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In vitro effects of nitazoxanide on *Echinococcus granulosus* protoscoleces and metacestodes

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Objectives: Infection of humans and domestic ruminants with the larval stage (metacestode) of *Echinococcus granulosus* results in cystic echinococcosis (CE). The metacestode causes a space-occupying lesion in visceral organs, most commonly in the liver. Benzimidazole carbamate derivatives, such as mebendazole and albendazole, are currently used for chemotherapeutic treatment of CE. In human patients, benzimidazoles have to be applied in high doses for extended periods of time, and adverse side effects are frequently observed. In order to evaluate alternative treatment options, the *in vitro* efficacy of nitazoxanide, a broad-spectrum drug used against intestinal parasites and bacteria, was investigated.

Methods: Freshly isolated *E. granulosus* protoscoleces were subjected to nitazoxanide treatment (1, 5 and 10 µg/mL), and the effects on parasite viability were monitored by Trypan Blue staining and scanning electron microscopy. Protoscolex cultures were maintained further, until metacestode development took place. Metacestodes were then subjected to nitazoxanide treatment (10 µg/mL), and corresponding effects were visualized by scanning and transmission electron microscopy.

Results: Dose-dependent protoscolex death within a few days of nitazoxanide treatment was observed. Subsequent *in vitro* culture of drug-treated protoscoleces confirmed the non-viability of parasites, while further cultivation of non-treated protoscoleces for a period of at least 3 months resulted in stage conversion and the formation of small metacestodes 3–4 mm in diameter. Nitazoxanide had a deleterious effect on these metacestodes, which was comparable to that of albendazole.

Conclusions: Our study indicates a potential for nitazoxanide as an alternative treatment option against CE.

Keywords: *E. granulosus*, cystic echinococcosis, *in vitro* drug treatment, albendazole

Introduction

Cystic echinococcosis (CE) is caused by the metacestode (larval) stage of *Echinococcus granulosus*. The disease is found worldwide, and affects humans as well as domestic livestock including cattle, sheep, camels, pigs, horses and others.¹ In Central Europe and other industrialized countries, measures such as mandatory meat inspection have reduced the incidence of the disease, whereas in countries with lower hygiene standards, *E. granulosus* infections are commonly observed.¹ The definitive host is the dog, in which adult tapeworms attach to the intestinal epithelium and undergo sexual reproduction, leading to the development of eggs. These eggs are shed into the environment with the faeces.

The eggs contain an oncosphere, which upon ingestion by a suitable intermediate host and subsequent passage through stomach and intestine become activated, penetrate the mucosa, enter blood and lymphatic vessels and are disseminated in the body.²

After an undefined incubation period, *E. granulosus* metacestodes are formed. These are single, fluid-filled cysts, which are surrounded by a thick, host-derived layer of connective tissue (adventitia). The parasite tissue is delineated by the laminated layer, an acellular, carbohydrate-rich outer surface structure of considerable thickness, which separates the living parasite tissue at the inside from the outer host environment. Most human patients harbour a solitary cyst within a single organ. In 70% of patients, infection occurs in the liver, the lung being affected in

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20% of patients. Other sites of infection include kidney, spleen, muscle, skin, abdominal and pelvic cavity, mediastinum, heart, brain, spinal cord and others.³ Within fertile metacestodes, brood capsules are formed out of distinct cells originating from the germinal layer, which themselves contain protoscoleces. Infertile metacestodes only contain the germinal layer. Often the metacestode contains one or more daughter cysts, which give rise to the septate appearance in diagnostic imaging.⁴ The cysts slowly expand over several years, often without being noticed. Spread of infection into other sites, such as the peritoneal cavity, has been described, and this can be caused by cyst rupture and spillage of protoscoleces. These have the potential to develop into metacestodes in the intermediate host, and into adult parasites when ingested by the final host.²

Treatment of human CE by chemotherapy has been developed following a series of animal studies, which demonstrated the efficacy of benzimidazole carbamates against *E. granulosus* and *Echinococcus multilocularis*.^{5–10} Albendazole and mebendazole are considered to be equally effective. When evaluated up to 12 months after initiation of benzimidazole treatment, 10%–30% of patients exhibit cyst disappearance, 50%–70% show degeneration of cysts and 20%–30% show no morphological changes in cyst appearance.^{11,12} Continuous or intermittent treatment with albendazole is recommended for a period of 6 months. Praziquantel, shown to exhibit protoscolicidal activity, has been reported to enhance the effect of benzimidazoles, especially when cyst spillage was considered to pose a problem. In about 75% of patients, metacestodes exhibit clear signs of degenerative changes.¹¹ However, adverse side effects have been observed with both benzimidazoles, including abnormalities in liver function, leucopenia and alopecia, and albendazole has been shown to be teratogenic in rats and rabbits. It should therefore be avoided during pregnancy and lactation.^{3,11,12} As a consequence, the development of alternative means of treatment of CE is necessary.

Nitazoxanide [2-acetolyloxy-N-(5-nitro-2-thiazoly) benzamide], originally developed as a veterinary anthelmintic, was first described in 1984 as a human cestocidal drug.¹³ To date, nitazoxanide is known as a broad-spectrum drug against a wide variety of intestinal parasites and enteric bacteria infecting animals and humans. Specifically for the treatment of chronic diarrhoea in immunocompromised patients (e.g. AIDS), nitazoxanide has become widely used.¹⁴ More recently, we have reported on the efficacy of nitazoxanide against *E. multilocularis* metacestodes *in vitro*,¹⁵ and upon egg infection in the murine model.¹⁶ We have also shown that combined nitazoxanide/albendazole treatment of mice infected intraperitoneally with *E. multilocularis* metacestodes results in a treatment efficacy that is superior to albendazole alone. This effect was partially attributed to the fact that combined application of the two drugs leads to increased albendazole sulphoxide serum levels for extended periods of time.¹⁶ In this study, we report on the *in vitro* efficacy of nitazoxanide against *E. granulosus* protoscoleces and metacestodes.

Materials and methods

Biochemicals and drugs

If not otherwise stated, all tissue culture media were purchased from Gibco-BRL (Zürich, Switzerland), and biochemical reagents were

from Sigma (St Louis, MO, USA). Nitazoxanide used in this study was obtained from Romark Laboratories (Tampa, FL, USA). Albendazole was purchased from GlaxoSmithKline (Germany).

In vitro culture of *E. granulosus* protoscoleces and metacestodes

E. granulosus hydatid cysts containing protoscoleces were removed under aseptic conditions from infected sheep presented for routine slaughter in abattoirs in Almaty, Kazakhstan. The largely intact cysts were stored for no longer than 1 week at 4°C, during which time they were transported to Switzerland. *In vitro* cultivation of *E. granulosus* protoscoleces and metacestodes was carried out as previously described for *E. multilocularis* metacestodes.¹⁷ Briefly, the hydatid cysts (2–5 cm in diameter) were cut open and vesicle fluid (containing protoscoleces) was separated from the metacestode tissue and host adventitia. Protoscoleces were allowed to settle in a 50 mL Falcon tube, were washed twice in Hanks balanced salt solution and placed into culture medium (RPMI 1640 containing 12 mM HEPES, 2 mM glutamine, 200 U/mL of penicillin, 200 µg/mL of streptomycin and 0.50 µg/mL of amphotericin B) supplemented with 10% fetal calf serum and phenol red. Cultures were kept in culture flasks (200 mL) placed in an upright position in an incubator at 37°C, 5% CO₂, with medium changes every 4–8 days.

Drug treatment of *E. granulosus* protoscoleces

Treatment of protoscoleces was initiated within 10 days of *in vitro* culture. Three T25 tissue culture flasks containing at least 500 protoscoleces in 20 mL culture medium were supplemented with 1, 5 and 10 µg/mL nitazoxanide [stock solution in 10 mg/mL in dimethyl sulphoxide (DMSO)], and one control culture was supplemented with 20 µL DMSO alone. The vitality/viability of protoscoleces was assessed by observation of motile behaviour and a Trypan Blue exclusion test, respectively. The corresponding numbers of viable/non-viable protoscoleces were determined in 10 randomly chosen fields by phase contrast microscopy using a Leitz DMIL inverted microscope at 10× magnification. At day 5 following initiation of drug treatment, the medium±nitazoxanide was removed, parasites were washed twice in 20 mL Hanks balanced salt solution and culture was continued in fresh culture medium without nitazoxanide. Of each culture, a small sample was processed for scanning electron microscopy (SEM) (see below).

Drug treatment of *E. granulosus* metacestodes

E. granulosus metacestode formation was achieved by continuing the culture of protoscoleces for a period of 3–4 months. Parasites that had undergone stage conversion into small (1–4 mm) hydatid cysts were collected, split into groups of 40, and resuspended in 5 mL of culture medium supplemented with nitazoxanide (10 µg/mL) and albendazole (10 µg/mL). Two control cultures were set up in culture medium and medium supplemented with the corresponding amount of DMSO, respectively. Samples for SEM and transmission electron microscopy (TEM) inspection were taken on days 0, 4 and 7 following initiation of drug treatment.

SEM and TEM

Protoscoleces and metacestodes were processed as described by Hemphill & Croft.¹⁸ Briefly, parasites were washed once in 100 mM sodium cacodylate buffer pH 7.2, and placed into the primary fixative (2.5% glutaraldehyde in 100 mM sodium cacodylate buffer) for 2 h at room temperature. After addition of the fixative, vesicles were

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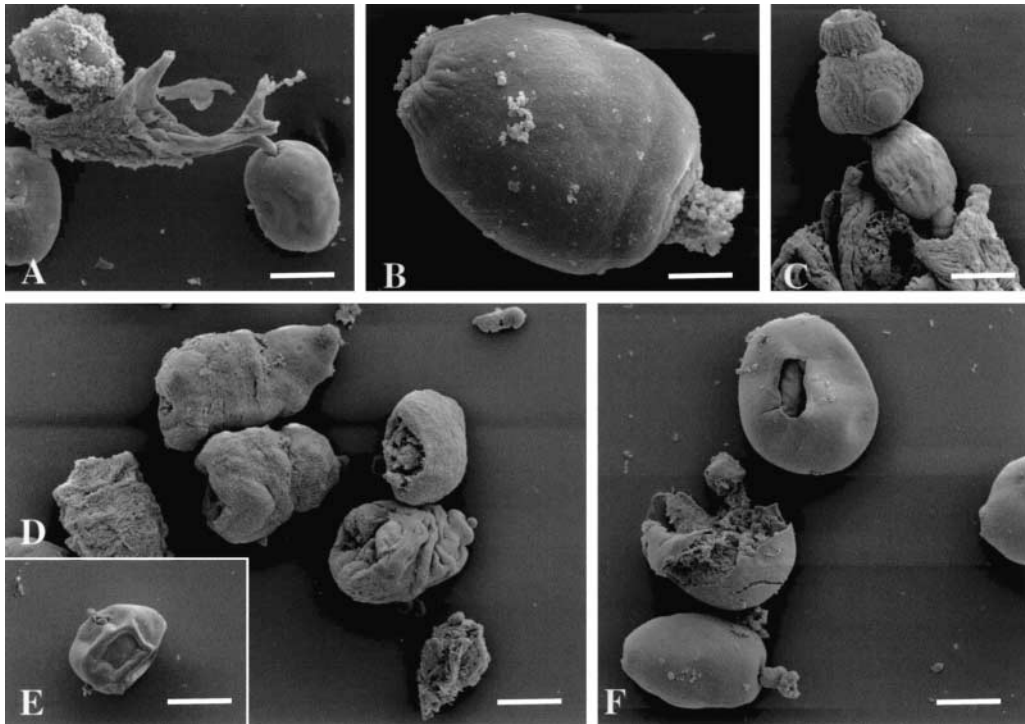


Figure 1. Scanning electron microscopy of *E. granulosus* protoscoleces cultured for 7 days in the presence or absence of nitazoxanide (NTZ; 5 $\mu\text{g}/\text{mL}$). A–C are protoscoleces cultured in medium containing DMSO (1:1000), D–F are protoscoleces cultured in the presence of NTZ. Note the evaginated protoscolex in C, and the extensive drug-induced damage in D and E. Bars in A = 100 μm , B = 30 μm , C = 76 μm , D = 62 μm , E = 90 μm , F = 66 μm .

gently broken up with a pipette. After several washes in cacodylate buffer, samples were post-fixed in 2% OsO_4 in cacodylate buffer. The specimens were then extensively washed in distilled water.

For SEM analysis, the specimens were dehydrated by sequential incubations in increasing concentrations of ethanol (50%–70%), and were finally immersed in hexamethyl-disilazane and air-dried under a fume hood. They were then sputter-coated with gold, and inspected on a JEOL 840 scanning electron microscope operating at 25 kV.

For TEM, fixed metacystode vesicles were pre-stained in 1% uranyl acetate for 30 min at 4°C, followed by several washes in water. They were dehydrated in a graded series of ethanol and subsequently embedded in Epon 812. Polymerization of the resin was carried out at 65°C overnight. Sections 1–2 μm thick were cut on a Reichert and Jung ultramicrotome, loaded onto poly-L-lysine-coated glass coverslips, and stained with Methylene Blue-Fuchsin. Eighty nanometre sections were loaded onto 300-mesh copper grids (Plano GmbH, Wetzlar, Germany), and contrasting with uranyl acetate and lead citrate was performed, as described previously.¹⁸

Results

Effects of nitazoxanide on E. granulosus protoscoleces

Following the isolation of protoscoleces from hydatid cysts, the Trypan Blue exclusion test revealed that >85% of the parasites were still viable, and >60% of protoscoleces exhibited distinct movements. Of those, the majority was invaginated (Figure 1A and B), but ~10%–15% were evaginated; rostellum and suckers were clearly visible (see Figure 1C), and these regions of the parasites were the most motile compartments (data not shown).

The decrease in the vitality of the protoscoleces became evident after 3–4 h following the addition of nitazoxanide, since movements had ceased, regardless of whether protoscoleces were invaginated or evaginated. However, Trypan Blue exclusion failed to detect a significant change in viability (data not shown). Loss of protoscolex viability in nitazoxanide-treated cultures became clearer after 24 h, with a 50% reduction in the number of viable parasites at 5 and 10 $\mu\text{g}/\text{mL}$ of nitazoxanide (see Figure 2). The number of dead protoscoleces increased with time, and from day 3 onwards, viable parasites could no longer be seen in cultures treated with 5 and 10 $\mu\text{g}/\text{mL}$ of nitazoxanide. Whereas nitazoxanide at 1 $\mu\text{g}/\text{mL}$ exhibited a less dramatic

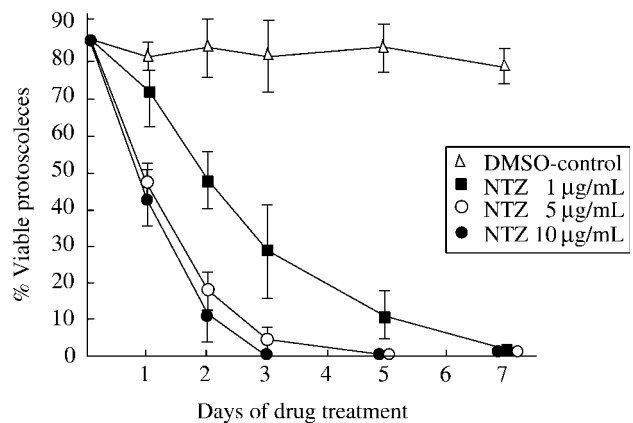


Figure 2. Loss of viability of *E. granulosus* protoscoleces during *in vitro* nitazoxanide (NTZ)-treatment. Viability was determined through Trypan Blue staining. Note the dose-dependent effect of NTZ.

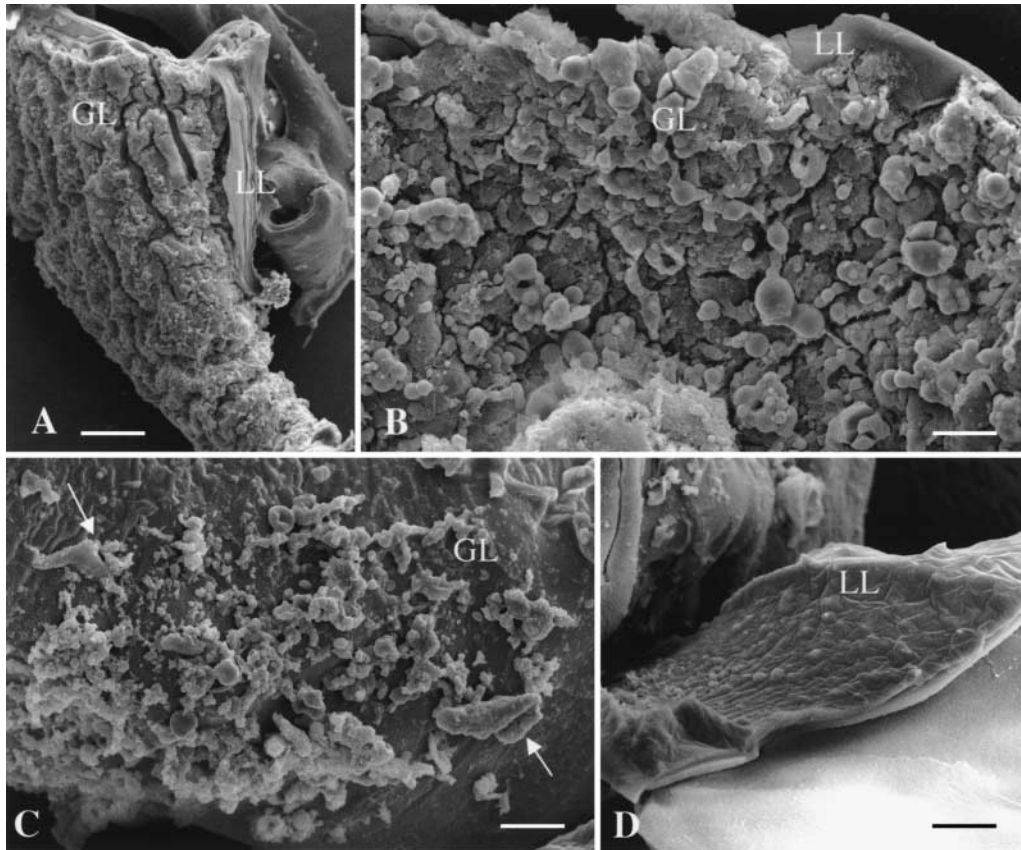


Figure 3. Scanning electron microscopy of *E. granulosus* metacystodes. Metacystodes were either kept for 7 days in medium containing DMSO (A, B), or were treated with 10 µg/mL nitazoxanide (NTZ) (C, D). Note the extensive NTZ-induced damage, affecting the germinal layer, with either only residues of germinal cells present (arrows in C), or only shells of laminated layer (D). GL=germinal layer, LL=laminated layer. Bars in A=620 µm, B=260 µm, C=260 µm, D=330 µm.

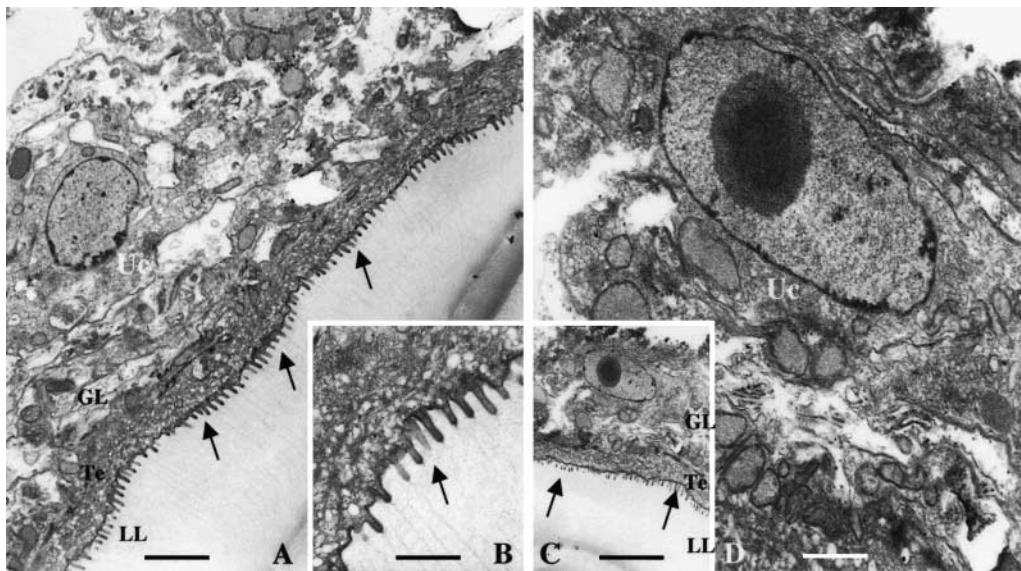


Figure 4. Transmission electron microscopy of *E. granulosus* metacystode tissue at either timepoint 0 (A, B) or after 7 days of culture in DMSO diluted 1:1000 in medium (C, D). Uc=undifferentiated cell, LL=laminated layer, Te=tegument, GL=germinal layer. Arrows point towards microtriches. Bars in A=3.8 µm, B=1.9 µm, C=7.8 µm, D=1.8 µm.

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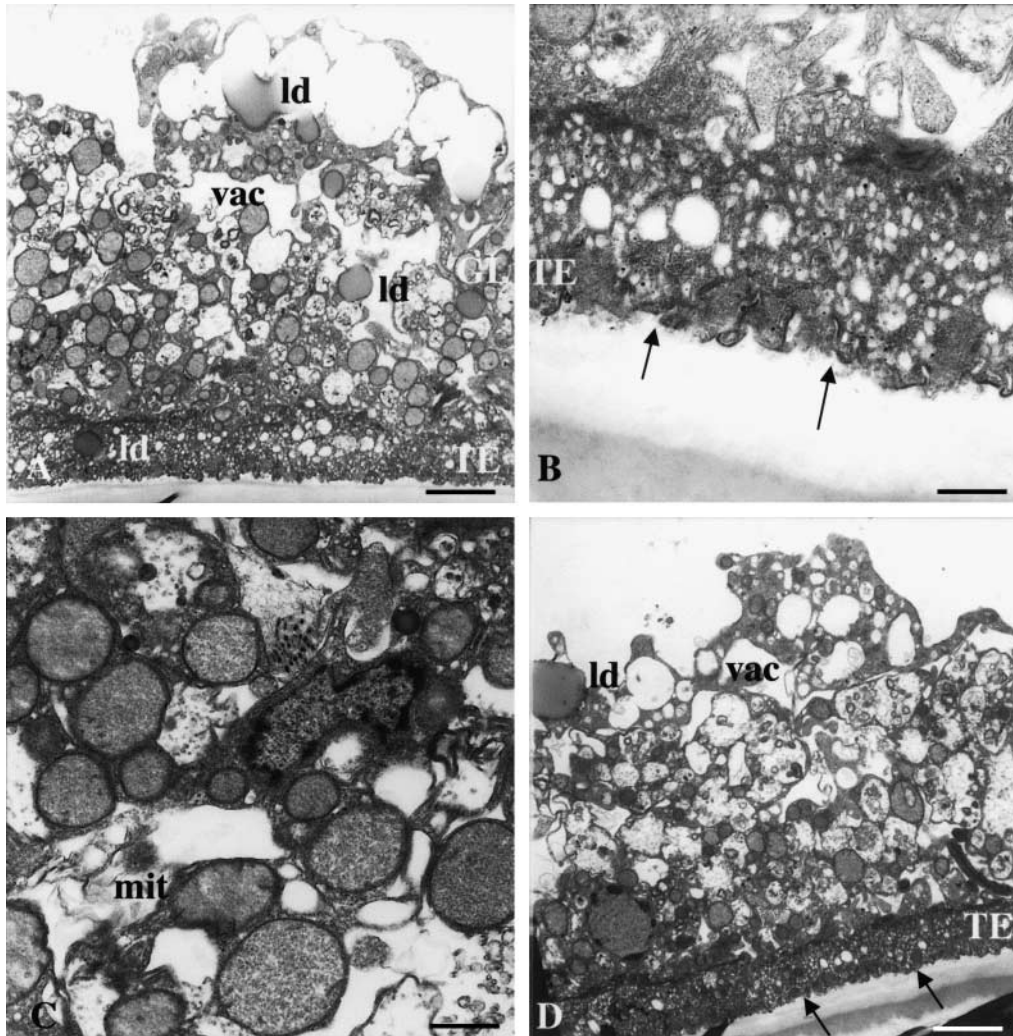


Figure 5. Transmission electron microscopy of metacystodes treated with nitazoxanide (A–C) or albendazole (D) for a period of 4 days. Note that the tegument (TE) remains, but effects include increased vacuolization (vac) of the germinal layer (GL), occurrence of lipid droplets (ld), the truncated microtriches (arrows), and aberrant mitochondria (mit). Bars in A = 1.9 μm , B = 0.8 μm , C = 0.6 μm , D = 2.0 μm .

decrease, complete loss of protoscolex viability was observed at day 7. In contrast, those parasites cultured in the absence of nitazoxanide were not significantly altered. SEM demonstrated the drug-induced morphological and structural damage imposed upon nitazoxanide-treated protoscoleces (Figure 1D–F).

Resuspension of nitazoxanide-treated protoscoleces in fresh medium for a period of several weeks without the drug did not result in any changes, indicating that none of the parasites had survived the treatment. These results demonstrated the dose-dependent protoscolicidal effect of nitazoxanide on *E. granulosus*. In contrast, a considerable fraction (~20%) of protoscoleces, which were not treated with the drug, had undergone vesiculation, eventually resulting in metacystode stage conversion (see below).

In vitro effects of nitazoxanide treatment on *E. granulosus* metacystodes

E. granulosus metacystodes were obtained by continuous *in vitro* maintenance of the cultures initiated with protoscoleces for a

period of 3–4 months. This resulted in the formation of vesiculated parasites of 1–4 mm in diameter. Inspection of these vesicles by SEM revealed that they exhibited features typical of *E. granulosus* metacystodes, with a distinct acellular outer laminated layer and an intact germinal layer comprised of a multitude of different, morphologically intact, cell types (Figure 3A and B). Upon treatment with nitazoxanide and albendazole for 3 days, macroscopic morphological changes became evident in >50% of metacystodes, most evident through the loss of turgidity. No damage could be seen in the control vesicles. At days 5–7, all vesicles were extensively distorted upon drug treatment, but control vesicles showed no alteration. Corresponding effects are illustrated by SEM in Figure 3(C and D). Clearly, at day 7 of treatment with both nitazoxanide and albendazole, in many areas, only cellular debris of the former germinal layer could be seen.

These results were confirmed on the ultrastructural level by TEM. In non-treated control cultures, the external surface of the metacystodes is comprised of an acellular, amorphous and heavily glycosylated, laminated layer which surrounds the entire

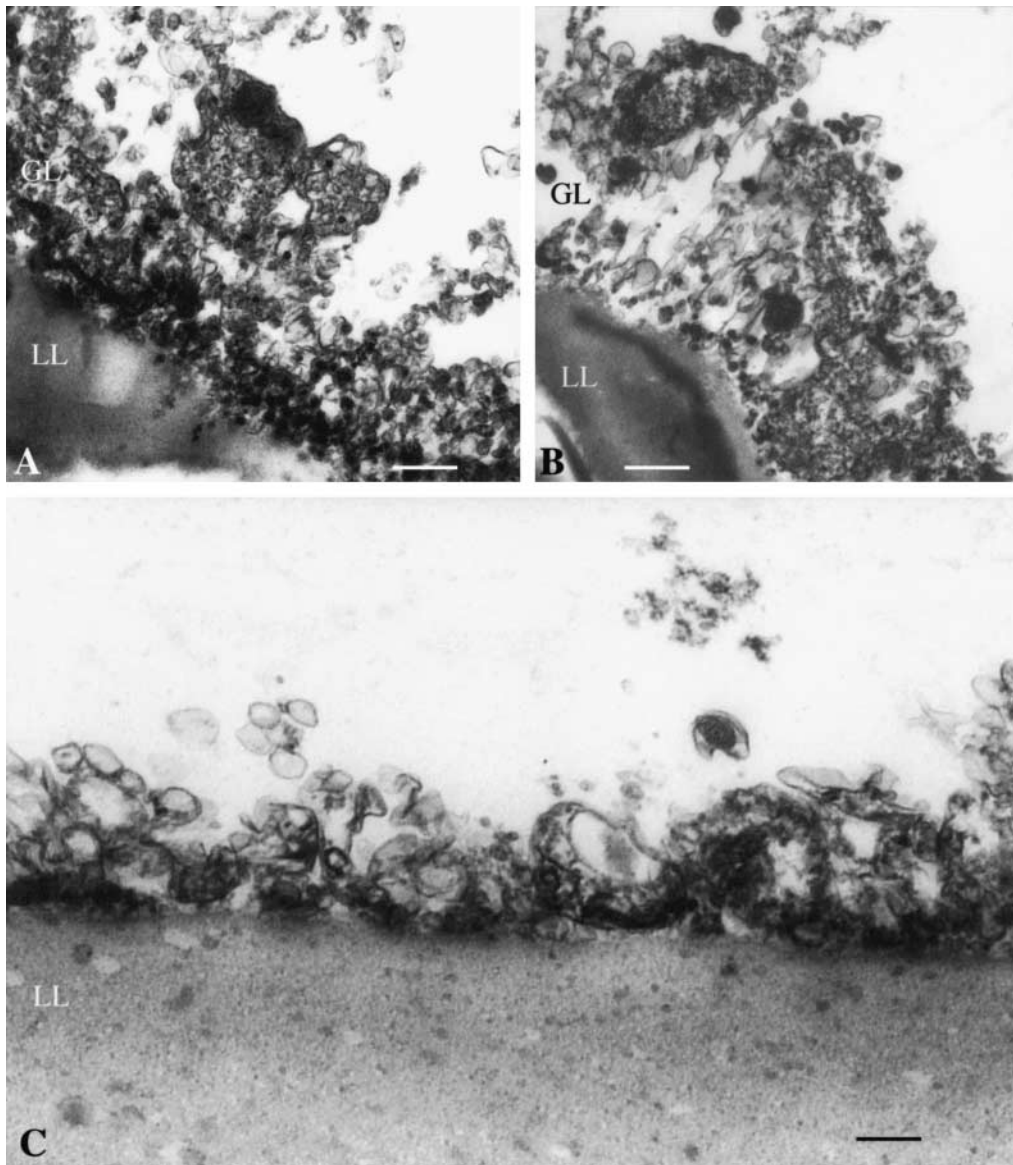


Figure 6. Transmission electron microscopy of metacestodes treated with nitazoxanide (A, B) and albendazole (C) for 7 days. Note the complete deterioration of the structural integrity of the germinal layer (GL), while the laminated layer (LL) still appears intact. Bars in A = 1.5 μm , B = 1.4 μm , C = 0.9 μm .

parasite (Figure 4). Attached to the interior surface of the laminated layer is the tegument, a syncytial parasite tissue with numerous microtriches protruding into the laminated layer and thus significantly enhancing the resorbing surface of the parasite. More towards the interior, the tegument is replaced by the germinal layer, which contains a number of different cell types, including a number undifferentiated cells with a large nucleus and nucleolus (Figure 4A and B). Control- and DMSO-treated cultures exhibited no ultrastructural alterations in parasite tissue during the entire incubation period of 7 days (Figure 4C and D).

In contrast, both nitazoxanide and albendazole-treated metacestodes had undergone considerable degenerative changes following 4 days of *in vitro* treatment, including the extensive truncation of microtriches. However, the rest of the tegument was still present, but other structural aberrations became evident. Treatment with both drugs resulted in the formation of lipid droplet inclusions, the appearance of rounded mitochondria and

increased vacuolization of the germinal layer (Figure 5). At day 7 of drug treatment, both drugs had induced complete destruction of the parasite tissue, and only residues of the germinal layer could be seen still associated with the laminated layer (Figure 6). In conclusion, *in vitro* drug treatment with nitazoxanide and albendazole exhibited similar, and clearly parasiticidal, effects.

Discussion

The aim of this study was to investigate the efficacy of nitazoxanide against the cestode parasite *E. granulosus*, the causative agent of CE. *In vitro* culture of protoscoleces and metacestodes was employed to demonstrate the parasiticidal effects of nitazoxanide on both protoscoleces and metacestodes. Recent studies demonstrating the *in vitro* and *in vivo* efficacy of this drug against *E. multilocularis* metacestodes^{15,16} implied that

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nitazoxanide could also be a promising candidate drug for corresponding treatment against *E. granulosus* infections. For this species, it is relevant to investigate these effects on both metacestodes and protoscoleces, since protoscoleces will not only develop into adult tapeworms in the intestine, but also exhibit the potential to differentiate into metacestodes when they reach other organs.² Thus, they are largely responsible for metastases formation through spillage of cyst fluid during cyst rupture, puncture or surgery.³ Prospectively, the introduction of new drugs could help patients suffering from CE undergo improved chemotherapy.

Nitazoxanide is a 5-nitrothiazole analogue, with structural similarities to the benzimidazole antihelminthics such as albendazole and its metabolic derivatives albendazole sulphoxide and albendazole sulphone, with a 5-nitrothiazole ring substituting the benzimidazole ring. However, the mode of action of the two drugs appears to be different. Benzimidazole derivatives bind to beta-tubulin, and prevent the uptake of glucose by disrupting cellular microtubular structures. This leads to a reduction in glucose uptake, depletion of glycogen storage, degenerative alterations in the germinal layer and cellular autolysis.¹⁹ In bacteria and in the protozoan parasite *Cryptosporidium*, nitazoxanide has been postulated to be reduced to a toxic radical when the 5-nitro-group on the nitroheterocyclic ring reacts with pyruvate: ferredoxin oxidoreductase.²⁰ Nothing is known about a possible mode of action of nitazoxanide in helminths. However, the enzymes of anaerobic electron transport also could be considered as potential targets.

E. granulosus protoscoleces had been shown earlier to be susceptible to treatment with the albendazole metabolite albendazole sulphoxide.²¹ Another drug, which is widely used for inactivation of protoscoleces, is praziquantel. Praziquantel is a pyrazinoisoquinoline derivative with a wide antihelminthic spectrum including schistosomes, hermaphroditic flukes and cestodes. Whereas it is very effective against adult tapeworms, as well as against *E. granulosus* protoscoleces *in vitro*, *in vivo* studies on *E. granulosus* metacestode infections have been inconclusive.^{22,23} However, more recently it was suggested that praziquantel exerts a substantial effect against metacestodes and protoscoleces when applied in combination with albendazole.^{24,25}

In addition to its efficacy against protoscoleces, nitazoxanide also exhibits a considerable damaging effect on the metacestode stage. The high parasitocidal efficacy of nitazoxanide is not surprising, as this compound was shown to exhibit a uniquely wide spectrum of antiparasitic and antimicrobial activities,¹⁴ including *in vitro* and *in vivo* activity against *E. multilocularis* metacestodes.^{15,16} *In vivo* pharmacokinetic studies in humans and mice have shown that nitazoxanide is substantially absorbed following oral administration, and that it is rapidly hydrolysed to tizoxanide, which is extensively bound to plasma albumin, and later to tizoxanide glucuronate.^{16,26,27}

Previous studies on *E. multilocularis* metacestodes treated with nitazoxanide and albendazole, respectively, had been able to differentiate between the effects of the two drugs during *in vitro* and *in vivo* treatment.^{15,16} For instance, the progressive destruction of *E. multilocularis* metacestode tissue through albendazole treatment was characterized primarily by shortening and distortion of microtriches, followed by other alterations (vacuolization of the germinal layer, aberrant mitochondria, lipid droplets, inclusion bodies and loss of cell-cell contacts within a time frame of 6–10 days).²⁸ In contrast, nitazoxanide treatment

leads first to a marked release of small vesicles from the tegument into the laminated layer, and the formation of large cytoplasmic vacuoles in undifferentiated cells, accompanied by progressive changes within the germinal layer.¹⁵ This indicated that in *E. multilocularis* metacestodes the two drugs exhibited two different modes of action. In contrast, in this study on *E. granulosus* metacestodes, we were not able readily to distinguish between the alterations imposed upon the parasites through albendazole and nitazoxanide treatment, respectively. However, a more detailed analysis, involving shorter time frames, will be necessary to provide respective information.

In conclusion, we here demonstrate the *in vitro* efficacy and parasitocidal activity of nitazoxanide against *E. granulosus* protoscoleces and metacestodes. In a next step, we will investigate the efficacy of nitazoxanide and its derivatives in an animal model. There, the active compound has to reach the infected organ and the parasite tissue in a sufficiently high concentration.

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