In vitro and in vivo treatment of *Echinococcus* protoscoleces and metacestodes with artemisinin and artemisinin-derivatives

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In vitro treatment of *E. multilocularis* and *E. granulosus* larval stages with the anti-malarials dihydroartemisinin (DHA) and artesunate (10-40 µM) exhibited, while 6 weeks in vivo treatment of mice infected with *E. multilocularis* metacestodes (200 mg/kg/day) had no effect. However, combination treatments of both drugs with albendazole lead to a substantial, but statistically not significant, reduction in parasite weight compared to albendazole alone.
Cystic echinococcosis (CE), caused by *Echinococcus granulosus*, is distributed worldwide. Alveolar echinococcosis (AE), caused by *Echinococcus multilocularis* is generally confined to the Northern hemisphere (2). Growth and/or proliferation of *Echinococcus* metacestodes, mainly in the liver, but also lungs and other organs, leads to the development of space-occupying lesions, organ malfunction, and will eventually cause death (10, 23). The preferred treatment option is radical resection of the parasitic mass. Surgery is accompanied by chemotherapy, and in inoperable cases, chemotherapy is the only option. Albendazole and mebendazole are currently used (8, 10). For AE, these compounds were shown to act parasitostatic rather than parasitocidal, with high recurrence rates after interruption of therapy. Improved drug treatments are needed (8, 24).

Most malaria endemic countries have now adopted artemisinin-based combination therapy as first-line treatment for *P. falciparum* infection (34), and activities of artemisinins against other protozoans were reported (1, 12). Trematodes including schistosomes (31) and others have proven susceptible to artemisinins and semi-synthetic derivatives (13-16, 26), and anti-tumor activities of artemisinins were reported (11, 18, 35). *E. multilocularis* metacestodes also exhibit tumor-like properties, including potentially unlimited growth and proliferation (17). These findings have prompted us to investigate the potential of artemisinins for anti-echinococcal treatment.

We first assessed the in vitro activities of artemisinin, artesunate, artemether and dihydro-artemisinin (DHA) against *E. granulosus* and *E. multilocularis* larval stages. These were evaluated in comparison to albendazole and nitazoxanide as reference drugs (8). All compounds were dissolved as stock solutions of 10 mM in DMSO. *E. granulosus* protoscoleces were isolated and maintained and tested in vitro as described earlier (21, 32). Compounds were added at 4, 10 and 40 μM.
Protoscoleces viability was assessed microscopically by trypan blue exclusion test (Fig. 1). At 40 µM, artesunate and DHA exhibited similar activity as nitazoxanide (32), but the action of DHA was delayed by two days (90% viability reduction occurring on day 6.) Artemisinin and artemether were ineffective (data not shown). At 10 µM, artesunate and DHA showed strongly decreased efficacies compared to nitazoxanide (Fig. 1).

E. multilocularis metacestode drug assays were carried out as described (7, 9, 21, 27, 28, 30). Artemisinins and albendazole were added to the cultures at a concentration of 40 µM. During the 12 days treatment, 200 µl of culture supernatant were collected daily and stored at -20°C to measure E. multilocularis alkaline phosphatase (EmAP) activity (30). Artesunate treatment led to a rapid increase of EmAP activity in medium supernatants within 4 days (Fig. 2). DHA exhibited a delayed effect, with an increased EmAP activity coming up at day 8. Artemisinin and artemether treatments did not result in a high-level EmAP release (Fig. 2), as earlier reported by Reuter et al. (25). No elevated EmAP levels were observed at 10 µM drug concentrations (data not shown). EmAP activity has been identified earlier as a marker indicating the loss of viability of in vitro drug-treated vesicles (28, 30). Our findings correlated well with SEM and TEM analyses, confirming that in vitro exposure of E. multilocularis metacestodes with artesunate and DHA resulted in profound tissue alterations and loss of the characteristic multicellular structure of the germinal layer (data not shown). Similar observations were made when E. granulosus metacestodes were exposed to these compounds (Spicher and Hemphill, unpublished).

The effects of artesunate and DHA were further evaluated in the experimental Balb/c mouse model (27, 29). Mice were separated into 6 treatment groups of 10 animals each. Drug suspensions were prepared in 0.5% carboxymethylcellulose (CMC) and
were applied as follows: (i) artesunate at 200 mg/kg bodyweight (bw), (ii) a combination of artesunate (200 mg/kg bw) and albendazole (50 mg/kg bw), (iii) DHA (200 mg/kg bw), (iv) a combination of DHA (200 mg/kg bw) and albendazole (50 mg/kg bw), (v) albendazole at 200 mg/kg bw, and (vi) 0.5% CMC alone (control group). Treatment began at eight weeks post-infection and the drug- and control-suspensions were applied by intra-gastric inoculation (100 \mu l / mouse / day) for 6 weeks. Finally, mice were sacrificed by CO2 euthanasia, parasite tissue removed from the peritoneal cavity, and the parasite weight was determined (Fig. 3). Parasite weights within the CMC control- (5.71 +/- 1.79 g), artesunate- (4.60 +/- 2.28 g) and DHA-group (4.11 +/- 2.03 g) were consistently high with minor differences. As expected, continuous treatment of mice with albendazole (2.96 +/- 1.10 g) resulted in a significant reduction in parasite weight. In addition, the combination of artesunate and albendazole (1.39 +/- 0.81 g) and the combination of DHA and albendazole (1.38 +/- 1.25 g) resulted in an even more pronounced reduction of the parasite weights compared to the control (Fig. 3). The improvements obtained with albendazole, artesunate/albendazole, and DHA/albendazole were highly significant (one-way ANOVA, \( F = 44.66, P = 0.0000 \)). The artesunate/albendazole and DHA/albendazole treatments resulted in lower mean parasite weights compared to the albendazole treatment alone, but the differences closely missed statistical significance. (Kruskal-Wallis multiple-comparison z-value test: significant difference if z-value > 1.96, z-value for artesunate/albendazole 1.89, z-value for DHA/albendazole 1.92).

No adverse effects were observed in the drug-treated groups, with the exception of one mouse found dead in the DHA/albendazole-group at day 30 and one mouse in the artesunate/albendazole group found dead at day 32. These two mice could potentially be attributed to the described toxicity and neurotoxicity of artemisinin.
derivatives in laboratory animals (3, 4, 20). However, none of the mice exhibited any aberrant behaviour during the treatments, and histopathological examination of liver-, kidney- and brain-tissue did not show any signs of toxicity, indicating that the cause of death of these two mice could be possibly attributed to other causes.

The promising in vitro results that were achieved with artesunate and DHA (Figs. 1 and 2) could not be completely translated to the in vivo mouse model (Fig. 3). There are several potential explanations for this. First, artemisinins are primarily converted to DHA via ester hydrolysis and further to inactive metabolites by hepatic cytochrome P-450 and other enzyme systems (19, 34), and DHA exhibits a low bioavailability after oral administration with a short elimination half-life (19, 34). Secondly, the ways of drug delivery to the parasite target tissue in vivo are obviously very different compared to in vitro situations. Thirdly, *Echinococcus* metacestodes are surrounded by a highly glycosylated acellular laminated layer that exhibits immunomodulating properties (5), and it is not clear to what extent this barrier contributes to the action of anti-parasitic drugs. Thus, the drugs and their metabolites used here are perhaps not delivered and accumulated in the parasite tissues in adequate quantities.

In contrast, the albendazole-combination treatments resulted in consistently lower parasite weights compared to albendazole monotherapy. This already represents a promising result. However, since the improvement closely missed statistical significance, there is ample room for optimization (modulation of application route, dosage, treatment duration etc). The slightly improved result after combination therapy could be due to the fact that the two drugs altered the pharmacokinetics of albendazole, thus retarding the metabolic conversion of the primary metabolite albendazole-sulphoxide to albendazole-sulphone. Similar finding were obtained during in vivo treatment of *E. multilocularis*-infected mice with albendazole/nitazoxanide combination therapy (29), albendazole/cimetidine (33) and
albendazole/2-methoxyestradiol treatments (27). Novel synthetic artemisinin derivatives have been developed, which are characterized by improved pharmacokinetic profiles (16). Further studies are underway to elucidate the anti-echinococcal efficacy of these and other molecules.

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Figure legends

Fig. 1. Protoscolicidal activity of artemisinin-derivatives. *E. granulosus* protoscoleces were cultured in vitro in the presence of artesunate, DHA, and NTZ as positive control (10 and 40 µM). Note the dose-dependent killing of protoscoleces by both artesunate and DHA. This experiment was repeated three times with virtually identical outcomes. One representative result is shown.

Fig. 2. EmAP activity in culture supernatant of drug-treated *E. multilocularis* metacestodes. Artesunate, dihydroartemisinin (DHA), artemisinin and artemether were applied to in vitro cultured vesicles at 40 µM, and EmAP activity was measured in culture supernatants at different timepoints as indicated. Albendazole (ABZ) and corresponding amounts of the solvent dimethylsulfoxide (DMSO) were added as positive and negative control, respectively.

Fig. 3. Experimental chemotherapy in *E. multilocularis* infected mice. In vivo treatment of *E. multilocularis*-infected mice was carried out with albendazole (ABZ), artesunate (AS), dihydroartemisinin (DHA), and combinations of ABZ/AS and ABZ/DHA. CMC = solvent control (0.5% carboxymethylcellulose in PBS). The box plots indicate the distribution of parasite weights in the different treatment groups. Significant reductions of parasite weights in relation to the CMC-control group were achieved by treatment with ABZ, ABZ/AS and ABZ/DHA. Although the combination treatments were most efficient, the reduction in both groups in relation to ABZ alone was not significant (Kruskal-Wallis multiple-comparison z-value test: significant difference if z-value > 1.96, z-value for artesunate/ABZ 1.89, z-value for DHA/ABZ 1.92).
Spicher et al., Fig. 1
Spicher et al., Fig. 2
Spicher et al., Fig. 3