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# Induction of tachyzoite egress from cells infected with the protozoan *Neospora caninum* by nitro- and bromo-thiazolides, a class of broad-spectrum anti-parasitic drugs

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**Abstract**

*Neospora caninum* represents an important pathogen causing stillbirth and abortion in cattle and neuromuscular disease in dogs. Nitazoxanide (NTZ) and its deacetylated metabolite tizoxanide (TIZ) are nitro-thiazolyl-salicylamide drugs with a broad-spectrum anti-parasitic activity in vitro and in vivo. In order to generate compounds potentially applicable in food and breeding animals, the nitro group was removed, and the thiazole-moiety was modified by other functional groups. We had shown earlier that replacement of the nitro-group by a bromo-moiety did not notably affect in vitro efficacy of the drugs against *N. caninum.* In this study we report on the characterization of two bromo-derivatives, namely Rm4822 and its de-acetylated putative metabolite Rm4847 in relation to the nitro-compounds NTZ and TIZ. $IC_{50}$ values for proliferation inhibition were 4.23 and 4.14 μM for NTZ and TIZ, and 14.75 and 13.68 μM for Rm4822 and Rm4847, respectively. Complete inhibition ($IC_{99}$) was achieved at 19.52 and 22.38 μM for NTZ and TIZ, and 18.21 and 17.66 μM for Rm4822 and Rm4847, respectively. However, in order to exert a true parasiticidal effect in vitro, continuous culture of infected fibroblasts in the presence of the bromo-thiazolide Rm4847 was required for a period of 3 days, while the nitro-compound TIZ required 5 days continuous drug exposure. Both thiazolides induced rapid egress of *N. caninum* tachyzoites from their host cells, and egress was inhibited by the cell membrane permeable $Ca^{2+}$-chelator BAPTA-AM. Host cell entry by *N. caninum* tachyzoites was inhibited by Rm4847 but not by TIZ. Upon release from their host cells, TIZ-treated parasites remained associated with the fibroblast monolayer, re-invaded neighboring host cells and resumed proliferation in the absence of the drug. In contrast, Rm4847 inhibited host cell invasion and respective treated tachyzoites did not proliferate further. This demonstrated that bromo- and nitro-thiazolides exhibit differential effects against the intracellular protozoan *N. caninum* and bromo-thiazolides could represent a valuable alternative to the nitro-thiazolyl-salicylamide drugs.


*Keywords:* *Neospora caninum*; Intracellular parasite; Nitazoxanide; Thiazolides; Egress

## 1. Introduction

*Neospora caninum* is an apicomplexan parasite causing neosporosis that affects mainly cows and dogs, although this protozoan has been detected in many other species worldwide (Dubey et al., 2002; Hemphill and Gottstein, 2000; Innes et al., 2005; Hemphill et al., 2006a). Dogs and other canids serve as definitive hosts (Gondim et al., 2004). Bovine neosporosis causes abortion, stillbirth or birth of chronically infected calves that are weak, exhibit neuromuscular disturbances, and occasionally show severe birth defects. In addition, calves can be born infected but lacking any clinical signs, and they can then vertically transmit the parasite to their own offspring at a later time point. This mode of endogenous transplacental transmission from the mother to the fetus is the most important

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infectious route (Innes et al., 2005; Williams and Trees, 2006).

Besides abortion and the loss of calves, costs associated with neosporosis include reduced milk yield (Hernandez et al., 2002), premature culling (Thurmond and Hietala, 1996) and reduced post-weaning weight gain in beef calves (Barling et al., 2000). This causes significant economic losses to the cattle industry worldwide and there is a keen interest in developing strategies for control of the disease (Innes et al., 2005). A recent financial analysis of potential control strategies indicated that two options, namely discontinued breeding with offspring from seropositive cows, and chemotherapeutical treatment of female offspring, resulted in a positive net benefit to cost ratio (Hasler et al., 2006). While considerable efforts have been concentrating on vaccine development (Hemphill et al., 2006a; Williams and Trees, 2006; Innes and Vermeulen, 2006), no successful therapies are available for the treatment of bovine neosporosis, despite the fact that this disease has been recognized as one of the most important infectious causes of abortion in cattle worldwide (Innes et al., 2005).

Recent studies have shown the in vitro efficacy of thiazolides, in particular nitazoxanide (NTZ), against *N. caninum* tachyzoites (Esposito et al., 2005, 2006). In humans, NTZ (Alinia™) has been approved in the United States by the Food and Drug Administration (FDA) for the treatment of diarrhea caused by *Giardia lamblia* and the apicomplexan parasite *Cryptosporidium parvum*. NTZ has also been FDA-approved for the treatment of equine myeloencephalitis caused by another member of the apicomplexans, *Sarcocystis neurona* (McClure and Palma, 1999). However, there can be major side effects in horses treated with NTZ, such as severe enterocolitis (Navigator™ prescribing information, IDDEX Pharmaceuticals Inc., Greensboro, NC, USA), since the drug is also active against microaerophilic and anaerobic bacteria of the gastrointestinal flora. This will inevitably cause significant problems in the digestive system of ruminants, which in turn could be more serious than the disease itself (Fox and Saravolatz, 2005; Hemphill et al., 2006b). It has been speculated that gastrointestinal problems could be due to the action of the enzyme pyruvate ferredoxin oxidoreductase (PFOR) or other nitroreductases which have been hypothesized to reduce the thiazole ring-associated nitro group, and thus kill bacteria through the production of free radicals (Sisson et al., 2002; Hoffman et al., 2007; reviewed in Hemphill et al., 2006b). This speculated process could also potentially generate mutagens, which in the application of the drug in food and breeding animals for the treatment of *N. caninum* may make it incompatible with standards set by food regulatory bodies.

It has recently been shown that NTZ-bromo-derivatives, in which the thiazole-associated nitro group has been replaced with a bromo-moiety, exhibit no effect against anaerobic bacteria (Pankuch and Appelbaum, 2006), and are thus unlikely to harm the intestinal microbial flora. However, with few exceptions, most bromo-derivatives maintained considerable in vitro activity against intracellular protozoa such as *N. caninum* (Esposito et al., 2006) and *Besnoitia besnoiti* (Cortes et al., 2007). Thus, thiazolide bromo-derivatives lacking the nitro-thiazole could be a safer alternative to the parent compound NTZ. In this study, we directly compared the in vitro characteristics of NTZ with another promising drug candidate, Rm4822, against *N. caninum* tachyzoites, and investigated their respective deacetylated metabolites tizoxanide (TIZ) and Rm4847. Rm4822 and Rm4847 were selected based on computational chemistry-derived criteria from a collection of 29 NTZ-derivatives previously screened against *N. caninum* in vitro (Esposito et al., 2006). We demonstrate that these thiazolides induce egress of *N. caninum* tachyzoites, in a $Ca^{2+}$-dependent manner. We also show that only Rm4847 negatively affects host cell entry. These effects could be largely responsible for the anti-parasitic activities observed for these compounds. Bromo-thiazole-derivatives lacking the potentially harmful nitro-group may represent an important addition to the anti-parasitic arsenal for food animal production, especially in cattle.

## 2. Materials and methods

### 2.1. 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, TIZ, Rm4822 and Rm4847 (see Fig. 1) were synthesized either at the Department of Chemistry at the University of Liverpool, or at the Department of Chemistry at the University of Bern. Drugs were kept as stock solutions at 10 mg/ml in dimethyl sulfoxide (DMSO) and were stored at −20 °C.

### 2.2. Tissue culture and parasite purification

Cultures of Vero cells were maintained in RPMI 1640 medium (Gibco-BRL, Basel, Switzerland) supplemented with 5% 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. *Neospora caninum* tachyzoites (Nc-Liverpool isolates) were maintained in Vero cell monolayer cultures (Hemphill, 1996), 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 (Hemphill, 1996).