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PLGA nanoparticles and nanosuspensions with amphotericin B: Potent *in vitro* and *in vivo* alternatives to Fungizone and AmBisome

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ABSTRACT

This paper describes the development of poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles (NPs) and nanosuspensions with the polyene antibiotic amphotericin B (AmB). The nanoformulations were prepared using nanoprecipitation and were characterised with respect to size, zeta potential, morphology, drug crystallinity and content. Standard *in vitro* sensitivity tests were performed on MRC-5 cells, red blood cells, *Leishmania infantum* promastigotes and intracellular amastigotes and the fungal species *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum*. The *in vivo* efficacy was assessed and compared to that of Fungizone and AmBisome in the acute *A. fumigatus* mouse model at a dose of 2.5 and 5.0 mg/kg AmB equivalents. The developed AmB nanoformulations were equivalently or more effective against the different *Leishmania* stages and axenic fungi in comparison with the free drug. The *in vitro* biological activity, and especially hemolytic activity, clearly depended on the preparation parameters of the different nanoformulations. Further, we demonstrated that the superior *in vitro* antifungal activity could be extrapolated to the *in vivo* situation. At equivalent dose, the optimal AmB-loaded PLGA NP was about two times and the AmB nanosuspension about four times more efficacious in reducing the total burden than AmBisome. The developed AmB nanomedicines could represent potent and cost-effective alternatives to Fungizone and AmBisome.

1. Introduction

The polyene antibiotic amphotericin B (AmB) is still the gold standard therapy for invasive fungal infections [1,2] and, additionally, has been used as second-line drug in the treatment of visceral leishmaniasis (VL) since the 1960s. Its liposomal formulation AmBisome is currently the first-choice treatment for VL in southern Europe endemic countries as well as in other developed countries [3]. The formation of pores permeable to cations, anions and small nonelectrolytes in the plasma membrane lies at the basis of AmB's fungicidal and leishmanicidal action [4,5]. Membrane sterols are considered indispensable for the mechanism of pore formation and ergosterol-containing membranes are selectively targeted by AmB over cholesterol-containing ones [6]. Further, AmB was shown to inhibit the fungal proton ATPase, to induce lipid peroxidation of cell membranes and to trigger apoptosis-like responses in fungal cells and *Leishmania* promastigotes [5,7].

AmB's major drawback is its poor water solubility at physiological pH, notably <1 mg/l [2,8], limiting oral delivery and complicating formulation strategies for parenteral administration. Fungizone, a micellar dispersion of AmB with sodium deoxycholate, has been available since 1958 for the treatment of fungal infections and is considered the classical formulation of AmB with, unfortunately, serious disadvantages [2]. Approximately 80% of patients show various acute side effects such as fever, chills, vomiting, headache or nausea during intravenous (IV) therapy with Fungizone and about 30% exhibit renal malfunctions [4]. Fungizone's dose-dependent nephrotoxicity is thought to be the consequence of its extensive association with low-density lipoproteins (LDL) and subsequent uptake by kidney cells bearing internalisable LDL receptors [7]. In the 1990s a second generation of AmB formulations was introduced to market: (i) AmBisome that consists of small unilamellar liposomes, (ii) Abelcet, which is composed of ribbon-like AmB-phospholipid complexes and (iii) Amphocil, a colloidal dispersion of AmB with sodium cholesteryl sulphate existing in thin discoid structures [1,2,9]. The AmB lipid preparations all exhibit an improved therapeutic index in comparison with Fungizone, allowing higher doses to be administered, and as a result provide better overall treatment efficiency. However, the fact that these products are

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among the most expensive anti-infective agents based on a daily defined dose constrains their success [10,11]. Taken together, the search for safer, more potent and especially more cost-effective AmB delivery systems still continues. Most recent literature examples of novel drug delivery systems (DDS) for AmB include lipid nanoparticles (NPs) [12], polymersomes [13] and functionalised carbon nanotubes [14].

In this study we propose poly(D,L-lactide-co-glycolide) PLGA NPs and nanosuspensions with the polyene antibiotic as potent and cost-effective alternatives to Fungizone and AmBisome. Polymeric NPs as well as nanosuspensions could show clear advantages over the lipid formulations such as long shelf-life at room temperature and low production costs [8]. In addition, we must emphasise the growing interest in nanosuspensions in pharmaceutical R&D, probably due to the explicit simplicity of the nanosuspension formulation strategy compared to other DDS. Nanosuspensions are already considered second most successful nanomedicines, after the liposomes, with a high number of products on the market and in clinical phases [15]. The present paper reports on the preparation and physicochemical characterisation of AmB-loaded PLGA NPs and AmB nanosuspensions. All formulations were assessed *in vitro* with respect to leishmanicidal, fungicidal and hemolytic activity and the two most promising ones were assessed in the *Aspergillus fumigatus* mouse model with respect to efficacy.

2. Materials and methods

2.1. Test organisms and cell cultures

Leishmania infantum MHOM/MA(BE)/67 was kindly provided by the Institute of Tropical Medicine in Antwerp (Belgium) and was maintained in the laboratory by serial passage in golden hamsters (*Mesocricetus auratus*). Fresh *ex vivo L. infantum* amastigotes and promastigotes were obtained as previously described [16]. *Candida albicans* B63195, *Aspergillus fumigatus* B42928 and *Trichophyton rubrum* B68183 were obtained from the Scientific Institute of Public Health (IHEM, Brussels, Belgium). *In vitro* maintenance and culture of primary peritoneal macrophages (PPM) and MRC-5_{SV2} cells were performed as previously described [17].

2.2. Preparation of PLGA NPs

The PLGA NPs were prepared from 40 kDa PLGA 50:50 (Resomer RG 503, Boehringer Ingelheim, Ingelheim, Germany) by means of a solvent displacement (nanoprecipitation) method. An organic phase consisting of 120.0 mg PLGA and a suitable amount of AmB (Sigma, Bornem, Belgium) dissolved in 4 ml of DMSO or a mixture of DMSO/acetone (1:1) (Sigma) was injected in 50 ml aqueous stabiliser solution. The injection procedure was carried out, using a 19G-needle, under sonication (Branson Sonifier Model S-450D, Branson, UK) for 5 min at 22–24 W (amplitude 40%) on ice. The stabiliser solution consisted of 0.5% (w/v) PVA (87–90% hydrolysed with MW 30–70 kDa, Sigma), which still remains the most commonly used stabiliser for PLGA NPs [18], or surfactant mixtures of poloxamer 188 (P188), poloxamer 338 (P338) (Pluronic, Ludwigshafen, Germany) with polysorbate 80 (P80, Tween 80) (non-ionic) or sodium cholate (anionic) (Sigma). AmB was entrapped into the PLGA NPs at an initial drug loading of 10% or 20% w/w to PLGA. The obtained NPs were filtered two times over regenerated cellulose membranes with a MWCO of 100 kDa (Vivaflow 50, Sartorius) using a Masterflex L/S pump (model 7518–00) and tubing (Sartorius, Göttingen, Germany) to remove excess stabiliser, non-incorporated AmB and DMSO [19]. Collection of the NPs was done using 50 ml Milli-Q water (Millipore Co., Bedford, MA, USA) or stabiliser solution. The purified NP formulations were cooled down to −18 °C and subsequently freeze-dried (FreeZone 1 lyofilisator, Labconco Corporation, Kansas City, MO, US coupled to the vacuum pump type 302101, Ilmvac Gmbh, Ilmenau, Germany). In addition, a separate batch of each AmB-loaded PLGA NP was produced with the purpose of studying the nanoformulations' physicochemical properties following freeze-drying in the absence as well as presence of different concentrations of mannitol. The freeze-dried NPs were stored at +4 °C.

2.3. Preparation of AmB nanosuspensions

Nanosuspensions containing 0.7% (w/v) of AmB and 71.4% (w/w to compound) of PVA as stabiliser were prepared by solvent-antisolvent precipitation. We chose to use this bottom-up method due to the high degree of similarity with the solvent displacement technique for the PLGA NPs. In brief, an organic phase consisting of 175.0 mg AmB dissolved in 2 ml of DMSO was injected in 25 ml 0.5% (w/v) stabiliser solution. The injection procedure and purification by means of cross-flow filtration was carried out as described for the PLGA NPs in 2.2.

2.4. Physical characterisation of nanoformulations

The mean particle size (Z_{ave}), size distribution and zeta potential of the NPs were determined with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Freshly prepared PLGA NPs and nanosuspensions were diluted with Milli-Q water, and freeze-dried NPs were reconstituted in Milli-Q water using ice-cooled sonication. The average value of three size measurements was used to determine the ratio of mean particle size (S_f) of reconstituted freeze-dried samples and the initial mean particle size (S_i) before freeze-drying as described by Holzer et al. [20]. The reported zeta potential value is the average of 10 consecutive measurements on the same sample.

2.5. Determination of drug loading and entrapment efficiency

The drug loading of the PLGA NPs was determined directly by measuring the amount of AmB entrapped in the NPs. Briefly, 50.0 mg of the freeze-dried NPs was dissolved in 5 ml DMSO and filled up to 20.0 ml with methanol (MeOH) (Sigma). From this solution 5.0 ml was transferred and diluted to 25.0 ml with MeOH. Absorbance of properly diluted stock solutions was measured at 407 nm (GENESYS 10 UV, Analis, Ghent, Belgium) and the AmB concentration was calculated using calibration curves (correlation coefficient > 0.999) over a concentration range of 0.75–6.00 μg/ml AmB. Blanks and AmB standard solutions were spiked with DMSO to obtain a final in-test concentration of 5% (v/v). Validation of the method according to ICH guidelines showed that it was precise ($RSD_{between\ days}$ 1.2%) and accurate (mean recovery 101.4%). The same procedure was followed for the determination of drug content of the AmB nanosuspension. All samples were measured in triplicate.

2.6. In vitro release of AmB

Initially, a conventional *in vitro* drug release test was set up: an amount of freeze-dried nanoformulation equivalent to 50 μg of AmB was dispersed in 1.0 ml of release medium and the screw-capped vial subsequently incubated at 37 °C in a shaking water bath. The release medium consisted of phosphate-buffered 5.0% w/v glucose to which sodium deoxycholate (final concentration of 0.08%, 1.0% or 2.0% w/v) was added to maintain sink conditions. At regular time intervals, the NPs were separated from the supernatant through centrifugation (30 min at 21460 × *g* and 37 °C), the absorbance of the supernatant was measured at 407 nm and the concentration of AmB released was calculated using calibration curves (the AmB standard solutions were prepared in the respective release medium).

Additionally, monomeric AmB release from the PLGA NPs and nanosuspension was monitored by the absorbance change at 407 nm as described by Legrand et al. [21]. Briefly, the nanoformulations were diluted to 0.1 μg/ml AmB equivalents and incubated in a shaking