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In vitro effect of new formulations of amphotericin B on amastigote and promastigote forms of *Leishmania infantum*

Lara Ordóñez-Gutiérrez [a], Raquel Espada-Fernández [b], María Auxiliadora Dea-Ayuela [c], Juan José Torrado [b], Francisco Bolás-Fernandez [c,*], José María Alunda [a]

[a] *Department of Animal Health, Faculty of Veterinary Medicine, Avda. Puerta de Hierro s/n, Complutense University, Madrid 28040, Spain*
[b] *Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, Avda. Complutense s/n, Complutense University, Madrid 28040, Spain*
[c] *Department of Parasitology, Faculty of Pharmacy, Avda. Complutense s/n, Complutense University, Madrid 28040, Spain*

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Abstract

The in vitro antileishmanial activities of various new amphotericin B (AMB) formulations were investigated, including microspheres of hydrophilic albumin with three AMB aggregation forms (monomeric, dimeric and multiaggregate) and the polymers of polylactic-co-glycolic acid, Resomer RG502 and RG503 with the multiaggregate AMB form. This in vitro study was performed on the extracellular promastigote form and the intracellular amastigote form of a canine strain of *Leishmania infantum* (UCM 20) using the infected J774 murine macrophage-like cell line. Albumin-encapsulated forms did not show any toxicity for murine cells and had lower median effective concentration (EC_{50}) values (ca. 0.003 μg/mL) for *L. infantum* amastigotes than free formulations (0.03 μg/mL). In addition, the aggregation state of AMB had a notable effect on the antileishmanial activity of the drug. Results obtained in vitro point towards interest in monomeric AMB encapsulated in microspheres in the chemotherapeutic control of leishmaniasis.

Keywords: Amphotericin B; In vitro; *Leishmania infantum*; Microencapsulation; Albumin; Amastigote

1. Introduction

Leishmania infantum is the causative agent of human and canine leishmaniasis in the western Mediterranean. A rise in human cases has been observed in recent years linked to human immunodeficiency virus (HIV) infection [1,2]. Dogs are the main domestic reservoir of this parasite and play a central role in the transmission cycle to humans via phlebotomine sandflies, but dogs are also affected by the disease [3]. The infection affects 6–9% of the total dog population of the area, reaching prevalences of 30% in some selected areas [4,5].

Canine leishmaniasis is characterised by a long asymptomatic phase before the infection is clinically patent. In addition, infection levels are probably higher since most asymptomatic canine infections are undiagnosed in endemic areas [6]. Despite the results obtained with some antigens [7], vaccination is not yet available and vector control is difficult. Chemotherapy is therefore currently the main approach to limit disease extension. Most commonly used drugs (antimonials, allopurinol) are, for the most part, toxic and do not result in parasitological cure of infected individuals. The search for new drugs, drug combinations and/or administration schedules is still an open area of research.

The polyene antibiotic amphotericin B (AMB) is a standard drug used for the treatment of systemic fungal infections. The mechanism of action of the compound is related to its binding to fungal membrane sterols. Since *Leishmania* membranes contain ergosterol, the drug impairs cell permeability of the parasites, with loss of small cations, particularly K^+,

* Corresponding author. Present address: Department of Parasitology, Faculty of Pharmacy, Plaza de Ramon y Cajal s/n, Complutense University, Madrid 28040, Spain. Tel.: +34 91 394 1818; fax: +34 91 394 1815.
E-mail address: francisb@farm.ucm.es (F. Bolás-Fernandez).

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causing cell death [8,9]. Thus, its use has been extended to leishmaniasis chemotherapy as a second-line treatment for emerging strains resistant to commonly used products [10]. AMB presents a notable effect against *Leishmania*, although its toxicity, related to the similarity between leishmanial ergosterol and mammalian cell cholesterol, has limited its use in clinical practice.

Among possible solutions to reduce the toxicity of the antibiotic, some vehicles such as lipid emulsions, liposomes and nanoparticles have already been assayed and are presently in use [11–13], especially in cases of antimony resistance. However, their high cost, related to the high production costs of the available AMB preparations (i.e. liposomes) [14], preclude them from widespread use in developing countries and in canine treatment.

Microcapsules are an effective and cheap carrier system and are particularly good to act on phagocytic cells. Among them, microcapsules of albumin, a highly conserved biodegradable molecule, have already been studied [15], used and marketed (Optison^{TM}, albumin microcapsules with octafluoropropane).

The aim of this investigation was to evaluate in vitro the possible advantages of a new formulation with different aggregation states of AMB in albumin compared with polylactic-co-glycolic acid (PLGA) microspheres.

2. Material and methods

2.1. Parasite

An autochthonous isolate of *L. infantum* (UCM 20), obtained from an ill dog in Madrid, Spain, by the Department of Animal Health of the Facultad de Veterinaria, Universidad Complutense de Madrid (UCM), was routinely maintained as promastigotes in Schneider medium (Sigma, St Louis, MO) at 26 °C supplemented with heat inactivated (30 min at 56 °C) foetal bovine serum (FBS) (Sera Laboratories International, Horsted Keynes, UK) and 100 U/mL penicillin + 100 μg/mL streptomycin (BioWhittaker, Verviers, Belgium) in 25 mL culture flasks.

2.2. Cells

J774 cells (murine monocyte-like cell line), donated by the Department of Pharmacology (Faculty of Pharmacy, UCM) were grown in 25 mL flasks in Minimum Essential Medium Eagle (Sigma) supplemented with FBS and antibiotics as above in a humidified 5% CO_2/air atmosphere at 37 °C.

2.3. Compounds tested

The compounds studied in the present work were three aggregation states of AMB (monomeric, dimeric and multiaggregate) free in aqueous solutions and encapsulated in albumin microspheres as well as the multiaggregate form encapsulated in two commercial polymers of PLGA, Resomer RG502 and RG503. Aggregation states were prepared in our laboratory following Sánchez-Brunete et al. [16].

2.4. Promastigote assay

Promastigotes (10^5 parasites/well) were cultured in 96-well plastic plates. Various dilutions of the compounds (0.002, 0.004, 0.02, 0.04, 0.1, 0.4, 1, 2 and 4 μg/mL for free AMB and 0.002, 0.0032, 0.004, 0.02, 0.032, 0.04, 0.2, 0.4, 1.6 and 3.2 μg/mL for encapsulated forms) up to 200 μL final volume were added. After 48 h at 26 °C, 20 μL of AlamarBlue reagent (Serotec Ltd., Oxford, UK) was added and the absorbance (570 nm and 600 nm) was determined to calculate growth inhibition (%) [17].

2.5. Cell cytotoxicity assay

J774 cells were counted in an improved Neubauer chamber (using vital staining Trypan blue) and 10^4 cells/well were placed in a 96-well plate with different dilutions of the compounds (0.002, 0.004, 0.02, 0.04, 0.1, 0.4, 1 and 2 μg/mL for free AMB and 0.002, 0.0032, 0.004, 0.02, 0.032, 0.04, 0.2, 0.4, 1.6 and 3.2 μg/mL for encapsulated forms). After 48 h, medium was eliminated and 200 μL of fresh supplemented medium plus 20 μL of AlamarBlue were added and the absorbance was measured as above. Cultures were performed at least in triplicate. Negative control cultures without AMB and with empty microspheres of the three types tested (human serum albumin and PLGA RG502 and RG503) were included.

2.6. Amastigote assay

In the amastigote assay, 10^4 J774 cells/well were cultured in 8-well Lab-Tek chambers (Nunc, Roskilde, Denmark) using a modification of the method described by Méndez et al. [18]. Briefly, once macrophages were adhered, 10^5 stationary phase *Leishmania* promastigotes/well were added and maintained at 33 °C in 5% CO_2 overnight. Non-internalised promastigotes were eliminated and dilutions of compounds (five encapsulated forms and three free forms of AMB) were added for 48 h. Slides were fixed and stained (Giemsa) and the number of amastigotes/100 cells was determined. Cultures were performed at least in triplicate.

2.7. Statistics

Results obtained (mean ± standard deviation) were analysed by two-tailed analysis of variance.

3. Results and discussion

None of the three aggregation states (monomeric, dimeric and multiaggregate) of free AMB were able to reduce