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Oral treatments of *Echinococcus multilocularis*-infected mice with the antimalarial drug mefloquine that potentially interacts with parasite ferritin and cystatin

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ABSTRACT

This study investigated the effects of oral treatments of *Echinococcus multilocularis*-infected mice with the antimalarial drug mefloquine (MEF) and identified proteins that bind to MEF in parasite extracts and human cells by affinity chromatography. In a pilot experiment, MEF treatment was applied 5 days per week and was intensified by increasing the dosage stepwise from 12.5 mg/kg to 200 mg/kg during 4 weeks followed by treatments of 100 mg/kg during the last 7 weeks. This resulted in a highly significant reduction of parasite weight in MEF-treated mice compared with mock-treated mice, but the reduction was significantly less efficacious compared with the standard treatment regimen of albendazole (ABZ). In a second experiment, MEF was applied orally in three different treatment groups at dosages of 25, 50 or 100 mg/kg, but only twice a week, for a period of 12 weeks. Treatment at 100 mg/kg had a profound impact on the parasite, similar to ABZ treatment at 200 mg/kg/day (5 days/week for 12 weeks). No adverse side effects were noted. To identify proteins in *E. multilocularis* metacestodes that physically interact with MEF, affinity chromatography of metacestode extracts was performed on MEF coupled to epoxy-activated Sepharose®, followed by SDS-PAGE and in-gel digestion LC–MS/MS. This resulted in the identification of *E. multilocularis* ferritin and cystatin as MEF-binding proteins. In contrast, when human cells were exposed to MEF affinity chromatography, nicotinamide phosphoribosyltransferase was identified as a MEF-binding protein. This indicates that MEF could potentially interact with different proteins in parasites and human cells.

1. Introduction

The parasite *Echinococcus multilocularis* is an endoparasitic flatworm of the family Taeniidae. The life cycle of *E. multilocularis* is based on a predator–prey relationship. The definitive hosts are wild carnivores such as the red fox (*Vulpes vulpes*) and the arctic fox (*Alopex lagopus*), but the tapeworm also infects, and develops within the intestine of, domestic dogs and cats, increasing the infection pressure for humans [1,2]. The definitive host sheds eggs that contain a first larval stage, the oncosphere. When taken up orally, oncospheres hatch as they reach the intestine, penetrate the intestinal wall and use the blood and lymphatic system for dissemination. They typically invade the liver, where they develop into the metacestode stage, which causes human alveolar echinococcosis (AE). AE is distributed in the Northern hemisphere, with endemic areas stretching from Northern America through Central and Eastern Europe to Central and East Asia including Northern parts of Japan [1]. The increase in the urban fox populations in Central Europe, together with the high prevalence rate of *E. multilocularis* in foxes, has resulted in increased environmental contamination with *Echinococcus* eggs and, as a consequence, has led to an increased risk of transmission to humans [2].

Human AE manifests itself by tumour-like infiltrative growth of metacestodes mainly in the liver, but other organs may also be affected. AE is often compared with a slow-growing liver cancer and, if untreated, the disease is usually lethal. The current strategy for treatment consists of surgical measures complemented by chemotherapy with mebendazole or albendazole (ABZ). In inoperable cases, chemotherapy has been shown to inhibit parasite proliferation with a parasitostatic effect, but benzimidazoles are rarely curative, resulting in a life-long duration of treatment, high

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costs and an elevated risk of side effects [3]. However, benzimidazoles have prolonged the average life expectancy of European patients at diagnosis from 3 years to 20 years [3]. Nevertheless, alternative options for chemotherapy with parasiticidal activity are needed [4].

Mefloquine (MEF) is a synthetic analogue of quinine commonly used in the treatment and prophylaxis of chloroquine-resistant *Plasmodium falciparum* malaria [5,6]. The mechanism of action of MEF against *Plasmodium* spp. has not been completely elucidated, but several investigations indicated a disturbance of haemoglobin metabolism. Degradation of haemoglobin is usually followed by the formation of an insoluble polymer, hemozoin. However, MEF binds to haem, inhibits hemozoin formation and the oxidative and glutathione-dependent degradation of haem. The resulting MEF–haem complexes are toxic for the parasite, just like free haem is, causing parasite death [5]. It is not known whether a haem-related mode of action is relevant for the anti-echinococcal activity of MEF in vitro.

MEF also exhibits considerable efficacy against other helminths such as *Schistosoma mansoni*, *Schistosoma japonicum*, *Opisthorchis viverrini*, *Brugia patei* and *Brugia malayi* [6–8]. We have previously demonstrated the efficacy of MEF against AE in experimentally infected mice when the drug was applied intraperitoneally but not when applied orally [9]. In this study, we present two distinct oral MEF treatment protocols in *E. multilocularis*-infected mice and show that MEF treatment, when applied orally as a suspension in honey at a dosage of 100 mg/kg twice a week, exhibits anti-echinococcal activity comparable with ABZ applied orally at 200 mg/kg/day. We also show that (i) the iron-binding protein ferritin and (ii) cystatin, a potential immunomodulator in parasite infections, bind to MEF and thus possibly could be targeted by MEF in *E. multilocularis* metacestodes. In contrast, MEF affinity chromatography of the human cancer cell line Caco2 results in binding of the enzyme nicotinamide phosphoribosyltransferase (NAPRT).

2. Materials and methods

2.1. Biochemicals and compounds

Unless stated otherwise, all culture media and reagents were purchased from Gibco BRL (Zürich, Switzerland) and biochemical reagents were from Sigma (St Louis, MO). MEF was kindly supplied by Mepha Pharma AG (Aesch, Switzerland).

2.2. In vitro culture

Culture of *E. multilocularis* isolate H95 was carried out as previously described [10]. The human colon carcinoma cell line Caco2 was maintained as previously described by Müller et al. [11].

2.3. Experimental infection of mice and treatments with mefloquine or albendazole

Female BALB/c mice (age 9 weeks; mean body weight 25 g) were housed in a temperature-controlled daylight/night cycle room with food and water ad libitum. Experiments were carried out according to the Swiss Federal Animal Welfare regulations (TschV, SR 455) under licence number BE_103/11.

The metacestode solid fraction was obtained by extensive washing of in vitro-cultured parasites with phosphate-buffered saline (PBS), breaking the vesicles mechanically, followed by centrifugation [10]. Animals were randomly divided into experimental groups and were then infected by intraperitoneal injection of 100 μL of metacestode solid fraction in 100 μL of PBS.

The pilot experiment was performed with 15 mice divided into three groups of 5 animals each. Compounds were formulated in 1% carboxymethyl cellulose (CMC) at the concentrations indicated below. Starting at 6 weeks post-infection, mice were treated by gavage for a period of 11 weeks with either 100 μL of honey/CMC 1% (control group), 100 μL of ABZ (200 mg/kg in honey/CMC 1%; ABZ group) or 100 μL of MEF at different concentrations (from 12.5 mg/kg up to 200 mg/kg in honey/CMC 1%; MEF group), basically 5 days per week and according to the treatment regimen depicted in Supplementary Fig. S1. During treatment, animals were carefully monitored and checked for clinical signs of impaired health such as weight loss, ruffled coat, hunched back and changes in behaviour (inactivity, nervousness), and the treatment regimen was adjusted accordingly. At the end of the 11-week treatment period, animals were euthanised. After necropsy, parasite tissue was collected and the parasite weight was measured. After pressing this material through a metal tea strainer, 100 μL of the resulting metacestode solution was re-injected into two mice per group to observe whether the parasite was still viable and re-growing.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2015.07.016.

Experiment 2 was performed with 39 mice (age 9 weeks) divided into four treatment groups of 8 animals each and a control group of 7 animals. Starting at 6 weeks post-infection, mice were treated as follows: the control group received 100 μL of honey/CMC 1%; the ABZ group received ABZ (200 mg/kg) emulsified in honey/CMC 1%; and the MEF treatment groups were treated by gavage of MEF emulsified in honey/CMC 1% at 25, 50 and 100 mg/kg, respectively. Treatments were performed for a period of 12 weeks on 5 days/week for the ABZ-treated group and twice weekly for the MEF-treated groups. On those days where no MEF, but ABZ, was administered, mice from the MEF groups received honey/CMC 1%. At the end of the study animals were euthanised and, after necropsy, parasite tissue was collected and the parasite weight was measured. Parasiticidal activity was assessed by in vitro cultivation of resected parasite material.

Data were analysed using the software R v.3.0.1 (https://www.r-project.org/). Upon removal of Grubbs' outliers, the data were analysed by one-way analysis of variance (ANOVA) and Bonferroni-adjusted *P*-values were calculated by pairwise *T*-test. Data were visualised by boxplot in Microsoft Office Excel 2010 (Microsoft Corp., Redmond, WA).

2.4. Coupling of mefloquine to epoxy-activated Sepharose® matrix

MEF was coupled to an epoxy-activated Sepharose matrix with a 12C spacer [11]. For this, 0.5 g of Sepharose was extensively washed with distilled water and was sedimented, followed by a two-step wash with coupling buffer (100 mM $NaHCO_3$, pH 9.5). Then, 20 mg of MEF–HCl, solubilised in 1 mL of dimethyl sulphoxide (DMSO), was added to 1 mL of the washed epoxy-Sepharose matrix and was incubated for 72 h on a slow horizontal shaker at 37 °C in order to allow coupling to the epoxy group. The resulting matrix was then transferred to a chromatography column (Novagen; Merck, Darmstadt, Germany) and was washed with 20 mL of coupling buffer, followed by 1 M ethanolamine (pH 9.5) for 4 h at room temperature in the absence of light in order to block residual groups. Subsequently, the column was extensively washed with PBS and PBS/DMSO (1:2) to remove unbound MEF. In addition to the MEF–Sepharose column, a mock column was prepared containing epoxy-Sepharose treated identically as described above but in the absence of MEF. The columns were stored in PBS containing 0.02% NaN_3 at 4 °C.