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ORIGINAL PAPER

Dose-response relationships and tegumental surface alterations in *Opisthorchis viverrini* following treatment with mefloquine in vivo and in vitro

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Abstract The treatment and control of opisthorchiasis relies on a single drug, praziquantel; hence, there is a need to develop novel opisthorchicidal drugs. We investigated the in vitro and in vivo activity of the antimalarial mefloquine against *Opisthorchis viverrini*. Hamsters infected with *O. viverrini* for 2 weeks (juvenile infections) and 4 weeks (adult infections) were treated orally with single 200–400-mg/kg oral mefloquine. Worm burden reductions were assessed against untreated control hamsters. Worms were incubated in the presence of 10 and 100 μg/ml mefloquine. Scanning electron microscopy was used to examine adult *O. viverrini* after recovery from hamsters and following in vitro incubation. A single oral dose of 300-mg/kg mefloquine resulted in worm burden reductions of 88.5% (juvenile infection) and 96.0% (adult infections), respectively. Incubation with 10 and 100 μg/ml mefloquine resulted in rapid death of *O. viverrini*. Extensive tegumental disruption such as blebbing, sloughing, and furrowing was seen on worms incubated in vitro and on flukes recovered 48 h posttreatment. In conclusion, we have documented promising opisthorchicidal activities in hamsters and in vitro with the tegument being an important drug target. Proof-of-concept studies with mefloquine could be considered in opisthorchiasis patients.

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Introduction

Opisthorchiasis is endemic in Cambodia and Vietnam and is a major public health problem in Thailand and Lao People's Democratic Republic. It is estimated that 67.3 million people are at risk and nine million are infected (Keiser and Utzinger 2007). Caused by the liver fluke *Opisthorchis viverrini*, the disease is associated with abdominal and hepatobiliary symptoms. Serious manifestations such as obstructive jaundice and ascending cholangitis are observed (Mairiang and Mairiang 2003; Sithithaworn et al. 2009). Chronic infections are associated with cholangiocarcinoma, the malignant bile duct cancer (Honjo et al. 2005; Sripa et al. 2007; Sripa and Pairojkul 2008).

Praziquantel is the drug of choice for treating infections with *O. viverrini*. Single-dose treatment with praziquantel is highly effective, causes few adverse events, and is cheap (Keiser and Utzinger 2004; Sithithaworn et al. 2007). Treatment failures of praziquantel in *O. viverrini*-infected patients have not yet been reported. Nonetheless, with increasing drug usage in large-scale mass treatment programs in *O. viverrini* endemic settings (Montresor et al. 2008), praziquantel-resistant isolates might develop. It is therefore dangerous to rely on a single drug for treatment and control of this neglected tropical disease.

An often successful fast-track approach to develop novel drugs for tropical diseases is to piggyback on

marketed drugs, for which a wealth of clinical information is already available, hence, which would not need to undergo the long and expensive process of drug development (Nwaka and Hudson 2006). Following this strategy, we have already documented the opisthorchicidal properties of the antimalarials artemether and artesunate (Keiser et al. 2006) and the Chinese anthelmintic tribendimidine in the *O. viverrini* hamster model (Keiser et al. 2008).

The aim of the present work was to investigate the activity of mefloquine against *O. viverrini*. We have recently reported that this drug, widely and effectively used in the treatment and prophylaxis of malaria, is highly effective against schistosomes, the related blood flukes: high worm burden reductions were achieved in mice infected with *Schistosoma mansoni* and *Schistosoma japonicum* and treated with single oral doses of mefloquine (Keiser et al. 2009). In the present study, we evaluate the dose–response relationship of oral mefloquine administered to hamsters infected with adult *O. viverrini*. The minimal effective mefloquine dose in adult *O. viverrini*-infected hamsters was tested in a next step against juvenile *O. viverrini* in vivo. Finally, we assessed tegumental alterations in adult *O. viverrini* recovered from hamsters following mefloquine treatment and in worms incubated with mefloquine in vitro using a scanning electron microscope (SEM).

Materials and methods

Mefloquine

Mefloquine was kindly provided by Mepha (Aesch, Switzerland). We prepared a homogenous suspension in 7% Tween-80 and 3% ethanol before oral administration.

Ethical clearance and *O. viverrini* hamster model

All animal studies presented here were approved by the local government according to Swiss national regulations.

Metacercariae of *O. viverrini* were obtained from cyprinid fish caught in Khon Kaen province, Thailand, as described previously (Keiser et al. 2006).

Twenty-seven male Syrian Gold hamsters (age 3 weeks, weight ~100 g) were purchased from Charles River (Sulzfeld, Germany). Hamsters were kept in groups of four in environmentally controlled conditions (temperature ~25°C; humidity ~70%; 12-h light and 12-h dark cycle). Hamsters had free access to water and rodent diet. The hamsters were each infected intragastrically with 45 *O. viverrini* metacercariae.

Dose–response relationship of mefloquine against adult *O. viverrini*

Four weeks postinfection groups of four hamsters were treated orally with single doses of 200–400-mg/kg doses of mefloquine. Four untreated infected hamsters served as control. Hamsters were killed and dissected 10 days posttreatment. All *O. viverrini* were removed from the gall bladder and bile ducts and counted.

Effect of mefloquine on juvenile *O. viverrini*

Four hamsters were treated with a single oral dose of 300 mg/kg mefloquine at day 14 postinfection. An infected but untreated group of four hamsters served as controls. On day 32 postinfection hamsters were killed; the livers were removed and all flukes present in the bile ducts and gall bladder were counted.

Statistical analysis

The Kruskal–Wallis test (KW) was used to compare the medians of the treatment and control groups at a significance level of 5%. Statsdirect statistical software (version 2.7.2, Statsdirect Ltd., Cheshire, UK) was used for the statistical analysis.

In vivo SEM observations

Three hamsters were treated with single oral 300 mg/kg mefloquine 5 weeks postinfection. After 24, 48, and 72 h posttreatment, one hamster each was killed by CO_2, and flukes were collected from the gall bladder and bile ducts. *O. viverrini* collected from one hamster, which had not been treated, served as control specimens. The flukes were rinsed in 0.9% (*v*/*v*) saline and fixed with 2.5% glutaraldehyde in a phosphate-buffered saline buffer for 24 h at room temperature. Following fixation, *O. viverrini* were dehydrated and critically point-dried (Bomar SPC-900; Tacoma, USA). After mounting the trematodes on aluminum stubs, they were 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.

In vitro SEM observations

O. viverrini collected from untreated hamsters were incubated in 2-ml minimal essential medium (MEM; Invitrogen, Carlsbad, USA) in the presence of 10 and 100 μg/ml mefloquine. Control flukes were incubated in MEM in the absence of drug. Cultures were kept at 37°C in an atmosphere of 5% CO_2 and observed after 1 h of

exposure under a dissecting microscope. Subsequently, all flukes were collected and processed for SEM as described above.

Results

Dose–response relationship of mefloquine against adult *O. viverrini*

In Table 1, we present the dose–response relationship of mefloquine against adult *O. viverrini* harbored in hamsters.

At the lowest dose investigated (200-mg/kg), no effect on the worm burden was observed. A worm burden reduction of 96% was found when a 300-mg/kg single oral dose of mefloquine was administered to hamsters. The highest dose administered (400-mg/kg) achieved a complete elimination of worms. The mean worm burden observed in untreated control hamsters was 12.5 worms. There was a significant difference (KW=3.86, P=0.049) in the medians of the total worm burden between treated and untreated hamsters.

Effect of mefloquine on juvenile *O. viverrini* in hamsters

The effect of a 300-mg/kg single oral dose of mefloquine against juvenile *O. viverrini* is summarized in Table 1. At this dose, a worm burden reduction of 88.5% was obtained, which was statistically significant (KW=5.39, P=0.02). In this experiment, the control group harbored a mean of 6.5 *O. viverrini*.

SEM observations of control *O. viverrini*

The tegument of adult *O. viverrini* obtained from untreated hamsters had a normal appearance. In Fig. 1, the oral sucker is presented and in Fig. 2 the anterior part of an adult *O. viverrini* is shown.

In vivo SEM investigations

Seven worms were recovered from a hamster 24 h after treatment with a single 300-mg/kg oral dose of mefloquine. These flukes had a normal appearance and showed active movements. SEM observations of these flukes revealed no damage of the tegument (no picture shown).

Forty-eight hours after drug administration, ten worms were removed from a hamster's bile duct. The worms appeared pale and were inactive. Substantial damage on the ventral and dorsal tegument as well as on the anterior and posterior regions of the trematodes was visible on eight flukes by means of SEM. Two worms revealed no damage. Extensive sloughing was seen near the oral and ventral suckers (Figs. 3 and 4) and the ventral and dorsal anterior and posterior surfaces (Fig. 5). Blebbing was visible on the entire surfaces and the oral and ventral suckers (Figs. 3 and 4). Extensive furrowing was visible on the ventral and dorsal surfaces (Figs. 3, 4, and 5). One fluke showed a severely sloughed and collapsed tegument leading to structural disorganization (Fig. 6).

Seventy-two hours following treatment with mefloquine, the majority of flukes had been expelled and only three *O. viverrini* were recovered from the bile duct of the one hamster designated at this time point. All *O. viverrini* were alive but showed reduced mobility. Tegumental damage of these flukes was less severe than observed at the 48-h time point. Small areas of sloughing and blebbing were observed on all flukes at the dorsal or ventral surfaces (no images shown).

In vitro SEM observations

All *O. viverrini* had died 1 h after incubation in the presence of 10 and 100 μg/ml of mefloquine. Extensive tegumental alterations were observed by SEM. Worms exposed to 10 μg/ml of mefloquine showed severe blebbing on the entire body surface area (Figs. 7 and 8). Swelling of

Table 1 Effect of mefloquine against adult and juvenile *O. viverrini*

Stage of infection	Treatment	Dose (mg/kg)	No. of hamsters investigated	No. of hamsters cured	Mean worm burden (SD)	Total worm burden reduction (%)	Kruskal–Wallis	P value
Adult infection	Control	–	4	0	12.5 (5.8)	–	–	–
	Mefloquine	400	4	4	0	100	3.85	0.049
		300	4	3	0.5 (1.0)	96.0		
		200	4	0	14.0 (4.1)	0		
Juvenile infection	Control		4	0	6.5 (3.1)	–		
	Mefloquine	300	4	2	0.75 (0.96)	88.5	5.39	0.02

SD standard deviation

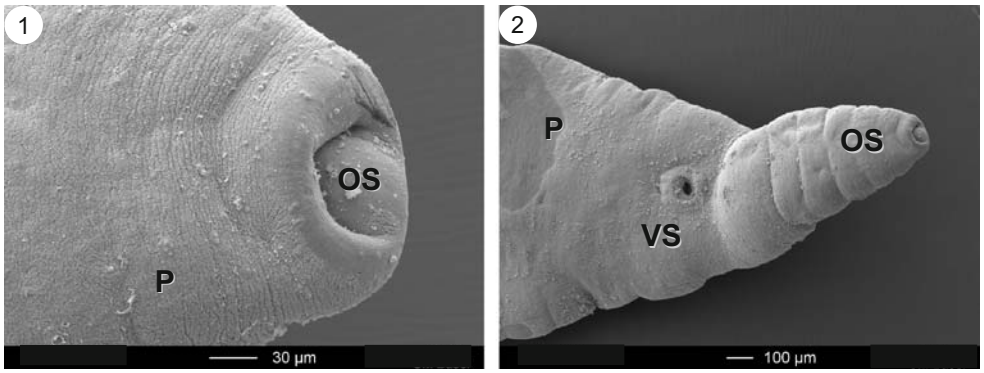

Fig. 1 SEM of the oral sucker (*OS*) and tegument with papillae (*P*) of an adult *O. viverrini* removed from an untreated control hamster

Fig. 2 SEM of the oral sucker (*OS*), ventral sucker (*VS*), and tegument with papillae (*P*) of an adult *O. viverrini* removed from an untreated control hamster

the tegument was evident on the dorsal and ventral surfaces (Fig. 7). *O. viverrini* incubated with 100 µg/ml showed sloughing and the midbody regions of several flukes were carpeted in blebs (no picture shown). The anterior parts of the worms were highly affected, revealing a badly disrupted tegument. The tegumental apex of several worms had been removed from the anterior half of the flukes, exposing the basal lamina (Figs. 9 and 10).

Discussion

To our knowledge, we have documented for the first time that the antimalarial drug mefloquine possesses interesting opisthorchicidal properties in hamsters. Single oral doses of 300-mg/kg of mefloquine resulted in worm burden reductions of 88.5% (juvenile infections) and 96% (adult infections), respectively. In addition, *O. viverrini* incubated with 10 and 100 µg/ml mefloquine in vitro died rapidly.

Promising activities of the antimalarial mefloquine against biologically related trematodes, the schistosomes, have been described recently: diminished egg fecundity was observed in mice treated with a low dose of mefloquine (150-mg/kg; Van Nassauw et al. 2008). At a dose of 400-mg/kg, complete or very high total and female worm burden reductions were observed against adult and juvenile stages of *S. mansoni* and *S. japonicum* (Keiser et al. 2009). Morphological studies on adult *S. japonicum* worms following mefloquine treatment showed a pronounced dilatation of the gut, accompanied by focal or extensive peeling of gut epithelial cells or even focal collapse of the gut wall as well as swelling of tegument, muscles, or even parenchymal tissues (Zhang et al. 2009). Finally, SEM studies on *S. mansoni* following mefloquine administration

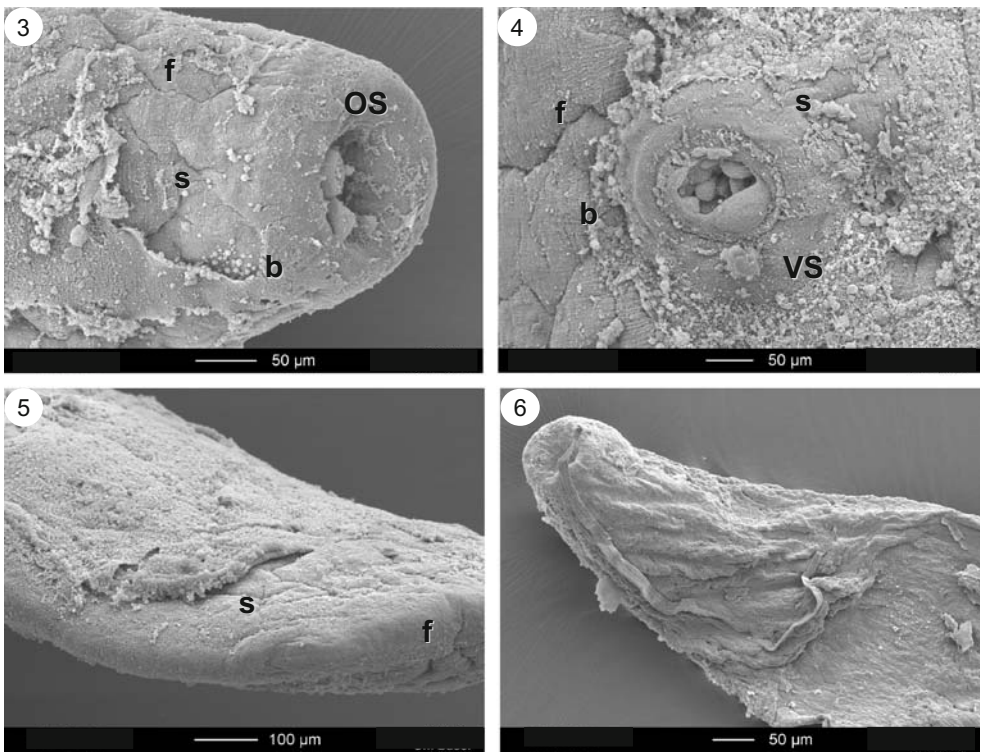

Fig. 3 SEM of an adult *O. viverrini* 48 h after the administration of a single dose of mefloquine (400-mg/kg) showing furrowing (*f*), sloughing (*s*), and blebbing (*b*) of the tegument near the oral sucker (*OS*)

Fig. 4 SEM of an adult *O. viverrini* 48 h after the administration of a single dose of mefloquine (400-mg/kg) with furrowing (*f*), blebbing (*b*), and sloughing (*s*) near the ventral sucker (*VS*) visible

Fig. 5 SEM of an adult *O. viverrini* 48 h after the administration of a single dose of mefloquine (400-mg/kg) showing furrowing (*f*) and sloughing (*s*) on the ventral tail region

Fig. 6 SEM of an adult *O. viverrini* 48 h after the administration of a single dose of mefloquine (400-mg/kg) depicting a collapsed anterior region of the fluke

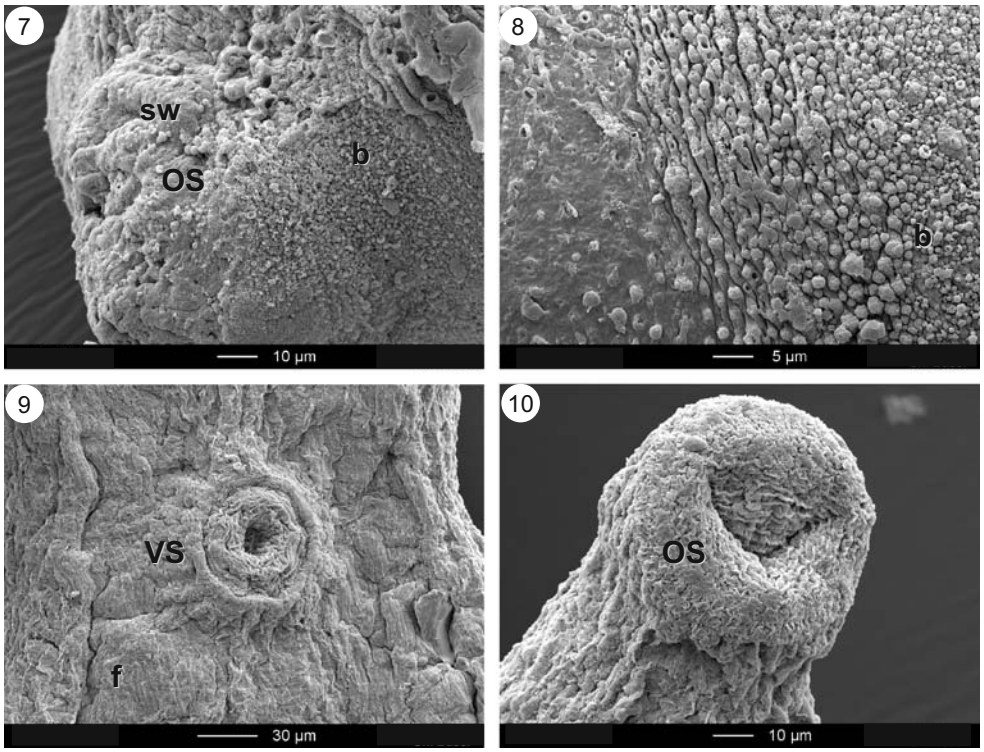

Fig. 7 SEM of an adult *O. viverrini* 1 h after the incubation with 10 µg/ml mefloquine. Blebbing (*b*) and swelling (*sw*) observed on the oral sucker (*OS*) region

Fig. 8 SEM of the dorsal midbody region of an adult *O. viverrini* 1 h after the incubation with 10 µg/ml mefloquine. Extensive blebbing (*b*) visible

Fig. 9 SEM of an adult *O. viverrini* 1 h after the incubation with 100 µg/ml mefloquine. Extensive disruption of the tegument near the ventral sucker (*VS*)

Fig. 10 SEM of the oral sucker (*OS*) region of an adult *O. viverrini* 1 h after the incubation with 100 µg/ml mefloquine. The tegument has been sloughed off, exposing the basal lamina

in vivo and incubation with mefloquine in vitro showed that the tegument seems to be a main drug target as extensive tegumental disruption was observed (Manneck et al., submitted for publication).

Interestingly, the liver flukes *Fasciola hepatica* and *Clonorchis sinensis* harbored in rats are not affected by mefloquine, even when high dosages are used (unpublished observations).

Our SEM study revealed severe tegumental alterations as sloughing, furrowing, and blebbing, which occurred rapidly following incubation of *O. viverrini* with mefloquine in vitro. In several flukes, the tegument had been sloughed off exposing the basal membrane. Interestingly, both *O. viverrini* as well as *S. mansoni* (Manneck et al., submitted for publication) incubated with mefloquine die in the absence of red blood cells or hemin in vitro; thus, an interference with the hemoglobin digestion, the supposed mode of action against *Plasmodium* sp. (Brocks and Mehvar 2003), might not be the sole mechanism of action of mefloquine against trematodes.

A slower onset of action was observed when mefloquine was administered to *O. viverrini*-infected hamsters. Tegumental alterations on *O. viverrini* were first detected 48 h posttreatment with mefloquine. All surfaces and both the anterior and posterior part of the flukes were affected, with similar tegumental alterations observed when compared to the in vitro incubation of *Opisthorchis* with mefloquine. A slow onset of action was also observed on the tegument of *S. mansoni* collected from mice posttreatment with mefloquine (Manneck et al., submitted for publication). Interestingly, tegumental changes did not increase further in severity; in contrast, less damage was observed on *O. viverrini* collected 72 h posttreatment from hamsters when compared to the 48-h time point. However, affected *O. viverrini* might have already been eliminated at this time point. The differences between the fast drug action on *O. viverrini* in vitro and the slower action in vivo cannot be explained at the moment; however, drug concentrations are most likely much lower in the hamster bile ducts when compared to the in vitro concentrations used.

The mechanism of how *O. viverrini* are attacked by praziquantel seems to be different from mefloquine: extensive blebbing was also observed when *O. viverrini* were incubated with praziquantel in vitro. However, a disruption of the vacuoles resulting in crater-like lesions was a common feature seen on the tegument of *O. viverrini* incubated with praziquantel (Apinhasmit and Sobhon 1996; Mehlhorn et al. 1983; Sirisinha et al. 1984). Both the ventral as well as the dorsal tegument were affected by praziquantel treatment, whereas the anterior parts of the worms tended to be damaged less than the posterior parts. Similar tegumental changes were observed when worms were collected following praziquantel treatment in vivo. Tegumental damage was observed already 4 h posttreatment of hamsters with praziquantel (Sirisinha et al. 1984), thus much earlier when compared to mefloquine.

Another drug which was recently analyzed for its opisthorchicidal properties is the Chinese anthelmintic drug tribendimidine (Keiser et al. 2008). In contrast to mefloquine and praziquantel, SEM analysis of *O. viverrini* incubated in vitro showed only mild damage of the tegument. A complete closure of the oral sucker was a common feature observed on *O. viverrini* recovered posttreatment of hamsters with tribendimidine. Additional tegumental alterations seen were sloughing, furrowing, and blebbing. Similar to mefloquine-treated specimens in vivo, no clear dorsal–ventral and anterior–posterior difference in tegument disruption was evident for *O. viverrini* exposed to tribendimidine and the severity of damage did not increase further at 48–72 h posttreatment (Keiser et al. 2008).

Hence, SEM observations made on *O. viverrini* collected from mefloquine-, praziquantel-, and tribendimidine-treated hamsters point to different mechanisms of actions. Drug combinations, characterized by independent modes of action, are in many therapeutic areas seen as a possibility to enhance efficacy while ensuring mutual protection against resistance. An evaluation of the potential of combinations of mefloquine, praziquantel, and tribendimidine in *O. viverrini*-infected hamsters has been initiated in our laboratories.

In conclusion, we have strengthened the current evidence base of the interesting trematocidal properties of mefloquine documenting promising opisthorchicidal activities in hamsters and in vitro. The tegument of *O. viverrini* seems to be a potential drug target of this antimalarial drug. Proof-of-concept studies, which have already been launched in African settings to assess the effect of mefloquine in infections with *S. mansoni* and *Schistosoma haematobium*, could also be considered in opisthorchiasis patients.

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