron microscopy (SEM).

Materials and methods

Ethical clearance, drug, parasites and host–parasite models

All animal studies presented here were approved by regulatory authorities following Swiss national regulations (permission no. 2070) and carried out at the Swiss Tropical Institute (Basel, Switzerland).

The chemical structure of OZ78 is depicted in Figure 1. OZ78 was synthesized at the College of Pharmacy, University of Nebraska Medical Center (Nebraska, USA). OZ78 was prepared in suspensions in 7% (v/v) Tween 80 and 3% (v/v) ethanol before oral administration.

Metacercarial cysts of *E. caproni* were removed from the kidney-pericardial region of infected snails of the species *Biomphalaria glabrata* at our laboratories as described previously.[11] Metacercariae (Cullompton isolate) of *F. hepatica* were purchased from Mr G. Graham (Addlestone, UK).

Female NMRI mice (n = 18, age: 5 weeks, weight: ~25 g) and female Wistar rats (n = 48, age: 5 weeks, weight: ~100 g) were purchased from RCC (Itingen, Switzerland). Animals were kept in groups of 5 in macrolon cages in environmentally controlled conditions (temperature: ~25°C; humidity: ~70%; 12 h light/dark cycle) and acclimatized for 1 week. They had free access to water and rodent diet.

In vivo studies E. caproni

Eighteen mice were infected intragastrically with 35 metacercarial cysts of *E. caproni* each. Two weeks post-infection, 3 groups of 4 mice were treated intragastrically with OZ78 at single doses of 400, 800 and 1000 mg/kg. The remaining 6 mice were left untreated, and hence they served as control. Three days post-treatment, mice were euthanized by CO_2. At necropsy, the intestines were removed from the pylorus to the ileocaecal valve, placed in a Petri dish and opened longitudinally. All *E. caproni* worms were removed and counted.

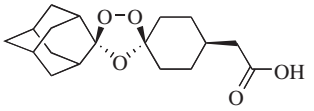

Figure 1. Chemical structure of OZ78.

In vivo studies F. hepatica

Adult infection. Thirty-two rats were infected intragastrically with 25 metacercarial cysts of *F. hepatica* each. Eight to nine weeks post-infection, 4 groups of 5 rats were treated orally with OZ78 at single doses of 50, 100, 200 and 400 mg/kg. Twelve untreated rats served as a control group. Ten days post-treatment, rats were euthanized by CO_2. At necropsy *F. hepatica* were harvested from the excised bile ducts and counted.

Juvenile infection. Fifteen rats were infected intragastrically with 25 metacercarial cysts of *F. hepatica* each. Three weeks post-infection, 2 groups of 5 rats were treated orally with OZ78 at a single oral dose of either 50 or 100 mg/kg. Five untreated rats served as controls. Eight weeks post-infection rats were killed with the CO_2 method and all *F. hepatica* were recovered from the bile ducts.

In vivo SEM observations on F. hepatica

One rat, infected intragastrically with 25 metacercarial cysts of *F. hepatica*, was administered a single oral dose of 100 mg/kg OZ78 at week 12 post-infection. The rat was killed by CO_2 at 3 days post-treatment. At necropsy *F. hepatica* were recovered from the excised bile ducts and processed for SEM studies as described below. Four flukes were examined.

In vitro studies

F. hepatica recovered from the rats of the control group were quickly washed with 0.9% (w/v) NaCl and incubated in 6-well plates (Costar), placing two *F. hepatica* per well. Culture medium in each well contained 5 mL of NCTC 135 (Gibco), which was supplemented with antibiotics (50 µg/mL streptomycin and 50 U/mL penicillin; Gibco). For one series of experiments 10% (v/v) fetal calf serum was added to the medium. Four trematodes were used for each control and experimental group.

Stock solutions of OZ78 were prepared in 60% (v/v) DMSO. The flukes were incubated with three serial drug dilutions of 1, 10 and 100 µg/mL for up to 72 h. The control well contained the highest concentration of solvent—0.06% DMSO. Cultures were kept at 37°C in an atmosphere of 5% CO_2 and observed after exposure for 24, 48 and 72 h under a dissecting microscope. *F. hepatica* were considered dead if no movement was observed for 2 min. Flukes incubated in the medium further supplemented with calf serum were collected at 24, 48 and 72 h and prepared for SEM observations as described below.

SEM observations

F. hepatica were rinsed with 0.9% (w/v) NaCl and fixed with 2.5% (v/v) glutaraldehyde in a PBS buffer for 24 h at room temperature. After rinsing with PBS buffer, the specimens were washed with distilled water, dehydrated and critically point dried (Bomar SPC-900; Tacoma, USA). After sputter-coating with gold of 20 nm (Baltec Med 020, Tucson, USA) *F. hepatica* were mounted on aluminium stubs and observed in a high-resolution SEM (Philips XL30 ESEM; Eindhofen, The Netherlands) at an accelerating voltage of 5 kV.

Statistical analysis

Average worm burdens were expressed as arithmetic means, including values of zero for animals with no worms. The Kruskal–Wallis (KW) test was used 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%. Statistical analyses were done with version 2.4.5 of Statsdirect statistical software (Statsdirect Ltd, Cheshire, UK).

Results

Effect of OZ78 on adult E. caproni harboured in mice

The effects of OZ78 on adult *E. caproni* harboured in mice were assessed by worm burden reductions and the results are summarized in Table 1. OZ78 given at a dose of 1000 mg/kg killed all the worms (KW = 7.01; $P = 0.008$). At doses of 800 and 400 mg/kg, OZ78 reduced worm burdens insignificantly by 65.4% (KW = 2.2; $P = 0.129$) and 10.1% (KW = 0.75; $P = 0.386$).

Effect of OZ78 on adult F. hepatica harboured in rats

The results of the effects of OZ78 on adult *F. hepatica* harboured in rats are summarized in Table 2. Administration of single oral doses of 100 mg/kg and above resulted in worm burden reductions of 100% (KW = 10.32; $P = 0.001$). Even at 50 mg/kg, the lowest dose investigated, a statistically significant worm burden reduction of 52.7% was obtained (KW = 4.53; $P = 0.033$).

Effect of OZ78 on juvenile F. hepatica harboured in rats

In Table 3 we present the results of the effects of 50 and 100 mg/kg single oral doses of OZ78 on juvenile *F. hepatica* harboured in rats. A 100% worm burden reduction was observed with 100 mg/kg OZ78 (KW = 7.81; $P = 0.005$). Administration of OZ78 at a dose of 50 mg/kg achieved a 66.7% worm burden reduction (KW = 5.47; $P = 0.019$).

In vitro studies

Serum-free medium. Exposure of *F. hepatica* in a serum-free medium containing OZ78 at 100 μg/mL for 24 h resulted in the death of all trematodes. They showed extensive blebbing. *F. hepatica* exposed for 24 h to 10 μg/mL OZ78 showed normal movements. After exposure for 48 h, the flukes showed reduced activity. Another 24 h later, all trematodes had died and numerous blebs could be observed on their surface. Finally, *F. hepatica* exposed to 1 μg/mL OZ78 for 72 h showed slightly reduced movement, but there were no signs of surface changes.

Medium supplemented with 10% (v/v) fetal calf serum. F. hepatica exposed to 100 μg/mL OZ78 in a medium supplemented with 10% (v/v) fetal calf serum were still alive after 24 h, but they showed reduced movements and occurrence of blebs on the tegument. The flukes died 48–72 h after incubation at this concentration: they showed extensive blebbing and a coiled appearance. SEM pictures taken from flukes 24 h after treatment with 100 μg/mL revealed sloughing and blebbing at the ventral and dorsal surfaces (Figure 2a). *F. hepatica* exposed to 10 μg/mL

Table 1. Worm burden reductions of adult *E. caproni* harboured in mice following the administration of OZ78 at different doses

Treatment	Dose (mg/kg)	No. of mice investigated	No. of mice cured	Mean worm burden (SD)	Total worm burden reduction (%)
Control	–	6	–	21.7 (6.0)	–
OZ78	400	4	0	19.5 (6.1)	10.1
	800	4	3	7.5 (15.0)	65.4
	1000	4	4	0	100

Table 2. Worm burden reductions of adult *F. hepatica* harboured in rats following the administration of OZ78 at different doses

Treatment	Dose (mg/kg)	No. of rats investigated	No. of rats cured	Mean worm burden (SD)	Total flukes recovered		Total worm burden reduction (%)
					live	dead	
Control	–	12	–	5.5 (2.6)	66	0	–
OZ78	50	5	0	2.6 (2.0)	13	0	52.7
	100	5	5	0	0	2	100
	200	5	5	0	0	6	100
	400	5	5	0	0	3	100

Table 3. Worm burden reductions of juvenile *F. hepatica* harboured in rats following the administration of OZ78 at different doses

Treatment	Dose (mg/kg)	No. of rats investigated	No. of rats cured	Mean worm burden (SD)	Total flukes recovered		Total worm burden reduction (%)
					live	dead	
Control	–	5	–	4.8 (2.2)	24	0	–
OZ78	50	5	0	1.6 (0.8)	8	0	66.7
	100	5	5	0	0	0	100

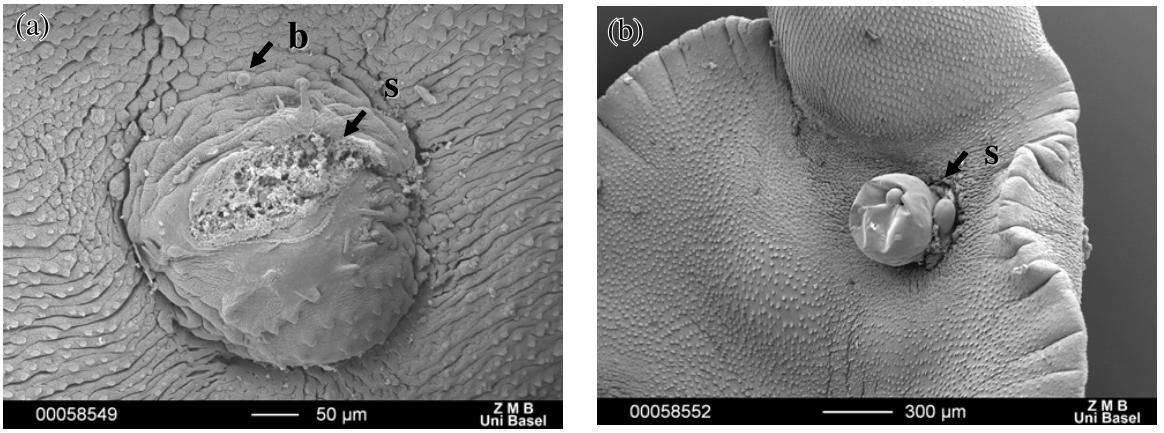

Figure 2. SEM of adult *F. hepatica* after incubation with 10 and 100 µg/mL OZ78 in NTCT-135 medium supplemented with 10% (v/v) fetal calf serum. (a) Blebbing (indicated by '**b**') and sloughing (indicated by '**s**') on the dorsal tegument 24 h after incubation with 100 µg/mL OZ78. (b) Severe swelling and sloughing (indicated by '**s**') visible on the dorsal tegument 48 h after incubation with 10 µg/mL OZ78.

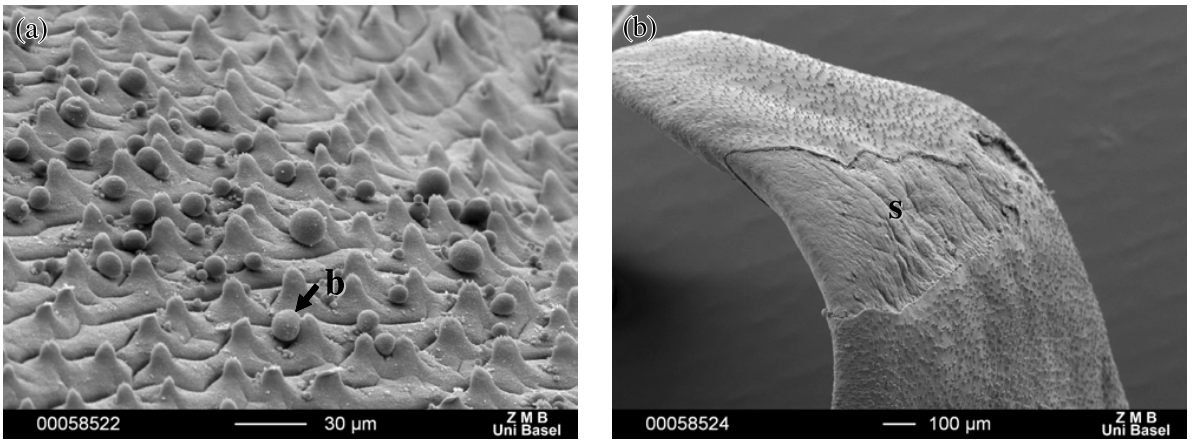

Figure 3. SEM observation of adult *F. hepatica* recovered from rat bile ducts 3 days after the administration of OZ78 at a dose of 100 mg/kg. (a) Blebbing (indicated by '**b**') visible on the dorsal tegument. (b) Extensive sloughing (indicated by '**s**') of the ventral tegument.

OZ78 for 48 h showed a slightly reduced movement and also blebbing and sloughing on the dorsal tegument (Figure 2b), but the trematodes were still alive after 72 h. Flukes incubated for 72 h with 1 µg/mL OZ78 were still moving normally, but showed tegumental alterations including sloughing (image not shown).

In vivo SEM observations

F. hepatica recovered from the central bile duct of rats 3 days after oral administration of a single dose of 100 mg/kg OZ78 were either dead ($n = 2$) or showed only feeble activity ($n = 4$). Blebs were visible on the ventral and dorsal tegument of both dead and alive specimens (Figure 3a). Many flukes showed sloughing of the tegumental surfaces (Figure 3b).

Discussion

We recently reported that the artemisinins exhibit activity against *E. caproni* in the mouse model;[11] subsequently these findings were confirmed against one of the major liver flukes, i.e. *F. hepatica,* in the rat model.[9] In view of these results, we were motivated to investigate further whether other peroxide compounds possess fasciocidal properties. We selected the antimalarial 1,2,4-trioxolanes (OZs) because these compounds are characterized by improved chemical and biopharmaceutical parameters compared with the artemisinins.[10]

It is widely acknowledged that *E. caproni* is a suitable laboratory model to study intestinal trematodiasis.[12] We have demonstrated the utility of the *E. caproni*–mouse model to screen compounds for their trematocidal activity.[11] In view of the ease of maintenance and relatively short life cycle, this host–parasite model has become our primary screen. Similar to the artemisinins[11] high doses of OZ78 were required to show an effect against *E. caproni.* Since OZ78 achieved a 100% worm burden reduction in *E. caproni*-infected mice, this compound progressed to the *F. hepatica*–rat model.

We found that OZ78 was highly efficacious against adult *F. hepatica*; complete worm burden reductions were obtained at a single oral dose of 100 mg/kg. At half this dose we still found a statistically significant worm burden reduction exceeding 50%. For comparison, a slightly lower dose (40 mg/kg) of triclabendazole, the drug of choice for fascioliasis, reduced adult worm burdens by 99% in *F. hepatica*-infected rats.[13] Similar to

triclabendazole, but in contrast to several existing fasciolicides such as closantel or closurlon[14] OZ78 showed excellent activity against juvenile *F. hepatica*; a single oral dose of 100 mg/kg cured juvenile *F. hepatica* infections harboured in rats.

The improved pharmacokinetic properties of OZ78 might explain why much lower doses of OZ78 cured *F. hepatica*-infected rats when compared with the artemisinins. For example, a 4-fold higher dose of artesunate (400 mg/kg) was necessary to achieve complete worm burden reductions in rats infected with adult *F. hepatica*.[9] OZ78 in rats has an oral bioavailability of 74.1%, which is several-fold higher than the oral bioavailabilities of either artemether (1.4%) or artesunate (23.3–32.3%). The half-life of OZ78 in rats is 2 h, which is more than double that of artesunate (0.47 h).[10] Importantly, OZ78 was better tolerated by the *F. hepatica*-infected rats than artesunate; at an artesunate dose of 400 mg/kg, 3 of 5 rats died within 24–96 h while no fatalities occurred following OZ78 administration at the same dose.[9]

Our temporal SEM investigations have shown that, very similar to artesunate and artemether, OZ78 acts very slowly on *F. hepatica*. Seventy-two hours post-treatment some flukes recovered from the bile duct still showed feeble activity. Compared with the artemisinins, tegumental damage was more pronounced, as an extensive swelling and sloughing was detected on *F. hepatica* 3 days following the administration of OZ78. Damage and loss of the spines of *F. hepatica* has been observed frequently following treatment with several fasciolicides.[15] The tegument of *Fasciola* spp. is the interfacing layer that helps the parasite to maintain its homeostasis, and to escape the host's immune attacks.[16] Hence, once the tegument or the spines of *F. hepatica* are damaged, drugs can easily penetrate to the deep tissues causing substantial and widespread damage.[15] We observed an increased susceptibility to OZ78 *in vitro* in a non-serum supplemented medium. A similar observation was made in a previous study, which evaluated the fasciocidal properties of phenolic, halogenated diphenyl, salicylanilide, benzimidazole and diaminophenoxyalkane anthelmintics in serum-free and serum-supplemented media.[17] The increased potency of the fasciolicides in the absence of serum has been explained by drug serum protein binding, which renders the drugs less accessible to the flukes.[17]

In conclusion, we have presented the first evidence of the potent fasciocidal properties of a 1,2,4-trioxolane and this is supported by *in vivo* and *in vitro* studies and underscored by SEM observations. Our results call for additional studies in larger mammals, e.g. sheep infected with *F. hepatica* or *F. gigantica*. These investigations could go hand-in-hand with a vigorous multidimensional screening and lead optimization process with the aim to gain a better insight into the structural characteristics necessary for optimal trematocidal activity of the synthetic 1,2,4-trioxolanes.

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Transparency declarations

None to declare.

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