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Activities of fenbendazole in comparison with albendazole against *Echinococcus multilocularis* metacestodes in vitro and in a murine infection model

Tatiana Küster, Britta Stadelmann, Denise Aeschbacher, Andrew Hemphill*

Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggassstrasse 122, CH-3012 Berne, Switzerland

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ABSTRACT

The current chemotherapeutic treatment of alveolar echinococcosis (AE) in humans is based on albendazole and/or mebendazole. However, the costs of treatment, life-long consumption of drugs, parasitostatic rather than parasiticidal activity of chemotherapy, and high recurrence rates after treatment interruption warrant more efficient treatment options. Experimental treatment of mice infected with *Echinococcus multilocularis* metacestodes with fenbendazole revealed similar efficacy to albendazole. Inspection of parasite tissue from infected and benzimidazole-treated mice by transmission electron microscopy (TEM) demonstrated drug-induced alterations within the germinal layer of the parasites, and most notably an almost complete absence of microtriches. On the other hand, upon in vitro exposure of metacestodes to benzimidazoles, no phosphoglucose isomerase activity could be detected in medium supernatants during treatment with any of these drugs, indicating that in vitro treatment did not severely affect the viability of metacestode tissue. Corresponding TEM analysis also revealed a dramatic shortening/retraction of microtriches as a hallmark of benzimidazole action, and as a consequence separation of the acellular laminated layer from the cellular germinal layer. Since TEM did not reveal any microtubule-based structures within *Echinococcus* microtriches, this effect cannot be explained by the previously described mechanism of action of benzimidazoles targeting β-tubulin, thus benzimidazoles must interact with additional targets that have not been yet identified. In addition, these results indicate the potential usefulness of fenbendazole for the chemotherapy of AE.

1. Introduction

Echinococcus multilocularis is an endoparasitic flatworm of the family Taeniidae. The life cycle is typically sylvatic and 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 can also infect domestic dogs and cats, which shed eggs containing an infective larval stage within their faeces. The disease is distributed in the Northern hemisphere with endemic areas stretching from North America through Central and Eastern Europe to Central and East Asia including Northern parts of Japan [1].

Oral ingestion of *E. multilocularis* eggs and subsequent infection predominantly of liver tissue can cause human alveolar echinococcosis (AE). Upon uptake of eggs, the first larval stage (oncosphere) hatches and penetrates the intestinal wall, disseminates via blood and lymphatic vessels and reaches the liver, subsequently transforming to the metacestode stage that exhibits tumour-like proliferation and infiltrative and potentially unlimited growth. The liver represents the predilection site for infection but other organs might also be affected, either through primary infection or metastasis formation [2]. If left untreated, AE is usually lethal. However, current chemotherapy has had a major impact in that in Europe the average life expectancy of AE patients at diagnosis has increased from 3 years to 20 years [1].

The current strategies for treating human AE are surgical resection of the parasite mass complemented by chemotherapy with benzimidazoles such as mebendazole or albendazole, and for inoperable cases chemotherapy alone is applied. Albendazole treatment has been proven to inhibit parasite proliferation but is rarely curative, resulting in a long duration of treatment, high costs and an elevated risk of adverse effects [1,2].

The benzimidazoles are a large chemical family with a heterocyclic ring structure representing fusion of a benzene and an imidazole ring. Benzimidazole compounds include albendazole, mebendazole, flubendazole, fenbendazole, oxfendazole,

* Corresponding author. Tel.: +41 316 312384; fax: +41 316 312477.
E-mail address: andrew.hemphill@vetsuisse.unibe.ch (A. Hemphill).

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oxibendazole, thiabendazole and triclabendazole as well as the prodrugs of fenbendazole and albendazole (febantel and netobimin, respectively) [3]. Febantel is metabolised to the active compound fenbendazole and subsequently to fenbendazole sulfoxide (oxfendazole) and oxfendazole sulfone [4]. The drug is broadly used for the treatment of a variety of gastrointestinal helminth infections in animals, either as monotherapy or combined with other substances such as pyrantel, praziquantel and metrifonate. There are no reports on the use of febantel or fenbendazole in humans. In an earlier report, febantel in combination with praziquantel was successfully employed in the treatment of dogs experimentally infected with pre-adult stages of *Echinococcus granulosus* and *E. multilocularis*, yielding 100% parasite clearance [5]. Fenbendazole has also been shown previously to be efficient in inhibition of metacestode formation as well as in reducing worm burden and the number of protoscoleces in *E. multilocularis*-infected mice, but no direct comparison with albendazole treatment was made [6].

Like albendazole and other benzimidazoles, fenbendazole and its metabolites are believed to interfere with microtubule formation by binding to free β-tubulin of the parasite, thus interfering with microtubule-dependent uptake of glucose [3]. In support of this notion, molecular genetics revealed that sensitivity to benzimidazoles in evolutionary distant organisms such as fungi, nematodes, Platyhelminthes and various protozoa was correlated with the presence of specific alleles of β-tubulin genes (reviewed in [7]). Differences in reversibility, kinetics and stability of β-tubulin binding result in the differences in sensitivity to these drugs between species [3,7].

In this study, the profound anti-metacestode activities of fenbendazole were compared with albendazole in experimentally infected mice, and their modes of action were further investigated by transmission electron microscopy (TEM).

2. Materials and methods

Unless otherwise stated, all culture media and reagents were purchased from Gibco BRL (Zürich, Switzerland) and biochemical reagents were from Sigma (St Louis, MO).

2.1. In vitro culture of Echinococcus multilocularis metacestodes

Echinococcus multilocularis isolate H95 was cultured as previously described [8]. In short, metacestodes dissected from experimentally infected BALB/c mice were pressed through an autoclaved tea sieve. The sedimented material was washed with phosphate-buffered saline (PBS) and 1 mL was added to a cell culture flask containing 5×10^6 rat hepatoma cells (ATCC CRL-1600) per 50 mL of cultivation medium [Dulbecco's Modified Eagle Medium (DMEM), 10% foetal calf serum, 100 U/mL penicillin G and 100 μg/mL streptomycin sulphate (Biochrom, Berlin, Germany)]. These co-cultures were incubated at 37 °C in 5% CO_2 with medium changes once a week. Splitting of cultures was carried out when they exceeded 15 mL total metacestode volume. Metacestodes were used for in vitro assays when they reached a diameter of ca. 4 mm.

2.2. Experimental infection and in vivo treatment of mice with fenbendazole and albendazole

Twenty-four female BALB/c mice (age 9 weeks; mean body weight 25 g) were housed in a temperature-controlled light cycle room with food and water ad libitum. Experiments were carried out according to the Swiss Animal Welfare regulations. In vitro cultured metacestodes were washed extensively with PBS, broken mechanically and the tissue and vesicle fluid were separated by centrifugation. Experimental infection was carried out by intraperitoneal injection of 100 μL of sedimented metacestode material. After 6 weeks, animals were divided into three groups of eight animals each. For the treatments, albendazole and fenbendazole were formulated in a 1:1 mixture of honey (M-Budget; Migros, Bern, Switzerland) and carboxymethyl cellulose (CMC) (final concentration of 1%). The three groups received the following oral treatments: group 1 (untreated control) received diluted honey (honey/CMC 1%); group 2 received 200 mg/kg albendazole emulsified in diluted honey; and group 3 received 200 mg/kg fenbendazole emulsified in diluted honey. The treatments were performed daily for a period of 6 weeks. At the end of the study, animals were euthanised, necroscopy was performed and total parasite material was collected to determine the parasite weight. Experimental data were analysed with a box plot and outliers were identified by the extreme Studentised deviate method with a significance level of 0.05 (two-sided). No outliers were identified and the data were submitted to a two-tailed distributed Student's *t*-test, with two-sample equal variance between the untreated group and each of the treatment groups.

2.3. In vitro drug treatment of Echinococcus multilocularis metacestodes

Echinococcus multilocularis metacestodes were used after 1–2 months of culture as described by Stadelmann et al. [9]. In short, after extensive washing with PBS, 10–15 intact vesicles were distributed into 24-well plates in 1 mL of DMEM without phenol red containing 100 U/mL penicillin G, 100 μg/mL streptomycin sulphate and 2 mM L-glutamine. The benzimidazoles febendazole, oxfendazole and albendazole, the arylimidamide DB1127 [10] and the thiazolide nitazoxanide [9] were prepared as stock solutions (10 mM) in dimethyl sulphoxide (DMSO) and were added at a final concentration of 20 μM (0.2% DMSO). Non-treated controls were incubated with the corresponding concentration of DMSO. After 5 days and 10 days of culture at 37 °C in 5% CO_2, medium supernatants were collected and stored at −20 °C until they were used for phosphoglucose isomerase (PGI) assays, and the metacestode tissue was further processed for TEM.

2.4. Detection of phosphoglucose isomerase activity in medium supernatant

PGI assays [9–11] were performed according to Stadelmann et al. in 96-well microtitre plates (Sarstedt, Sevelen, Switzerland).

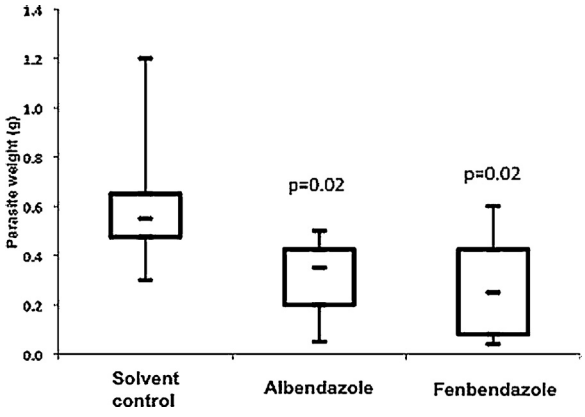

Fig. 1. Effects of fenbendazole and albendazole treatment in *Echinococcus multilocularis*-infected mice. Treatment was initiated 6 weeks following intraperitoneal infection with *E. multilocularis* metacestodes. Each treatment group comprised eight animals. Albendazole and fenbendazole emulsified in honey/carboxymethyl cellulose was applied orally at 200 mg/kg daily for a period of 6 weeks. Parasite weights are shown as box plots. Fenbendazole- and albendazole-treated mice exhibited a significant reduction in parasite weight compared with the solvent-treated control.