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

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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.
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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^+,

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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™, 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 Alamar-Blue 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

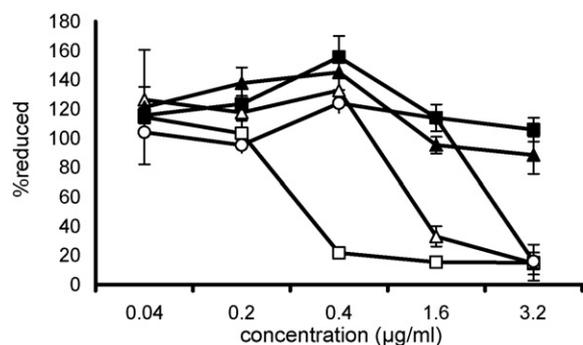None of the three aggregation states (monomeric, dimeric and multiaggregate) of free AMB were able to reduce

Fig. 1. Effect of encapsulated amphotericin B (AMB) on promastigotes of *Leishmania infantum*. Results are expressed as percentage reduction in culture medium with AlamarBlue reagent. (△) Monomeric AMB in albumin microspheres; (□) dimeric AMB in albumin microspheres; (○) multiaggregate AMB in albumin microspheres; (▲) multiaggregate AMB in RG502; and (■) multiaggregate AMB RG503.

Fig. 2. Effect of free amphotericin B (AMB) on amastigotes of *Leishmania infantum*. Results are expressed as number of amastigotes/100 cells. (♦) Monomeric AMB; (□) dimeric AMB.

Fig. 3. Effect of encapsulated amphotericin B (AMB) on amastigotes of *Leishmania infantum*. Results are expressed as number of amastigotes/100 cells. (△) Monomeric AMB in albumin microspheres; (□) dimeric AMB in albumin microspheres; (○) multiaggregate AMB in albumin microspheres; (▲) multiaggregate AMB in RG502; and (■) multiaggregate AMB in RG503.

the *Leishmania* extracellular promastigote population in the range of concentrations tested (not shown). However, when this parasitic stage was exposed to encapsulated AMB, both in albumin and in PLGA microspheres (Fig. 1), all AMB preparations significantly reduced promastigote numbers at drug concentrations >0.2 μg/mL and completely eliminated parasites in the culture medium at the highest drug concentration tested (3.2 μg/mL).

Despite the results obtained with *Leishmania* promastigotes, it should be kept in mind that this parasitic genus has two main forms (promastigotes and amastigotes) with different morphological and biochemical characteristics [19], including energy metabolism [20,21] and transport across membranes [20]. Most of the drug-screening experiments against *Leishmania* have been performed on promastigotes and not on the actual parasite phase causing infections in humans and dogs [22]. Therefore, these in vitro tests against promastigotes are of limited value since the physiological and biochemical differences between the parasite stages are not taken into account. Even in the case of drugs targeted against 'universal' metabolic pathways, the differences in the parasite lifecycle could mask the results obtained. Whilst the extracellular promastigote stage is similar to the form found in the gut of the sandfly vector, the parasite stage (the amastigote) affecting vertebrates, among them humans and dogs, is an intracellular form residing inside macrophages and this radical difference is responsible, among other reasons, for the antileishmanial activity of free AMB in dogs and humans, whereas it is not able to affect significantly promastigote
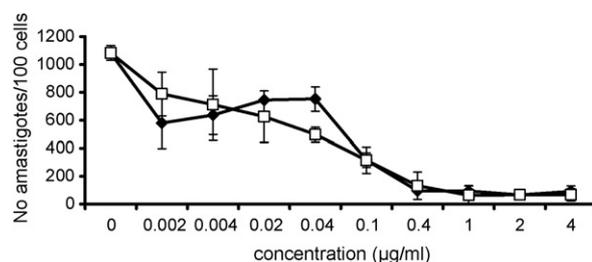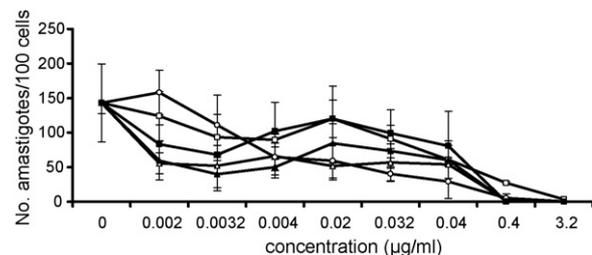
multiplication, as seen in our results and as widely reported [23]. On these grounds, the available AMB preparations were tested against amastigotes cultured in the macrophage-like murine cell line J774, using the in vitro infection model developed in our laboratory [18] with some modifications.

Preliminary assays on the possible toxicity of AMB for the host cells (J774) were carried out. In a similar way to that described for promastigotes, all preparations and drug concentrations were tested, including empty albumin and PLGA microspheres. In our experimental conditions, 4 μg/mL of free AMB, particularly the multiaggregate, was toxic for macrophage populations (Table 1). These results agree with the correlation found between the aggregation state of AMB and toxicity for red blood cells [24]. None of the encapsulated AMB preparations significantly affected J774 cellular viability, thus confirming the advantages of microencapsulation in reducing toxicity for mammalian cells.

The high toxicity for murine cells observed in this study with the multiaggregate AMB precluded its use in the efficacy study against *Leishmania* amastigotes infect-

Table 1
Mean ± standard deviation LD$_{50}$ (μg/mL) of free and encapsulated amphotericin B (AMB) on amastigotes of *Leishmania infantum*

	Albumin microspheres			PLGA polymer microspheres	
	Monomeric AMB	Dimeric AMB	Multiaggregate AMB	RG502 with multiaggregate AMB	RG503 with multiaggregate AMB
Encapsulated AMB	0.0037 ± 0.00232	0.037 ± 0.0113	0.0039 ± 0.0022	0.033 ± 0.0115	0.08 ± 0.0463
Free AMB	0.069 ± 0.01	0.033 ± 0.007	Toxic	N.D.	N.D.

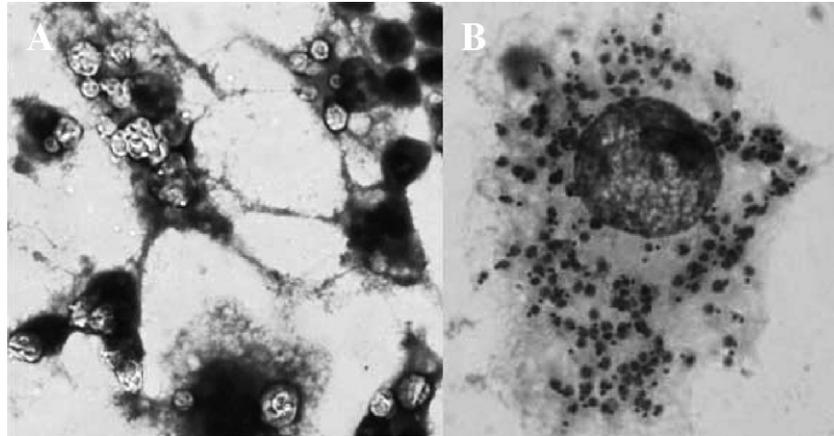LD$_{50}$, 50% lethal dose; PLGA, polylactic-co-glycolic acid; N.D., not determined.

Fig. 4. (A) J774 murine macrophage-like cells treated with polylactic-co-glycolic acid (PLGA) microspheres showing intracytoplasmic refringent bodies. (B) J774 cells infected with *Leishmania infantum*. Magnification 1000×.

ing J774 cells. Both monomeric and dimeric free AMB caused a significant reduction in parasite burden with AMB concentrations >0.4 μg/mL (Fig. 2), although in no case was complete clearance of intracellular parasites in the macrophages observed. The antileishmanial activity was improved by the encapsulated AMB preparations. Despite variations, all formulations completely eliminated the macrophage infections at the same drug concentration (0.4 μg/mL) (Fig. 3). Interestingly, it was found that the empty microspheres of albumin, PLGA RG502 and RG503 induced a certain leishmanicidal/leishmaniostatic effect with reductions of 57%, 20% and 14%, respectively, in parasite burden compared with the untreated cultures. The unspecific antileishmanial effect was related to the presence inside the macrophage cytoplasm of refringent particles of similar size to PLGA observed by microscopic observation (Fig. 4), irrespective of the empty/filled state of the microspheres. The possibility of this microsphere uptake triggering the respiratory burst or other antileishmanial mechanism should be explored given the low cell toxicity of microspheres.

From our results with *Leishmania*-infected macrophages (Table 1), it appears that encapsulation of AMB significantly reduces the toxicity of the drug for mammalian cells and, in addition, the 50% lethal dose (LD_{50}) values for *Leishmania* amastigotes were also lower, although the variations among cultures were not statistically significant ($P < 0.05$).

Encapsulation of AMB to reduce its toxicity and to improve its in vivo activity against *Leishmania* has been addressed previously (i.e. liposomes and other preparations) [25–28] as well against other pathogens (i.e. *Candida*) [29]. However, the in vitro experiments carried out testing Abelcet® and Fungizone® on the same parasite species (*L. infantum*) both with the free form and the encapsulated (liposomal) preparations of AMB, yielded LD_{50} values 10 times higher [30] than those observed here. Comparable results have been obtained with other *Leishmania* species, with LD_{50} values of 0.2–0.6 μg/mL for free AMB against *L. major* amastigotes [13,31], which are much higher values than those obtained by us.

From our experiments, it appears that albumin encapsulation of AMB, besides the low cost of production and preparation, clearly reduces toxicity for host cells and improves the efficacy of AMB in *Leishmania* amastigote clearance. Moreover, the aggregation-related efficacy of the drug points towards the potential interest of combining monomeric AMB in albumin microcapsules in the treatment of human and canine leishmaniasis. These results are in line with those obtained with *Candida* [29]. For ethical reasons, there is a need for in vitro assays when exploring new chemotherapeutic approaches; however, the unusual biopharmaceutical properties of AMB [32] and the complex interactions between drug uptake/drug toxicity [33] probably necessitate testing this AMB preparation in vivo to confirm the predictive value of in vitro tests with *Leishmania*-infected macrophages.

Acknowledgments

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