Parasitol Res (2008) 103:517–522
DOI 10.1007/s00436-008-0998-0

ORIGINAL PAPER

Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep

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Received: 13 March 2008 / Accepted: 10 April 2008 / Published online: 15 May 2008

Abstract Triclabendazole is the current drug of choice against *Fasciola* spp. infections in livestock, but resistance has become a major problem. In this study, we assessed the efficacy and safety of artemether, a derivative of artemisinin, in sheep with a low natural *Fasciola hepatica* infection. Artemether was administered orally or intramuscularly; sheep were monitored for 8 h posttreatment and then once daily for adverse events, and drug efficacy was estimated by fecal egg count reductions and worm burden reductions. Single 40- and 80-mg/kg oral doses of artemether showed no effect on *F. hepatica* egg and worm burden. Treatment with a single 160-mg/kg intramuscular dose of artemether significantly reduced the egg burden (64.9%) and worm burden (91.3%). At half this dose, a worm burden reduction of 65.3% was obtained, which was still statistically significant ($P<0.05$). The lowest intramuscular dose of artemether investigated (40 mg/kg) yielded no effect on egg counts and worm burden. There were no adverse events due to artemether; however, two abortions were observed 7 days posttreatment. In conclusion, artemether shows interesting fasciocidal properties in sheep, but embryotoxicity is of concern. Further studies are warranted to assess the potential of additional artemisinin derivatives and other peroxidic compounds for the treatment of *Fasciola* spp. infections in different ruminants.

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Introduction

Fascioliasis (fasciolosis), caused by liver flukes of the species *Fasciola hepatica* and *F. gigantica*, is a significant livestock and an emerging public health problem (McManus and Dalton 2006; Keiser and Utzinger 2007). An estimated 350 million cattle, 250 million sheep, and 91 million people are at risk of acquiring an infection from *Fasciola* spp. (Keiser and Utzinger 2007; Rioux et al. 2007). The annual economic loss due to fascioliasis in the veterinary field has been estimated at US$ 2–3 billion, as a result of animal weight loss, reduction of milk yield and fertility declines (Schweizer et al. 2005; McManus and Dalton 2006; Rioux et al. 2007).

The control of fascioliasis is crucial for the health, and hence productivity of farm animals, most notably cattle and sheep. As there is presently no vaccine available for the prevention of fascioliasis (McManus and Dalton 2006)

effective control rests on chemotherapy. Triclabendazole is the current drug of choice. It is safe and efficacious against both juvenile and adult flukes. Other veterinary fasciolicides (e.g. clorsulon, albendazole, rafoxanide, and closantel) lack activity against the young migrating *F. hepatica* stages, which cause extensive hemorrhaging and fibrosis of the liver (Fairweather and Boray 1999). However, triclabendazole resistance has been developed and is spreading; indeed, resistance has been reported in livestock farms across Europe (Ireland, The Netherlands, Spain, and the UK) and in Australia (Keiser et al. 2005; Alvarez-Sanchez et al. 2006). In addition, triclabendazole has a number of shortcomings. First, the drug has a relatively long withholding period, i.e., 28 days before slaughtering animals and 7 days before milking for human consumption (http://www.ah.novartis.com). Triclabendazole is therefore not an ideal drug for use in dairy sheep. Second, no injectable formulation of triclabendazole is currently available. Third, triclabendazole is presently not marketed in all *Fasciola*-endemic countries. In Italy, for example, triclabendazole is not readily available (Cringoli 2006).

The development of novel fasciocidal drugs that are effective against juvenile and adult stages of *Fasciola* spp. and triclabendazole-resistant *Fasciola* strains would be a major advance. We have recently reported fasciocidal properties of artemether and artesunate (derivatives of artemisinin), which are best known for their antimalarial (Davis et al. 2005) and antischistosomal properties (Utzinger et al. 2007). Administration of single-dose oral artemether (200 mg/kg) and artesunate (400 mg/kg) to rats infected with adult *F. hepatica* resulted in 100% worm burden reductions (Keiser et al. 2006a). Moreover, single-dose oral artemether and artesunate (200 mg/kg) achieved worm burden reductions of 82% and 46%, respectively, in rats with a 3-week immature *F. hepatica* infection (Keiser et al. 2006a).

In light of the promising fasciocidal activities of the artemisinins in the rat model, particularly artemether, we were motivated to assess the efficacy and safety of artemether in sheep naturally infected with *F. hepatica*. In the current proof-of-concept study, we administered single doses of artemether at different concentrations and routes of administration, and monitored adverse events, and drug efficacy by means of egg and worm burden reductions.

Material and methods

Study animals and ethical clearance

The study was carried out on a dairy sheep farm in the Salerno province of southern Italy (geographical coordinates: 40°33′30″ N latitude and 15°04′17″ E longitude). Details of the study area have been described elsewhere (Cringoli 2006). The flock contains approximately 700 mixed breed dairy sheep, which are raised and pastured on the farm all year long. The study animals were separated from the rest of the flock and maintained on designated pasture for the current study.

The study was approved by the institutional review board of the Swiss Tropical Institute (Basel, Switzerland). Ethical clearance was granted by the Centre for Veterinary Service of the University of Naples Federico II, which is the central ethical committee for the control of animal diseases and the conduct of clinical trials in this part of Italy (Ref. no. 116/07).

Drug and solvents

Artemether was kindly provided by Dafra Pharma (Turnhout, Belgium) and Kunming Pharmaceutical Cooperation (Kunming, People's Republic of China). For the oral drench, a homogeneous suspension of 7% Tween-80 and 3% ethanol was prepared shortly before oral administration. Artemether was dissolved in 10–30 ml peanut oil purchased from Roth (Basel, Switzerland) for the intramuscular application.

Study design, parasitological examination, treatment, and safety assessment

In May 2007, we collected stool samples from 100 randomly selected sheep on the above-mentioned farm. The samples were subjected to the FLOTAC method, which allows an analytic sensitivity of one egg per gram of stool (EPG). The flotation solution employed was zinc sulfate plus potassium iodomercurate (specific density 1.45) (Cringoli et al. 2004). The FLOTAC technique is a novel multivalent fecal egg count method, facilitated by the FLOTAC apparatus, which is designed to carry out flotation in a centrifuge, followed by a transversal cut (i.e., translation) of the apical portion of the floating suspension (Cringoli 2006).

All *F. hepatica*-positive sheep were included in the study. Four additional stool samples were collected from these sheep over consecutive days and egg counts determined as before. For each sheep, the geometric mean egg count was determined based on the five stool examinations.

Sheep were randomly allocated to five treatment groups and one control group, in blocks of five to seven, according to weight and the geometric mean egg count. Animals in group 1 (n=7) remained untreated, serving as control. Groups 2 and 3 (n=6 sheep each) were treated orally with single-dose artemether at 40 and 80 mg/kg, respectively. Groups 4–6 (n=5 sheep each) received single 40, 80, and 160 mg/kg intramuscular doses of artemether, respectively. Sheep were monitored for the occurrence of adverse events for 8 h and then once daily posttreatment. Between days 10

and 27 post-treatment, three to four stool samples were collected from each sheep on consecutive days for examination of the *F. hepatica* egg burden.

All sheep were slaughtered after the final stool collection. Livers were removed and transferred to the laboratory in thermal containers. All *F. hepatica* flukes were harvested from the gall bladder, liver and excised bile ducts, counted, and recorded separately. The morphology and movement of flukes were monitored. All procedures followed the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) for evaluation of the efficacy of anthelminthic drugs in ruminants (Wood et al. 1995).

Statistical analysis

Statistical analyses were done with version 2.4.5 of Statsdirect statistical software (Statsdirect Ltd; Cheshire, UK). The fasciocidal effect of artemether was estimated by analyzing the reduction of the geometric mean of fecal egg counts pretreatment with that posttreatment. Analyses of EPG values were performed on logarithmically transformed log [count+1] data. The pretreatment and posttreatment differences were analyzed by using an unpaired two-tailed Student's *t*-test, allowing for unequal variance. Data were considered significant with a significance level of 0.05.

The responses between the medians of the treatment and control groups regarding *F. hepatica* in the liver and bile ducts were analyzed with the Kruskal–Wallis test. Differences in medians were considered to be significant at a significance level of 0.05.

Results

Safety evaluation

There were neither physical nor clinical signs of toxicity in sheep treated orally or intramuscularly with artemether within 8 h after treatment. Local injection site reactions were not observed. However, two out of the 27 treated sheep, one in the low (40 mg/kg) and one in the high (160 mg/kg) intramuscular treatment group, aborted 7 days posttreatment.

Effect of artemether on *F. hepatica* fecal egg count

Table 1 shows the effect of artemether treatment on *F. hepatica* egg counts in stool samples. Prior to artemether administration, sheep passed between 1 and 72 EPG. At doses of 40–80 mg/kg artemether, administered either orally or intramuscularly, we found no or only a low and statistically non-significant reduction of *F. hepatica* egg counts (0–39%). At the highest intramuscular route of administration (i.e., 160 mg/kg), we noted a significant egg count reduction (64.9%; $P<0.001$).

Effect of artemether on *F. hepatica* worm burden

Table 2 summarizes the effect of oral and intramuscular artemether on *F. hepatica* in terms of worm burden reduction after dissection of sheep. We observed low worm burden reductions of 17.4% and 39.2% after administration of single 40- and 80-mg/kg oral doses of artemether, respectively, with no statistical significance (KW=2.13; $P=0.354$). A single 160-mg/kg intramuscular dose of artemether resulted in a high worm burden reduction of 91.3%. At half this dose, using the same route of administration, a worm burden reduction of 65.3% was found, which still showed statistical significance. At the lowest intramuscular dose investigated (40 mg/kg), there was no apparent effect on the *F. hepatica* worm burden. There was a significant difference between the number of *F. hepatica* (KW=13.76; $P=0.003$) recovered from the intramuscularly treated and non-treated control sheep.

No morphological alterations were detected among *F. hepatica*, and the worm motor activity was similar among *F. hepatica* recovered from treated compared to non-treated sheep.

Table 1 Effect of single-dose oral and intramuscular artemether on *F. hepatica* fecal egg counts in sheep, expressed as geometric mean and range of eggs per gram of stool (EPG)

Treatment group	Dose (mg/kg)	Route of administration	Number of animals	Pretreatment geometric mean (EPG)	Range	Posttreatment geometric mean (EPG)	Range	% reduction
1 (Control)	–	–	7	11.3	1–72	16.3	6–48	–
2	40	Oral	6	15.8	6–48	9.6	0–60	39.0
3	80	Oral	6	12.4	1–42	15.0	6–72	0
4	40	Intramuscular	5	5.0	2–22	14.7	6–74	0
5	80	Intramuscular	5	6.3	1–30	4.7	0–36	24.2
6	160	Intramuscular	5	6.5	2–20	2.3	0–14	64.9*

Student's *t*-test comparing treatment groups 2–6 to control; *$P<0.001$

Table 2 Effect of single-dose oral and intramuscular artemether on *F. hepatica* worm burden reductions

Treatment group	Dose (mg/kg)	Route of administration	Mean worm burden (SD)	Total worm burden reduction (%)	KW	*P*
1 (Control)	–	–	4.6 (2.6)	–	–	
2	40	Oral	3.8 (2.7)	17.4		
3	80	Oral	2.8 (3.1)	39.2	2.13	0.354
4	40	Intramuscular	5.8 (4.6)	0		
5	80	Intramuscular	1.6 (0.9)	65.3		
6	160	Intramuscular	0.4 (0.9)	91.3	13.76	0.003

KW Kruskal Wallis test, *SD* standard deviation

Discussion

We have recently reported that the two semi-synthetic artemisinin derivatives, artemether and artesunate, exhibit interesting activity against *F. hepatica* harbored in the rat (Keiser et al. 2006a). In addition, a recent clinical study in Vietnam has shown that artesunate might also play a role in the treatment of acute human fascioliasis (Hien et al. 2008). In view of our findings in the *F. hepatica*-rat model, we were motivated to investigate whether the artemisinins also exhibit fasciocidal properties in a larger animal. In the current proof-of-concept study, we have chosen artemether, as it is better tolerated by rats than artesunate (Keiser et al. 2006a). While artesunate has been administered to cows aiming to elucidate pharmacokinetic and metabolism properties of this compound (Huo et al. 1991), to our knowledge, this is the first report on the administration of artemether to a small ruminant. We found that single-dose intramuscular artemether (160 mg/kg) was efficacious against a low natural *F. hepatica* infection in sheep. At this high dose, we found a worm burden reduction of 91.3%. At half this dose, we still found a relatively high worm burden reduction of 65.3%. However, it should be noted that, based on egg counts, sheep harboring a low *F. hepatica* infection were overrepresented in these two treatment groups.

It is interesting to note that the effect of artemether on *Fasciola* egg counts in fecal samples was somewhat less prominent. Only the highest dose of artemether, administered intramuscularly, achieved a significant egg count reduction (64.9%; $P<0.001$). Single-dose intramuscular artemether (80 mg/kg) resulted in an egg count reduction of only 24.2% ($P>0.05$). Although a positive correlation between *Fasciola* worm burden and egg output has been described (Molina et al. 2005), this association might be less significant in low infection intensities. Nonetheless, the fecal egg count reduction test still is the most commonly applied technique to determine the effect of anthelminthic drugs. WAAVP guidelines advise to use the controlled slaughter test, as it represents the most reliable method for evaluating anthelminthic activity in ruminants (Wood et al. 1995).

Our study revealed that single-dose oral artemether at dosages of 40 and 80 mg/kg lacked activity against *F. hepatica* in sheep. In patients suffering from acute malaria, unfavorable pharmacokinetic parameters of intramuscular artemether (2 mg/kg) were found compared to single-dose oral artemether (2 mg/kg). For example, intramuscular artemether was characterized by a considerable delay of maximum plasma concentrations (C_{max}), a threefold lower bioavailability of antimalarial activity and a threefold lower area under the plasma concentration–time curve (AUC) when compared to oral artemether (Silamut et al. 2003). In ruminants, however, the bioavailability after oral administration of artemether might be much lower compared to humans as this parameter is closely related to the digestive physiology of the rumen. For example, the microflora of the rumen, characterized by a large number of anaerobic bacteria and protozoa is well-known to inactivate drugs. The rumen furthermore prolongs the absorption period, and hence residence time of the drugs (Vandamme and Ellis 2004; Formentini et al. 2005). This characteristic is a likely explanation why the AUC of oral albendazole sulfoxide was found to be almost fourfold lower compared to subcutaneous administration of the same compound (Formentini et al. 2005). Studies are ongoing in our laboratories to elucidate the pharmacokinetic parameters after oral administration of artemether in rats and sheep. If the sheep studies confirm a substantial first pass metabolism of artemether, ruminal drug delivery systems (Vandamme and Ellis 2004) might be considered.

Artemether, even at the highest dose of 160 mg/kg administered intramuscularly, was well tolerated by sheep. However, an abortion was noted in two sheep 7 days following a single 40- and 160-mg/kg intramuscular dose of artemether. Several studies, which evaluated the safety of the artemisinins in pregnant malaria patients, found that birth outcomes did not differ significantly to community rates for abortion, stillbirth, or congenital abnormality (McGready et al. 2001; Adam et al. 2004; Clark et al. 2004). For example, the overall abortion rate for women treated with artemisinins in Thailand during the first trimester was 18.9% compared to an overall community

rate of 12.3% (McGready et al. 2001). However, the total number of women pregnant in the first trimester and exposed to an artemisinin derivative analyzed to date is not sufficiently large for issuing a final recommendation regarding administration of these drugs during the first trimester of pregnancy (Clark et al. 2004). On the other hand, embryotoxicity has been observed in laboratory animals following high multiple doses with the artemisinins (Chen et al. 1984; Clark et al. 2004). Hence, we currently cannot rule out that artemether is toxic to sheep embryos, particularly when administered at high doses as in the present proof-of-concept study. In addition, intramuscular administration of high doses of artemether has been shown to produce selective damage to the brain stem center in rodents, but only when multiple high doses of the drug were administered. For example, intramuscular artemether (50–100 mg/kg/day for 28 days) caused dose-dependent neuropathologic damage to the brain stem of mice (Nontprasert et al. 2002).

In conclusion, we have presented the first evidence that an artemisinin derivative possesses fasciocidal properties in small ruminants. Our results call for additional studies with other peroxidic drugs in *F. hepatica*-infected sheep, ideally in experimental infections characterized by high infection intensities. Due to the problematic biopharmaceutical (e.g., solubility) and pharmacokinetic parameters of artemether, we suggest that future work should concentrate on artesunate or the synthetic 1,2,4 trioxolanes (OZ) (Vennerstrom et al. 2004). As we have recently shown that OZ78 is highly efficacious in rats harboring *F. hepatica* infections and is well tolerated (Keiser et al. 2006b), further studies with OZ78 in *F. hepatica*-infected sheep are warranted.

Acknowledgments We are grateful to the farmer Mr. Massimo Lullo for his kind collaboration, and thank Dafra Pharma and Kunming Pharmaceutical Cooperation for the supply of artemether powder. We thank Prof. Xiao Shuhua for his collaboration and many discussions on the anti-trematocidal activities of the artemisinins. This investigation received financial support from the Swiss Tropical Institute, the Swiss National Science Foundation via projects PPOOA-114941 (to J. Keiser) and PPOOB-102883 and PPOOB-119129 (to J. Utzinger).

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