International Journal of Pharmaceutics 436 (2012) 106–110

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Aerosolized liposomal amphotericin B: Prediction of lung deposition, *in vitro* uptake and cytotoxicity

Mélanie Fauvel[a], Cécile Farrugia[b,c], Nicolas Tsapis[a], Claire Gueutin[a], Odile Cabaret[b,c], Christian Bories[c,d], Stéphane Bretagne[c,e], Gillian Barratt[a,*]

[a] Faculty of Pharmacy, Paris-Sud XI University, UMR CNRS 8612, IFR 141, 5 rue J.B. Clément, 92296 Chatenay-Malabry, France
[b] Université Paris-Est-Créteil, UMR BIPAR 956, Créteil, France
[c] AP-HP, Groupe hospitalier Henri Mondor-Albert Chenevier, laboratoire de Parasitologie-Mycologie, 51, avenue du Maréchal-de-Lattre-de-Tassigny, 94010 Créteil, France
[d] Faculty of Pharmacy, Paris-Sud XI University, UMR CNRS 8076, IFR 141, 5 rue J.B. Clément, 92296 Chatenay-Malabry, France
[e] Centre National de Référence des Mycoses et des Antifongiques, Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris, France

ARTICLE INFO

Article history:
Received 16 May 2012
Received in revised form 6 July 2012
Accepted 9 July 2012
Available online 17 July 2012

Keywords:
Liposomal amphotericin B
A549
Nebulization
MTT

ABSTRACT

To predict the efficacy and toxicity of pulmonary administration of liposomal amphotericin B (L-AMB) for the treatment or the prevention of pulmonary invasive aspergillosis, a multistage liquid impinger was used to estimate the concentrations of drug that could be attained in different lung compartments after nebulization. The highest concentration of amphotericin B was found in the alveolar compartment, where it was calculated that the concentration in the lung surfactant could reach 54 μM or more when 21.6 μmoles of drug as liposomes was nebulized. The uptake and toxicity of L-AMB were studied *in vitro* using the A549 human lung epithelial cell line. Uptake was time and concentration-dependent and reached intracellular concentrations exceeding the minimal inhibitory concentrations for most *Aspergillus* species. The toxicity of L-AMB toward these cells, estimated by the MTT reduction assay, was reduced compared with the conventional form, deoxycholate amphotericin B (D-AMB), with an IC_{50} value of about 120 μM after 24 h of exposure for D-AMB, but only a 13% reduction in viability for 200 μM L-AMB at 24 h. These results indicate that aerosol therapy with nebulized L-AMB could be efficient but that doses need to be carefully controlled to avoid toxicity.

1. Introduction

Invasive pulmonary aspergillosis (IPA) is an important source of morbidity and mortality in immunocompromised individuals, such as patients undergoing allogeneic stem cell transplantation for hematological malignancies (Segal, 2009). The inhalation of spores, or conidia, is the first step in the pathogenesis of invasive pulmonary aspergillosis (IPA), followed by failure of macrophages and neutrophils to inhibit their development into mycelia. Since the respiratory epithelium and alveoli are the first tissues encountered by inhaled conidia, the delivery of drugs by aerosol would be an interesting alternative to intravenous administration of AMB to ensure that the drug concentration in the alveoli is adequate while reducing systemic toxicity (Kuiper and Ruijgrok, 2009).

Several studies have already addressed the possible use of AMB as an aerosol, mainly in a prophylactic setting (Kuiper and Ruijgrok, 2009). However, human studies have recorded serious side-effects after the administration of the conventional form of AMB (hereafter D-AMB), which contains sodium deoxycholate as a solubilizing agent (Behre et al., 1995; Dubois et al., 1995; Erjavec et al., 1997). AMB is also available in lipid-based formulations, including liposomal AMB (L-AMB), small unilamellar liposomes containing AMB embedded in the lipid bilayer. This formulation, which does not contain deoxycholate, has been shown to be better tolerated by the pulmonary route in neutropenic patients (Rijnders et al., 2008; Slobbe et al., 2008) and in lung transplant recipients (Monforte et al., 2009, 2010).

The aim of this work was to determine which doses of AMB can reach the different lung compartments (alveoli and respiratory epithelium) when using aerosols of L-AMB and the possible toxic effects on the cells. In the first step, a multi-stage liquid impinger was used to estimate the amounts of AMB delivered to different parts of the respiratory tract after nebulization of L-AMB. Although the efficacy of aerosols of L-AMB can be empirically studied (Monforte et al., 2009; Rijnders et al., 2008) or modelized (Vecellio et al., 2009), aerosol concentration can be measured experimentally as an alternative. This method has the advantage of allowing the concentration of the active pharmaceutical ingredient

* Corresponding author at: UMR CNRS 8612, Faculté de Pharmacie, Univ. Paris-Sud, 5 rue J.B. Clément, 92296 Chatenay-Malabry, France. Tel.: +33 1 46 83 56 27; fax: +33 1 46 83 58 32.
E-mail address: gillian.barratt@u-psud.fr (G. Barratt).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2012.07.012

in the aerosol to be measured at different stages of the respiratory tract. In the second step, a cell culture system with A549 human pulmonary cells was used to study AMB toxicity as a function of concentration.

2. Materials and methods

2.1. Reagents and solvents

Two formulations of AMB were tested. D-AMB was obtained from GIBCO (Invitrogen, Cergy Pontoise, France). L-AMB was a gift from Gilead (Foster City, USA). It is a liposomal formulation of AMB for intravenous administration, consisting of small unilamellar liposomes (SUV) between 50 and 100 nm in diameter prepared from hydrogenated soy phosphatidylcholine, cholesterol, distearoylphosphatidylglycerol and amphotericin B in a molar ratio of 2:1:0.8:0.4. After reconstitution of the lyophilized material, the suspension was stored at +4 °C without light during the study. Stability after reconstitution was shown for at least 6 months (Morand et al., 2007).

Hydrogenated soy phosphatidylcholine (HSPC) and distearoylphosphatidylglycerol (DSPG) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Dimethylsulfoxide (DMSO), bovine serum albumin (BSA), cholesterol (Chol), alpha-tocopherol (AT), sucrose, disodium succinate hexahydrate, Triton X-100 and gentamicin were obtained from Sigma–Aldrich (Lyon, France). Methanol, tetrahydrofuran (THF) and acetonitrile were from Carlo Erba (Val de Reuil, France). Formic acid and triethylamine were from VWR International (West Chester, PA, USA). DMEM and fetal bovine serum were from PAA (Cölbe, Germany). Isotonic phosphate-buffered saline (PBS) and L-glutamine were from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

The human alveolar basal epithelial cell line A549 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and cultured as previously described (Bellanger et al., 2009). Cell cultures were checked for the presence of *Mycoplasma* spp. every month using the PCR kit Venor® GeM (Biovalley, Marne La Vallée, France).

2.3. Preparation of empty liposomes

For toxicity studies, empty liposomes were prepared to serve as controls. HSPC (213 mg), DSPG (84 mg), Chol (52 mg) and AT (0.64 mg) were dissolved in chloroform in a round-bottomed flask. The organic solvent was removed by evaporation under reduced pressure to leave a lipid film. This was hydrated with 12 mL of a solution of sodium succinate (27 mg) and sucrose (900 mg), at 70 °C, to form multilamellar liposomes. The size was then reduced by extrusion through calibrated polycarbonate filters (Whatman, Maidstone, Kent, UK): five passages through a 200-nm pore size followed by 10 passages through a 100-nm pore size. The mean diameter of the final liposomes was measured by quasi-elastic light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) at a detection angle of 173°. Three measurements were made on the same sample. The size of the commercial L-AMB used in the study was also measured for comparison. The *Z*-average diameter and polydispersity index are reported for each preparation.

2.4. Nebulization of L-AMB

Nebulization was achieved using a commercially available compressor, used in pneumatic mode, (Atomisor AOBOX, La Diffusion Technique Française, Saint Etienne, France) and a nebulizer (Atomiser NL9M, La Diffusion Technique Française, Saint Etienne, France) connected by a 10 cm polyethylene tube (internal diameter, 10 mm, external diameter 15 mm) to a multistage liquid impinger (MSLI, Asking and Olsson, 1997) (Copley Scientific, Nottingham, UK) and a pump (Critical Flow Controller TPK, Copley Scientific, Nottingham, UK) with an air flow rate of 15 L min^{-1} in order to simulate inspiration. The nebulizer was filled with 4 mL of an aqueous suspension of L-AMB at 5.4 mM (5 mg mL^{-1}). The dose (21.6 μmoles) was chosen to be of the same order of magnitude as those used in human studies: 12.5 mg (13.5 μmoles) in neutropenic patients (Rijnders et al., 2008; Slobbe et al., 2008) and 25 mg (27.1 μmoles) in lung transplant recipients (Monforte et al., 2009, 2010). The MSLI comprised 5 stages. Stages 1 to 4 each contained 20 mL methanol/DMSO (50/50, v/v), and the fifth was a filter paper. They were all separated by filters with decreasing cut-off diameters. At the settled air flow rate we used, the cut-off diameters were, respectively, 6.2 μm and 3.4 μm between stages 3 and 4 and between stages 4 and 5 (filter), thus, these two last stages correspond to lungs. The fourth stage corresponds more precisely to the bronchi and the filter to the alveoli.

Nebulization was carried out for 20 min. This time was chosen to correspond with the nebulization times reported in different clinical studies, which ranged between 7 and 30 min. Afterwards, the AMB accumulated at each stage was collected by extracting the filter paper with methanol/DMSO (50/50, v/v) and by rinsing stages 1 to 4, the nebulizer and the connections with methanol/DMSO (50/50, v/v). The AMB concentration was then determined by HPLC as described below. Two independent experiments were performed, with two samples from each stage analyzed in each experiment.

2.5. Quantification of AMB by HPLC

AMB was quantified by reversed phase high-performance liquid chromatography in accordance with a previously validated method (Ménez et al., 2006). Chromatographic separation was performed with a Hypersil ODS C18 column (150 mm × 4.6 mm ID, 5 μm) (Thermo Fisher Scientific, Villebon, France) thermostated at 30 °C (7956R, Grace, Deerfield, USA). The mobile phase was a mixture of 1% triethylamine in water adjusted to pH 5.2 with formic acid, acetonitrile and tetrahydrofuran (1000/385/154, v/v/v). The pump flow (600 Controller, Waters, Guyancourt, France) was fixed at 1 mL min^{-1}. Before injection, samples were diluted in methanol/DMSO (50/50, v/v) and 100 μL were injected by an automatic injector (717 Autosampler, Waters, Guyancourt, France). AMB was detected by its absorbance at 409 nm (2996 Photodiode Array Detector, Waters, Guyancourt, France) and the peaks were automatically integrated (Empower software, Waters, Guyancourt, France). Under these conditions, the retention time for AMB was approximately 6 min. This method showed a good linearity between 0.02 and 10 μg mL^{-1} (0.022–10.82 μM) with a mean correlation coefficient of 0.9985. The limit of quantification was 0.01 μg mL^{-1} (0.0108 μM). No internal standard was used.

2.6. Measurement of cell-associated AMB

A549 cells were harvested from culture plates by trypsination and seeded in 2 mL aliquots into 12-well plates at 2.3×10^5 cells/well (well surface = 3.8 cm). Cells were allowed to adhere in drug-free complete DMEM during 24 h. The culture medium was then removed and 1 mL/well of different dilutions of L-AMB in complete culture medium was added. Cells were incubated for a further 2, 4 or 24 h. At the end of the exposure, the culture medium was removed and the cells were washed three times with 2 mL PBS. They were lysed by adding 200 μL/well of cold methanol