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Experimental treatment of *Neospora caninum*-infected mice with the arylimidamide DB750 and the thiazolide nitazoxanide

Karim Debache [a,*], Christophe Guionaud [a], Christian Kropf [a], David Boykin [b], Chad E. Stephens [c], Andrew Hemphill [a,*]

[a] Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland
[b] Department of Chemistry, Georgia State University, P.O. Box 4098, Atlanta, GA 30302-4098, USA
[c] Department of Chemistry and Physics, Augusta State University, Augusta, GA 30904-2200, USA

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ABSTRACT

The cationic arylimidamide DB750 and the thiazolide nitazoxanide had been shown earlier to be effective against *Neospora caninum* tachyzoites *in vitro* with an IC_{50} of 160 nM and 4.23 μM, respectively. In this study, we have investigated the effects of DB750 and nitazoxanide treatments of experimentally infected Balb/c mice, by applying the drugs either through the oral or the intraperitoneal route. In experiment 1, administration of DB750 (2 mg/kg/day) and nitazoxanide (150 mg/kg/day) started already 3 days prior to experimental infection of mice with 2×10^6 tachyzoites. Following infection, the drugs were further administrated daily for a period of 2 weeks, either orally or intraperitoneally. Intraperitoneal injection of DB750 was well tolerated by the mice, but treatment with nitazoxanide resulted in death of all mice within 3 days. Upon intraperitoneal application of DB750, the cerebral parasite load was significantly reduced compared to all other groups, while oral application of DB750 and nitazoxanide were not as effective, and resulted in significant weight loss. In experiment 2, mice were infected with 2×10^6 tachyzoites and at 2 weeks post-infection, DB750 (2 mg/kg/day) was applied by intraperitoneal injections for 14 days. In the DB750-treated group, only 2 out of 12 mice succumbed to infection, compared to 7 out of 12 mice in the placebo-group. DB750 treatment also resulted in significantly reduced cerebral parasite burden, and reduced numbers of viable tachyzoites. Our data suggest that DB750 exerted its activity also after crossing the blood–brain barrier, and that this class of compounds could be promising for the control of *N. caninum*-associated disease.

1. Introduction

Neospora caninum is a cyst forming apicomplexan parasite originally described in a domestic dog and was initially recognized in Norway as a *Toxoplasma gondii*-like organism associated with hind limb paralysis (Bjerkas et al., 1984). The parasite (Apicomplexa: Eimeriina: Sarcocystidae) was named by Dubey et al. (1988a,b) and soon researchers associated *Neospora* infection with bovine abortion (Dubey and Lindsay, 1996). Subsequently, *N. caninum* was reported in various species of livestock, including cattle, sheep, goats, horses and deer (Dubey and Lindsay, 1996, 2007; Hemphill, 1999; Hemphill and Gottstein, 2000). Today *N. caninum* is known as the most frequently diagnosed cause of abortion and stillbirth in cattle worldwide (Gondim et al., 2005; Dubey et al., 2007), and represents an important veterinary health problem, which leads to severe economic losses. In Switzerland 30% of all bovine abortions might be related to *Neospora* infection (Hasler et al., 2006a,b). The negative economical impact of neosporosis on the dairy industry, including reduced milk yield (Hernandez et al., 2002), premature culling (Strohbusch et al., 2009) and reduced post-weaning weight gain in beef calves (Reichel, 2000; Hasler et al., 2006a,b), justifies the research on the development of strategies for prevention and treatment of *N. caninum* infection. No efficient vaccine or safe compound has been made available to date, but both have been identified as economically viable options (Hasler et al., 2006a,b; Reichel and Ellis, 2006).

Ideally, a drug against neosporosis should fulfill several criteria. It should cross the blood–brain barrier and be effective against the bradyzoites stage and the chronic phase of infection, since many *Neospora*-associated abortions occur in chronically infected cattle. In addition, treatments should be cost-effective in bovines, and leave no residues in meat or milk. Although these conditions are difficult to meet, a wide range of compounds has been tested as *Neospora* inhibitors employing the tachyzoite culture model. These include, besides others, lasalocid, monensin, pirithrexim, pyrimethamine, clindamycin, robenidine and trimethoprim (Lindsay

* Corresponding authors.
E-mail addresses: karim.debache@vetsuisse.unibe.ch (K. Debache), andrew.hemphill@vetsuisse.unibe.ch (A. Hemphill).

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et al., 1994, 1996), artemisinin (Kim et al., 2002), depudecin (Kwon et al., 2003), toltrazuril, ponazuril (Darius et al., 2004), nitro-and bromo-thiazolides (Esposito et al., 2005, 2007a,b). Alcoholic herbal extracts (Youn et al., 2004) have also been reported to exhibit anti-*N. caninum* tachyzoite activities. Only few drugs have been evaluated in small animal models. Sulfadiazine and amprolium were investigated, and sulfadiazine administered at 1 mg/ml prevented disease in experimentally infected mice, but did not eliminate the parasite (Lindsay and Dubey, 1990). Several studies in mice focussed on toltrazuril (Gottstein et al., 2005; Strohbusch et al., 2008), showing that (i) inclusion of toltrazuril into the drinking water eliminated parasites in the central nervous system but that cell-mediated immunity was required to achieve its full efficacy in mice, and (ii) that toltrazuril treatment controlled dia-placental *N. caninum* transmission in experimentally infected pregnant mice. In addition, studies on prophylactic toltrazuril administration in newborn calves suggested that this treatment regime could exhibit a certain degree of protective efficacy, but further studies are required (Kritzner et al., 2002; Haerdi et al., 2006). Treatments in dogs were successful in eliminating clinical signs in 10 of 27 cases of canine neosporosis, using clindamycin, potentiated sulphonamides and pyrimethamine (Barber and Trees, 1996).

Nitazoxanide [2-acetyloxy-*N*-(5-nitro-2-thiazolyl) benzamide] was first described in 1984 as a human cestocidal drug (Rossignol and Maisonneuve, 1984). To date, nitazoxanide is known as a broad-spectrum anti-infective drug against a wide variety of intestinal cestodes, protozoa and nematodes infecting animals and humans, and also acts against a range of anaerobic and microaerophilic bacteria and rotavirus, influenza A/B/HIN1 and hepatitis B/C (Rossignol et al., 2006, 2009; Müller et al., 2008). The broad activities of this drug also include intracellular apicomplexan parasites such as *Cryptosporidium parvum*, *Sarcocystis neurona*, *N. caninum* and *T. gondii* (Esposito et al., 2005, 2007a,b).

Leepin et al. (2008) reported on the efficacy of DB750, a dicationic arylimidamide against tachyzoite stages of *N. caninum* and *T. gondii* in the submicromolar range ($IC_{50's}$ of 0.23 and 0.16 μM, respectively). The drug did not affect extracellular tachyzoites, thus did not affect host cell invasion, but severely impaired the intracellular proliferation of tachyzoites. In addition, exposure of fibroblasts to 1.7 μM DB750 for 24 h, followed by infection with *N. caninum* tachyzoites and subsequent culture in the absence of DB750, resulted in a significantly delayed parasite proliferation, indicating that either these compounds or respective active metabolites were still present after removal of the drugs, or that drug treatments reversibly impaired fibroblast activities essential for parasite proliferation and/or survival.

Both compounds had been postulated to represent interesting candidates for the development of a chemotherapeutical approach to combat *N. caninum* infection. In this study, we investigated whether the promising *in vitro* activities of DB750 and nitazoxanide could also be translated *in vivo*, employing a non-pregnant mouse model for monitoring cerebral infection and acute disease.

2. Materials and methods

2.1. Chemicals, reagents and drugs

Unless otherwise stated, all cell culture reagents were supplied by Gibco-BRL (Zurich, Switzerland) and biochemicals reagents were purchased from Sigma (St. Louis, MO, USA). DB750 was synthesised in the Centre for Biotechnology and Drug Design, Georgia State University and in the Department of Chemistry and Physics, Augusta State University. Nitazoxanide was obtained from Prof. Christian Leumann, Department of Chemistry and Biochemistry, University of Bern. Drugs were kept as stock solutions at 1 mg/ml or 10 mg/ml in DMSO and were stored −20 °C.

2.2. Cell culture and N. caninum purification

Monkey kidney epithelial (Vero cells) and human foreskin fibroblasts (HFF) were routinely cultured as previously described (Leepin et al., 2008) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U of penicillin/ml and 50 ug of streptomycin/ml at 37 °C/5% CO_2 in tissue culture flasks. Cultures were passaged at least once per week. *N. caninum* tachyzoites [Nc1 isolate, (Dubey et al., 1988b)] were maintained by serial passages in Vero cells or HFF during which time FCS was replaced with 5% immunoglobulin G (IgG)-free horse serum (HS). Parasites were harvested as described previously by Hemphill et al. (1996). Infected cells were trypsinized, washed twice in cold RPMI 1640 medium, and the resulting pellet was resuspended in 2 ml cold RPMI 1640 medium. Cells were repeatedly passaged through a 25 G-needle and liberated tachyzoites were purified by passage through Sephadex-G25 columns (Amersham Biosciences, Otelfingen, Switzerland), previously equilibrated with cold RPMI 1640 medium. Purified tachyzoites were obtained after centrifugation at 600*g*/10 min/4 °C, counted in a Neubauer chamber and were used for infection experiments as described below.

2.3. Experiment 1: in vivo treatment of Balb/c mice with DB750 and nitazoxanide starting prior to infection

Sixty female Balb/c mice between 8 and 9 weeks of age were purchased from Charles River Laboratories (Sulzheim, Germany) and were maintained in a common room under controlled temperature and a 14 h dark/10 h light cycle according to the standards set up by the animal welfare legislation of the Swiss Veterinary Office. At day zero, mice were randomly caged into 6 experimental groups of 10 mice as outlined in Table 1. Enzyme-linked immunosorbent assay (ELISA) was carried out to insure that mice were serologically *Neospora*-negative. Mice received either DB750 (2 mg/kg/day; Wang et al., 2010) or nitazoxanide (150 mg/kg/day; Stettler et al., 2004). Both drugs were suspended in 0.5% carboxymethyle-cellulose in water (CMC). The drugs were administered in a total volume of 100 μl, either orally (intragastric inoculation) or intraperitoneally (by injection). Placebo groups obtained the corresponding amount of the solvent only (see Table 1). The treatments were performed on a daily basis, starting at day 1. On day 3, all mice were infected by intraperitoneal injection of 2×10^6 freshly purified *N. caninum* tachyzoites in medium. If not indicated otherwise, treatments continued until day 18 (day 14 post-infection), after which the mice were sacrificed by CO_2-euthanasia. Mice exhibiting clinical signs of neosporosis (ruffled coat, apathy and hind limb paralysis) were euthanized at the onset of these clinical signs. From each animal, the brain was recovered, and the two hemispheres were processed separately for either DNA- or RNA-extraction (Debache et al., 2008, 2009b).

2.4. Experiment 2: in vivo treatment of Balb/c mice with DB750 starting 14 days following infection with N. caninum tachyzoites

Thirty-six female Balb/c mice were purchased and housed as described above. One group of eight animals was not infected and did not undergo any treatment. On day 1, 28 mice were infected by intraperitoneal injection of 2×10^6 *N. caninum* tachyzoites, and four mice exhibited clinical signs during the first two weeks and were euthanized. On day 14 post-infection, treatment was initiated on 12 surviving and asymptomatic animals by intraperitoneal administration of DB750 (2 mg/kg/day) in 100 μl 0.5% CMC per shot for a period of 14 days. Another 12 animals, also