In Vitro Parasiticidal Effect of Nitazoxanide against *Echinococcus multilocularis* Metacestodes

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When humans serve as inadvertent intermediate hosts for *Echinococcus multilocularis*, disease (alveolar echinococcosis [AE]) may result from the expanding parasite metacestode in visceral organs, mostly in the liver. Benzimidazole carbamate derivatives such as mebendazole and albendazole are used for chemotherapeutic treatment of AE. However, these treatments are, in most cases, parasitistatic rather than parasiticidal. As treatment is discontinued, a recurrence of parasite growth has been observed in many AE patients with nonradical resections. The only curative treatment for AE is radical surgical resection of the parasite tissue and support by chemotherapy. As there is a need for new treatment options for AE, the in vitro efficacy of nitazoxanide (NTZ), a broad-spectrum drug used against intestinal parasites and bacteria, was investigated. We showed that in vitro treatment of *E. multilocularis* metacestodes with NTZ induced high levels of alkaline phosphatase activity in the medium. Concurrently, distinct morphological and ultrastructural alterations were detected. Most significantly, two distinct types of alterations were observed as soon as after 3 h of NTZ treatment. At first, the drug induced a peripheral output of membranous vesicles from the tegumental membrane into the laminated layer. Simultaneously, germinal layer-associated undifferentiated cells produced large vacuoles filled with lipid-like and often electron-dense membranous segments. Other alterations were observed at later time points, including vacuolization of the germinal layer, accumulation of lipid droplets, and lastly, loss of microtriches and separation of the laminated and germinal layers. The pattern of damage induced by NTZ was different from the alterations earlier observed in albendazole sulfoxide-treated vesicles. The nonviability of NTZ-treated metacestodes was confirmed through bioassay, i.e., inoculation of treated and untreated parasites into mice. These experiments demonstrate the in vitro parasiticidal effect of NTZ on *E. multilocularis* metacestodes.

Alveolar echinococcosis (AE) is caused by the metacestode (larval) stage of *Echinococcus multilocularis* and is a rare but life-threatening disease. The reported incidence rate per year is 0.02 to 1.4/100,000 population (7, 8). The geographical distribution of *E. multilocularis* is confined to Palearctic regions of the northern hemisphere extending from central Europe throughout northern and central Eurasia to the Far East, including Japan, and to North America (Alaska, Canada, and the northern and central United States). In central Europe, recent surveys on *E. multilocularis* infection in red foxes have revealed that the parasite has a much wider geographical distribution than previously reported. The prevalences of *E. multilocularis* in red foxes differ extensively within and between areas of endemicity, from about 1% to over 60% (5, 8).

The adult worm exists as an enteric parasite in the fox and a few other carnivores, such as the wolf, cat, and dog. Parasite eggs are shed into the environment with the feces of tapeworm-infected canids. The eggs contain an oncosphere, which upon ingestion by a suitable intermediate host and subsequent passage through the stomach and intestine get activated, penetrate the mucosa, enter the blood and lymphatic vessels, and

end up in the liver. Oncospheres then encyst in the liver parenchyma and develop over time to form mature metacestodes, which exhibit typical features such as tumor-like proliferation and occasionally metastatic formation into other organs (20).

Mice and other small mammals act as natural intermediate hosts for *E. multilocularis*, while humans accidentally acquire AE, also by ingesting viable parasite eggs. Detection of AE lesions at an early stage may allow its radical resection like that performed in tumor surgery. Depending on the postsurgical status, chemotherapy must be provided for at least 2 years and may be up to lifelong. The antiparasitic drugs presently used include mebendazole and albendazole. Both may effectively stop the growth of the parasite, but they do not appear to be parasiticidal in vivo. This is the reason why many patients must inevitably take the drugs on a lifelong basis to prevent recurrences of AE (8).

As a consequence, the development of new means of treatment of AE is anticipated. In vitro culture of *E. multilocularis* metacestodes allows growth and maintenance of infective parasites under standardized conditions (12, 13), and detection of increased alkaline phosphatase (EmAP) activity in the culture supernatant was shown to correlate with a progressive destructive effect of drug treatment (28). Thus, this model was used to assess the parasiticidal activities of several drugs, including nitazoxanide (NTZ).

NTZ [2-acetolyloxy-*N*-(5-nitro 2-thiazolyl) benzamide] was

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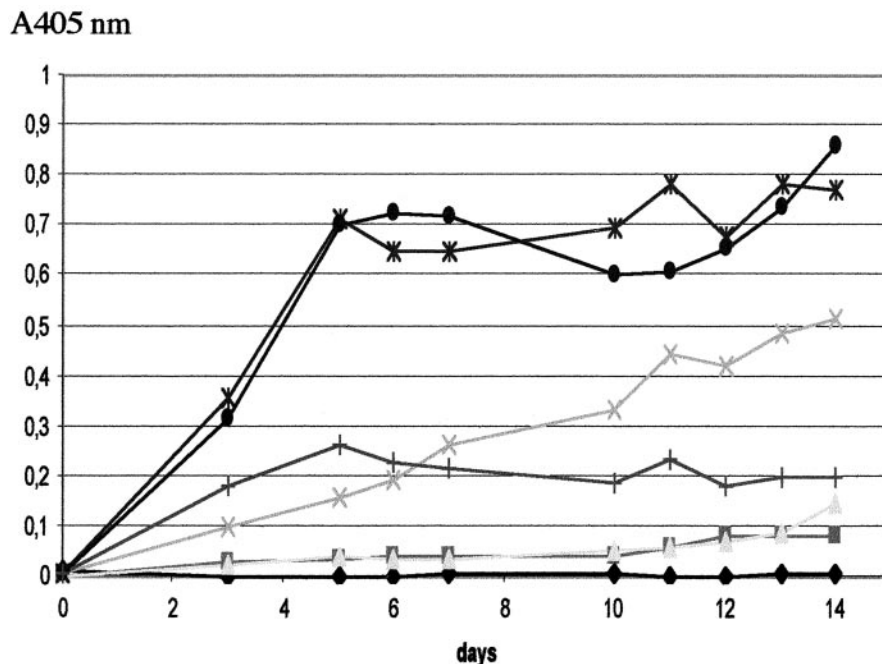
A405 nm

FIG. 1. Time course experiment demonstrating elevated EmAP activity in culture supernatants of *E. multilocularis* metacestodes treated with 1 (+), 5 (●), and 10 (*) μg of NTZ/ml or 10 μg of ABZSO/ml (×). Controls include RPMI 1640 medium alone without parasites (designated medium) (♦), RPMI 1640 medium plus metacestodes (control) (■), or RPMI 1640 medium plus metacestodes plus the corresponding amount (0.1%) of DMSO (▲). Note the increase in EmAP levels starting at day 4 in cultures treated with 5 and 10 μg of NTZ/ml.

originally developed as a veterinary antihelminthic and was first described in 1984 as a human cestocidal drug (26). To date, NTZ is known as a drug with a broad spectrum of activity against a wide variety of intestinal parasites and enteric bacteria infecting animals and humans. NTZ has become widely used specifically for the treatment of chronic diarrhea in immunocompromised patients (e.g., patients with AIDS) (1, 2, 9, 10, 21–24). In addition, clinical studies suggest that NTZ is effective for treatment of human fascioliasis (18, 25).

In this study we report on the in vitro efficacy of NTZ against *E. multilocularis* metacestodes and demonstrate the powerful parasiticidal effect of this drug using the EmAP assay, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and reinoculation of metacestodes treated in vitro into mice.

MATERIALS AND METHODS

Biochemicals and drugs. If not otherwise stated, all tissue culture media were purchased from Gibco-BRL (Zurich, Switzerland), and biochemical reagents were from Sigma (St. Louis, Mo.). The NTZ used in this study was obtained from Pharmacia Animal Health, Kalamazoo, Mich. Tizoxanide (TIZ) and TIZ glucuronide (TIZ gluc) were obtained from Romark Laboratories, Tampa, Fla. Albendazole sulfoxide (ABZSO) was kindly provided by R. J. Horton, SmithKline Beecham, London, United Kingdom.

In vitro culture of *E. multilocularis* metacestodes. In vitro cultivation of *E. multilocularis* metacestodes was carried out as described previously (15–17). Briefly, gerbils (*Meriones unguiculatus*) were infected intraperitoneally with *E. multilocularis* clone KF5 and isolate IM280. After 1 to 2 months, the animals were euthanized and the parasite tissue was recovered from the peritoneal cavity under aseptic conditions. The tissue pieces were cut into small tissue blocks (volume, 0.5 cm³), which were washed twice in Hanks balanced salt solution. Two pieces of tissue were placed in 75 ml of culture medium (RPMI 1640 containing 12 mM HEPES, 2 mM glutamine, 200 U of penicillin/ml, 200 μg of streptomycin/ml, 0.50 μg of amphotericin B/ml) supplemented with 10% fetal calf serum and

phenol red. Tissue blocks were kept in tightly closed culture flasks (200 ml) placed in an upright position in an incubator at 37°C with 5% CO_2, with medium changes every 2 to 4 days.

Drug treatments and recovery of medium supernatants. Free-floating vesicles with diameters of 1 to 5 mm were harvested after 3 to 4 weeks of culture. The time of vesicle collection was selected in order to obtain actively growing and proliferating parasites. The metacestodes were pooled, washed three times in sterile water, and again divided into separate cultures with approximately 50 vesicles in 15 ml of culture medium lacking fetal calf serum and phenol red. NTZ, TIZ, TIZ gluc, and ABZSO were prepared as stock solutions of 10 mg/ml in dimethyl sulfoxide (DMSO). These reagents were added to the cultures, yielding final concentrations of 1, 5, and 10 μg/ml. For each experiment, the appropriate controls included (i) a culture containing an equal amount of DMSO and (ii) a culture in culture medium alone. The parasites were incubated at 37°C with 5% CO_2. After defined time points, as indicated in Fig. 1, 300 μl of the culture supernatant was collected and centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was recovered and stored at −20°C prior to measurement of EmAP activity.

Determination of EmAP activity. The procedure described by Stettler et al. (28) was used for the quantitative assessment of EmAP activity. Briefly, 30 μl from each culture supernatant was mixed with 170 μl of alkaline phosphatase enzyme-linked immunosorbent assay (ELISA) substrate buffer (0.5 M ethanolamine, 0.5 mM $MgCl_2$ [pH 9.8]) containing *p*-nitrophenyl phosphate (1 mg/ml). A total of 200 μl of each sample was pipetted into the wells of a 96-well ELISA plate, and the plate contents were incubated for 30 min at 37°C. A_{405} values were read on a Dynatech MR7000 ELISA reader.

SEM and TEM. Metacestodes cultured in vitro were processed as described by Hemphill and Croft (14). Briefly, freshly isolated vesicles were gently broken up by using a pipette and were fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer for 2 h at room temperature, followed by postfixation in 2% OsO_4 in phosphate buffer. The samples were extensively washed in distilled water and dehydrated in ethanol. For SEM analysis, the specimens were dried by sublimation in Peldri II (Plano GmbH, Marburg, Germany), placed onto glass coverslips, sputter coated with gold, and inspected on a JEOL 840 scanning electron microscope operating at 25 kV. For TEM, the vesicles were fixed as described above. The samples were then extensively washed in distilled water and incubated 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 concentrations and subse-

quently embedded in Epon 812 (14). Polymerization of the resin was carried out at 65°C overnight. Sections were cut on a Reichert and Jung ultramicrotome and were loaded onto 300-mesh copper grids (Plano GmbH). Staining with uranyl acetate and lead citrate was performed as described previously (14).

Assessment of metacestode viability by bioassay in C57BL/6 mice. In order to investigate drug-treated metacestodes for viability or nonviability, female C57BL/6 mice (seven animals per group; age, 8 weeks; body weight, 20 ± 5 g) were infected by intraperitoneal injection with three to five drug-treated vesicles. Before inoculation the vesicles had been treated for 14 days in vitro with either NTZ (group 1) or ABZSO (group 2) dissolved in DMSO or with DMSO alone (group 3) as a control. The drug concentration in the medium was 10 μg/ml for both NTZ and ABZSO. Animals were housed in a temperature-controlled light-cycle room. At 5 months postinoculation the mice were euthanized with CO_2 and the parasite tissue was inspected by light microscopy and TEM (see above).

RESULTS

Increase in EmAP activity in culture supernatants of drug-treated *E. multilocularis* metacestodes. We investigated the EmAP activity in culture supernatants at different time points following the addition of NTZ, TIZ, TIZ gluc, or ABZSO into the culture medium. Time course experiments (as shown in Fig. 1) demonstrated that the EmAP activity in the culture supernatants of NTZ-treated parasites was immediately and dramatically enhanced compared to that in the corresponding supernatants of control cultures. The results obtained for TIZ and TIZ gluc were essentially identical (data not shown). After 12 to 14 days of in vitro culture, distinct increases in EmAP activity were also observed in the supernatants of ABZSO-treated cultures and in the medium supernatants of control vesicles, although to a much lower extent. These experiments were repeated six times, and all provided essentially identical results.

Morphological and ultrastructural alterations induced by drug treatment investigated by SEM and TEM. In order to correlate this dramatic increase in EmAP activity in the culture supernatants of NTZ-treated parasites with alterations of parasite morphology, both control and drug-treated parasites were examined by SEM. Nontreated control metacestodes exhibited an intact germinal layer, with a multitude of different, morphologically intact cell types (Fig. 2A and B). In contrast, after 4 days of in vitro NTZ treatment, the germinal layer of the parasites had lost its characteristic multicellular structure and was largely disintegrated in many areas, and only a fraction of the metacestode tissue appeared to be still attached to the interior surface of the acellular laminated layer (Fig. 2C). At day 7 of treatment with NTZ, in many areas, only cellular debris of the former germinal layer could be seen (Fig. 2D).

The ultrastructure of vesicle walls of metacestodes cultivated in vitro has been described previously (12, 13). The external surface of the parasite larvae is comprised of an acellular, heavily glycosylated, laminated layer which surrounds the entire parasite (20). Attached to the interior surface of the laminated layer is the tegument, a syncytial parasite tissue with numerous microtriches that protrude into the laminated layer and that thus significantly enhance the resorbing surface of the parasite. More toward the interior, the tegument is replaced by the germinal layer, which contains a number of different cell types such as muscle cells, connective tissue, and glycogen storage cells. In actively proliferating vesicles a large number of undifferentiated cells each with a large nucleus and nucleolus is found. Control and DMSO-treated cultures did not exhibit any ultrastructural alterations of parasite tissue during the entire incubation period of 14 days (Fig. 3A and B).

The first signs of tissue alterations due to NTZ treatment were already seen after 3 h of treatment (Fig. 3C and D). On the one hand, these alterations concerned the undifferentiated cells, the cytoplasms of which contained large vacuoles filled with electron-dense bodies, granular particles, membrane-like leaflets, and small vesicles (Fig. 3C). These vacuoles occupied a large portion of the cytoplasms of these cells. On the other hand, alterations also concerned the distal portion of the tegument, where a distinct increased concentration of small vesicles, apparently budding off from the tegumental membrane, was observed (Fig. 3D). This process of membrane budding was a lot more apparent in metacestodes fixed and processed after 1 day of NTZ treatment (Fig. 3E). At this time point vesicles and larger membrane leaflets were released from both the tegumental base and the tips of the microtriches and were deposited into the matrix of the laminated layer.

At day 4 of NTZ treatment, significant reductions in the relative numbers and lengths of the microtriches became evident (Fig. 4A). However, in areas where microtriches were still present, the matrix of the laminated layer was completely infiltrated with small vesicles a few nanometers in diameter (Fig. 4B). Alterations which then became more apparent in the tissue of the germinal layer included increased vacuolization, caused largely by the loss of cell-cell contacts, and the formation of large lipid droplets. After 6 days, NTZ treatment resulted in the formation of aberrant, rounded mitochondria which had abnormally increased in size, and the cellular disintegration process had advanced, rendering the tissue largely necrotic. Microtriches disappeared, and in many areas separation of the laminated layer and the tegument had taken place. Thus, NTZ treatment resulted in complete necrosis of the germinal layer-associated tissue within 6 days of treatment (Fig. 4C and D). No further changes became evident after this time point until day 14, when the experiment was terminated. Treatments of vesicles with TIZ and TIZ gluc yielded essentially identical results (data not shown).

Viability assays with C57BL/6 mice. Vesicles were treated in vitro with either NTZ and ABZSO or were incubated in DMSO-containing medium (controls) for 14 days, and mice were infected with this material by intraperitoneal injection. Infected mice were killed after 5 months and investigated for the occurrence of viable metacestodes, and parasites were examined by light and electron microscopy (Fig. 5). The criteria for cyst viability assessment of implanted drug-treated metacestodes after recovery included features such as cyst turgidity, as well as the presence of a germinal layer which was visible by histological examination.

Intact parasite vesicles were recovered from the peritoneal cavities from six of seven mice that had received DMSO-treated control metacestodes. All of the vesicles were up to 3 mm in diameter. One of these metacestodes was partially encapsulated in connective tissue, but it was later shown by histological and ultrastructural examination to contain an intact, apparently viable germinal layer, with microtriches protruding well into the laminated layer (Fig. 5A and B). In contrast, in all seven mice that had received NTZ-treated metacestodes, only small (diameters, 1 to 2 mm) compact nodules were found. Histology and TEM failed to detect any of the classical fea-
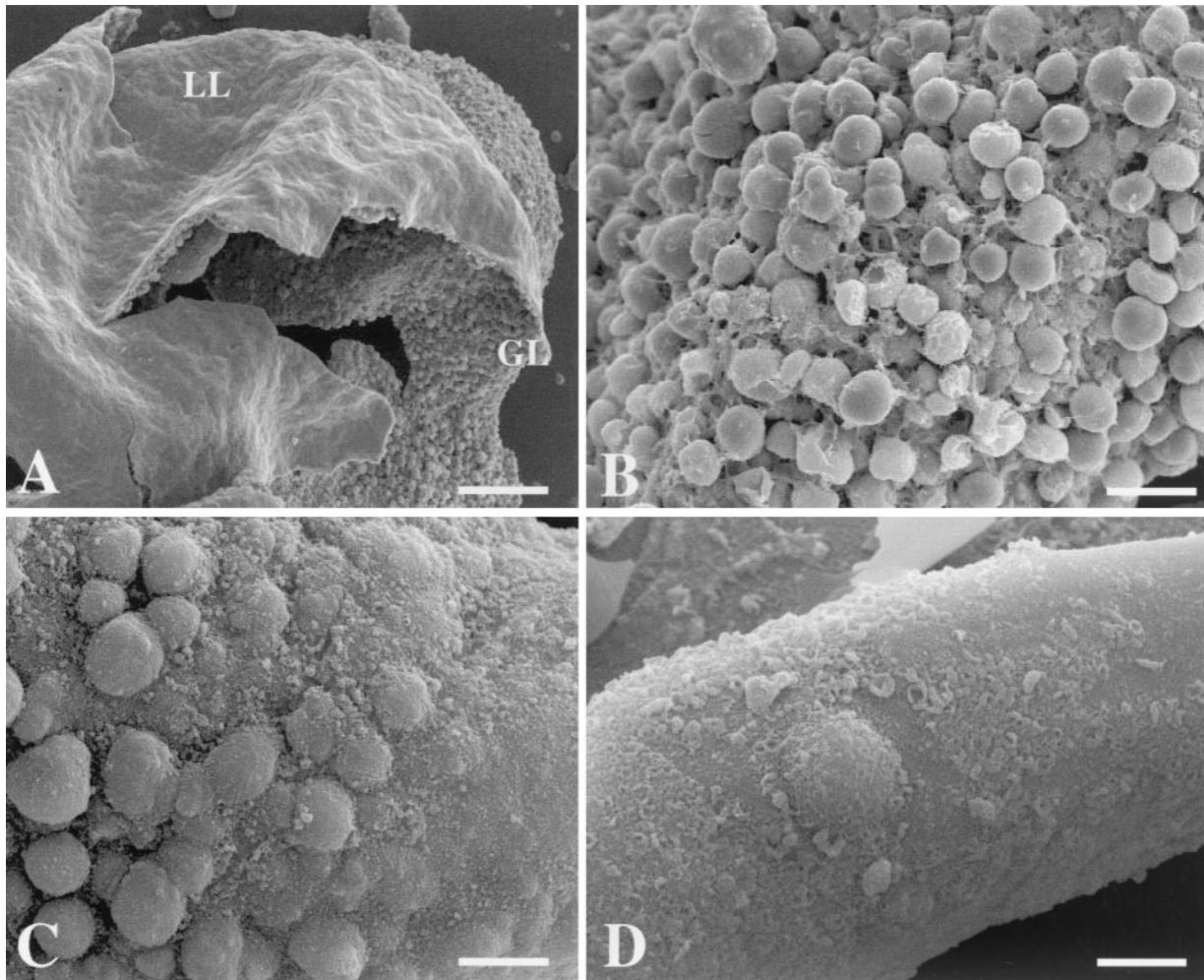
FIG. 2. SEM of nontreated (A and B) or NTZ-treated (C and D) *E. multilocularis* metacestodes. (A and B) Control metacestodes cultured in vitro in the presence of DMSO (1:1,000) but in the absence of any drugs. Note that most cells exhibit an intact morphology. LL, laminated layer; GL, germinal layer. (C and D) Metacestodes cultured in vitro in the presence of 10 µg of NTZ/ml for 4 days (C) and 7 days (D). Substantial portions of the germinal layer already show massive signs of cellular destruction after 4 days of drug treatment but more clearly show massive signs of cellular destruction after 7 days of drug treatment and are detached from the laminated layer. Bars, 800 µm (A), 280 µm (B), 240 µm (D), and 320 µm (E). Similar results were obtained for parasites treated with 5 µg of NTZ/ml or TIZ and TIZ gluc (data not shown).

tures of viable *E. multilocularis* tissue, with the exception of the acellular laminated layer, which was still present and completely encapsulated by host tissue (Fig. 5C and D). Six of seven mice infected with ABZSO-treated metacestodes contained both viable and nonviable encapsulated parasite tissue, sometimes within the same animal: in two mice, only viable metacestodes had been produced, in two mice only small nodules containing an encapsulated laminated layer were recovered, and in another two animals, both viable and nonviable parasites were found (data not shown).

DISCUSSION

The in vitro model for the culture of *E. multilocularis* metacestodes, in association with measurement of EmAP activity in culture supernatants (28), was used to perform first-round assays of drug activity. The goal of this project was to identify novel candidate drugs for the development of a future treat-

ment of AE which would prospectively help patients suffering from this disease to undergo improved chemotherapy (15). In this study we investigated NTZ and its human metabolites, TIZ and TIZ gluc; and our results documented the parasiticidal effects of NTZ and its metabolic products during in vitro drug treatment, as assessed by measurements of EmAP activity, histology and electron microscopy, and viability tests with mice.

NTZ, a 5-nitrothiazole analogue, was shown earlier to exhibit structural similarities to the benzimidazole antihelminthics such as albendazole and its metabolic derivatives ABZSO and albendazole sulfone, with a 5-nitrothiazole ring substituting for the benzimidazole ring. However, while the benzimidazole derivatives apparently bind to beta-tubulin and prevent the uptake of glucose by disrupting cellular microtubular structures (3, 11), the mode of action of NTZ is postulated to be different. In anaerobic bacteria as well as in the intestinal protozoan parasite *Cryptosporidium*, NTZ is reduced to a toxic
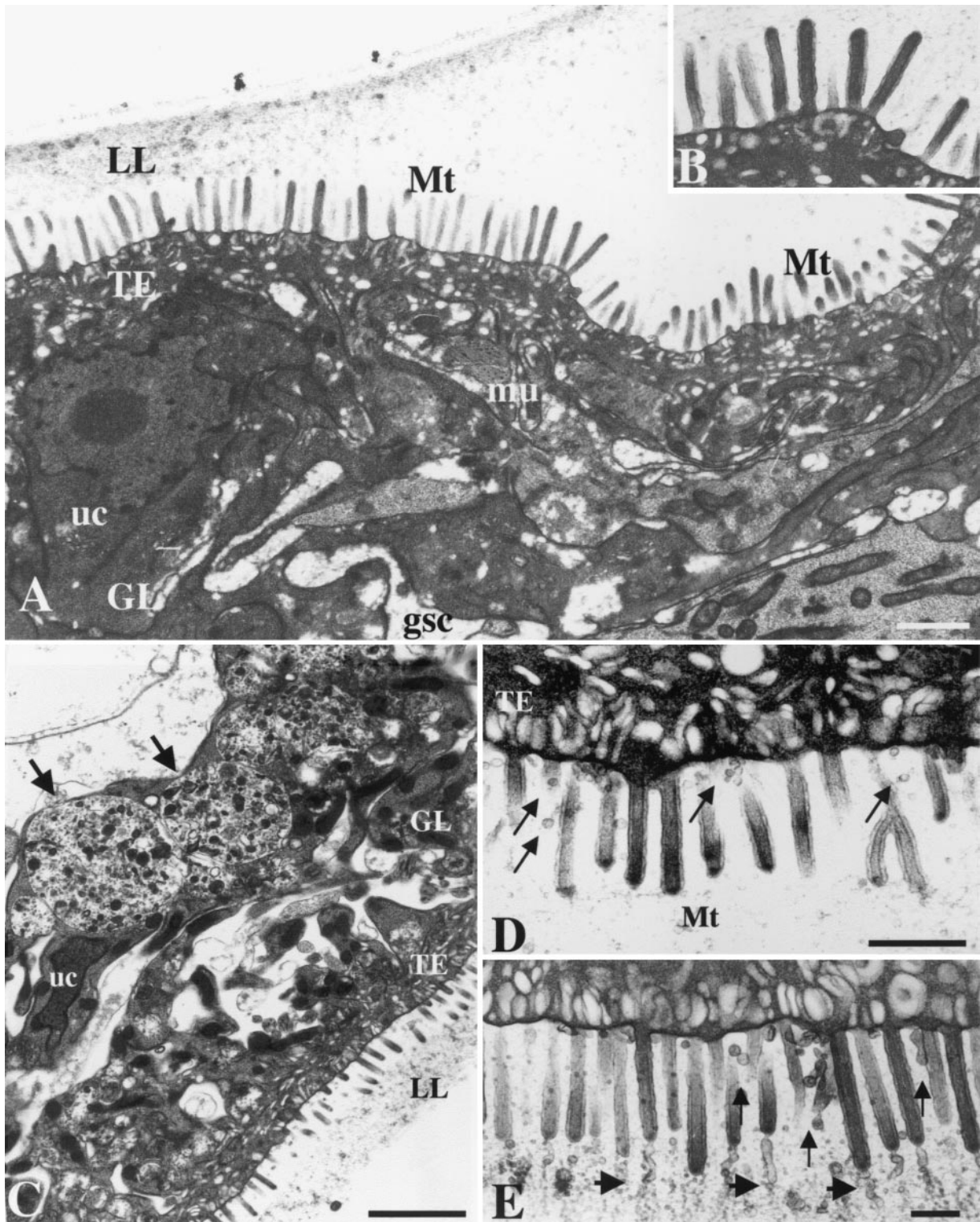
FIG. 3. TEM of *E. multilocularis* metacestode tissue after 14 days of in vitro culture in the presence of DMSO (A and B) and in the presence of NTZ for 3 h (C and D) and 24 h (E). (A) Note the distinct features of the metacestode tissue such as the acellular laminated layer (LL), the tegument (TE), and the germinal layer (GL) which comprises a whole range of different cell types such as muscle cells (mu), glycogen storage cells (gsc), connective tissue, and undifferentiated cells (uc) with a large nucleus and nucleolus. (B) A higher magnification of intact microtriches (Mt) which protrude well into the laminated layer. After 3 h of NTZ exposure (C), large vacuolar bodies are found in undifferentiated cells (arrows), as well as infiltration of the laminated layer with small vesicles budding from the tegumental membrane, marked with arrows (D). (E) Increased infiltration of the laminated layer after 24 h of NTZ treatment with vesiculated bodies originating from the tegumental membrane (vertical arrows) and with membrane leaflets budding off from the microtriches (horizontal arrows). Bars, 2.5 μm (A), 0.8 μm (B), 3.4 μm (C), 1.1 μm (D), and 0.5 μm (E).
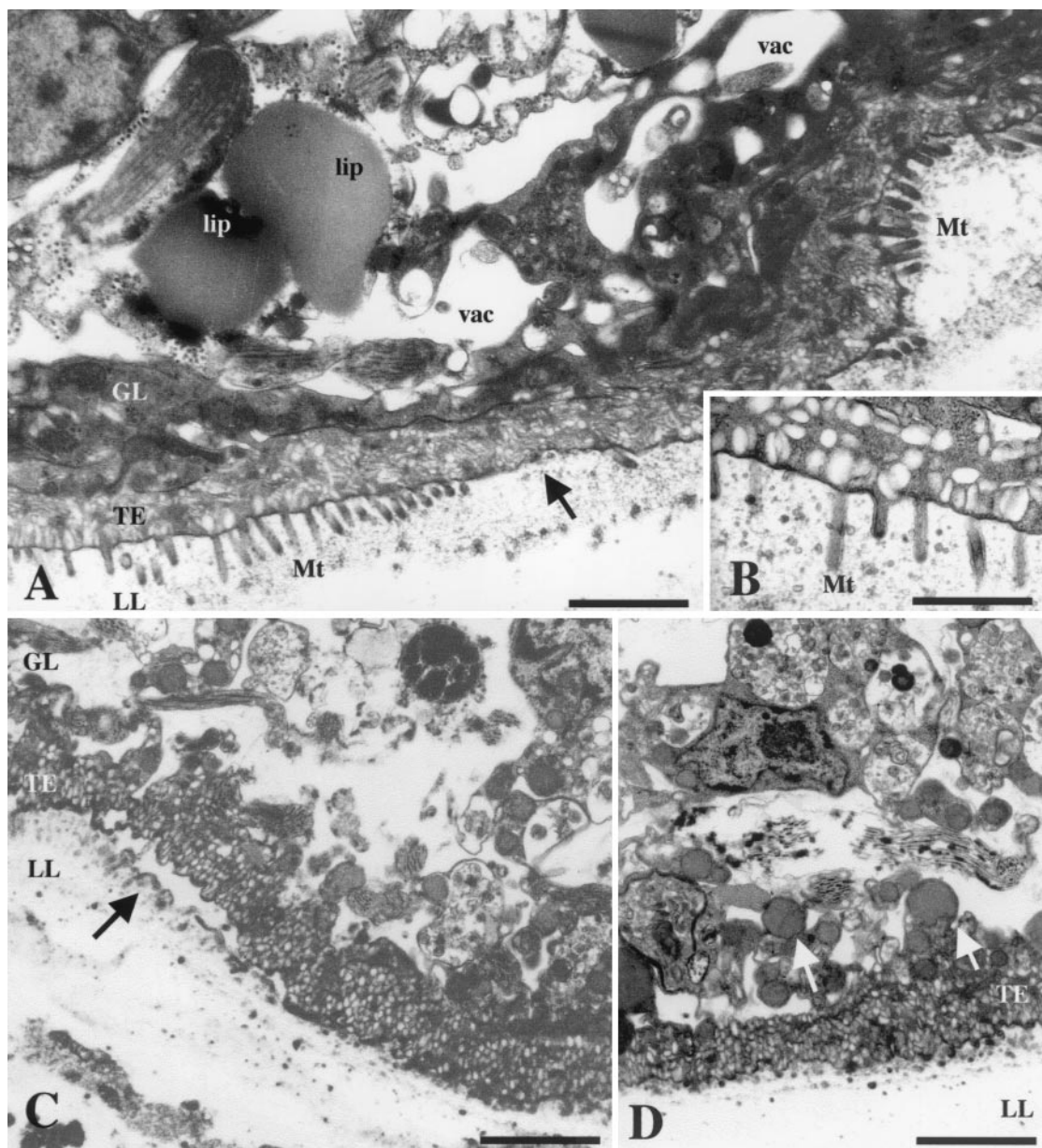
FIG. 4. TEM of *E. multilocularis* metacestode tissue fixed at 4 to 6 days of NTZ treatment. (A) Four days of NTZ exposure results in partial loss of microtriches (arrow), increased vacuolization (vac), loss of cell-cell contacts, and accumulation of lipid droplets (lip) within the germinal layer. (B) High density of small vesicular bodies within the laminated layer of NZT-treated metacestodes after 4 days of NTZ treatment. (C) Necrotic parasite tissue after 6 days. Note the large amount of cellular debris, the complete absence of microtriches, and the separation of the laminated layer and the tegument (arrow). (D) The white arrows point to rounded mitochondria. Bars, 3 μm (A), 1.7 μm (B), 4.1 μm (C), and 3.9 μm (D). GL, germinal layer; TE, tegument; LL, laminated layer; Mt, microtriches.

radical when the 5-nitro group on the nitroheterocyclic ring reacts with the pyruvate ferredoxin oxidoreductase enzyme (4, 10, 27). Nothing is known about a possible mode of action of NTZ against helminths; however, the enzymes of anaerobic electron transport could also be considered potential targets.

In our experiments, measurements of EmAP activity in culture supernatants indicated distinct differences between NTZ and ABZSO. Treatment of metacestodes with NTZ at 5 and 10 μg/ml yielded a relatively fast increase in EmAP activity, with maximum levels being achieved as soon as after 5 days of

treatment and remaining relatively constant for another 9 days, while the drug appeared to be largely ineffective at 1 μg/ml. In comparison, ABZSO treatment (at 10 μg/ml) led to a slower but continuous and steady increase in EmAP activity within a period of 14 days.

A comparison of the progressive destruction of parasite tissue after NTZ treatment (this study) with the alterations imposed on the parasite after ABZSO treatment, as investigated by Ingold et al. (17), shows that distinct differences are obvious with regard to the morphology and ultrastructural character-
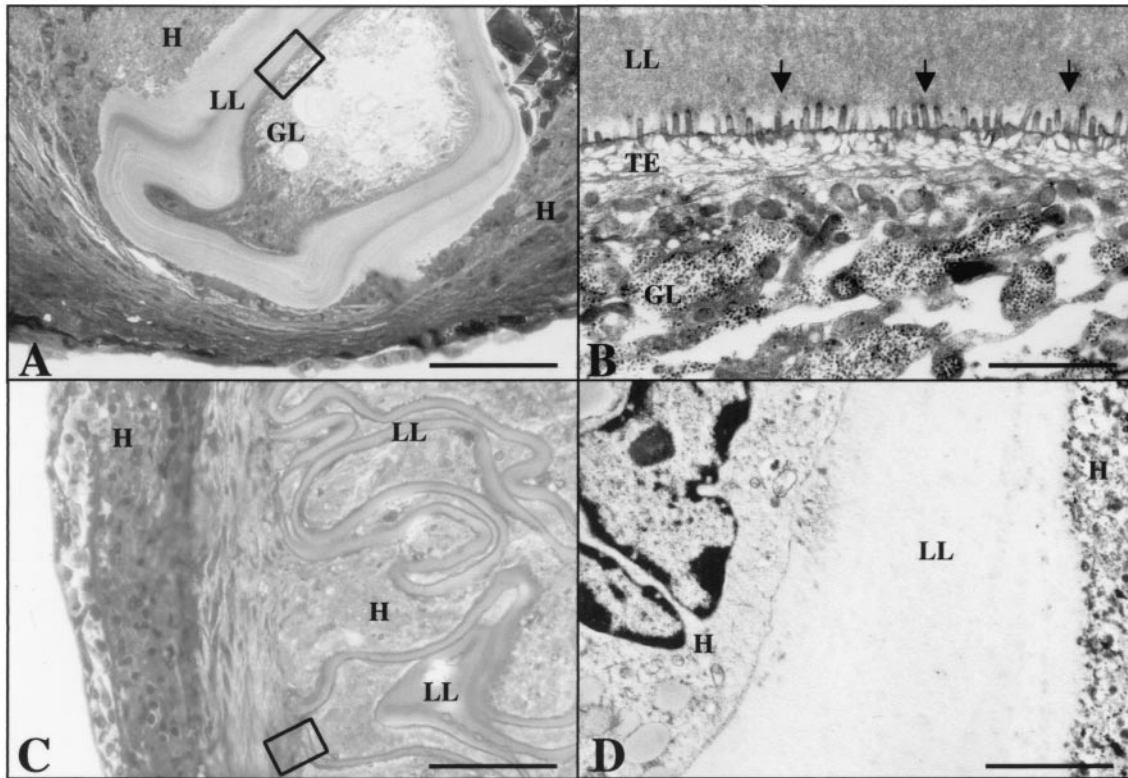
FIG. 5. Light microscopy (A and C) and corresponding TEM (B and D) of tissue recovered from mice which had been infected with DMSO-treated metacestodes (A and B) or NTZ-treated metacestodes (C and D). Note the presence of viable parasite in panel A, and a region similar to the one framed in panel A is shown by TEM in panel B. (C) The laminated layer is completely encapsulated by host tissue, and a region similar to the one framed in panel C is shown by TEM in panel D. Note that no germinal layer is visible in tissue recovered from mice infected with NTZ-treated metacestodes. Bars, 120 μm (A), 4.2 μm (B), 60 μm (C), and 1.2 μm (D). GL, germinal layer; TE, tegument; LL, laminated layer; H, host tissue.

istics of drug-induced alterations, and this also points toward completely different modes of action of albendazole and NTZ. ABZSO and albendazole sulfone treatment of *E. multilocularis* metacestodes was shown to lead, as soon as after 6 h of treatment, to a shortening and distortion of the microtriches, followed by other alterations including vacuolization of the germinal layer, aberrant mitochondria, lipid droplets, inclusion bodies, and loss of cell-cell contacts within 6 to 10 days (17). In contrast, NTZ treatment led 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, both of which started as soon as 3 h of NTZ treatment. The infiltration of the laminated layer with small vesicles persisted and was still evident at day 4 of NTZ treatment and was accompanied by progressive changes within the germinal layer.

Differences between NTZ- and ABZSO-treated metacestodes were also noted following inoculation of drug-treated vesicles into mice. While no viable parasite could be recovered from mice injected with NTZ-treated vesicles, a fraction of the ABZSO-treated vesicles still appeared to be viable, since they could be recovered from mice as intact metacestodes. Thus, in vitro, NTZ exhibits a higher parasiticidal activity against *E. multilocularis* metacestodes.

The high parasiticidal efficacy of NTZ is not surprising, as this compound was shown to exhibit an uniquely wide spectrum of antiparasitic and antimicrobial activities (1, 6, 10, 11, 18, 19, 21–27, 30). In vivo pharmacokinetic studies have shown that NTZ is substantially absorbed following oral administration and that it is rapidly hydrolyzed to TIZ, which is extensively bound to plasma albumin (2, 29). The final aim of our studies will be to investigate the efficacy of NTZ and its derivatives against metacestodes of *E. multilocularis* developing in the human liver or other visceral organs. In conclusion, we have demonstrated here the in vitro efficacy and parasiticidal activity of NTZ against *E. multilocularis* metacestodes. In a next step, we will investigate the efficacies of NTZ and its derivatives in an animal system in which the active chemical compound must reach the infected organ and the parasite tissue in a sufficiently high concentration.

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