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Evaluation of the inhibitory effects of miltefosine on the growth of *Babesia* and *Theileria* parasites

Mahmoud AbouLaila[a,b], Davasorin Batadoj[a], Akram Salama[a,c], Tserendorj Munkhjargal[a], Madoka Ichikawa-Seki[a], Mohammad A. Terkawi[a], Naoaki Yokoyama[a], Ikuo Igarashi[a,*]

[a] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*
[b] *Department of Parasitology, Faculty of Veterinary Medicine, University of Sadat City, Sadat City 32897, Minoufiya, Egypt*
[c] *Department of Infectious Diseases and Internal Medicine, Faculty of Veterinary Medicine, University of Sadat City, Sadat City 32897, Minoufiya, Egypt*

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ABSTRACT

Miltefosine, a membrane-active synthetic ether-lipid analogue, has antiproliferative and antiparasitic effects. In this study, the inhibitory effects of miltefosine were evaluated against three *Babesia* species and *Theileria equi in vitro* and against *Babesia microti* in mice. The drug showed significant growth inhibition from an initial parasitemia of 1% for *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *T. equi* with IC50 values of 25, 10.2, 10.4, and 99 μM, respectively. Complete inhibition was observed at 200 μM of miltefosine on the third day of culture for the three *Babesia* species and 400 μM on the fourth day for *T. equi*. Reverse-transcription PCR (RT-PCR) showed that miltefosine inhibited the transcription of choline-phosphate cytidylyltransferase in *B. bovis*. Miltefosine at a dose rate of 30 mg/kg resulted in a 71.7% inhibition of *B. microti* growth in BALB/c mice. Miltefosine might be used for drug therapy in babesiosis.

1. Introduction

Babesia, a major pathogen infecting erythrocytes in animals, is transmitted by ticks to vertebrates and results in serious economic losses in the livestock industry worldwide. The clinical signs of babesiosis include malaise, fever, hemolytic anemia, jaundice, and hemoglobinuria (Kuttler, 1988). *Babesia microti* usually causes infection in rodents and sometimes humans in North America and other areas (Kogut et al., 2005). Several babesicidal drugs that have been in use for years have proven ineffective due to problems related to their toxicity (Vial and Gorenflot, 2006). Therefore, the development of new drugs that have a chemotherapeutic effect against babesiosis with high specificity to the parasites and low toxicity to the hosts is urgently needed.

In most eukaryotic organisms, phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) are the major phospholipids of cellular membranes, whereas PtdCho and PtdEtn represent 40–50% and 35–40%, respectively, of the total phospholipids in *Plasmodium falciparum* (*P. falciparum*) (Vial et al., 1992). PtdCho and PtdEtn are the most abundant phospholipids in *Babesia bovis* (31.8% ± 2.8 and 27.8 ± 2.5 of the total phospholipids, respectively) (Florin-Christensen et al., 2000).

In *P. falciparum*, PtdCho and PtdEtn are synthesized through three major routes: *de novo* cytidine

* Corresponding author. Tel.: +81 155 49 5641; fax: +81 155 49 5643.
E-mail addresses: igarcpmi@obihiro.ac.jp, protozoa@obihiro.ac.jp (I. Igarashi).

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diphosphate (CDP)-choline (Kennedy pathway), serine decarboxylation-phosphoethanolamine methylation (SDPM), and the CDP-diacylglycerol pathway (Ben Mamoun et al., 2010). The *de novo* CDP-choline pathway is the major route for the synthesis of PtdEtn in *Toxoplasma gondii* (Sampels et al., 2012) and mammalian cells (Lykidis and Jackowski, 2001).

In the *P. falciparum* Kennedy pathway, choline is phosphorylated to phosphocholine by a *P. falciparum*-specific choline kinase (PfCK), subsequently coupled to phosphocholine cytidylyltransferase (CTP) to generate CDP-Cho by a CDP-choline cytidylyltransferase (PfCCT), and further converted into PtdCho by a *P. falciparum*, CDP-diacylglycerol-cholinephosphotransferase (PfCEPT), in the presence of diacylglycerol (Ben Mamoun et al., 2010).

In the *P. falciparum* SDPM pathway, phosphatidylcholine is synthesized from serine and ethanolamine. Serine is either transported from the host or obtained from the degradation of host hemoglobin. Ethanolamine can be obtained in limited amounts from plasma and in larger quantities from serine decarboxylation by a parasite-encoded serine decarboxylase. Ethanolamine formed *via* this reaction is subsequently phosphorylated into phosphoethanolamine, which serves as a substrate for PtdEtn biosynthesis or is converted into phosphocholine and incorporated into PtdCho (Ben Mamoun et al., 2010). The CDP-diacylglycerol pathway initiates from serine and CDP-diacylglycerol to form phosphatidylserine, which is then converted into PtdEtn *via* the activity of phosphatidylserine decarboxylase enzymes. PtdEtn is subsequently methylated into PtdCho by PtdEtn methyltransferase (Pessi et al., 2004).

P. falciparum retains various enzymes that are important for synthesis of phospholipids from precursors produced by the parasite metabolic machineries or scavenged from human serum (fatty acids, serine, inositol, and choline) (Ancelin and Vial, 1989). The enzymes for the synthesis of the major phospholipids are critical for the rapid multiplication of the parasite within human erythrocytes, and they display properties that differ sufficiently from their human counterparts to be considered good targets for chemotherapy (Wengelnik et al., 2002). A gene encoding for choline-phosphate cytidylyltransferase (CCT), the second enzyme in the Kennedy pathway, is found in the genome sequence database of *B. bovis* (Accession number: EDO06474) (Brayton et al., 2007) and *Theileria equi* (formerly *Babesia equi*) (Accession number: XM_004830243) (Kappmeyer et al., 2012). The parasite enzyme amino acid sequences have low identities of 15% and 14.3% with the bovine and equine homologues, respectively. CCT is the target of miltefosine in cancer cells (Jimenez-Lopez et al., 2002). Furthermore, phosphoethanolamine N-methyltransferase (PMT) is the target of miltefosine in *P. falciparum* (Pessi et al., 2004), but PMT was found in neither the *B. bovis* nor the *T. equi* genome sequence databases. This may indicate that *Babesia* species and *T. equi* might only use the *de novo* CDP-choline pathway as the main source of PtdCho biosynthesis.

Miltefosine (1-*O*-hexadecylphosphocholine) is a membrane-active synthetic ether-lipid analogue. Miltefosine displays antitumor (Hilgard et al., 1988; Leonard et al., 2001), antifungal (Widmer et al., 2006), and antibacterial (Llull et al., 2007) activities. Miltefosine has shown antiprotozoal activity against a wide range of parasites in a number of genera (Azzouz et al., 2005; Croft et al., 1996; Pessi et al., 2004; Saraiva et al., 2002; Seifert et al., 2001; Walochnik et al., 2009; Blaha et al., 2006; Eissa and Amer, 2012). The aim of the present study was to evaluate the inhibitory effects of miltefosine on the *in vitro* growth of three *Babesia* species and *T. equi* and on the *in vivo* growth of *B. microti*. A further aim was to study the effect of miltefosine on the transcription of *B. bovis* choline-phosphate cytidylyltransferase by RT-PCR.

2. Materials and methods

2.1. Chemical reagents

Miltefosine was purchased from Sigma–Aldrich (Saint Louis, USA). Stock solutions of 100 mM in double-distilled water (DDW) were prepared and stored at −30 °C until use. Diminazene aceturate (Ganaseg) was purchased from (Ciba-Geigy Japan Ltd., Tokyo, Japan) and used as a positive control drug. A working stock solution of 10 mM dissolved in DDW was prepared and stored at −30 °C until required for use.

2.2. Rodent Babesia and mice

The Munich strain of *B. microti* was maintained by serial passage in the blood of BALB/c mice (AbouLaila et al., 2012). Thirty female BALB/c mice (8 weeks old) were purchased from CLEA Japan (Tokyo, Japan) and used for the *in vivo* studies.

2.3. In vitro cultivation of Babesia parasites

Miltefosine was evaluated for its chemotherapeutic effect against *B. bovis* (Texas strain) (Hines et al., 1995), *Babesia bigemina* (Argentina strain) (Bork et al., 2004b), *Babesia caballi* (Bork et al., 2004b), and *T. equi* (U.S. Department of Agriculture) (AbouLaila et al., 2010a). Parasites were cultured in bovine or equine red blood cells using a continuous micro-aerophilous stationary phase culture system (AbouLaila et al., 2010b). The culture medium, M199, applied to *B. bovis*, *B. bigemina*, and *T. equi* (obtained from Sigma–Aldrich, Tokyo, Japan), was supplemented with 40% bovine or equine serum and 60 U/ml of penicillin G, 60 μg/ml of streptomycin, and 0.15 μg/ml of amphotericin B (Sigma–Aldrich, Tokyo, Japan). TES-hemisodium salt (229 mg/ml) N-tris-(hydroxymethyl)-methyl-2-aminoethansulfonic acid; 2-[(2-hydroxy-1,1-bis- [hydroxymethyl] ethyl)amino] ethane sulfonic acid (Sigma–Aldrich, Saint Louis, USA) was added to bovine *Babesia* parasite cultures as a pH stabilizer (pH 7.2) (AbouLaila et al., 2010c).

Hypoxanthine (ICN Biomedicals, Inc., Aurora, OH) was added to the *T. equi* culture as a vital supplement at 13.6 mg/ml. For *B. caballi*, the culture medium RPMI 1640 was supplemented with 40% horse serum, antibiotics, and amphotericin B (AbouLaila et al., 2010a).