

# Effects of miltefosine treatment in fibroblast cell cultures and in mice experimentally infected with *Neospora caninum* tachyzoites

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## SUMMARY

Miltefosine was investigated for its activity against *Neospora caninum* tachyzoites *in vitro*, and was shown to inhibit the proliferation of *N. caninum* tachyzoites cultured in human foreskin fibroblasts (HFF) with an IC<sub>50</sub> of 5.2 µM. Treatment of infected cells with 25 µM miltefosine for a period of 10 h had only a parasitostatic effect, while after 20 h of treatment parasitocidal effects were observed. This was confirmed by transmission electron microscopy of *N. caninum*-infected and miltefosine-treated HFF. Administration of miltefosine to *N. caninum*-infected Balb/c female mice at 40 mg/kg/day for 14 days resulted in 6 out of 10 mice exhibiting weight loss, ruffled coat and apathy between days 7 and 13 post-infection. In the group that received placebo, only 2 out of 8 mice succumbed to infection, but the cerebral burden was significantly higher compared to the miltefosine treatment group. In a second experiment, the time-span of treatment was reduced to 5 days, and mice were maintained without further treatment for 4 weeks. Only 2 out of 9 mice in the miltefosine treatment group exhibited signs of disease, while 8 out of 10 mice succumbed to infection in the placebo group. These results showed that miltefosine hampered the dissemination of parasites into the CNS during experimental *N. caninum* infection in mice.

Key words: miltefosine, *Neospora caninum*, tachyzoites, cultured fibroblasts, proliferation.

## INTRODUCTION

*Neospora caninum*, an apicomplexan parasite that was initially described associated with severe neuromuscular disease in dogs (Dubey *et al.* 1988), represents one of the most important causative agents of infectious bovine abortion. Neosporosis is responsible for important economic loss in both dairy and beef cattle worldwide, also involving reduced milk yield and diminished post-weaning weight gain in beef calves (Dubey *et al.* 2007; Dubey and Schares, 2011). The worldwide serious economic impact of neosporosis upon the cattle industry has inflamed the interest for an effective control strategy as well as for prophylactic or metaphylactic measures to prevent and treat bovine neosporosis infection. At the present time, there is no vaccine available to induce complete protective immunity (Monney *et al.* 2011). Therefore, chemotherapeutic measures using pharmacologically active compounds might represent economically viable options, provided that suitable compounds are identified (Häsler *et al.* 2006a, b).

To date, no specific treatment against neosporosis is available. However, several studies on chemotherapeutically interesting compounds have been carried out. These include lasalocid, monensin, pirithrexim, pyrimethamine, clindamycin, robenidine and

trimethoprim (Lindsay *et al.* 1994), 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), pentamidine analogues (Leepin *et al.* 2008; Debache *et al.* 2011) and alcoholic herbal extracts (Youn *et al.* 2004). Only few compounds have been investigated for anti-*Neospora* activity using experimental *in vivo* models. A low efficacy in *Neospora*-infected mice was reported for sulfadiazine and amprolium (Lindsay and Dubey, 1990). Toltrazuril, a triazinone derivate that inhibits the electron transfer in the mitochondrial respiratory chain, was shown to exhibit effective anti-parasitic activity against *N. caninum* in mice (Gottstein *et al.* 2001), while results in bovines did not allow definitive conclusions (Kritzner *et al.* 2002; Haerdi *et al.* 2006). Arylimidamides also seem to be interesting compounds for chemotherapeutical intervention. For instance, DB750, a phenylated pentamidine analogue, significantly improved the survival rate and reduced the cerebral parasite burden in experimentally infected mice when applied intraperitoneally. In contrast, nitazoxanide, which had shown promising activity against *N. caninum in vitro*, was not active *in vivo*, (Debache *et al.* 2011).

Miltefosine (hexadecylphosphocholine, HePC) is an alkylphospholipid originally developed as an anti-cancer agent for local treatment of skin metastases. It was later found to show potent leishmanicidal activity as a consequence of its interference in the parasite's metabolic pathways (Unger *et al.* 1989; Barratt *et al.*

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2009). Miltefosine is commercialized in Germany as Impavido(R) for leishmaniasis treatment, is also registered for the treatment of visceral and cutaneous leishmaniasis in India and Colombia, and is undergoing clinical trials for registration in several other countries, including Brazil and Guatemala. It currently represents the only effective oral treatment for leishmaniasis. The mode of action of miltefosine against *Leishmania* parasites involves the interaction with membrane phospholipids and sterols, and the drug interferes with the functionality of a number of enzymes involved in phospholipid metabolism such as protein kinase C and the phospholipases A, C and D, finally leading to apoptosis (Barratt *et al.* 2009). Hexadecyltrimethylammonium bromide, a compound structurally similar to miltefosine, was demonstrated to exhibit potent *in vitro* activity against *Plasmodium falciparum* (Walochnik *et al.* 2002; Schuster *et al.* 2006; Choubey *et al.* 2007).

In this study, we investigated the effects of miltefosine against *N. caninum* tachyzoites in cultured fibroblasts. In experimentally infected mice, miltefosine treatment at 40 mg/kg/day for 17 days affected the dissemination of the parasite into the CNS, but 50% of the mice succumbed to infection, indicating that miltefosine treatment rendered mice more susceptible to neosporosis. However, reducing the treatment time to 5 days severely inhibited *N. caninum* dissemination and cerebral infection.

#### MATERIALS AND METHODS

Unless otherwise stated, cell culture reagents were supplied by Gibco-BRL (Zürich, Switzerland) and chemicals were purchased from Sigma (St Louis, MO, USA).

##### *Cell culture and Neospora caninum purification*

Vero cells (green monkey kidney epithelial) and human foreskin fibroblasts (HFF) were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U of penicillin/ml and 50 µg of streptomycin/ml at 37 °C/5% CO<sub>2</sub> in tissue-culture flasks. Cultures were passaged at least once per week. *N. caninum* tachyzoites of the Nc1 strain (Dubey *et al.* 1988) 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 (Hemphill, 1996). Infected cells were trypsinized, washed twice in cold RPMI 1640 medium, and the resulting pellet was re-suspended in 2 ml of 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, and re-suspended in 1 ml of RPMI 1640 containing 5% FCS, 50 U of penicillin/ml and 50 µg of streptomycin/ml. They were counted in a Neubauer chamber and used to infect HFF monolayers as described below.

##### *In vitro miltefosine treatments*

*In vitro* drug treatment assays were carried out in triplicate essentially as described by Leepin *et al.* (2008). HFF cell monolayers were grown to confluency in 24-well tissue-culture plates, and infection was carried out for 1 h by addition of  $2 \times 10^4$  cell culture-derived *N. caninum* tachyzoites per well. Infected monolayers were washed 3 times with cold RPMI 1640, and fresh medium was added, containing miltefosine, previously prepared as a stock solution in dimethyl sulfoxide (DMSO), at concentrations ranging from 0.1 µg/ml to 50 µg/ml. Controls contained DMSO solvent alone. The cultures were maintained for 72 h at 37 °C/5% CO<sub>2</sub> and were inspected daily by light microscopy. After 72 h the medium was removed, and attached cells were suspended in 200 µl of lysis buffer (Qiagen, Basel, Switzerland) and stored at -20 °C for subsequent *N. caninum* real-time PCR analysis (Müller *et al.* 2002).

IC<sub>50</sub> values were calculated after logit-log-transformation of the relative growth data (RG; control = 1) according to the formula  $\ln[(RG)/(1 - RG)] = a \times \ln(\text{drug concentration}) + b$ . Subsequent regression analysis was performed using the corresponding software tool contained in the Excel software package (Microsoft, Seattle, WA, USA).

To determine the time-span required for miltefosine to exert a true parasitocidal activity, infected HFF monolayers were treated with 25 µM miltefosine (5 times IC<sub>50</sub>) for 10 h or 20 h. The drug-containing medium was then removed and replaced with fresh culture medium without drug and changed every 2 days. Samples were collected on a daily basis by adding 200 µl of Lysis buffer (Qiagen, Basel, Switzerland) until up to 9 days post-treatment and stored at -20 °C for subsequent *N. caninum* real-time PCR analysis.

##### *Transmission electron microscopy (TEM)*

HFF cell layers were grown to confluency in Corning cell-culture flasks, infected with *N. caninum* tachyzoites for 2 days and treated with miltefosine at 24.5 µM. After 10 h or 20 h, respectively, samples were rinsed with sterile PBS, washed in 100 mM sodium cacodylate buffer (pH 7.2), and fixed in cacodylate buffer containing 2.5% glutaraldehyde for 2 h at room temperature. Cells were scraped

with a rubber policeman and centrifuged at 1000 g for 10 min at 4 °C and, and the resulting pellet was further fixed at 4 °C overnight, followed by post-fixation in 2% OsO<sub>4</sub> in cacodylate buffer for 4 h at 4 °C. Subsequently, specimens were washed in water and pre-stained in 1% uranyl acetate in water for 1 h at 4 °C, followed by extensive washing in water. Following dehydration in a graded series of ethanol (30, 50, 70, 90, and 100%), they were embedded in Epon 820 epoxy resin over a period of 2 days with 3 resin changes. The resin was polymerized at 65 °C for 24 h. Ultrathin sections were cut on a Reichert and Jung ultramicrotome and were loaded onto 300-mesh copper grids (Plano GmbH, Marburg, Germany). Staining with uranyl acetate and lead citrate was performed as described by Leepin *et al.* (2008).

#### *In vivo Experiment 1: long-term miltefosine treatments in Neospora caninum-infected Balb/c mice*

Female Balb/c mice (6 weeks of age) were purchased from Charles River Laboratories (Sulzheim, Germany) and housed under conventional day/night conditions according to the standards set up by the animal welfare legislation of the Swiss Veterinary Office. At the age of 8–9 weeks, mice were randomly divided into 4 groups of 8 or 10 mice each (Table 1) and the serological status (*Neospora*-negative) was checked by enzyme-linked immunosorbent assay (ELISA). Miltefosine suspensions were prepared in 0.5% carboxy-methylcellulose (CMC) at 20 and 40 mg/kg/day, respectively, controls received CMC without drug. The drug and control suspensions were sterile filtered and applied by intragastric inoculation (100 µl per mouse/day). At days 1–3, all mice received the corresponding dose of miltefosine or CMC, at day 4 all mice were infected intraperitoneally with freshly purified *N. caninum* tachyzoites ( $2 \times 10^6$ ) resuspended in 100 µl RPMI, with the exception of the group receiving miltefosine 40 mg/kg/day without infection (see Table 1). Treatments were continued until 14 days post-infection (p.i.). Mice were weighed daily to assess potential toxicity. Animals exhibiting signs of acute disease (ruffled coat/inability to reach up for food and water/apathy) or neuronal symptoms (head tilt, running in circles, hind limb paralysis) were euthanized at the onset of these symptoms. Latest on day 18 p.i., all mice were sacrificed by CO<sub>2</sub>-euthanasia. Brains were collected using individual sterile instruments and immediately frozen at –20 °C for subsequent quantitative PCR analysis.

#### *In vivo Experiment 2: short-term miltefosine treatments in Neospora caninum-infected Balb/c mice*

Female mice (Charles River Laboratories, Sulzheim, Germany) were housed under conditions as described

above, and were divided into 2 groups of 10 (placebo) and 9 (miltefosine, 40 mg/kg/day), respectively. Treatments and infection were done as described above, but miltefosine treatment was discontinued at day 5 p.i., and mice were observed for another 30 days. Animals exhibiting signs of disease were euthanized at the onset of these symptoms and on day 35 p.i. at the latest, all mice were sacrificed by CO<sub>2</sub>-euthanasia. Collection of tissue samples and storage was done as for Experiment 1

#### *DNA extraction and real-time-PCR*

DNA purification from *in vitro* cultures and cerebral tissues was performed using the DNeasy® Blood & Tissue Kit (Qiagen, Basel, Switzerland) according to the standard protocol suitable for animal tissues. Frozen samples were lysed overnight at 56 °C in 1 ml of ATL buffer containing proteinase K, and 100 µl of the suspension were used for DNA purification. DNA was eluted in 50 µl of AE buffer and boiled at 95 °C. The DNA concentrations in all samples were determined by UV spectrophotometry and adjusted to 100 ng/µl with sterile DNase-free water. The assessments of *N. caninum* tachyzoite loads were performed using a LightCycler™ Instrument (Roche Diagnostic, Basel, Switzerland) as previously described (Cannas *et al.* 2003a,b; Alaeddine *et al.* 2005; Debache *et al.* 2009). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 parasites included in each run.

#### *Statistical analysis*

The significance of the differences between values of the control and experimental assays in parasite load assessment *in vitro* assay was determined by Student's *t*-test using the Microsoft Excel program. *P* values of <0.01 and <0.05 were considered statistically significant. Cerebral parasite loads in different treatment and control groups were analysed by Tukey-Kramer Multiple-Comparison (*P*<0.05). To determine the significance of mean weight loss prior and after treatment in the different experimental groups, one-way ANOVA followed by Duncan's multiple range test were employed.

## RESULTS

#### *In vitro activity of miltefosine in HFF and Vero cells*

In order to investigate potential susceptibility of the host cells used for culture of *N. caninum* tachyzoites, namely Vero cells and HFF, respectively, non-infected confluent monolayers were incubated with different concentrations of miltefosine (1.5–122 µM) for 48 h, and cultures were stained with Trypan blue. Light microscopical inspection revealed that miltefosine had no effect on HFF monolayers, while Vero cell

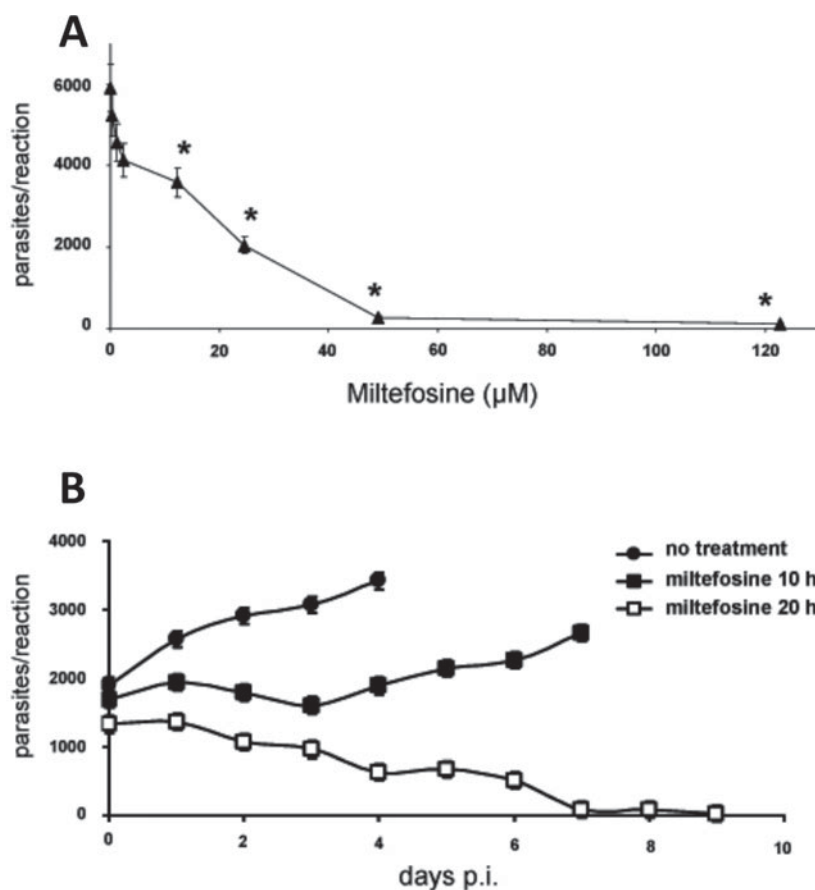


Fig. 1. Effects of miltefosine on the proliferation of *Neospora caninum* tachyzoites in HFF monolayers *in vitro*. (A) Dose-dependent inhibition of tachyzoite proliferation in HFF. The experiment was carried out in triplicate for each concentration and results are presented as means of number of tachyzoites  $\pm$  S.D. Asterisks indicate those parasite numbers significantly differing from the parasite as assessed in the infection control ( $0 \mu\text{g/ml}$ ) ( $P < 0.01$ ). (B) Parasiticidal efficacy of miltefosine against *N. caninum* tachyzoites proliferation. Miltefosine ( $25 \mu\text{M}$ ) was applied for 10 h and 20 h respectively, cultures were washed and further incubated in medium for up to 9 days. From day 6 onwards, all parasite numbers assessed from 20 h post-treatment samples were significantly lower compared to tachyzoite numbers in the infection control and the 10 h post treatment samples. Results are presented as means of number of tachyzoites  $\pm$  S.D.

monolayers contained numerous (up to 50%) Trypan blue positive cells even at the lowest miltefosine concentration used (data not shown). Thus, HFF monolayers were used for further assessments on the effects of miltefosine against *N. caninum*.

#### *In vitro* activity of miltefosine against *Neospora caninum* tachyzoites

Different concentrations of miltefosine ( $1.5$ – $122 \mu\text{M}$ ) were added to *N. caninum*-infected HFF monolayers, and *N. caninum* proliferation was analysed after 3 days. Miltefosine inhibited the multiplication of tachyzoites with an  $\text{IC}_{50}$  of  $5.2 \mu\text{M}$ , and parasite proliferation was completely abolished at  $50 \mu\text{M}$  miltefosine (Fig. 1A).

Subsequently, infected cells were treated with  $25 \mu\text{M}$  miltefosine for 10 h and 20 h, respectively and, after washing in medium, cultures were maintained for an additional 9 days (Fig. 1B). A pronounced inhibition of parasite proliferation was observed in both treatment groups during a period

of 3 days following treatment. Subsequently, tachyzoites in those cultures treated for 10 h resumed proliferation. In contrast, in those specimens treated for 20 h the numbers of tachyzoites decreased continuously until day 9, indicating that in this case miltefosine exhibited parasiticidal efficacy.

#### Effects of miltefosine treatment on the ultrastructure of *Neospora caninum* tachyzoites

The morphological and structural alterations associated with miltefosine treatments in *N. caninum*-infected HFF were visualized by TEM. In non-treated control cultures, intact *N. caninum* tachyzoites were localized within a parasitophorous vacuole, surrounded by a parasitophorous vacuole membrane, and characteristic organelles such as micronemes, rhoptries and dense granules, were clearly identified (Fig. 2A). Upon miltefosine treatment of infected cultures for 10 h, prominent alterations were noted within the tachyzoite cytoplasm, such membrane stacks and an increasing



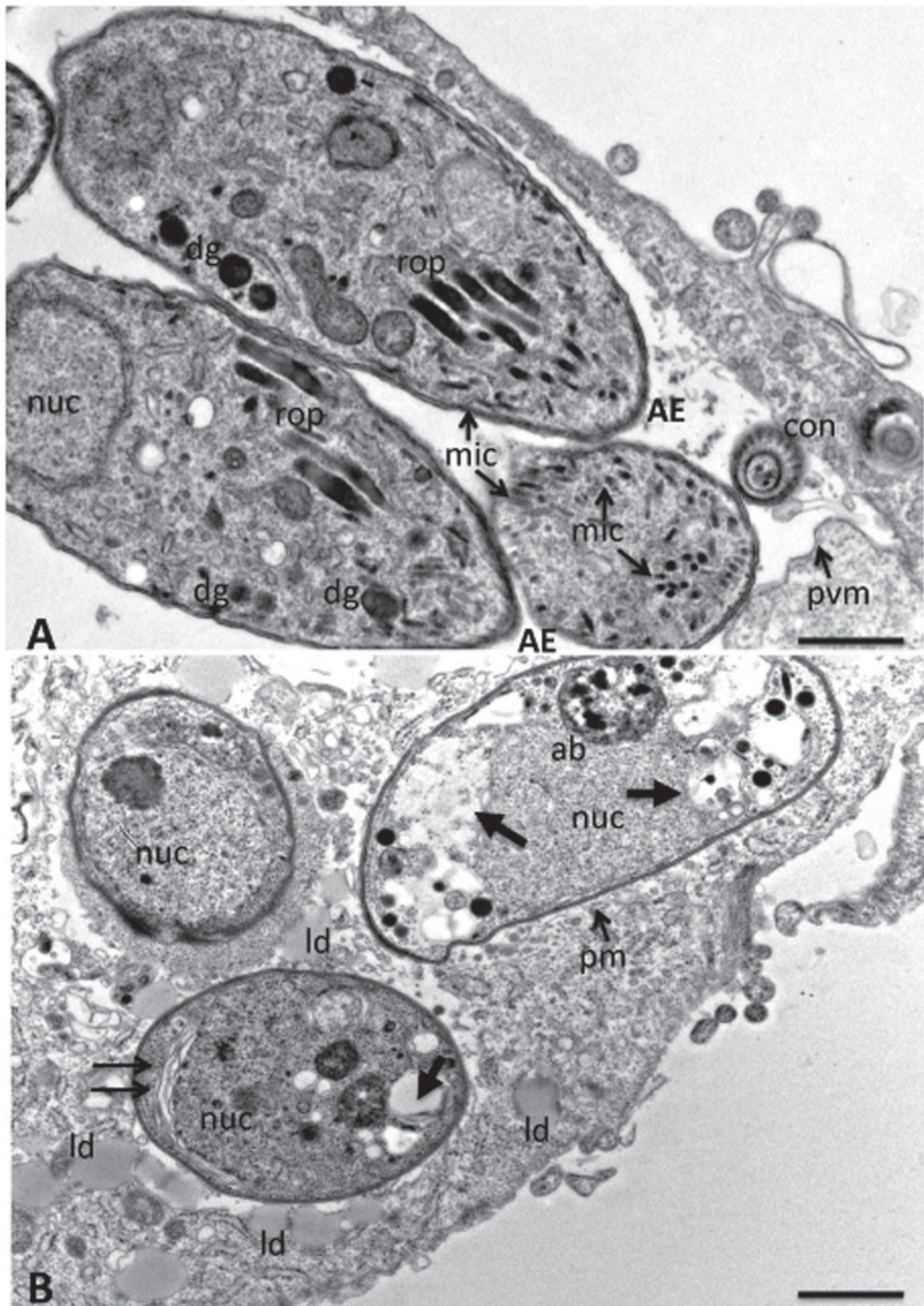


Fig. 2. TEM of *Neospora caninum* tachyzoites grown in HFF. (A) Non-treated control culture, with tachyzoites within a parasitophorous vacuole that is delineated by a parasitophorous vacuole membrane (pvm). The hallmarks of apicomplexan parasites such as rhoptries (rop), micronemes (mic) and dense granules (dg) are clearly visible. AE, anterior end; con, cross-section through a conoid; nuc, nucleus. Scale bar = 0.32  $\mu\text{m}$ . (B) TEM after treatment of infected monolayers with 25  $\mu\text{M}$  miltefosine for 10 h. Note the presence of an apoptotic body (ab) in one tachyzoite, membrane stacks are indicated by 2 thin horizontal arrows, increased vacuolization is indicated by thick arrows, ld, lipid droplets; pm, plasma membrane. Scale bar = 0.38  $\mu\text{m}$ .



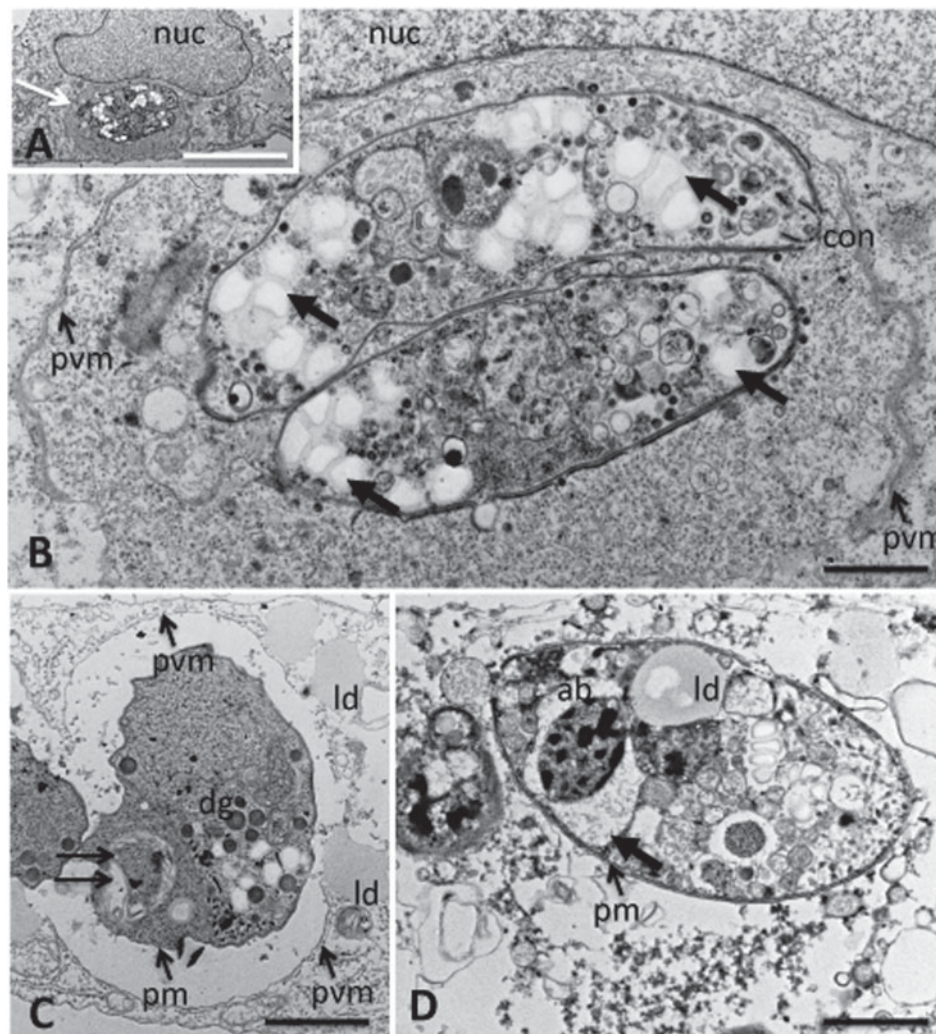


Fig. 3. TEM of *Neospora caninum* tachyzoites grown in HFF and treated with 25  $\mu$ M miltefosine for 20 h. (A) Overview and (B) corresponding higher magnification view of 2 intracellular tachyzoites. Parasites are largely surrounded by a parasitophorous vacuole (pvm), and embedded in a dense granular matrix. Thick arrows indicate extensive vacuolization, con, conoid. Scale bar in (A) = 8  $\mu$ m; in (B) = 0.4  $\mu$ m. (C) Intracellular tachyzoite with alterations such as cytoplasmic membrane stacks (horizontal arrows), still intact plasma membrane (pm) and parasitophorous vacuole membrane (pvm), and lipid droplets (ld) just adjacent to the pvm; dg, dense granules. Scale bar = 0.38  $\mu$ m. (D) Extracellular, non-viable tachyzoite with distorted cytoplasmic organization, containing apoptotic body (ab), lipid droplet (ld) and cytoplasmic vacuoles filled with material of unknown origin (thick arrow). The plasma membrane still appears intact (pm). Scale bar = 0.38  $\mu$ m.

number of vacuoles within the parasite cytoplasm, some of which were filled with membranous and granular material of unknown origin, electron-dense inclusions, and the presence of structures resembling apoptotic bodies in some parasites (Fig. 2B). In addition, lipid droplets formed in the close vicinity of the parasitophorous vacuoles, although clearly localized within the cytoplasm of the host cells (Fig. 2B). Such lipid droplets were not found in uninfected fibroblasts (not shown). Upon incubation of infected cultures with miltefosine for 20 h, the alterations became much more pronounced. Most host cells harboured remnants of parasitophorous vacuoles that were filled with a dense granular matrix, containing completely distorted tachyzoites, and the micronemes, rhoptries and dense granule organelles

were not discernible anymore. The parasite cytoplasm was lacking any distinct subcellular organization; however, in all cases the parasite membrane surrounding the tachyzoites still appeared to be intact (Fig. 3A, B). Few tachyzoites were found that were still localized within a parasitophorous vacuole surrounded by a distinct parasitophorous vacuole membrane, but they also contained vacuoles filled with membrane stacks and other unidentified material, and large lipid droplet were localized just adjacent to the parasitophorous vacuole membrane (Fig. 3C). Many liberated, but clearly distorted tachyzoites were identified in the extracellular space, often surrounded by cellular debris of unknown origin (Fig. 3D). Thus, miltefosine treatment had a profound effect on the ultrastructure of *N. caninum* tachyzoites.

Table 1. *In vivo* studies: summary of experimental groups and outcome of experiments in terms of symptomatic mice, time of death and median body weights at day 0 and at the end of the experiments, day 14 for Exp. 1 and day 30 for Exp. 2

(Significant weight loss is found only in the experimentally infected mice treated with miltefosine (40 mg/kg/day for 14 days) and indicated by \*.)

Groups	Number of mice	Number of sympt. mice	Time of death (days p.i.)	Weight at day p.i.	
				0	14 or 30
Experiment 1	Non-infected miltefosine 40 mg/kg/day 14 days	8	–	–	18.95 ± 0.80 18.86 ± 0.56
	Infected placebo CMC only 14 days	8	2	11/12	19.07 ± 0.99 18.18 ± 0.56
	Infected miltefosine 20 mg/kg/day 14 days	8	1	12	17.9 ± 0.92 17.3 ± 1.59
	Infected miltefosine 40 mg/kg/day 14 days	10	6	7/10/11/12/12/13	20.09 ± 0.73 17.47 ± 1.52*
Experiment 2	Placebo CMC only 5 days	10	8	16/19/22/22/25/26/27/31	19.5 ± 0.86 18.9 ± 0.42
	Miltefosine 40 mg/kg/day 5 days	9	2	5/26	18.85 ± 0.82 18.41 ± 0.61

#### *Miltefosine treatment in mice experimentally infected with Neospora caninum tachyzoites*

In Experiment 1, miltefosine was orally applied to *N. caninum*-infected Balb/c mice at 20 and 40 mg/kg/day for a period of 14 days p. i., respectively, a placebo control group received PBS/CMC only, and a fourth group remained uninfected and was treated with 40 mg/kg/day of miltefosine to assess a potential impact of medication on the health of the animals. After the 14 days treatment period, mice were euthanized and the cerebral parasite burden was assessed. In the placebo group, 2 out of 8 mice succumbed to infection at days 11 and 12 p.i., while in the group treated with miltefosine at 20 mg/kg/day 1 mouse died at day 12 p.i. No significant average weight loss was recorded during the 14 days of treatment (Table 1). Comparison of the cerebral parasite load in the 2 groups did not reveal any major differences (Fig. 4A, Supplementary Table 1, online version only). However, when *N. caninum*-infected mice were treated with 40 mg/kg/day of miltefosine, 6 out of 10 mice exhibited ruffled coat, general weakness and apathy, and they were not able to feed anymore. Thus they had to be euthanized at the onset of these symptoms between days 7 and 13 p.i.. None of the mice exhibited neurological disorders such as tilting and hind limb paralysis. In addition, a significant mean weight loss of over 10% was noted for that group within that 14-day period. Application of miltefosine at 40 mg/kg/day in non-infected mice, however, did not result in any weight loss or signs of toxicity, indicating that the drug *per se* did not exert any toxic effects (Table 1). Mice treated with

40 mg/kg/day for 14 days exhibited significantly lower cerebral parasite burdens compared to the placebo group (Fig. 4A). This result suggested that miltefosine treatment, although not causing any harm by itself, rendered mice more susceptible to acute *N. caninum* infection, and the cause of death of these animals was due to infection of other organs.

In Experiment 2, *N. caninum*-infected mice were treated with 40 mg/kg/day, but only for a period of 5 days p.i., and were then left untreated for 30 days. As indicated in Table 1, miltefosine treatment had a profound impact, with only 2 out of 9 mice succumbing to infection on days 5 (due to weakness and apathy) and 26 (neurological signs such as head tilt and running in circles), respectively. On the other hand, 8 out of 10 mice had to be euthanized in the placebo group between days 16 and 31. These mice exhibited neurological symptoms (head tilt, hind limb paralysis, circular movements, ruffled coat), indicative for CNS involvement. The overall cerebral parasite burden in the miltefosine-treated mice was significantly lower compared to the placebo group (Fig. 4B, Supplementary Table 1, online version only). This showed that the drug inhibited the proliferation of *N. caninum* and dissemination of tachyzoites into the cerebral tissue.

#### DISCUSSION

Miltefosine, originally developed as an anti-cancer agent, is used for the treatment of skin metastases of mammary carcinoma (Leonard *et al.* 2001). Subsequently it was found that miltefosine is also

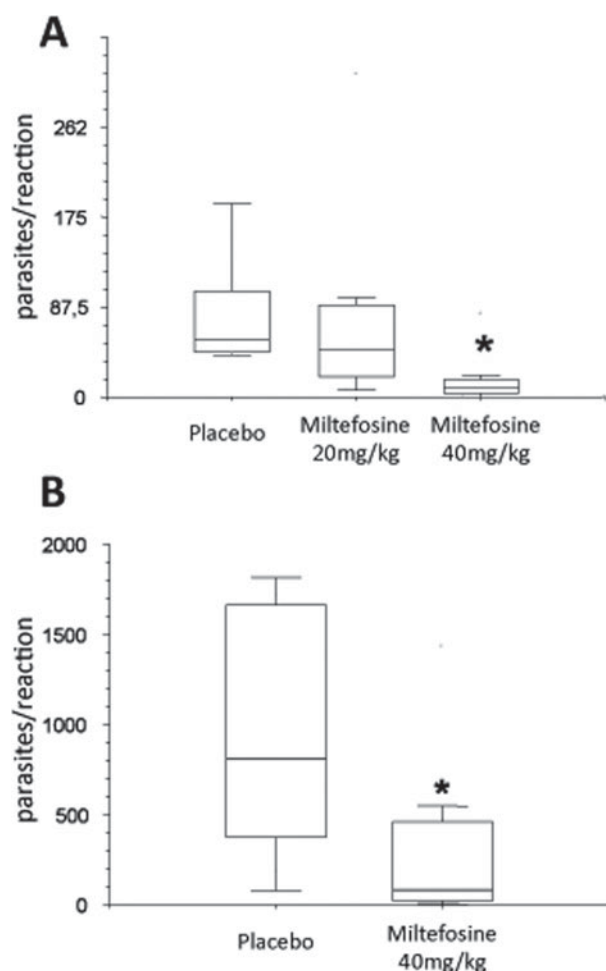


Fig. 4. Box plots showing the effects of miltefosine treatments on the cerebral parasite burden in mice experimentally infected with *Neospora caninum* tachyzoites. (A) Treatment with miltefosine (20 or 40 mg/kg/day for 14 days) or placebo. The y-axis depicts the number of detected parasites by real time PCR /100 ng of DNA. The placebo group was treated identically but with the solvent carboxy-methylcellulose (CMC) only. \* Indicates a significant reduction in parasite load ( $P < 0.05$ ). (B) Treatment with miltefosine (40 mg/kg/day) for a period of 5 days p.i. only, followed by maintenance of mice for 30 days without treatment. \* Indicates a significant reduction in parasite load ( $P < 0.05$ ) in the treatment group. For more details on cerebral parasite burden of each individual mouse refer to supplementary Table 1, online version only.

active against visceral and cutaneous leishmaniasis, and can be applied orally or topically, respectively (Croft and Engel, 2006; Bhattacharya *et al.* 2007). The drug was also active against *Trypanosoma brucei*, *Entamoeba histolytica*, *Acanthamoeba* spp. and *T. cruzi* (Croft *et al.* 1996; Konstantinov *et al.* 1997; Seifert *et al.* 2001; Walochnik *et al.* 2002), and it was shown that miltefosine affects apicomplexan parasites such as *Plasmodium falciparum* and *Cryptosporidium* (Pessi *et al.* 2004; Shahiduzzaman *et al.* 2009). Similar to many of the above-mentioned protozoan parasites, *Neospora* occupies an intracellular niche,

indicating that miltefosine might also be useful for the treatment neosporosis.

*In vitro* cytotoxicity assays demonstrated that Vero cells, which represent an immortalized and highly proliferative cell line that does not undergo contact inhibition, are highly susceptible to the drug. In contrast, human foreskin fibroblasts, which do not proliferate further once a confluent monolayer is formed, were not affected by miltefosine treatment at concentrations up to 122  $\mu\text{M}$ . This reflects the anti-proliferative properties of miltefosine (Leonard *et al.* 2001). The drug inhibited the proliferation of *N. caninum* tachyzoites with an  $\text{IC}_{50}$  of 5.2  $\mu\text{M}$ , which is in a similar range to that reported for the thiazolide nitazoxanide ( $\text{IC}_{50}$  = 4.23  $\mu\text{M}$ ; Esposito *et al.* 2007a), but substantially higher than the dicationic pentamidine derivative DB750 ( $\text{IC}_{50}$  = 0.23  $\mu\text{M}$ ; Leepin *et al.* 2008). Miltefosine appeared to act swiftly: at 25  $\mu\text{M}$  an incubation period of 20 h was sufficient to exert a parasitocidal activity within *N. caninum*-infected HFF. In comparison, nitazoxanide added to infected HFF at 30  $\mu\text{M}$  required a treatment duration of 5 days in order to exert parasitocidal activity *in vitro* (Esposito *et al.* 2005), and for toltrazuril, a 10-day treatment at a concentration of 70.5  $\mu\text{M}$  had only a parasitostatic effect, and a total of 14 days were required to exert a parasitocidal effect (Strohbusch *et al.* 2009).

Toltrazuril (Darius *et al.* 2004), nitazoxanide and other thiazolides (Esposito *et al.* 2005, 2007a, b), and DB750 (Leepin *et al.* 2008), exerted profound ultrastructural changes in *N. caninum* tachyzoites *in vitro*. The same accounted for miltefosine in this study. While we could not detect any major differences between treated and untreated uninfected HFF monolayers (data not shown), clear signs of *N. caninum* tachyzoite deterioration were already detectable after 10 h of treatment, which included profound alterations in the organization of some of the membrane-based compartments of the cytoplasm. Rhoptries and micronemes were mostly no longer visible after 10 h of treatment, while the electron-dense granules were apparently affected by a lower degree, and in many cells rounded structures resembling apoptotic bodies were evident. The tachyzoite cytoplasm exhibited increased vacuolization but, in most cases, no damage could be detected on nuclear membranes and parasite plasma membranes. In several tachyzoites layers of membrane stacks localized near the parasite nucleus were observed. An impressive number of lipid droplets accumulated within the host cell cytoplasm in close vicinity of the parasitophorous vacuole membrane. After 20 h of miltefosine treatment, the majority of tachyzoites were found either intracellularly, obviously non-viable and often embedded in a rather electron-dense granular matrix filling the parasitophorous vacuole, or were present in the extracellular space, without any clearly discernible organized



cytoplasmic features, with the exception of lipid droplets. For the parasites located intracellularly, the electron-dense parasitophorous vacuolar matrix closely resembled the cyst-wall associated structures observed during *in vitro* development of *N. caninum* bradyzoites induced by sodium nitroprusside treatment (Vonlaufen *et al.* 2002, 2004). This matrix could be formed due to stress-related increased secretory activity, most notably from the parasite dense granule organelles (Vonlaufen *et al.* 2004). The presence of extracellular tachyzoites could be a result of increased, drug-induced egress and the inability of the parasite to subsequently invade another host cell successfully, as has previously been demonstrated for a series of thiazolides (Esposito *et al.* 2007a).

The presence of increased numbers of lipid droplets within the host cell and tachyzoite cytoplasm could be caused by disturbances in fatty acid and sterol metabolism as previously reported for *L. donovani* by Rakotomanga *et al.* (2007). It is not clear whether the increased presence of lipid droplets in the vicinity of parasitophorous vacuoles reflects a similar effect in host cells, or whether they are a consequence of stress that is induced by the presence of tachyzoites. More prolonged TEM investigations covering more time-points would be required to study the effects of deteriorating parasites on host cell survival. Similar studies undertaken with nitazoxanide and related compounds (Esposito *et al.* 2005) had suggested that parasite death also had a negative impact on host cell ultrastructure and survival. However, in specimens incubated with miltefosine for 20 h, the vast majority of observed tachyzoites exhibited a clearly discernible plasma membrane, which would be indicative of apoptotic events taking place during drug treatment. This would be in accordance with previous studies on *L. donovani* (Paris *et al.* 2004).

Perez-Victoria *et al.* (2006) had reported that uptake of miltefosine is mediated by a plasma membrane P-type ATPase aminophospholipid translocase. Suggested targets of miltefosine in *Leishmania* include perturbation of ether-lipid metabolism, glycosylphosphatidylinositol (GPI) anchor biosynthesis and signal transduction as well as inhibition of the glycosomal located alkyl-specific acyl-Co-A acyltransferase, an enzyme involved in lipid remodeling (Lux *et al.* 2000). However, more detailed investigations are required in order to define the mechanisms that account for the effects of miltefosine against *N. caninum* tachyzoites.

The relatively low host cell toxicity and clear parasitocidal activity *in vitro* prompted us to undertake an *in vivo* study on the effects of miltefosine treatment on *Neospora* infection in mice. A first set of experimental groups were treated with either 20 mg/kg/day or 40 mg/kg/day for a period of 14 days, after which the animals were euthanized and analysed, similar to previous studies employing the

dicationic compound DB750 (Debache *et al.* 2011). The goal was to investigate whether miltefosine affected cerebral infection. Our results show that in *N. caninum*-infected mice treatment with miltefosine at 20 mg/kg/day did not reduce the cerebral parasite burden compared to the placebo group, while treatment at 40 mg/kg/day did, but those mice could obviously not cope with the infection. In human patients, adverse side effects of miltefosine treatment have been reported such as vomiting, nausea, kintosis, headache, diarrhoea, and a mild to moderate increase in aminotransferases and creatinine (reviewed by Oliveira *et al.* 2011). In contrast, application of miltefosine at 40 mg/kg/day for only 5 days did not exert any adverse effects. Following treatment, mice were monitored for a period of 30 days, and subsequent analysis showed a significantly reduced number of fatalities and a significantly decreased cerebral parasite burden in the miltefosine treatment group. When used for the treatment of *Leishmania* parasites in Balb/c mice, miltefosine treatment for 5 consecutive days at 30 mg/kg/day was highly effective, as was the case when treatment was performed in *Leishmania*-infected *scid* mice (Escobar *et al.* 2001).

The results obtained in *N. caninum* infected Balb/c mice suggest that miltefosine treatment can limit the dissemination of tachyzoites into the CNS. However, further studies are needed to demonstrate the potential of this compound in cases where the CNS has already been invaded by the parasite. Since miltefosine is an amphipatic molecule, it is likely to cross the blood-brain barrier, and further studies will show whether miltefosine can also exert an effect against parasites within the CNS. Since this drug is already in use for the treatment of canine leishmaniasis, its application in cases of canine neosporosis might be an attractive option.

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