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In Vitro Efficacies of Nitazoxanide and Other Thiazolides against *Neospora caninum* Tachyzoites Reveal Antiparasitic Activity Independent of the Nitro Group

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The thiazolide nitazoxanide [2-acetolyloxy-*N*-(5-nitro-2-thiazolyl)benzamide] (NTZ) exhibits a broad spectrum of activities against a wide variety of intestinal and tissue-dwelling helminths, protozoa, and enteric bacteria infecting animals and humans. The drug has been postulated to act via reduction of its nitro group by nitroreductases, including pyruvate ferredoxin oxidoreductase. In this study, we investigated the efficacies of nitazoxanide and a number of other thiazolides against *Neospora caninum* tachyzoites in vitro. We employed real-time-PCR-based monitoring of tachyzoite adhesion, invasion, and intracellular proliferation, as well as electron microscopic visualization of the effects imposed by nitazoxanide. In addition, we investigated several modified versions of this drug. These modifications included on one hand the replacement of the nitro group on the thiazole ring with a bromide, thus removing the most reactive group, and on the other hand the differential positioning of methyl groups on the salicylate ring. We show that the thiazole-associated nitro group is not necessarily required for the action of the drug and that methylation of the salicylate ring can result in complete abrogation of the antiparasitic activity, depending on the positioning of the methyl group. These findings indicate that other mechanisms besides the proposed mode of action involving the pyruvate ferredoxin oxidoreductase enzyme could be responsible for the wide spectrum of antiparasitic activity of NTZ and that modifications in the benzene ring could be important in these alternative mechanisms.

Neospora caninum is an apicomplexan parasite which represents one of the most important causative agents of infectious bovine abortion, stillbirth, and birth of weak calves. In addition, *N. caninum* causes neuromuscular disease in dogs, and infection with this parasite has been demonstrated in a wide range of other species worldwide (7, 14). The economic importance of neosporosis, especially in cattle, has led to considerable investments on the part of governmental and pharmaceutical funding bodies, with the aim to develop strategies for prevention and treatment of neosporosis. Epidemiological studies have shown that dogs and coyotes act as definitive hosts that shed oocysts (6, 9, 21, 22). Recently, Gondim et al. (10) have conclusive demonstrated transplacental transmission and abortion in cows administered *N. caninum* oocysts.

Hence, several investigations have been focusing on therapeutic intervention as a possible means of preventing neosporosis. A wide range of compounds, including lasalocid, monensin, pirithrexim, pyrimethamine, clindamycin, robenidine, and trimethoprim, have been shown earlier to exhibit parasiticidal activity against *N. caninum* tachyzoites in cell culture-based assays (18, 20). More recently, artemisin and depudecin have been reported to exhibit antiparasitic activity in vitro (16, 17), and Youn et al. (34, 35) demonstrated the in vitro efficacy of alcoholic herbal extracts against *N. caninum* tachyzoites. Darius et al. (5) used electron microscopy to describe the in vitro effects of toltrazuril, a symmetrical triazinone, and its metabolic derivative ponazuril against *N. caninum* tachyzoites in cell culture. A number of studies employed the murine model. Sulfadiazine and amprolium were investigated (19), and sulfadiazine administered at 1 mg/ml prevented disease in experimentally infected mice but did not eliminate the parasite. A number of studies with mice focused on toltrazuril (1, 11, 12), showing that inclusion of toltrazuril in the drinking water abrogated parasite detection in the central nervous system but that cell-mediated immunity was required to achieve its full efficacy in mice. In addition, toltrazuril treatment controlled diaplacental *N. caninum* transmission in experimentally infected pregnant mice. With regard to the natural hosts, dogs and cattle, attempts to treat neosporosis have remained in their initial stages, and no efficient treatment strategies have been elaborated so far.

In this study, we investigated the in vitro efficacy of nitazoxanide (NTZ) [2-acetolyloxy-*N*-(5-nitro-2-thiazolyl)benzamide] (29) against *N. caninum* tachyzoites. NTZ is known to exhibit a broad spectrum of activity against a wide variety of intestinal parasites and enteric bacteria infecting animals and humans (8, 33). The broad applicability of this drug also includes the treatment of human patients suffering from diarrhea caused by

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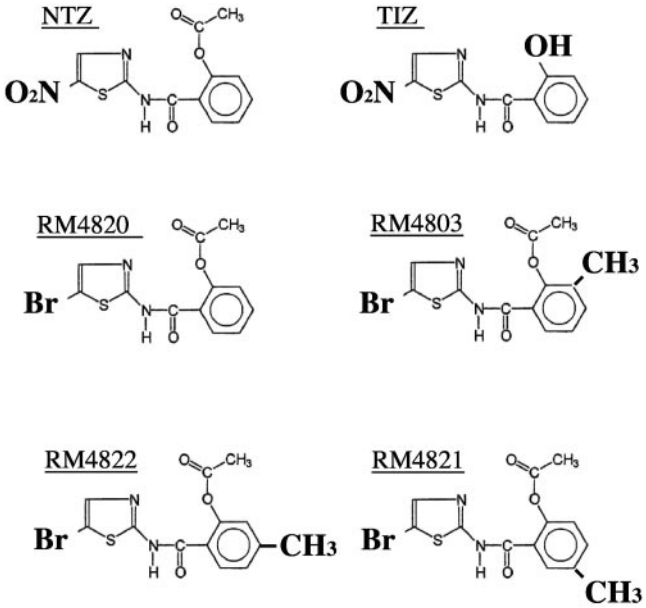

FIG. 1. Drugs investigated in this study. NTZ is composed of a nitrothiazole moiety and a salicylic acid moiety, Rm4820 is a de-nitro-NTZ with a bromide replacing the nitro group, Rm4803 has a methyl group at the *ortho* position on the salicylic acid ring, Rm4822 has a methyl group at position 4, and Rm4821 has a methyl group at position 5. TIZ, tizoxanide.

infection with the apicomplexan *Cryptosporidium parvum* as well as the treatment of equine myeloencephalitis caused by *Sarcocystis neurona*, for which this drug has gained Food and Drug Administration approval. NTZ has been postulated to exhibit a mode of action based upon reduction of its nitro group by nitroreductases, including pyruvate ferredoxin oxidoreductase (PFOR), but in contrast to metronidazole (MTZ), it has been shown to nonmutagenic (31). We employed real-time-PCR-based monitoring of tachyzoite adhesion, invasion, and intracellular proliferation, as well as electron microscopic visualization of the effects imposed by NTZ. In addition, we investigated the effects of several modified versions of this drug. Our findings indicate that other mechanisms, besides the proposed mode of action involving PFOR, could be responsible for the wide spectrum of NTZ, with the benzene ring being important in achieving antiparasitic activity.

MATERIALS AND METHODS

Tissue culture media, 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). NTZ, tizoxanide, tizoxanide glucuronide, and NTZ derivatives (Rm4803, Rm4820, Rm4821, and Rm4822) (Fig. 1) were synthesized at the Department of Chemistry at the University of Liverpool. They were kept as stock solutions at 10 mg/ml in dimethyl sulfoxide and were stored at −20°C.

Tissue culture and parasite purification. Cultures of Vero cells were maintained in RPMI 1640 medium (Gibco-BRL, Basel, Switzerland) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 50 U of penicillin/ml, and 50 μg of streptomycin/ml at 37°C with 5% CO_2 in tissue culture flasks. Cultures were trypsinized at least once a week. Human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) containing the same additives and were treated identically. *N. caninum* tachyzoites (Nc-1 and Nc-Liverpool isolates) were maintained in Vero cell monolayer cultures (13), during which time FCS was replaced with immunoglobulin G-free horse serum. Parasites were harvested when they were still intracellular by trypsinization of infected Vero cells, repeated passage through a 25-gauge needle at 4°C, and separation on Sephadex G25 columns as described previously (13).

Infection of HFF and in vitro drug treatment assays. In vitro drug assays were performed to assess the effects of drugs on *N. caninum* tachyzoite proliferation. HFF were grown to confluent monolayers in 24-well tissue culture plates (Sarstedt, Newton, MA). Purified *N. caninum* tachyzoites (5×10^4) were suspended in 1 ml of DMEM containing 5% fetal calf serum, 50 U of penicillin/ml, and 50 μg of streptomycin/ml and were added to the monolayers and left for 2 h at 37°C with 5% CO_2. Subsequently, unbound parasites were removed by washing in DMEM, and infected monolayers were maintained in DMEM-FCS-penicillin-streptomycin containing the drugs as indicated for the individual experiments. Controls contained the appropriate amounts of DMSO alone. In order to assess selective toxicity, uninfected fibroblast monolayers were treated identically. The cultures were maintained at 37°C with 5% CO_2 for various periods of time as indicated below, with medium changes every 2 days, and were inspected by light microscopy on a daily bases. In some experiments, NTZ was added prior to the infection of host cells. Samples for monitoring of parasite proliferation were taken at different time points following initiation of drug treatment. For this, the medium was removed, and the cellular material was taken up in 180 μl of lysis buffer, 20 μl proteinase K (DNAeasy kit; QIAGEN), and 200 μl phosphate-buffered saline. The specimens were transferred to Eppendorf tubes and were frozen at −20°C prior to DNA extraction. Each assay in a given experiment was carried out in quadruplicate, and the outcome of one representative experiment of at least three independent experiments, all producing virtually identical results, is shown.

PDTC-based adhesion/invasion assay. Adhesion/invasion assays were done in order to assess the effects of drug treatments on parasite host cell adhesion and invasion. Assays were carried out essentially as previously described (26). In short, HFF monolayers were grown in 96-well flat bottom tissue culture plates (Sarstedt). *N. caninum* tachyzoites (5×10^4) were resuspended in 100 μl of DMEM containing 5% horse serum and were incubated with NTZ (10 μg/ml) for 2 h, 6 h, or 24 h and added to the monolayers. They were allowed to invade for 30 min at 37°C with 5% CO2. Unbound parasites were removed by washing in DMEM, and infected monolayers were incubated with DMEM containing 100 μM pyrrolidine dithiocarbamate (PDTC), 0.2 μM $CuSO_4$, and a polyclonal rabbit hyperimmue serum raised against entire *N. caninum* tachyzoites (1:200) (13) for 2 h at 37°C. In parallel, control incubations in DMEM were performed. Subsequently, the wells were washed once with DMEM, and DMEM containing 1 mg/ml DNase I was added. The preparations were incubated for 1 h at 37°C. Control wells were also washed and incubated with DMEM alone. Finally, all wells were washed with medium containing 1 mM EDTA to inhibit DNase I activity, and the cellular material was taken up in 180 μl of lysis buffer (DNAeasy kit; QIAGEN). The specimens were transferred to Eppendorf tubes, heated for 5 min at 95°C, and stored at −20°C prior to further use. The assays were carried out in quadruplicate, and the experiment was repeated three times, producing essentially identical results. Results from one representative experiment are shown.

Processing of DNA samples and LightCycler-based quantitative PCR. DNA purification was performed using the DNAeasy kit (QIAGEN, Basel, Switzerland) according to the standard protocol suitable for tissue samples. DNA was eluted in 100 μl of AE buffer (elution buffer from the kit) and subsequently boiled for 5 min. For quantitative PCR, forward primer Np21plus and reverse primer Np6plus were used. These primers had been designed to amplify a 337-bp sequence of the Nc5 region of *N. caninum* (25). Detection of DNA amplification products and quantification of parasite numbers through fluorescence resonance energy transfer on the LightCycler instrument (Roche Diagnostics, Basel, Switzerland) were done as previously described (26), by assessing mean values (plus standard deviations) from triplicate determinations. As external standards, samples containing DNA equivalents from 100, 10, and 1 *N. caninum* tachyzoite(s) were included. Reproducibility of the test system was demonstrated by proving an overall low variation within three independent runs of the standard reactions, and PCR results were validated only when the differences in the secondary derivative maximum values within the triplicates did not extend for one cycle.

Statistical analysis. For time course experiments, the significance of the differences between end point values of the control and experimental assays was determined by Student's *t* test, using the Microsoft Excel program. *P* values of <0.05 were considered statistically significant. The same was true for PDTC-based adhesion/invasion assays.

Transmission electron microscopy. HFF monolayers were grown in six-well tissue culture plates, infected with *N. caninum* tachyzoites, and treated with NTZ (10 μg/ml) as described above. At different time points, monolayers were briefly

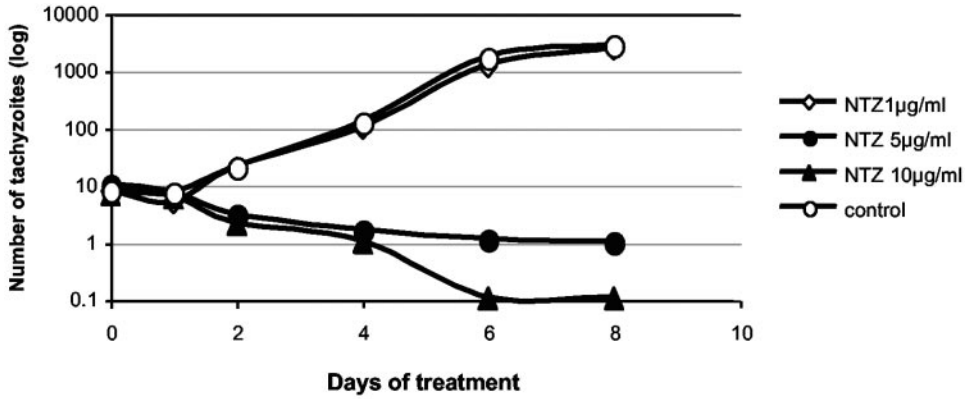

FIG. 2. NTZ inhibits *N. caninum* proliferation in HFF monolayers. HFF monolayers were infected with *N. caninum* tachyzoites, and at 2 h p.i., 1, 5, or 10 μg/ml NTZ was added. Samples were collected at 1, 2, 4, 6, and 8 days p.i and were processed for the quantitative assessment of *N. caninum* proliferation by real-time PCR. Note the severe inhibition of proliferation with NTZ at 5 and 10 μg/ml.

washed in 100 mM sodium cacodylate buffer, pH 7.2, and were fixed in 100 mM sodium cacodylate buffer containing 2.5% glutaraldehyde. Cells were scraped off using a rubber policeman and were centrifuged at 100 × *g* at 4°C for 10 min. The resulting pellet was further fixed for 2 h at room temperature, followed by postfixation in 1% OsO_4 for 4 h at 4°C. Subsequently, specimens were washed in water and were prestained in 1% uranyl acetate in water for 1 h at 4°C, followed by extensive washing in water. The specimens were then dehydrated in a graded series of ethanol solutions and were embedded in Epon 820 resin. The resin was polymerized at 65°C over a period of 48 h. Ultrathin 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 above. Finally, grids were viewed on a Phillips 300 transmission electron microscope operating at 60 kV.

RESULTS

NTZ inhibits the intracellular proliferation of *N. caninum* tachyzoites. In order to assess whether NTZ inhibits the proliferation of *N. caninum* tachyzoites, parasites were allowed to infect HFF monolayers for 1 h, and subsequently drug treatment (NTZ at 1, 5, and 10 μg/ml) was initiated. Parasite proliferation was monitored by real-time PCR at different time points until day 8 postinfection (p.i.) (Fig. 2). While 1 μg/ml did not exert any effect, 5 and 10 μg/ml were shown to be very effective in terms of inhibiting tachyzoite proliferation. Tizoxanide, the deacetylated metabolite (Fig. 1), exhibited properties identical to those of NTZ, while tizoxanide glucuronide was found to be ineffective (data not shown). In parallel, light microscopic inspection of infected and noninfected HFF monolayers did not reveal any drug-induced alterations, demonstrating the selective toxicity of NTZ (data not shown). Thus, further experiments were performed at a drug concentration of 10 μg/ml.

The antiparasitic activity of NTZ against *N. caninum* tachyzoites is based on different domains of the drug. Since the in vitro parasiticidal efficacy of NTZ on *N. caninum* tachyzoites was evident, a series of NTZ derivatives was investigated in order to elucidate which domains of this drug would act on *N. caninum* tachyzoites. It has been postulated that the antiparasitic activity of NTZ is based on the reduction of the nitro group on the thiazole ring by PFOR and other nitroreductases (31). One NTZ derivative, Rm4820 (Fig. 1), differs from NTZ by replacement of the thiazole-associated nitro group with a bromide. We found that Rm4820 was equally effective against *N. caninum* tachyzoites as NTZ in terms of inhibition of proliferation and parasiticidal activity (Fig. 3). In contrast, Rm4803, which is virtually identical to Rm4820 but with a methyl group at position 3 on the salicylate ring (Fig. 1), did not exhibit any efficacy at all. Moreover, Rm4821 (methylated

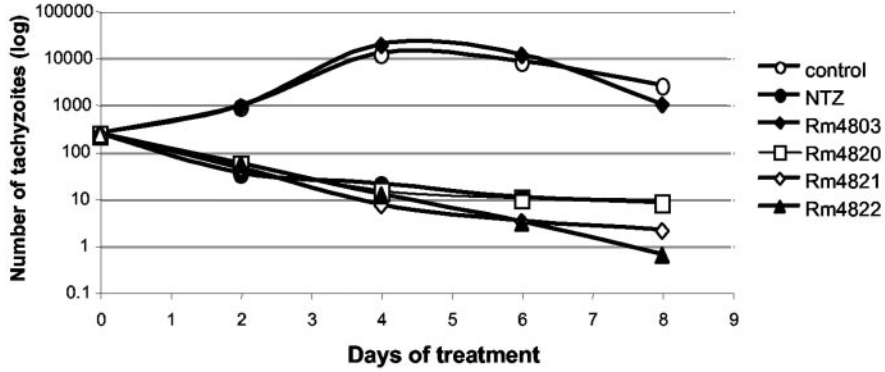

FIG. 3. Effects of several thiazolides on *N. caninum* tachyzoite proliferation in HFF. HFF monolayers were infected with *N. caninum* tachyzoites, and at 2 h p.i., 10 μg/ml NTZ, Rm4803, Rm4820, Rm4821, or Rm4822 was added and left for a period of 8 days. Note the severe inhibition of proliferation by Rm4820, Rm4821, and Rm4822, while Rm4803 has no effect.

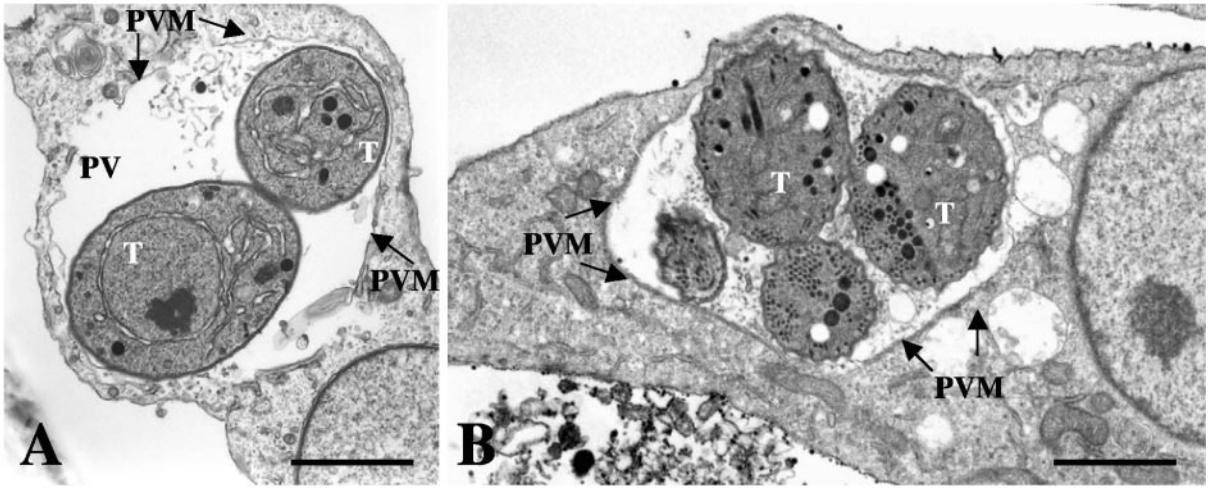

FIG. 4. Transmission electron microscopy of intracellular *N. caninum* tachyzoites at (A) 24 h p.i. and (B) 72 h p.i. Note that intracellular *N. caninum* tachyzoites (T) reside within a parasitophorous vacuole (PV), surrounded by a parasitophorous vacuole membrane (PVM). Bars, 0.9 μm (A) and 1 μm (B).

at position 5) and Rm4822 (methylated at position 4) were all as effective as NTZ and Rm4820 (Fig. 3). These findings indicate that the thiazole-ring-associated nitro group does not represent the only active domain of the molecule and that another, probably highly relevant, domain is located at position 3 of the salicylate ring.

Electron microscopy reveals ultrastructural effects of NTZ in *N. caninum*-infected HFF monolayers. The effects of NTZ in infected monolayers were studied in comparison to untreated cultures by transmission electron microscopy at different time points p.i. In control cultures (Fig. 4A), *N. caninum* tachyzoites were found within their host cells and localized within the parasitophorous vacuole, which is clearly separated from the host cell cytoplasm by the parasitophorous vacuole membrane (PVM), creating a compartment that allows tachyzoites to proliferate (Fig. 4B). In NTZ-treated cultures fixed at 6 h p.i., intracellular tachyzoites were also present, but a clearly delineated parasitophorous vacuole and corresponding membrane were largely missing (Fig. 5A). Although tachyzoites were situated rather freely within the host cell cytoplasm, they exhibited no signs of distortion. The PVM, normally entirely surrounding the parasite, was largely absent. This became more evident after 24 h (Fig. 5B). Parasites were located free in the host cell interior, but now the surrounding host cell cytoplasm was severely distorted or degraded. Lipid droplets were often observed in the vicinity of tachyzoites. The tachyzoites themselves started to show extensive alterations, with numerous vacuoles forming within the parasite cytoplasm, either appearing empty or filled with fine granular material. However, rhoptries and micronemes, two characteristic secretory organelles, as well as the mitochondrion, still appeared structurally normal. After 3 and 4 days of treatment (Fig. 5C and D, respectively), tachyzoites exhibited further alterations. These included the presence of a largely vacuolized cytoplasm, with vacuoles variable in size and content, filled with fine granular material but also more electron-dense membranous residues. Mitochondria appeared to be fragmented, and in most parasites the characteristic rhoptry and microneme organelles were no longer discernible. Tachyzoites were largely surrounded by host cell cytoplasmic debris and lipid droplets.

Characterization of antiparasitic activity of NTZ. To identify the minimal amount of time required for the drug to act permanently on the parasite in order to exert selective toxicity, we treated infected monolayers with NTZ for various time spans, followed by further culture without the drug for several days (Fig. 6). We found that the *Neospora*-infected HFF needed to be in contact with NTZ for 5 consecutive days for the proliferation-inhibitory effect to be maintained. Further incubation of infected and NTZ-treated monolayers in the absence of the drug for up to 20 days did not results in regrowth of parasites, showing that in vitro treatment of infected HFF for a period of 5 consecutive days exerted parasiticidal activity (data not shown).

We then investigated whether NTZ would also be effective if drug treatment was initiated at later stages of infection, when larger pseudocysts containing numerous tachyzoites had already formed. We found that NTZ exhibited a massive inhibitory effect also at a later stage of infection, such as at day 3 or 4 p.i. (Fig. 7). This inhibitory effect was evident shortly following initiation of treatment.

Further experiments were performed in order to investigate whether NTZ could affect host cell entry by *N. caninum* tachyzoites (Fig. 8). For this, freshly purified tachyzoites were resuspended in medium containing NTZ, and the drug remained present during the infection phase of 2 h. Subsequently, infected monolayers were cultured in either the presence or absence of NTZ. We found that the presence of NTZ during infection, and subsequent maintenance of cultures in the absence of NTZ, resulted in a growth curve identical to that for the untreated infection control (Fig. 8A). This indicates that the drug did not notably affect the host cell entry process. However, the presence of NTZ during infection, and subsequent continuous in vitro treatment for a period of 5 days, was slightly more effective than the procedure without pretreatment (Fig. 8A), but the values obtained were not statistically significant. We thus further investigated whether

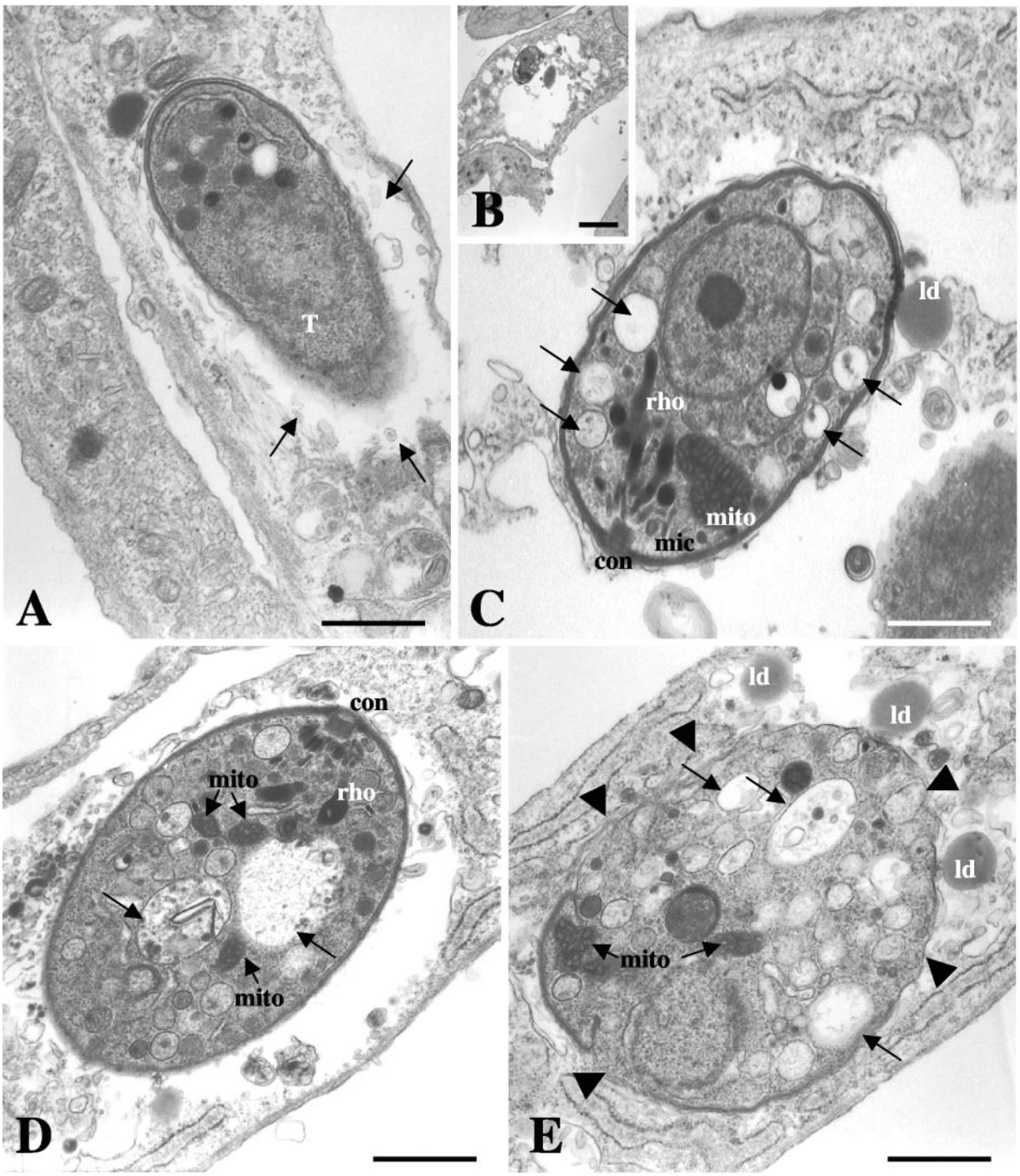

FIG. 5. Transmission electron microscopy of NTZ-treated, *N. caninum*-infected HFF monolayers. (A) At 6 h p.i., intracellular tachyzoites (T) are characterized by the absence of a parasitophorous vacuole membrane (arrows). Bar, 0.6 μm. (B) At 24 h p.i., severe alterations within the infected host cell cytoplasm are evident. Bar, 2 μm. (C) Higher magnification reveals distinct changes within the parasites, including increased cytoplasmic vacuolization (arrows). con, conoid; mito, mitochondrion; mic, micronemes; rho, rhoptries; ld, lipid droplets. Bar, 0.5 μm. (D and E) At 48 h (D) and 72 h (E), distinct changes and considerable damage are evident in drug-treated tachyzoites. Lipid droplets often surround the parasites, and the tachyzoite cytoplasm is heavily compartmentalized by vesicles, the contents of which are granular or membranous and electron dense (arrows). Note also the fragmented mitochondrion. At 72 h, the plasma membrane has lost its characteristic electron-dense appearance, indicating that membrane disintegration has taken place (triangles). Bars, 0.5 μm.

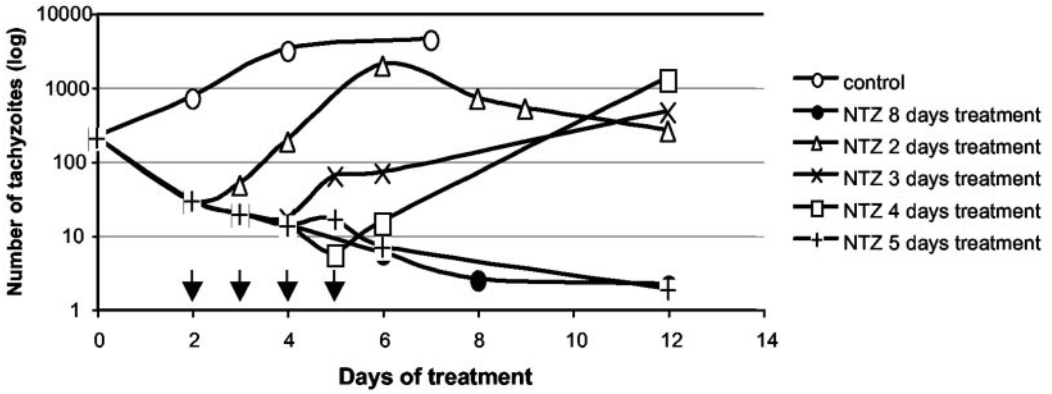

FIG. 6. Five days of continuous NTZ treatment is required to exert true parasiticidal activity. HFF monolayers were infected with *N. caninum* tachyzoites, and at 2 h p.i., 10 μg/ml NTZ was added. NTZ treatment was stopped after 2, 3, 4, or 5 days (arrows) by replacing the NTZ-containing medium with fresh medium devoid of NTZ. Note that parasite proliferation was irreversibly inhibited only after 5 days of continuous NTZ treatment.

more prolonged treatment of *N. caninum* tachyzoites would affect their ability to interact with HFF monolayers (Fig. 8B). For this, extracellular tachyzoites were resuspended in medium containing NTZ for different time spans (2, 6, and 24 h) and only then were added to HFF monolayers. The overall number of tachyzoites interacting with HFF and the respective number of invaded parasites were determined in relation to controls incubated in medium alone. We found that over a period of 24 h, NTZ exhibited no negative impact on extracellular tachyzoites with regard to their adhesive or invasive capacities (Fig. 8B), indicating that the drug acts on intracellular, proliferating parasites but does not affect the invasive potential of extracellular *N. caninum* tachyzoites.

DISCUSSION

In the United States, NTZ (Alinia) is currently used for the treatment of persistent diarrhea caused by *Cryptosporidium parvum* and *Giardia intestinalis* in adults, adolescents, and children from 1 year of age. In addition, the drug is marketed for the treatment of equine myeloencephalitis caused by *Sarcocystis neurona* (Navigator). Since both *Cryptosporidium* and *Sarcocystis* belong to the phylum Apicomplexa, we investigated the efficacy of this drug against another apicomplexan parasite, *N. caninum*.

Our results presented in this study indicate that NTZ treatment inhibits *N. caninum* proliferation and severely damages tachyzoites but that there are clear indications that the host cell, at least to some extent, is also involved in these processes. First, NTZ does not act on mechanisms involved in host cell invasion but acts on mechanisms affecting parasite proliferation. Second, a full 5 days of treatment was required to exert a true parasiticidal activity. During the first 4 days of in vitro treatment, the damage imposed on these parasites must be regarded as parasitostatic, allowing at least some tachyzoites to resume proliferation within just a few hours after removal of the drug. The fact that 5 days of continuous treatment was required to achieve a parasiticidal effect clearly indicates that NTZ does not exert an immediate toxic efficacy but that toxicity could be, at least partially, related to effects mediated by the host cell.

We observed that NTZ-treated infected cells are characterized by the absence of a parasitophorous vacuole and/or para-

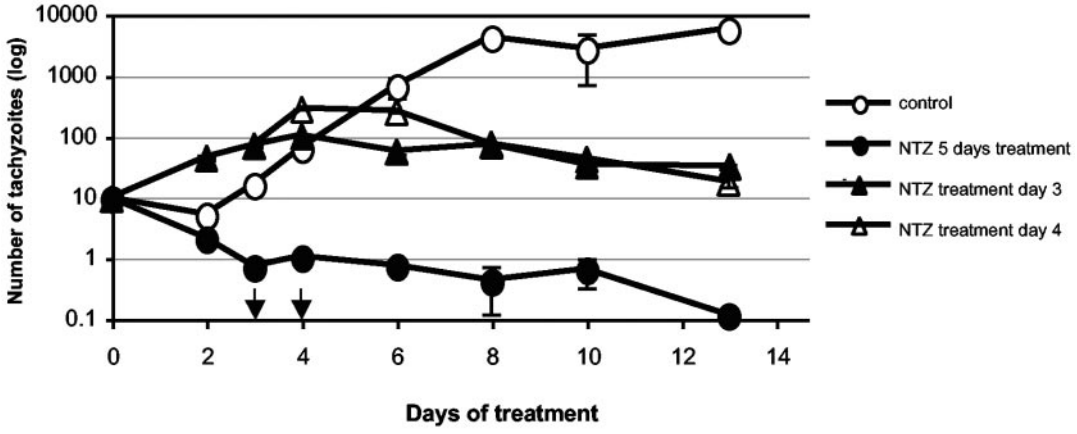

FIG. 7. NTZ inhibits proliferation at later stages of host cell infection. HFF monolayers were infected with *N. caninum* tachyzoites, and NTZ (10 μg/ml) was added at day 3 and day 4 p.i. (arrows). Note the immediate inhibition of proliferation of *N. caninum* tachyzoites.

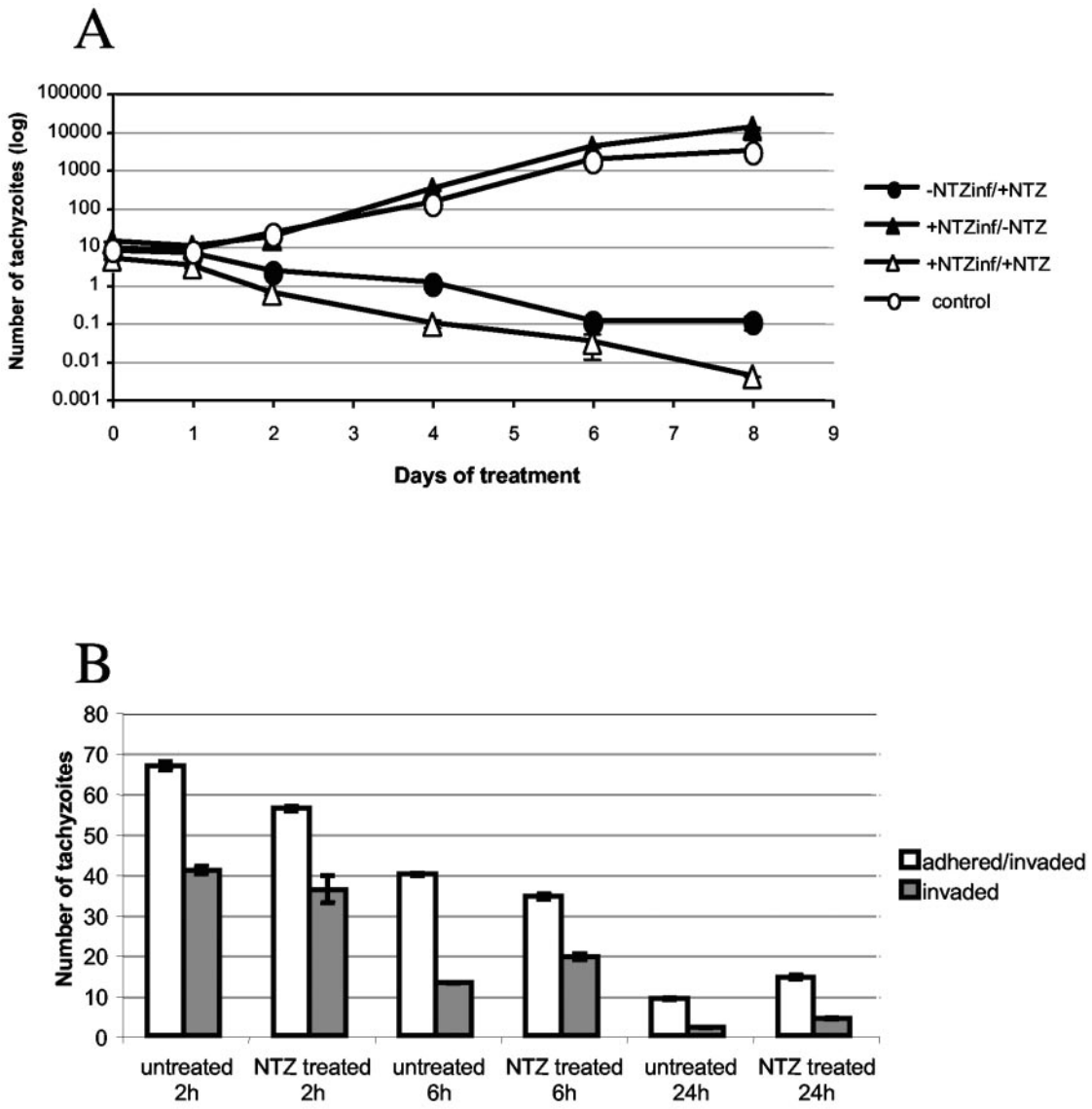

FIG. 8. NTZ acts on intracellular, but does not impair infectivity of extracellular, *N. caninum* tachyzoites. (A) HFF monolayers were infected with *N. caninum* tachyzoites. Four distinct conditions were assessed. First, no drug was added at any time (control). Second, NTZ (10 μg/ml) was present during the infection phase of 2 h, followed by further culture without the drug (+NTZinf/−NTZ). This was not effective at all. Third, NTZ was not present during infection but was added after 2 h upon completion of the infection phase (−NTZinf/+NTZ). Finally, NTZ was present during infection and also subsequently during the culture (+NTZinf/+NTZ). (B) Freshly purified *N. caninum* tachyzoites were incubated in medium in either the presence of absence of NTZ (10 μg/ml) for 2, 6, or 24 h. Parasites were then allowed to interact with HFF monolayers, and tachyzoites interacting with the monolayers (adhered/invaded) and the corresponding invaded parasites (invaded) were quantified by the PDTC adhesion/invasion assay. No significant impact on either adhesion or invasion was evident. Error bars indicate standard deviation.

sitophorous vacuole membrane, which normally separates tachyzoites from the host cell cytoplasm. It has been shown that *N. caninum* tachyzoites, once inside their host cell, secrete a wide range of molecules, aiming to modify the parasitophorous vacuole and its membrane according to their own needs; thus, this compartment is essential for survival, development, and proliferation (7). We do currently not know whether parasites located in the host cell cytoplasm without a surrounding parasitophorous vacuole membrane also undergo similar secretion events, but we found that the host cell cytoplasm in infected and drug-treated cells was severely altered and exhibited considerable damage. Most notably, this was not observed in uninfected host cells, indicating that these host cell alterations were mediated by the parasite or its secretory products rather than by the drug itself. Finally, at 72 and 96 h following the initiation of NTZ treatment, intracellular *N. caninum* tachyzoites are characterized by increased vacuolization of the parasite cytoplasm, fragmentation of the single tachyzoite mitochondrion, and accumulation of lipid droplets in the vicinity of the parasite. These are all signs of severely impaired metabolic activity and can be attributed to the action of the drug.

It is tempting to speculate that infected and drug-treated

host cells might undergo apoptosis, while uninfected ones do not. It is well known that intracellular parasites, including *Toxoplasma gondii* and *N. caninum*, are capable of modulating the survival status of their host cells (reviewed in reference 15). This can occur through a number of different pathways. For instance, Sinai et al. (30) found that *T. gondii* tachyzoites inhibited host cell apoptosis by inducing the activation of the transcription factor NF-κB, which in turn regulates the expression of inhibitors of apoptosis in the host cell. The activation of NF-κB pathway by *T. gondii* correlated with the localization of phosphorylated IκBα at the parasitophorous vacuole membrane (23). The lack of this membrane could potentially have serious consequences for the host cell itself, since Molestina and Sinai (24) detected a kinase activity at the *T. gondii* parasitophorous vacuole that was capable of phosphorylating host IκBα, thus demonstrating a direct link between the presence of this membrane and inhibition of host cell apoptosis. Although there is only limited information available to date (27, 28), similar mechanisms are likely to take place in *N. caninum*-infected cells. However, further and more detailed investigations are required in order to define the mechanism of the antiparasitic activity of NTZ against *N. caninum* tachyzoites, also taking into consideration possible effects mediated by the host cell.

The current knowledge suggests that the activity of NTZ is dependent upon intracellular reduction of its nitro group by nitroreductases in a manner similar to that of MTZ (31). However, the same authors (31) also noted distinct differences between MTZ and NTZ. For instance, analysis of mutation to rifampin resistance in *Helicobacter pylori* indicated that NTZ was not mutagenic and did not induce DNA breakage, in contrast to MTZ, which caused DNA damage and was strongly mutagenic. Our experiments, employing a number of defined NTZ derivatives, some lacking the thiazole-associated nitro group and some containing modified versions of the salicylic acid moiety, demonstrate the importance of the benzene ring located at the opposite end of the molecule. First, exchanging the nitro group for a bromide does not notably impair the parasiticidal activity of the molecule. This does not imply that the nitro group is not instrumental in terms of parasiticidal activity, but it clearly suggests that there are one or more additional active sites exerting an antiparasitic effect. One of these sites could be the salicylic acid moiety. We found that the parasiticidal activity of the molecule is completely lost if the salicylic acid ring is methylated at position 3, while methylation at position 4 or 5 does not have any effect. This implies that the unmodified *ortho* position is essential for the parasiticidal activity. Additional investigations will be necessary to determine whether the ultrastructural alterations induced by these modified NTZ derivatives correspond to what we observed with NTZ.

Further studies are required to investigate whether NTZ will be useful for in vivo treatment of *N. caninum* infections. Once orally applied, NTZ is rapidly deacetylated to tizoxanide, which shows equal parasiticidal activity in vitro. The second metabolite, tizoxanide glucuronide, exhibited no in vitro antiparasitic activity against *N. caninum* tachyzoites. The mouse model has been extensively used for studies on the pathology and immunology of experimental neosporosis (1, 3, 4, 11, 12). However, comparative analysis of the pharmacokinetics of NTZ metabolites in mice (32) and humans (2) has shown that in mice tizoxanide reaches only very low levels in serum (below 1 μg/ml for less than 1 h), while in humans peak tizoxanide serum levels reach 8 μg/ml and remain above 4 μg/ml for a time span of 5 to 6 h. Thus, the mouse model is essentially not a suitable model to investigate the efficacy of this drug against challenge infection with *N. caninum* tachyzoites. Therefore, it will be important to obtain detailed information on the pharmacokinetics of this drug in cattle or dogs prior to embarking on any further animal experimentation to study the in vivo efficacy of NTZ or is derivatives against neosporosis.

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