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Combined albendazole and amphotericin B against *Echinococcus multilocularis in vitro*☆

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ABSTRACT

Benzimidazoles, namely albendazole (ABZ) and mebendazole, are the only drugs licensed for the treatment of inoperable alveolar echinococcosis. In addition, amphotericin B (AMB) has shown effect against *Echinococcus multilocularis* as salvage treatment in humans. Both benzimidazoles and AMB are only parasitostatic against *E. multilocularis* and toxicity may limit long-term use. In the present study we examined the effect of combined treatment between ABZ and AMB on *E. multilocularis* larvae in an *in vitro* model.

Vesicles were grown in a tissue culture model of metacestodes and hepatocytes. Drugs were added to the culture and the destructive effect on the vesicles was visually observed.

Sequential application of ABZ and AMB yielded effective destruction of vesicles which was faster than the application of AMB alone. However, simultaneous application of ABZ and AMB had an inhibitory effect on vesicle destruction. After discontinuation of drug application, regrowth of vesicles occurred, hereby proving the parasitostatic effect of combined treatment against *E. multilocularis* larvae.

Due to an inhibitory effect between ABZ and AMB against *E. multilocularis* larvae, we discourage from the simultaneous application of both drugs. If our *in vitro* results hold true *in vivo*, sequential application of ABZ and AMB would be an effective means for long-term suppression of larval growth. Long-term tolerance of both drugs could be improved by a reduction of side effects.

1. Introduction

The larval stage of *Echinococcus multilocularis* causes alveolar echinococcosis (AE), a parasitic disease with tumor-like growth primarily affecting the liver. Human AE is endemic in regions of Western and Central Europe, Eastern Europe, North America and Asia. Untreated AE is fatal in over 90% of cases and surgical resection is often incomplete due to the diffuse infiltration of non-resectable structures (Brunetti et al., 2009; Torgerson et al., 2008).

The only drugs available for the treatment of human AE are benzimidazole carbamate derivatives, namely mebendazole (MBZ) and albendazole (ABZ). A disadvantage of the benzimidazoles is the fact that these drugs are parasitostatic rather than parasitocidal for *E. multilocularis* (Ammann et al., 1990, 1994; Wilson et al., 1987). This implies that the parasite is not depleted and may resume growth after discontinuation of treatment. Thus, treatment of AE implies lifelong application of benzimidazoles. The overall success-rate of benzimidazole treatment ranges between 55 and 97% (Ammann et al., 1994; Ishizu et al., 1997; Mesarina-Wicki, 1991; Reuter et al., 2000). Failure may be due to severe side effects, such as liver toxicity, forcing a discontinuation of the medication (Davis et al., 1986).

Amphotericin B (AMB) has been the most important fungicidal agent for decades. This drug is naturally derived from *Streptomyces nodosus* (Daneshmend and Warnock, 1983). Although its mode of action is not yet fully understood, the most important mechanism appears to be the selective and irreversible binding to sterols in cell membranes, hereby forming transmembrane channels. Furthermore, AMB was shown to form stable complexes with membrane phospholipids, to perturbate the fluidity of membranes and to influence anion transport and membrane-bound enzymes (Abu-Salah, 1996; Cohen, 1992; Hartsel and Bolard, 1996). AMB effectively destroyed *E. multilocularis* metacestodes *in vitro* (Reuter et al., 2003b). AMB was successfully used as salvage treatment in humans with AE, but adverse events such as nephrotoxicity and hypokalemia, as well as the necessity of intravenous application limited its long-term use (Reuter et al., 2003a).

Due to the above limitations, new treatment strategies are indispensable for patients with intolerance to benzimidazoles and/or AMB. The simultaneous or sequential application of different drugs is an appealing approach for potentially

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enhancing effectiveness and limiting toxicity. Both benzimidazoles and AMB are only parasitostatic against *E. multilocularis* and a parasitocidal drug or drug combination would shorten long-term use of these substances. In the present study we analyzed the effect of combined application of ABZ and AMB on *E. multilocularis* larvae *in vitro* tissue culture model.

2. Materials and methods

2.1. Culture system

E. multilocularis metacestodes, originally isolated from humans, were maintained in Mongolian gerbils (*Meriones unguiculatus*) by intraperitoneal injection of minced metacestode tissue. After 6–8 weeks the gerbils were euthanized. Metacestodes were isolated from the peritoneal cavity and cut into tissue blocks of 0.5 cm^3. After washing twice with PBS three tissue blocks were placed in each 25 cm^3 cell culture flask containing 20 ml of Dulbecco's Modified Eagles Medium (DMEM, Biochrom AG, Berlin). The culture medium was supplemented with 10% fetal calf serum (FCS, PAA Laboratories, Linz, Austria), 200 U of penicillin/ml, 200 μg streptomycin/ml (both Sigma–Aldrich, Taufkirchen, Germany) and 10 μg/ml of levofloxacin (Sanofi Aventis, Frankfurt, Germany). Cells of the human liver cell line HepG2 were grown adherent to the bottom of 25 cm^3 flasks. Tissue cultures were incubated at 37 °C with 5% CO_2. The culture medium was changed thrice weekly. Cultures were monitored for growth and integrity of parasitic vesicles by light microscopy. Average numbers ranged between 80 and 120 vesicles per flask. The basic features of this culture method for the metacestode stage of *E. multilocularis* were first described by Hemphill and Gottstein (1995) and Jura et al. (1996).

After 8–12 weeks, drugs were added to the cultures containing parasitic vesicles. The culture medium was changed thrice weekly and new drugs were added with each change of culture medium. For experiments with sequential application of drugs, the culture medium was changed prior to addition of the second drug, in order to clear the first drug. Follow-up parameters recorded were the number and integrity of vesicles under the influence of drugs. Vesicle damage was characterized by a loss of vesicle turgor leading to desintegration of the vesicle within less than 24 h. Integrity of vesicles was assessed every other day. After complete destruction of all vesicles, we recorded the number and size of secondary vesicles growing from the tissue block. All experiments were performed in duplicates.

2.2. Anthelminthic drugs

AMB (Biochrom AG, Berlin, Germany) was resuspended in 500 μl aqua dest and was used at concentrations of 2.5 and 0.25 μg/ml. ABZ and MBZ (Sigma–Aldrich, Taufkirchen, Germany) as well as ABZSO and ABZSN (kindly provided by R.J. Horten, SmithKline Beecham, London, United Kingdom) were resuspended in 40 μl DMSO/20 ml and were used at concentrations of 1 and 0.1 μg/ml. Control cultures for benzimidazoles contained 40 μl DMSO/20 ml.

2.3. Statistical analysis

All experiments were performed in duplicates and the inter-assay variation was <10%. Time courses of the number of vesicles in culture are depicted as line charts.

3. Results

3.1. Simultaneous application of ABZ and AMB

ABZ or its metabolites were tested alone and in combination with AMB *in vitro*. Drugs were added to the medium at the same time. The kinetics of vesicle destruction was fastest with AMB alone (2.5 μg/ml), with 70% of vesicles destroyed on day 5 and complete destruction after 2 weeks (Fig. 1a). A continuous destruction of vesicles was observed with ABZ and its metabolites ABZSO and ABZSN. However, compared to AMB, the kinetics of vesicle destruction was significantly delayed. As shown in Fig. 1b, with ABZSO a 75% destruction of vesicles was achieved after 20 days and complete destruction was observed after 31 days.

When combining AMB with the metabolites ABZSO or ABZSN a delay of vesicle destruction was observed as compared to AMB alone (Fig. 1b). The delayed vesicle destruction was most pronounced with AMB in combination with ABZSN (1 μg/ml).

In analogy to these findings, the combination of AMB (2.5 μg/ml) with ABZ (1 μg/ml) yielded a retarded effect of vesicle destruction as compared to AMB alone (Fig. 1a).

AMB and ABZ were both used at two different concentrations. AMB was used at 2.5 and 0.25 μg/ml and ABZ was used at 1 and 0.1 μg/ml. The lower doses resulted in a marked delay of several days until complete vesicle destruction (+20 days for AMB and +18 days for ABZ, results not shown).

3.2. Sequential application of ABZ and AMB

We evaluated the influence of initial treatment with ABZ on the subsequent kinetics of vesicle destruction under AMB (2.5 μg/ml). Metacestode cultures were pre-incubated with ABZ (1 μg/ml) for 4 and 24 h, respectively, and hereafter either AMB or the combination of AMB + ABZ was added. The addition of drugs was preceded by a complete change of medium. Fig. 2a shows that the time of pre-incubation with ABZ correlated with a more rapid destruction of vesicles under subsequent application of AMB. The fastest kinetics of vesicle destruction was seen after ABZ pre-incubation over 24 h. Here, vesicles were completely destroyed after 8 days, while after pre-incubation for only 4 h, complete destruction was observed after 10 days. Without ABZ pre-incubation, complete vesicle destruction under AMB was delayed until day 11.

Equally, the subsequent application of combined AMB + ABZ was dependent on pre-incubation with ABZ. A complete destruction of vesicles occurred after 12 days with ABZ pre-incubation for 24 h and after 14 days after ABZ pre-incubation for 4 h. In contrast, no pre-incubation delayed complete vesicle destruction until day 16. After pre-incubation with ABZ, vesicle destruction was more delayed with subsequent combination treatment (Fig. 2b) as compared to AMB alone (Fig. 2a). In AMB monotherapy, pre-incubation with ABZ for 4 and 24 h resulted in complete destruction of vesicles after day 8 and 10, while ABZ + AMB combination treatment delayed complete destruction until day 12 and 14, respectively.

After complete destruction of vesicles, drugs were applied for another 10 days and then discontinued. Cultures with metacestode tissue blocks were maintained by adding culture medium without drugs. The formation of new secondary vesicles from the tissue blocks was observed after 40–50 days in all experiments.

4. Discussion

Combination treatment is an appealing approach for the utilization of synergistic effects of two drugs and for the reduction of toxicity. For example, in clinical practice synergism is achieved with the combination of beta-lactam antibiotics and aminoglycosides. A