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Canine leishmaniosis: *in vitro* efficacy of miltefosine and marbofloxacin alone or in combination with allopurinol against clinical strains of *Leishmania infantum*.

Running Title: In vitro efficacy of antileishmanial drugs

AM. FARCA*, B. MINISCALCO*, P. BADINO**, R. ODORE**, P. MONTICELLI***, A. TRISCIUOGLIO**** & E. FERROGLIO****

Department of Animal Pathology, *section of Clinical Science, ** section of Pharmacology and Toxicology

*** Veterinary surgeon

**** Department of Animal Production, Epidemiology and Ecology, section of Parasitology

Faculty of Veterinary Medicine, University of Turin

Corresponding Author:

Anna Maria Farca:

Department of Animal Pathology

Via L. da Vinci 44

10095 Grugliasco (TO)

anna.farca@unito.it

tel +390116709070

fax + 39 6709083

Abstract

Despite the availability of different therapeutic options, Canine Visceral Leishmaniosis (CVL) remains a challenging disease to treat. Recently miltefosine has been registered for use in dogs and different studies have demonstrated its leishmanicidal effect. Moreover, it has been suggested that fluoroquinolones, compared to standard chemotherapeutic agents, could be an effective and pragmatic alternative to treat CVL.

The efficacy of miltefosine and marbofloxacin alone or in combination with allopurinol against clinical strains of *Leishmania infantum* was assessed *in vitro* by incubating increasing concentrations of the drugs with a standard parasite inoculum. Miltefosine was significantly more efficacious than marbofloxacin ($P<0.05$) against the two strains of *L. infantum* either alone or in combination with allopurinol. Both drugs were significantly ($P<0.05$) more efficacious when associated with allopurinol than alone.

Keywords: Visceral leishmaniasis; Dog, Miltefosine, Marbofloxacin; Allopurinol

1. Introduction

Leishmaniosis is an infectious disease affecting both humans and dogs caused by *Leishmania spp*., an intracellular protozoan that colonizes mononuclear cells.

In the Mediterranean, parts of Asia, and Latin America zoonotic Visceral Leishmaniosis (VL) is due to *Leishmania infantum* (*L. Chagasi*) (Maroli et al. 2010). The parasite is transmitted among dogs and between dogs and human beings by the bite of phlebotomine sand flies, although other vectors may be implicated (Gramiccia and Gradoni 2005; Ferreira et al. 2009).

Canis familiaris is the only confirmed reservoir of zoonotic VL in the Mediterranean area, Middle East and South America where *L. infantum* causes both human and CVL.

Drugs currently used for treatment of CVL are systemic agents like antimony, amminosidine, allopurinol and amphotericin. All these drugs allow to achieve clinical improvement in dogs, although rarely therapy is associated with elimination of parasite carriage or the prevention of clinical disease relapse (Xavier Roura 2010). Moreover, toxicity and the emergence of resistance often limit their efficacy. Therefore investigations are still in progress to discover new antileishmanial drugs (Bianciardi et al. 2004; Pujals et al. 2008; Zhang et al. 2010). Recently miltefosine has been registered for use in dogs and different studies have demonstrated its leishmanicidal effect (Mirò et al. 2009). Moreover, it has been suggested that fluoroquinolones, compared to standard chemotherapeutic agents, could be an effective and pragmatic alternative to treat CVL (Raether et al. 1989; Vouldoukis et al. 2006).

There is general consensus that the combination of meglumine antimoniate with allopurinol represents the most effective therapeutic protocol for treatment of CVL, although it does not allow complete parasitological cure (Torres et

al. 2010). Denerolle and Bourdoiseau (1999) demonstrated that dogs treated with the combination show a longer period of clinical remission than when receiving either drug alone.

Allopurinol**,** a structural analogue of hypoxanthine, is metabolised by *Leishmania* parasites to produce an inactive analogue of inosine. This is incorporated into leishmanial RNA causing faulty protein translation (Baneth and Shaw 2002). When administered to dogs as a single antileishmanial agent for a minimum period of 2-3 months, allopurinol almost always leads to moderate clinical improvement and partial restoration of some laboratory parameters, such as acute phase proteins of inflammation (Cavaliero et al. 1999). Similarly to antimonial drugs, allopurinol does not allow full parasitological cure, and relapses occur when treatment is interrupted. For this reason, allopurinol is usually administered for periods as long as several months. The tolerability of the drug is excellent and few adverse effects have been described (Noli and Auxilia 2005).

Miltefosine (hexadecylphosphocholine) belongs to the family of alkylphospholipids, stable analogues of lysophosphatidylcholine in which the ester linkage has been replaced by a more lipase-resistant ether bond (Barratt et al. 2009). They were first developed as anticancer agents (Unger et al. 1989) and it was later used to treat human leishmaniosis. The miltefosine molecular mechanism of action against cancer cells has been linked to apoptosis as well as lipid-dependent cell signalling pathways (Arthur and Bittman 1998). Similarly, rapid accumulation of miltefosine by *Leishmania* and subsequent apoptosis of the parasite suggest a direct action of the drug on the parasite, probably by impairing the membrane synthesis (Berkovic et al. 1995). Besides miltefosine direct toxicity on *Leishmania* parasites, Wadhone et al. (2009) recently postulated an immunomodulatory effect exerted by the drug via the nitroxyde (NO) pathways. Despite the fact that the drug has been registered for oral treatment of CVL in several European countries, at present few studies concerning the efficacy of miltefosine against *L. infantum* have been published (Xavier Roura 2010). The drug is administered alone or in combination with allopurinol. The clinical efficacy of miltefosine is improved when the drug is administered in combination with allopurinol. Similarly to other antileishmanial drugs, miltefosine is not able to fully eliminate the parasite from infected dogs, although a drastic and progressive reduction of the parasite load in lymph node aspirates has been documented (Manna et al. 2008).

Marbofloxacin is a broad spectrum synthetic third generation fluoroquinolone for veterinary use. Like other fluoroquinolones, marbofloxacin acts by inhibiting the bacterial enzyme DNA gyrase or topoisomerase II which shows structural differences from the mammalian enzyme (Slunt et al. 1996; Prescott et al. 2000). *L. infantum* topoisomerase resembles the bacterial one providing a good target for efficacious control of CVL (Chakraborty and Majumder 1998; Slunt et al. 1996). Furthermore, *in vitro* studies have demonstrated both direct and indirect leishmanicidal activity of marbofloxacin via tumor necrosis factor α (TNF α)) and NO synthase pathways. Vouldoukis et al. (2006) demonstrated

that after treatment with marbofloxacin, macrophages acquire resistance to infection and enhanced antileishmanial activity through the NO synthase pathway.

On these premises, the aim of the study was to compare *in vitro* the efficacy of miltefosine and marbofloxacin alone or in combination with allopurinol against clinical strains of *L. infantum*.

2. Materials and Methods

2.1 Parasites and drugs

L. infantum promastigotes (two Mon-1 strain, A and B, cultured from two infected dogs from North West Italy) were maintained at 27 °C in 199 medium supplemented with 20% HI-FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 40 mM Hepes, 0.1 mM adenine (in 50 mM Hepes), 5 µg/ml hemin (in 50% triethanolamine), and 1 µg/ml 6-biotin (in 95% ethanol). At the stationary phase of growth (Badolato et al. 1996), 1 ml of culture containing 1 x 10^6 parasites/ml were harvested and incubated at 27 °C with increasing concentrations (2.5 x 10^{-4} M – 2.5 x 10^{-6} M) of marbofloxacin (Marbocyl®, Vètoquinol Laboratoires), miltefosine and allopurinol alone or marbofloxacin and miltefosine in combination with allopurinol. Drug efficacy was assessed microscopically by counting and determining the percentage of killed parasites following Trypan blue staining after 24, 48 and 72 h of incubation. Assays were performed in triplicate and the results expressed as mean values ± SEM.

2.2 Drugs and chemicals

Marbofloxacin (Marbocyl®) was obtained by Vetoquinol Laboratoires. Miltefosine was purchased by Virbac, whereas allopurinol was purchased by Sigma Aldrich.

2.3 Statistical analysis

E_{max} was calculated using a computer program (GraphPad Prism) and expressed as percent of killed leishmania. To compare E_{max} between strains the Student's t-test has been used, whereas efficacy among drugs and associations has been evaluated by ANOVA followed by Tukey's Multiple Comparison Test (GraphPad InStat).

3. Results

The E_{max} values of miltefosine and marbofloxacin alone or in combination with allopurinol at different experimental times are reported in Table 1. No statistically significant difference in the efficacy of all drugs tested between the two clinical strains of *L. infantum* was observed (Table 1).

At each experimental time point, miltefosine was significantly more efficacious than marbofloxacin ($P<0.05$) against the two strains of *L. infantum* (Figure 1). This finding was confirmed when considering the association of the two drugs with allopurinol. Miltefosine associated with allopurinol caused a significantly higher percentage of killed parasites than marbofloxacin + allopurinol ($P<0.05$) (Figure 1). Both drugs were significantly ($P<0.05$) more efficacious when associated with allopurinol than alone, although increased efficacy for marbofloxacin was significant only at 72 hours (Figure 1).

4. Discussion

At present the CVL's preferred treatment is represented by meglumine antimoniate alone or in combination with allopurinol, with this last used for long-term maintenance therapy (Noli and Auxilia 2005). However, the treatment does not allow full elimination of the parasites from infected dogs. Indeed, few months after treatment parasites can still be demonstrated from several tissues of clinically cured dogs (Roura 2010). Furthermore, the protocol is expensive and it is associated with side effects. Gastrointestinal signs, pain and local swelling at the injection site are the most frequent side effects following antimonial therapy. Acute pancreatitis and renal lesions have also been described (Aste et al. 2005; Ikeda-Garcia et al. 2007; Bianciardi et al. 2009). Consequently, improved chemotherapy of canine leishmanial infection is still necessary and the need for new molecular targets on which to base future treatment strategies appears justified. Miltefosine is one of the few orally administered drugs effective against *Leishmania.* Despite the fact that it has recently been approved for use in dogs, few data concerning its *in vitro* efficacy have been published to date (Luz et al. 2009; Seifert et al. 2010). According to Luz et al. (2009), miltefosine shows excellent *in vitro* efficacy against *L. donovani* even on strains isolated from relapsed or non responder patients. By contrast, some studies describing its clinical efficacy are available (Manna et al. 2009; Mateo et al. 2009; Mirò et al. 2009).

Studies supporting the *in vitro* and *in vivo* efficacy of marbofloxacin against *Leishmania* have been published by Vouldoukis et al. (2006) and Rougier et al.(2008). Marbofloxacin shows direct and indirect leishmanicidal activity with the latest being the most relevant. The drug inhibits the ribonucleotide reductase involved in DNA replication and induces the production of nitrogen derivates and oxygen metabolites, which leads to parasite death (Vouldoukis et al. 1996).

Our results suggest that miltefosine is more potent *in vitro* against the two field strains tested than marbofloxacin. Miltefosine is known to affect membrane integrity directly or indirectly and is able to exert antileishmanial action independently of cell-mediated parasiticidal mechanisms, whereas marbofloxacin acts mainly via indirect mechanisms (Vermeersch et al. 2009). Other fluoroquinolones such as enrofloxacin were not found to be directly active against *L. infantum* although capable of stimulating a significant macrophage killing in the cells infected by the parasite (Bianciardi et al. 2004).

On the other hand, no *in vitro* studies considering the synergistic effect of both miltefosine and marbofloxacin associated with allopurinol on CVL have been published to date. Our data are consistent with an improved *in vitro* efficacy of the association respect to both miltefosine and marbofloxacin alone on clinically isolated *Leishmania* strains. This finding is in agreement with *in vivo* observations suggesting a longer survival for dogs with leishmaniosis treated with the combination of different anti-leishmania drugs and allopurinol (Noli and Auxilia 2005; Torres et al. 2010).

Either considering the two drugs alone or their association with allopurinol, the data obtained in the present study demonstrate a higher potency of miltefosine respect to marbofloxacin. This finding could be ascribed to the increase of both human- and veterinary-fluoroquinolone resistance observed among clinically isolates (Yooh et al. 2010). However, to date, the mechanisms and spreading of leishmania resistance to drugs have been studied mainly for antimonials (Mandal et al. 2009).

To our knowledge, the only study investigating the *in vitro* efficacy of marbofloxacin against CVL is the one by Vouldoukis et al. (2006). The Authors demonstrated the *in vitro* efficacy of marbofloxacin by exposing monocyte-derived macrophages to *L. infantum* promastigotes (strain MCAN/GR/82/LEM497) proposing the drug could be an effective oral route alternative to treat CVL. Our data seem not to be in line with these previous findings. However, the discrepancy could be ascribed both to the different *in vitro* model and to the parasite strain because we use "wild strains" isolated from naturally infected dogs, while Vouldoukis et al. (2006) used a laboratory maintained strain. Further studies are needed to investigate the *in vitro* efficacy of marbofloxacin and its association with allopurinol against both wild and laboratory strains of *Leishmania* spp.

To conclude, our results suggest that miltefosine alone or in association with allopurinol shows significant *in vitro* efficacy against clinical strains of *L. infantum*. The efficacy of marbofloxacin is significant only in association with allopurinol after 72 hours of incubation.

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Table 1

E_{max} of miltefosine and marbofloxacin alone or in combination with allopurinol at different experimental time points (24, 48 and 72 hours of incubation) against two clinical strains (A and B) of *L. infantum*. Mean values ± SEM of triplicate assays. Student's *t* test ($P<0.05$).

Drugs	**Strain A Emax (%)**	**Strain B Emax (%)**	**P**
Miltefosine 24h	35.37 ± 1.10	37.9 ± 4.41	ns
Miltefosine+ Allopurinol 24 h	55.75 ± 4.48	56.71 ± 1.30	ns
Marofloxacin 24h	20.52 ± 1.15	24.40 ± 1.77	ns
Marbofloxacin + Allopurinol 24h	30.24 ± 1.42	27.33 ± 0.50	ns
Miltefosine 48h	52.55 ± 1.14	55.66 ± 0.95	ns
Miltefosine+ Allopurinol 48h	75.2 ± 0.53	73.23 ± 0.75	ns
Marbofloxacin 48h	25.37 ± 3.20	22.01 ± 1.11	ns
Marbofloxacin + Allopurinol 48 h	29.95 ± 1.62	32.16 ± 0.59	ns
Miltefosine72h	54.06 ± 2.43	59.14 ± 1.95	ns
Miltefosine+ Allopurinolo 72h	78.23 ± 0.63	80.62 ± 1.20	ns
Marbofloxacin 72h	19.40 ± 1.03	20.23 ± 0.51	ns
Marbofloxacin + Allopurinol 72h	38.5 ± 4.54	37.55 ± 2.94	ns

Figure 1

Percent (%) of killed leishmania for miltefosine and marbofloxacin alone and in combination with allopurinol at different experimental time points (24, 48 and 72 hours of incubation). GraphPad Prism.