

## Artesunate and artemether are effective fasciolicides in the rat model and *in vitro*

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**Objectives:** To study the fasciocidal properties of artesunate and artemether in the rat model and *in vitro*.

**Methods:** Adult *Fasciola hepatica* were exposed *in vitro* to 1, 10 and 100 µg/mL of artesunate, artemether and dihydroartemisinin for 72 h. Female Wistar rats were administered a single oral dose of artesunate and artemether (100–400 mg/kg) commencing 3 or 10–14 weeks post-infection and worm burden reductions were assessed against infected but untreated control rats. *F. hepatica* were also observed by scanning electron microscopy (SEM) after recovery from bile ducts of rats given a single oral dose of 200 mg/kg artesunate 24 and 72 h post-treatment.

**Results:** *F. hepatica* exposed for 72 h to 10 µg/mL of artesunate, artemether and dihydroartemisinin *in vitro* showed poor mobility, swelling of the worm body, roughness, damage of the tegument and blebbing. Exposure to drug concentrations of 100 µg/mL resulted in the death of all *F. hepatica* by 72 h. One hundred per cent worm burden reductions were achieved in rats infected with adult *F. hepatica* after treatment with artesunate and artemether at 400 and 200 mg/kg, respectively. Administration of artesunate and artemether at a dose of 200 mg/kg to rats harbouring juvenile *F. hepatica* resulted in worm burden reductions of 46% and 82%, respectively. *F. hepatica* recovered from rats' bile ducts 24 h after administration of 200 mg/kg artesunate showed normal activity and SEM observations revealed that there was no visible damage. Seventy-two hours post-treatment *F. hepatica* displayed very poor mobility and there was focal swelling of the tegument and spines.

**Conclusions:** Artesunate and artemether exhibit promising fasciocidal activities, with the latter showing better tolerability by the hosts.

Keywords: *Fasciola hepatica*, food-borne trematodiasis, artemisinin, dihydroartemisinin, *in vitro* studies, *in vivo* studies, scanning electron microscopy

### Introduction

Fascioliasis (fasciolosis) is caused by liver flukes of the species *Fasciola hepatica* and *Fasciola gigantica*. It is a zoonotic disease of great veterinary importance and considerable public health significance. In humans, an infection with *Fasciola* spp. occurs through oral ingestion of metacercariae either through consumption of uncooked or unwashed aquatic plants, or through drinking contaminated water.<sup>1</sup> Chronic infections can lead to severe disabling diseases, including hepatic lesions, fibrosis and chronic inflammation of the bile ducts.<sup>1,2</sup> An estimated 91 million people are at risk of becoming infected mainly in the Andean countries of South America, Cuba, Western Europe, Egypt and the Islamic Republic of Iran.<sup>3</sup> It is estimated that between 2.4 and 17 million people are infected with *F. hepatica* and/or *F. gigantica*.<sup>4,5</sup>

Treatment options for fascioliasis are still unsatisfactory and hence there is a pressing need for new trematocidal drugs.<sup>6,7</sup> Bithionol, emetine and dehydroemetine are commonly used drugs for the treatment of these diseases, but all three agents can cause serious adverse effects.<sup>8,9</sup> Triclabendazole is currently the most efficacious and best tolerated drug for the treatment of fascioliasis, but the drug is so far registered in only four countries.<sup>10</sup>

Since the discovery of the outstanding antimalarial properties of artemisinin in the early 1970s by Chinese scientists,<sup>11,12</sup> followed by the synthesis of several semi-synthetic derivatives (e.g. arteether, artemether and artesunate), millions of malaria patients have been treated with these drugs.<sup>13,14</sup> Three studies are worth highlighting with regard to the effect of artemisinins on food-borne trematodiasis. First, administration of artemether and

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dihydroartemisinin-carbonate at daily doses of 30–60 mg/kg for five consecutive days to rats infected with *Clonorchis sinensis* resulted in worm burden reductions of 82–100%.<sup>12</sup> Second, administration of artemisinin at 500 mg twice daily for 5 days to *C. sinensis*-infected patients in northern Vietnam reduced infection intensity only marginally.<sup>15</sup> Finally, we have recently documented that artemisinin and its semi-synthetic derivatives are active against *Echinostoma caproni*, an intestinal trematode, both *in vitro* and in the mouse model. Administration of a single oral dose of artesunate or artemether at 700 and 1100 mg/kg respectively, cured the *E. caproni*-infected mice.<sup>7</sup>

In the light of the promising trematocidal activities of the artemisinins, this study aimed at: (i) evaluating the fasciocidal properties of artesunate and artemether and their metabolite dihydroartemisinin *in vitro*; (ii) assessing the therapeutic potential of a single oral dose of artesunate and artemether employing the *F. hepatica*–rat model; and (iii) investigating drug-induced alterations *in vivo* by means of scanning electron microscopy (SEM).

## Materials and methods

### Ethical clearance, drugs, parasites and rats

All animal studies presented here were approved by the local government based on Swiss national regulations (permission no. 2070).

Artesunate was obtained from Mepha AG (Aesch, Switzerland), artemether was provided by Kunming Pharmaceutical Cooperation (Kunming, P.R. China) and dihydroartemisinin was the product of Hoffman-La Roche (Basel, Switzerland). Drugs were prepared in homogeneous suspensions in 7% (v/v) Tween-80 and 3% (v/v) ethanol shortly before oral administration. *F. hepatica* metacercariae were purchased from G. Graham (Addlestone, UK).

Female Wistar rats ( $n = 64$ ; age: 5 weeks; weight: ~100 g) were purchased from RCC (Itingen, Switzerland). Rats were kept in groups of five in macrolon cages in environmentally controlled conditions (temperature: ~25°C; humidity: ~70%; 12 h light and 12 h dark cycle) and acclimatized for 1 week. They had free access to water and rodent food (Rodent Blox from Eberle NAFAG; Gossau, Switzerland).

### *In vitro* studies

A total of three rats were infected with 30 metacercariae by gavage. Twelve weeks post-infection rats were killed and all *F. hepatica* were recovered from the central bile duct. The trematodes were washed in several changes of 0.9% (w/v) NaCl solution and incubated in six-well plates (Costar) (two per well) containing 5 mL NCTC 135 culture medium (Gibco), supplemented with antibiotics (50 µg/mL streptomycin and 50 U/mL penicillin; Gibco). Two trematodes were used for each control and experimental group. Stock solutions of artesunate, artemether and dihydroartemisinin at 10 mg/mL were prepared with 60% (v/v) DMSO for immediate use. The flukes were incubated with three serial drug dilutions of 1, 10 and 100 µg/mL for 72 h. Each drug concentration was evaluated in duplicate. The control well contained the highest concentration of solvent; 0.06% (v/v) DMSO. Cultures were kept at 37°C in an atmosphere of 5% CO<sub>2</sub> 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.

### *In vivo* studies

To study the effect of the artemisinins on adult *F. hepatica*, 40 rats were each infected intragastrically with 30 metacercarial cysts of

*F. hepatica*. Ten to 14 weeks post-infection, six groups of five rats were treated orally with either artesunate or artemether at a single dose ranging from 100 to 400 mg/kg. Ten untreated rats served as control (control 1).

For drug evaluation on juvenile *F. hepatica* 18 rats were each infected with 30 *F. hepatica* metacercariae. Three weeks post-infection two groups of five rats each were treated with artesunate and artemether at a single oral dose of 200 mg/kg. Eight untreated rats served as control (control 2).

Twelve days post-treatment, rats were euthanized by CO<sub>2</sub>. At necropsy *F. hepatica* were harvested from the excised bile ducts and counted. The effect of the drugs was assessed by comparing the mean number of *F. hepatica* in any treatment group with that of the respective control group. Differences were tested for significance using an unpaired two-tailed Student's *t*-test, allowing for unequal variance. The data were considered significant with a *P* value <0.05. Statistical analyses were done with version 9.0 of the STATA software (StataCorp, College Station, TX, USA).

### SEM observations

Three rats were each infected intragastrically with 30 metacercarial cysts of *F. hepatica*. Fourteen weeks post-infection two rats were treated intragastrically with artesunate at a single dose of 200 mg/kg. The first rat was euthanized 24 h post-treatment and the second one at 72 h by CO<sub>2</sub>. At necropsy *F. hepatica* were harvested from the excised bile ducts. *F. hepatica* recovered from the untreated rat served as control. Four flukes were examined from each rat. The flukes were washed quickly in 0.9% (w/v) NaCl solution and immediately fixed with 2.5% glutaraldehyde (v/v) in a phosphate-buffered saline (PBS) buffer for 24 h at room temperature. After rinsing with PBS buffer the specimen 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; Eindhoven, The Netherlands) at an accelerating voltage of 5 kV.

## Results

### *In vitro* studies

Table 1 summarizes the observed mortality and morphological alterations of adult *F. hepatica* after exposure to artesunate, artemether or dihydroartemisinin at different concentrations *in vitro*. Exposure to artesunate, artemether or dihydroartemisinin at 100 µg/mL resulted in the death of all trematodes by 72 h. Flukes displayed a coiled appearance, indistinct internal structure, blebbing or roughness.

*F. hepatica* exposed for 24 h to a medium containing 10 µg/mL of artesunate, artemether or dihydroartemisinin showed normal movements. By 48 h they showed reduced activity and numerous blebs could be seen along the surface of those flukes exposed to artemether. Seventy-two hours after incubation two trematodes each were dead in the artemether and artesunate wells, and one trematode had died in the dihydroartemisinin well. The remaining flukes showed swelling, indistinction in internal tissues, but they still exhibited some activity.

Finally, *F. hepatica* exposed to 1 µg/mL of artesunate, artemether or dihydroartemisinin *in vitro* contracted less frequently than control specimens after an observation period of 72 h, but no tegumental damage could be seen.

## Fasciolicidal activity of artesunate and artemether

**Table 1.** Observed mortality and morphological features of adult *F. hepatica* after exposure *in vitro* to dihydroartemisinin, artemether and artesunate at three different concentrations

| Treatment          | Concentration (µg/mL) | No. of flukes observed | No. of flukes dead and morphological features after incubation for                                      |  |   |
|--------------------|-----------------------|------------------------|---|--|---|
|                    |                       |                        | 24 h  | 48 h   | 72 h  |
| Dihydroartemisinin | 1                     | 4                      | 0/4   | 0/4  | 0/4 slightly reduced mobility   |
|                    | 10                    | 4                      | 0/4   | 0/4 reduced mobility                             | 1/4 reduced mobility, roughness of body surface                               |
|                    | 100                   | 4                      | 4/4 flukes had coiled appearance, extensive blebbing in anterior part and indistinct internal structure | 4/4  | 4/4   |
| Artesunate         | 1                     | 4                      | 0/4   | 0/4  | 0/4 slightly reduced mobility   |
|                    | 10                    | 4                      | 0/4   | 0/4 reduced mobility                             | 2/4 reduced mobility, swelling, indistinct internal structure                 |
|                    | 100                   | 4                      | 0/4 strongly reduced mobility   | 4/4 flukes displayed extensive tegumental damage | 4/4   |
| Artemether         | 1                     | 4                      | 0/4   | 0/4  | 0/4 slightly reduced mobility   |
|                    | 10                    | 4                      | 0/4   | 0/4 reduced mobility, numerous blebs             | 2/4 reduced mobility, numerous blebs, swelling, indistinct internal structure |
|                    | 100                   | 4                      | 0/4 reduced mobility  | 0/4 roughness and blebs visible                  | 4/4 indistinct internal structure, swollen body                               |
| Control            | –                     | 4                      | 0/4 normal movement and no damage visible   | 0/4 normal movement and no damage visible        | 0/4 normal movement and no damage visible                                     |

**Tables 2.** Effect of artesunate and artemether against adult *F. hepatica* harboured in rats

| Treatment  | Dose (mg/kg)     | No. of rats investigated | No. of rats that died | No. of rats cured <sup>c</sup> | Mean worm burden (SD) | No. of flukes recovered |      | Total worm burden reduction (%) | P value |
|------------|------------------|--------------------------|-----------------------|--------------------------------|-----------------------|-------------------------|------|---------------------------------|---------|
|            |                  |                          |                       |                                |                       | live                    | dead |                                 |         |
| Control 1  | –                | 10                       | 0                     | 0                              | 8.4 (4.2)             | 84                      | 0    | –                               | –       |
| Control 2  | –                | 8                        | 0                     | 0                              | 5.6 (2.7)             | 45                      | 0    | –                               | –       |
| Artesunate | 100 <sup>a</sup> | 5                        | 0                     | 2                              | 5.8 (7.7)             | 28                      | 1    | 30                              | 0.799   |
|            | 200 <sup>a</sup> | 5                        | 1                     | 1                              | 2.5 (3.1)             | 10                      | 6    | 71                              | 0.021   |
|            | 400 <sup>a</sup> | 5                        | 3                     | 2                              | 0                     | 0                       | 0    | 100                             | <0.001  |
| Artemether | 100 <sup>a</sup> | 5                        | 0                     | 1                              | 5.8 (4.0)             | 29                      | 0    | 30                              | 0.561   |
|            | 200 <sup>b</sup> | 5                        | 0                     | 5                              | 0                     | 0                       | 6    | 100                             | <0.001  |
|            | 400 <sup>b</sup> | 5                        | 0                     | 5                              | 0                     | 0                       | 2    | 100                             | <0.001  |

<sup>a</sup>Worm burden reduction assessed by comparing the mean number of *F. hepatica* with that of control 1.

<sup>b</sup>Worm burden reduction assessed by comparing the mean number of *F. hepatica* with that of control 2.

<sup>c</sup>Signifies the number of rats without flukes.

Control flukes were moving normally with no evidence of damage 72 h after incubation.

### Effect of artesunate and artemether on adult *F. hepatica* *in vivo*

The effects of artesunate and artemether on 10 to 14-week-old adult *F. hepatica* harboured in rats are summarized in Table 2.

Administration of artesunate or artemether at a single oral dose of 400 mg/kg resulted in worm burden reductions of 100%. At half this dose, worm burden reductions of 100 and 71% were achieved with artemether and artesunate, respectively. The lowest oral dose investigated, i.e. 100 mg/kg was not effective, yielding only low worm burden reductions of 30% for both compounds.

Artemether tended to be better tolerated by the rats than artesunate; three rats died 24–96 h following an oral dose of

**Table 3.** Effect of artesunate and artemether against juvenile *F. hepatica* harboured in rats

| Treatment  | Dose (mg/kg) | No. of rats investigated | No. of rats that died | No. of rats cured <sup>a</sup> | Mean worm burden (SD) | Total worm burden reduction (%) | <i>P</i> value |
|------------|--------------|--------------------------|-----------------------|--------------------------------|-----------------------|---------------------------------|----------------|
| Control 2  | –            | 8                        | 0                     | –                              | 5.6 (2.7)             | –                               |                |
| Artesunate | 200          | 5                        | 0                     | 1                              | 3.0 (2.7)             | 46                              | 0.125          |
| Artemether | 200          | 5                        | 0                     | 1                              | 1.0 (0.7)             | 82                              | <0.001         |

<sup>a</sup>Signifies the number of rats without flukes.

400 mg/kg artesunate, while none of the rats died after administration of artemether at the same dose.

#### *Effect of artesunate and artemether on juvenile F. hepatica in vivo*

In Table 3, we present the effect of artesunate and artemether on juvenile (3-week-old) *F. hepatica* harboured in rats. A single oral dose of 200 mg/kg of artesunate and artemether yielded worm burden reductions of 46% and 82%, respectively.

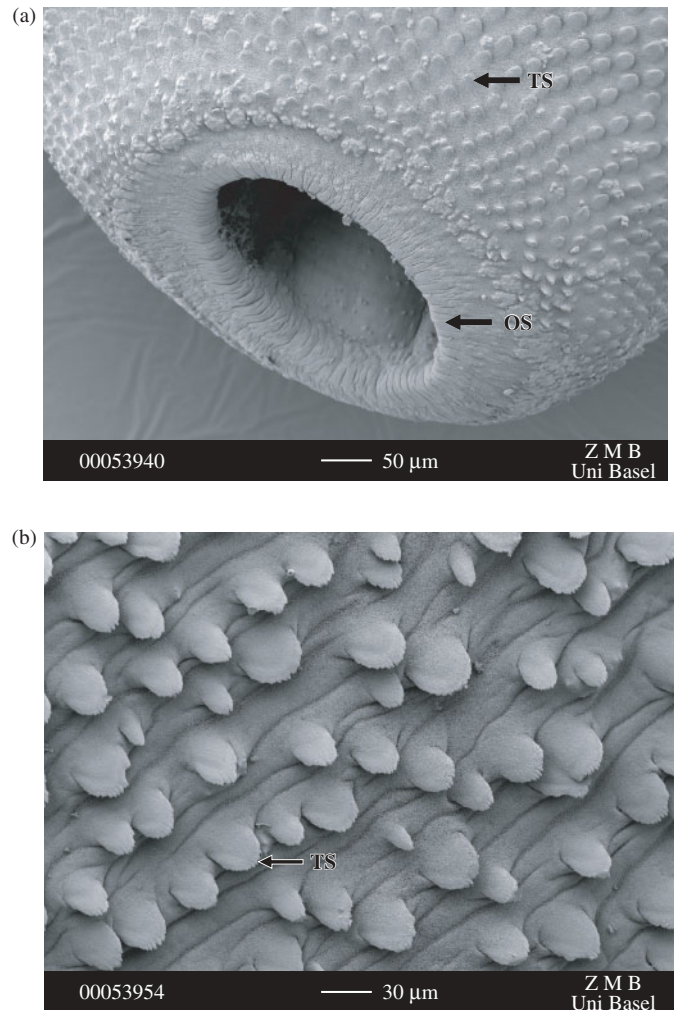
#### *SEM observations*

The morphological features of adult *F. hepatica* collected from untreated control rats were in agreement with previous reports.<sup>16,17</sup> Figure 1 shows the oral sucker (a) and the mid-ventral tegument (b) of *F. hepatica* recovered from these control rats.

We examined four *F. hepatica* recovered from rats 24 h after administration of a single dose of 200 mg/kg artesunate. They showed normal movement. There was no apparent alteration in the tegument of these flukes, with the exception of one specimen showing focal swelling of the tegument and some sunken spines near the tail (Figure 2a). Seventy-two hours post-treatment 4 *F. hepatica* were recovered from the bile duct, which showed only feeble activity or were without motility and appeared pale with no evidence of gut contents. Minor damage was visible as the tegument covering the spines appeared to be split open, and sunken spines were observed near the oral sucker rim or in the lateral surface of the oral sucker (Figure 2b and c). We also observed focal swelling and furrowing of the ventral tegument with some roughness and damaged spines (Figure 2d and e). No apparent damage of the dorsal tegument was observed.

#### **Discussion**

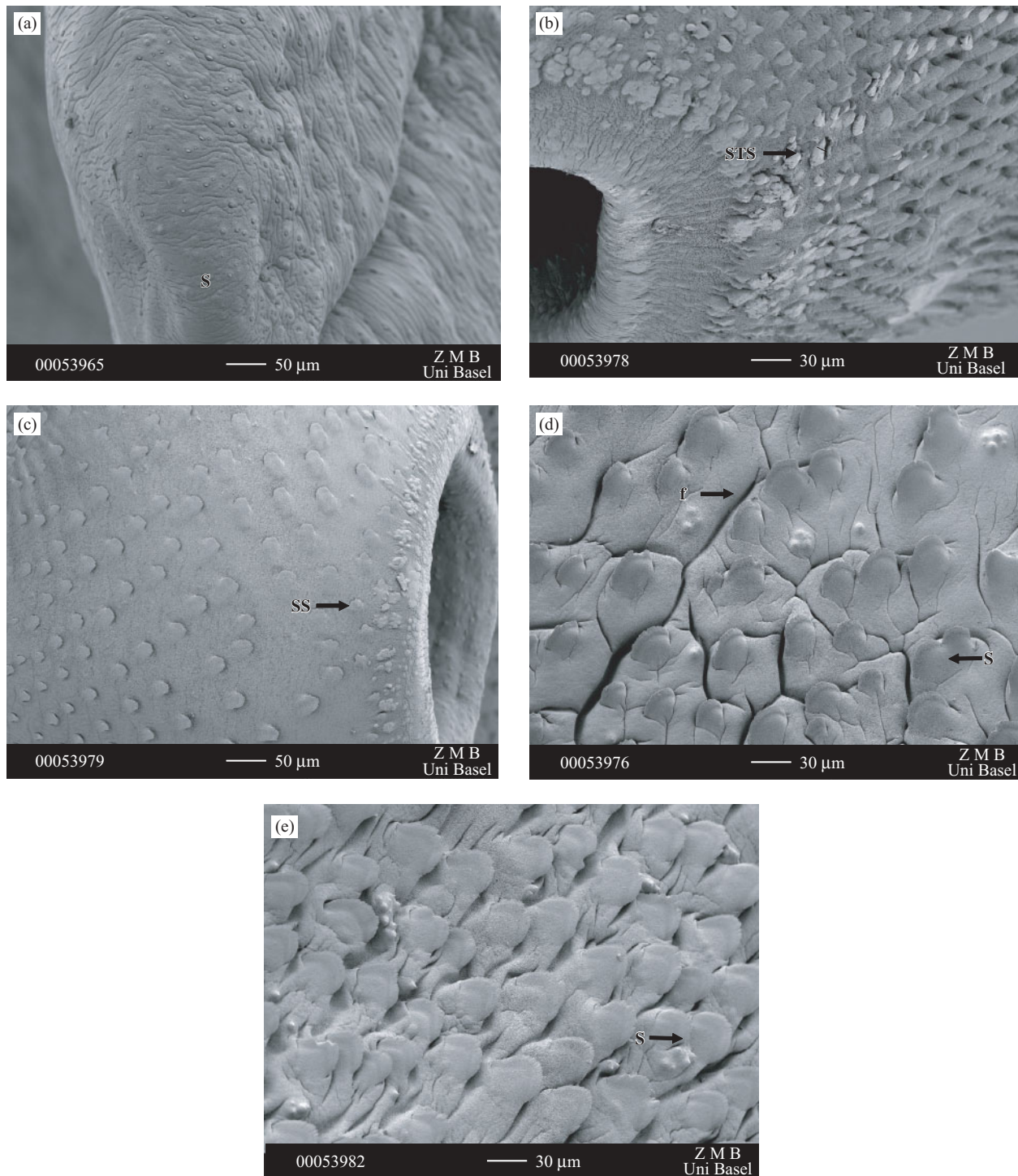
To our knowledge, this is the first time that derivatives of artemisinin have been tested for their effect against *F. hepatica*. We have assessed the dose–response relationships following administration of single oral doses in the *F. hepatica*–rat model. We found that both artesunate and artemether were highly efficacious against adult *F. hepatica*; complete worm burden reductions were obtained with artesunate and artemether administered at 400 and 200 mg/kg, respectively. In addition, administration of artemether at a dose of 200 mg/kg to rats infected with juvenile *F. hepatica* resulted in a worm burden reduction of 82%. Our results are important as there is an urgent need to



**Figure 1.** SEM observation of an oral sucker (OS) (a) and tegument with spines (TS) in the mid-ventral region (b) of adult *F. hepatica* recovered from a non-treated control rat.

develop new therapeutic agents against food-borne trematodiasis in general, and fascioliasis in particular. Triclabendazole is the only drug currently available for treatment of patients with fascioliasis.<sup>10</sup> However, triclabendazole is presently registered in only four countries and there is considerable concern that resistance might be developing; a phenomenon, which is already widespread in cattle and sheep in several countries.<sup>10,18</sup>

## Fasciolicidal activity of artesunate and artemether



**Figure 2.** SEM observation of adult *F. hepatica* following the administration of artesunate at a dose of 200 mg/kg. There is focal swelling (s) of the lateral ventral surface in the tail 24 h post-treatment (a), split tegumental spines (STS) near the rim of the oral sucker 72 h post-treatment (b), swelling with sunken spines (SS) near the rim of the oral sucker 72 h post-treatment (c), and swelling (s) and furrowing (f) of the tegument and spines in the mid-ventral region 72 h post-treatment (d and e).

*In vitro* exposure of *F. hepatica* to a high concentration of 100 µg/mL of dihydroartemisinin, the main metabolite of the artemisinins, resulted in a more rapid killing of the trematodes when compared with artesunate and artemether. These observations are in agreement with previous findings that dihydroartemisinin was the most rapidly acting artemisinin

derivative on *E. caproni in vitro*.<sup>7</sup> Hence, dihydroartemisinin might play an important role in the curing of *F. hepatica* infections in the rat. However, although the conversion of artesunate to dihydroartemisinin is 6-fold greater when compared with artemether,<sup>19</sup> artemether was slightly more active *in vivo*. Hence, most likely not only the primary metabolite but also the mother

compounds contribute to the observed *in vivo* activity. This claim is supported by the observation that all three derivatives showed an equal dose–response relationship *in vitro*. We did not include dihydroartemisinin in our *in vivo* investigations, as oral artesunate was found to have pharmacokinetic advantages over oral dihydroartemisinin.<sup>20</sup>

Our experiments revealed that artemether tended to be better tolerated than artesunate. While no adverse events could be observed following administration of a single oral dose of 600 mg/kg artemether (unpublished observation), three of five rats died 24–96 h after administration of a single oral dose of 400 mg/kg artesunate. *F. hepatica* infections have been described to dramatically impair the integrity and functionality of the microsomal drug metabolizing mono-oxygenase system,<sup>21</sup> which is involved in the elimination of the artemisinins.<sup>22–24</sup> Dihydroartemisinin, for example, is a potent inhibitor of CYP1A2,<sup>22</sup> one of the cytochrome enzymes which might be altered during chronic fascioliasis. As mentioned, dihydroartemisinin plasma levels are much higher following the administration of artesunate when compared with artemether, as artesunate is rapidly hydrolysed into dihydroartemisinin in the stomach.<sup>19,25</sup> It follows that toxic levels of this metabolite might accumulate in *F. hepatica*-infected rats after administration of a high dose of artesunate. Hence, the safety margin of artesunate might decrease during fascioliasis.

Considerably lower doses of artesunate and artemether achieved significant worm burden reductions in the *F. hepatica*–rat model, when compared with our previous results obtained in the *E. caproni*–mouse model. For example, while a dose of 200 mg/kg artemether achieved a worm burden reduction of 100% in *F. hepatica*-infected rats, no effect was seen with a 3-fold higher dose administered to *E. caproni*-infected mice. This result might be explained by differences in the pharmacokinetics and metabolism between the two rodent models as well as higher drug levels of the two artemisinin derivatives and its main metabolite dihydroartemisinin in the liver and bile duct, when compared with the small intestine. Knowledge on the absorption phase of the artemisinins is incomplete. While a low bioavailability of the parent drugs (19–35%) was observed when testing different formulations of all derivatives in rats,<sup>19</sup> the absolute bioavailability of artesunate was found to be 61% in malaria patients.<sup>26</sup>

Our *in vitro* and *in vivo* studies have shown that artesunate and artemether act rather slowly on *F. hepatica*. Twelve days post-treatment dead flukes could be recovered from the rat's bile ducts. Our SEM observations revealed that 24 h post-treatment with artesunate, trematodes collected from the bile duct showed activity and no external damage could be detected. Seventy-two hours post-treatment, flukes were still recovered from the bile duct but showed feeble activity. Tegumental damage was quite limited and hence both the tegument and suckers do not seem to be the main targets for artesunate to exert its fasciocidal effect. In contrast, after treatment of rats with clorsulon, a sulphonamide fasciolicide, flukes were dead by 25–30 h and expelled by 50–60 h.<sup>27</sup> Flukes that could be recovered 72 h post-treatment with clorsulon were severely damaged and showed, for example, a widespread disruption with sloughing of the apical membrane.<sup>16</sup>

Numerous studies carried out in different animal models and human clinical trials established an evidence base that artemether and artesunate are efficacious drugs against the juvenile stages of the biologically related digenans in the genus

*Schistosoma*.<sup>28,29</sup> Artemether seems to be more effective than artesunate against different developmental stages of both trematodes, *F. hepatica* in the rat and *Schistosoma mansoni* harboured in mice (reviewed in ref. 30). However, two marked differences between schistosomes and *F. hepatica* following administration of artemisinins to suitable animal models are worth highlighting. First, while we have demonstrated that both juvenile and adult *F. hepatica* are affected by the artemisinins, adult schistosomes are less susceptible to these drugs. For example, administration of six daily doses of 200 mg/kg artemether resulted in a worm burden reduction of only 39% in the adult *S. mansoni*–mouse model.<sup>31</sup> Finally, in contrast to our SEM findings, severe changes to the tegument of *S. mekongi*, characterized by vacuolization, collapse and peeling, were observed by means of SEM 1–3 days after administration of artesunate.<sup>32</sup> Hence, one might speculate that artesunate and artemether exert different mechanism of actions on these two genera of flukes.

In conclusion, our study shows the fasciocidal properties of artesunate and artemether. This finding raises hope for novel approaches towards trematocidal drugs.

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## Transparency declarations

None to declare.

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