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Polymeric carriers for amphotericin B: *in vitro* activity, toxicity and therapeutic efficacy against systemic candidiasis in neutropenic mice

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***Objective*: To study the toxicity and activity of two new amphotericin B formulations: poly(ε-caprolactone) nanospheres coated with poloxamer 188 (AmB-NP) and mixed micelles with the same surfactant (AmB-MM).**

***Materials and methods*: The toxicity of these formulations was evaluated in erythrocytes, J774.2 macrophages and LLCPK1 renal cells, as well as in mice. Activity was determined in clinical isolates and in neutropenic mice. Mice were made neutropenic with 5-fluorouracil, infected with *Candida albicans* and treated with the antifungal formulations for three consecutive days. AmB association in cells and accumulation in kidneys and liver of animals was quantified by HPLC.**

***Results*: Both formulations decreased between 8- and 10-fold the MIC of the polyene against clinical isolates of *C. albicans*. However, their activity was lower than or equal to that of AmB-deoxycholate when it was assessed against *C. albicans*-infected macrophages. When given as a single intravenous dose in mice, AmB-MM and AmB-NP had an LD_{50} of 9.8 and 18.6 mg/kg, respectively, compared with 4 mg/kg for AmB-deoxycholate. Comparison of residual infection burdens in the liver and kidneys showed that AmB-deoxycholate (0.5 mg/kg) was more effective and faster in eradicating yeast cells than polymeric formulations. This fact can be related to a lower AmB accumulation inside macrophages and in liver and kidneys (about 1.5 mg drug/g tissue) of mice, compared with those detected for AmB-deoxycholate (4 mg drug/g). Overall, the efficacy of these formulations at 2 mg/kg was equal to that of AmB-deoxycholate at 0.5 mg/kg.**

***Conclusions*: AmB-MM and AmB-NP decreased the *in vivo* antifungal activity of AmB, and higher concentrations were therefore necessary to obtain a similar therapeutic effect. However, these higher concentrations were achievable owing to the reduced toxicity of these formulations.**

Keywords: *Candida albicans*, micelles, nanospheres, poloxamer

Introduction

Infections due to fungi of the genera *Candida* and *Aspergillus* have become major nosocomial causes of morbidity and mortality in immunocompromised individuals. Their incidence has increased during the last three decades in parallel with the number of such patients and it is now a commonplace complication.[1,2] More recently, there has been a substantial increase in the frequency of candidaemia caused by species other than *Candida albicans*.[3]

Systemic candidiasis is associated with a high mortality rate and prolonged hospitalization.[4] The treatment options are extremely limited. Rapid treatment with amphotericin B (AmB) or fluconazole is required to reduce the mortality observed in these patients.[5] AmB is a very potent antifungal agent, but it has multiple severe toxic effects and about 36% rate of failure in the treatment of immunocompromised hosts.[6] Thus, several lipid formulations (AmBisome, Amphocil, Abelcet) have been developed and commercialized.[7,8] Although they have been proven to reduce AmB toxicity, their toxic effects and pharmacokinetic properties all differ and their use has been limited by their expense. Moreover, it has been recognized that the reduction in AmB toxicity was associated with a substantial reduction in AmB activity.[9–13] Various *in vivo* studies have demon-

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strated that higher doses of AmBisome (3 mg/kg) are often needed for a treatment to be as effective as 0.6 mg/kg of AmB-deoxycholate. It is difficult to understand the mechanism by which these lipid formulations modify the therapeutic index of AmB[14,15] as far as it implies multiple and different factors for each one. However, for all of them, it seems to be closely related to the strength of association between the drug and lipid compounds of the formulation.[8,16] Moreover, the drug toxicity depends on its aggregation state.[17,18] Aggregates are the forms responsible for drug toxicity but monomers are sufficient for the activity. The slow release of the drug from lipid formulations ensures that the drug remains as a monomer and is unable to form the aggregates responsible for drug toxicity and increased activity.[19] Other factors could also play a role.[16]

Obviously, the reduction in activity is a problem for treating severe fungal infections because high doses and rapid action are required in order to improve the survival rate. Thus, we do not yet have an ideal antifungal agent.

In previous work, we developed and characterized non-lipid formulations for AmB.[20–23] In spite of the weak association between the drug and the polymers, they decreased the acute toxicity of AmB in a mouse model.[20] Since the decrease in antifungal activity of AmB lipid formulations has been attributed to the stability of association between the drug and lipid components of the carriers,[10,13] in this study we evaluated the activity of these new polymeric formulations *in vitro* and *in vivo* in a mouse model of candidiasis. The toxicity of the formulations was also studied against mammalian cells and in mice after a single bolus administration to obtain information on the mechanism of action of AmB associated with these polymeric formulations.

Materials and methods

Chemicals

Squibb (Madrid, Spain) kindly supplied amphotericin B. Poly(ε-caprolactone) (mol. wt 42 500), was supplied by Sigma (Madrid, Spain) and poloxamer 188 (Pluronic F68) by Comercial Química Massó (Barcelona, Spain). All other chemicals were reagent grade. Culture media were obtained from Gibco (France).

Preparation of AmB dispersions

AmB nanoparticles (AmB-NP) and mixed-micelles (AmB-MM) were prepared by a solvent displacement method as previously described.[20–22] Briefly, 125 mg poly(ε-caprolactone) and 10 mg amphotericin B were dissolved in 30 mL of a mixture of organic solvents (methanol/acetone; 1:2 v/v) and acidified with 0.1 M HCl. This organic phase was heated at 50°C for 10 min and then poured into 40 mL of distilled water containing 125 mg poloxamer 188, under moderate magnetic stirring. The organic solvents were eliminated by evaporation under vacuum and the resulting nanoparticles (AmB-NP) concentrated to 10 mL.

Amphotericin B-mixed micelles (AmB-MM) were prepared as described above, in the absence of poly(ε-caprolactone).

An aqueous dispersion of AmB used as a control was prepared by solubilization of AmB in DMSO at a concentration of 10 mg/mL followed by dispersion of this organic stock solution in water to yield an initial concentration of 1 mg/mL (AmB).

Physicochemical characterization

Particle size analysis: The mean particle size of free dispersed AmB, AmB-MM and AmB-NP was determined by photon correlation spectroscopy (PCS) on a Zetasizer 4 instrument (Malvern Instruments, UK).

Stability of association upon dilution: CD spectroscopy: The aggregation state of the drug in AmB, AmB-MM and AmB-NP was analysed by UV–visible (Cary 219 Varian spectrophotometer) and CD (Jobin-Yvon V dichrograph) spectroscopy after dilution with distilled water to obtain a final drug concentration of 50, 10, 5, 1 and 0.5 μg/mL (path lengths of cuvettes 0.2, 1, 2, 5 and 10 cm, respectively). CD measurements were expressed in $\Delta\varepsilon$, the differential molar dichroic absorption coefficient ($M^{-1}\,cm^{-1}$), the amplitude of dichroic doublet characteristic of AmB self-association, and $\lambda\,(\varepsilon_\lambda - \varepsilon_r = 0)$, the wavelength of the centre of the dichroic doublet.

In vitro studies: toxicity of AmB formulations in human red blood cells (RBC)

Isolation of human erythrocytes: Human venous blood collected in tubes containing EDTA was centrifuged at 1500**g** for 10 min, and plasma and buffy coat were removed. Erythrocytes were then washed three times with phosphate-buffered saline (PBS; pH 7.4, 0.15 M), and finally, recovered in the same buffer at a haematocrit of 40%.

Measurement of antibiotic-induced haemoglobin and potassium (K^+) release from RBC: AmB dispersions were diluted to different concentrations (0.1–100 μM) with PBS and incubated for 5 min at 37°C. Freshly prepared erythrocytes were then added to a final haematocrit of 2% (approximately 2×10^8 cells per mL) and incubated at the same temperature for 1 h. The cell pellet was lysed with distilled water. K^+ was measured using a Flame Photometer 410 (Prolabo, Paris, France) and haemoglobin estimated from its absorption at 560 nm recorded with a spectrophotometer. Control RBC (2×10^8 cells per mL) incubated with PBS alone were used to estimate the total potassium and haemoglobin content. The remaining K^+ or haemoglobin was calculated as a percentage of the total content. Results are given as the mean of one experiment representative of three experiments carried out with each concentration in triplicate.

In vitro studies: toxicity against non-infected J774.2 macrophages and LLCPK1 renal cells (MTT viability assay)

The macrophage cell line J774.2 and renal cell line LLCPK1 were grown in monolayers in a humidified atmosphere containing 5% CO_2 at 37°C in 250 mL flasks containing Dulbecco's modified Eagle's Medium (DMEM, Gibco) or Medium 199, respectively containing 4 mM L-alanine-L-glutamine (Glutamax) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 μg/mL). The fetal bovine serum, penicillin and streptomycin were purchased from Gibco-BRL. For the experiments, cells in the pre-confluence phase were harvested with trypsin and plated at 4×10^4 cells per well in 96-well microplates. After adherence for 2 h at 37°C, the medium was replaced with different concentrations of AmB and AmB formulations. Then, the cell viability was evaluated after an 18 h incubation, by the colorimetric assay with MTT, as follows. After washing the cells, 200 μL of a 0.5 mg/mL MTT solution in DMEM medium was added to each well and incubated at 37°C for 4 h. The supernatants were removed and 200 μL extraction solution was added in order to solubilize the formazan salt. After 30 min at room temperature under shaking, the absorbance of each well was measured at 550 nm.

In vitro studies: association of AmB and AmB formulations by J774.2 and LLC-PK1 cells

Cells were plated as above and incubated in the presence of various AmB concentrations (0–100 μM) for 18 h at 37°C to evaluate the total association of AmB with cells or at 4°C to evaluate the adsorbed fraction. After incubation, the cells were rapidly cooled and washed three times with phosphate buffer pH 7.4 to remove unbound AmB. Cells were lysed with 50 μL Triton X-100 1% for 20 min at 37°C. The amount of AmB was determined by an HPLC method previously reported.[24] Briefly, the analytical separation was carried out on a reversed-phase, 250 × 4 mm end-capped column packed with 5 μm C_8 silica particles (Lichrospher 60 Select B, Merck, Darmstadt, Germany). The chromatographic system was a Hewlett-Packard HPLC (Waldbronn, Germany) equipped with a HP 1100 quaternary pump, a HP 1100 autosampler and a HP 1100 diode array detector set at 408 nm. The mobile phase consisted of acetonitrile (40% by vol.), water (14.5%), and a buffer solution (45.5%) of 0.05 M ammonium acetate, 0.02 M triethylamine and 4% acetic acid (pH 3). The flow rate was 0.6 mL/min and the injection volume 100 μL. The limit of quantification of amphotericin B in biological samples was 1 ng/mL and the method was linear from 1 to 25 000 ng/mL. The intra- and interassay coefficients of variation were 2.6 and 4.4, respectively.[24]

In vitro activity against extracellular Candida albicans and against Candida albicans inside J774.2 macrophages

The test organisms were clinical isolates of *Candida albicans* (Clínica Universitaria de Navarra, Dr Rubio) and ATCC number 44858 was used as the control strain in all the experiments. These strains were maintained on Sabouraud dextrose agar and passaged monthly. Before each experiment, cells were grown on agar at 37°C for 24 h. A broth microdilution technique in RPMI 1640 was chosen to test yeast susceptibility.[25] The inocula ranged from 10^4 to 10^5 cfu/mL and the range of concentrations tested was 0.001–10 μg/mL for AmB and AmB polymeric formulations. Studies were carried out in 96-well plates, at 37°C with agitation, and the incubation time was 24 h. The drug concentration in the well that completely suppressed all fungal growth, as judged by visual inspection, was recorded as the MIC. Each strain was tested at least three times.

Candida albicans ATCC 44858 was used for infecting macrophages. For this purpose, *C. albicans* was opsonized by incubation with fresh mouse serum for 1 h at 37°C under magnetic agitation. Macrophages J774.2 were plated in 24-well plates at 4×10^5 cells/well. After 2 h adherence, an inoculum of 3×10^5 cfu of opsonized *C. albicans* was then added to the monolayer. After a 30 min uptake period, 2×10^5 yeast particles were ingested and the non-ingested yeast was removed by washing the monolayer three times with the DMEM medium. The macrophages were reincubated for 24 h with the different AmB formulations. Percentage of infection was calculated by lysing macrophages with Triton X-100 (0.2%) and subculturing in TSA plates, compared with an untreated control. The amount of drug uptake by the cells was also determined by the HPLC method described above.

Acute toxicity of AmB formulations

Animal protocols were carried out in compliance with the regulations of the ethical committee of the University of Navarra in line with the European legislation on animal experiments (86/609/EU).

Male Swiss mice, average weight 20–25 g (CIFA, Universidad de Navarra, Spain), were housed under normal conditions with free access to food and water. The animals were injected with 0.2 mL of the different AmB formulations diluted in PBS in the lateral vein tail. In each experiment, eight mice per dose were used, and three different experiments were carried out with each AmB formulation. Mice were observed for death after injection and daily thereafter for 7 days. The results were pooled and the median LD_{50} at 7 days after treatment was calculated with Grafit 3.0 software.

Activity of AmB formulations in experimental candidiasis: efficacy studies

For these experiments, male Swiss mice (20–25 g) were treated with 5-fluorouracil (5-FU) to induce neutropenia,[26] then infected with *C. albicans* and finally, treated with the different AmB formulations. Neutropenia was induced in animals with a single intravenous (iv) injection of 100 mg/kg 5-FU. This reduced the peripheral blood absolute neutrophil count to <200 cells/mL, within 1 day after administration, and maintained it in that range for up to 10 days.[26,27] One day after inducing neutropenia, mice were infected with the *C. albicans* ATCC 44858 strain by injecting 0.2 mL of a dispersion of 10^6 cfu in the lateral tail vein. Finally, 24 h later, groups of 12 to 18 mice received iv injections of PBS buffer, AmB-deoxycholate (0.5 mg/kg per day), AmB-MM and AmB-NP (0.5 and 2 mg/kg per day) via the tail vein for three consecutive days.

Organ culture and AmB accumulation

Cultures were carried out on liver and kidney samples taken 3 and 10 days after the last treatment. The liver and kidneys of 3–5 mice were aseptically removed, weighed and homogenized in 10 mL of sterile saline. From here, we made serial 10-fold dilutions. We plated 0.1 mL from each one and from the undiluted sample on Sabouraud agar and they were incubated at 37°C for 24 h. Therefore, the limit of detection was 100 cfu per organ. The number of cfu per gram of tissue was calculated. Triplicate determinations were made for each time point and organ. The AmB accumulation was determined by HPLC in organs removed 24 h after the last iv administration. Briefly, the liver and kidneys were removed, weighed, homogenized in 1 mL of sterile saline and frozen at –40°C until assayed. Weighed samples (approximately 0.5 g of the homogenate) were placed in a glass tube along with 2 mL of methanol. The samples were vortex mixed for 1 min and then centrifuged at 4500 rpm for 10 min. A 200 μL sample of the supernatant was placed in an autoinjector vial, and 100 μL injected into the HPLC system. The analytical conditions were the same as previously described.[24] Comparisons of the mean AmB tissue concentrations were made between different formulations at two doses by the non-parametric Kolmogorov–Smirnov test. A P value of ≤ 0.05 was considered to indicate a significant difference.

Results

Physicochemical characterization: stability of interaction of AmB with poloxamer 188 or poly(ε-caprolactone) in mixed micelles or nanospheres

The aggregation state of AmB in drug delivery systems and the stability of the interaction between AmB and the carrier compounds have been previously reported to be the main factor in determining the toxicity of the drug at the cellular level[7,16–18] and also the exchange of the drug with plasma proteins and hence its biodistribution.[7,28–30] AmB forms aggregates in equilibrium with monomers at high AmB concentrations, above its critical micellar concentration. The progressive dilution of an AmB suspension leads to a progressive dissociation of the aggregates into the monomeric form. The evolution of the intensity of the dichroic doublet (% $\Delta\varepsilon/\Delta\varepsilon_{max}$ at 50 μg/mL) as a function of antibiotic concentration was used to evaluate the stability of association between AmB molecules in the aggregates (AmB), between the drug and poloxamer (AmB-MM) and/or poly (ε-caprolactone) (AmB-NP). The profile of the curves intensity of doublet as a function of antibiotic concentration (Figure 1) indicated a dissocia-

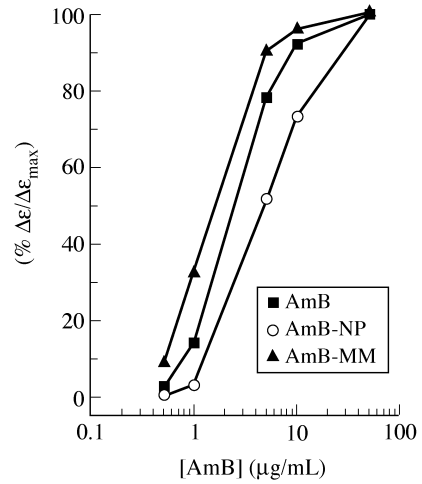

Figure 1. Evolution of the intensity of the dichroic doublet (% $\Delta\varepsilon/\Delta\varepsilon_{max}$) of AmB (filled squares), AmB-MM (filled triangles) and AmB-NP (open circles) as a function of the total AmB concentration (μg/mL) by dilution with distilled water of stock preparations 1 mg/mL AmB. $\Delta\varepsilon_{max} = \Delta\varepsilon$ at 50 μg/mL AmB.

Table 1. Mean size (nm), centre λ ($\varepsilon_\lambda - \varepsilon_r$=0) (nm) and intensity ($\Delta\varepsilon$) of dichroic doublet of AmB in AmB, AmB-MM and AmB-NP

		Dichroic doublet[a]	
	Mean size ± S.D. (nm) (polydispersity index)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	λ ($\varepsilon_\lambda - \varepsilon_r = 0$) (nm)
AmB	>1000 (1.0)	3970	335
AmB-MM	358 ± 62	5630	330
AmB-NP	282 ± 50 (0.10)	2340	333

[a]Measured at 50 μg/mL AmB.

tion of AmB that was concentration-dependent and a weak interaction between the drug and the polymer [poly(ε-caprolactone) or poloxamer]. Besides a strong interaction with carrier components, modifications in the AmB aggregation state were also reported to decrease the toxicity of the drug[31] and also seemed to play a pivotal role in drug activity.[32] In this sense, the association of AmB with poloxamer or poly(ε-caprolactone) in mixed micelles (AmB-MM) or nanospheres (AmB-NP) altered the conformation of the aggregates as revealed by modifications in UV–visible[21,22] and CD spectra (Table 1). Briefly, the adsorption of AmB onto nanospheres (AmB-NP) decreases AmB aggregation as reflected in the decrease in $\Delta\varepsilon$ and the red-shift of the dichroic doublet. In contrast, the dispersion of AmB with poloxamer in mixed micelles (AmB-MM) increased AmB aggregation as indicated by the increase in $\Delta\varepsilon$ and the blue shift of the dichroic doublet. Blue shift could be due to the presence of smaller and more homogeneous aggregates. More details of the spectroscopic properties of these formulations have been described.[21,22]

The size of AmB-MM and AmB-NP was also analysed (Table 1) because AmB self-aggregates have been associated with infusion-related side effects and precipitation of the drug causing obstruction of renal tubules.[33] AmB dispersion with poloxamer 188 (AmB-MM) or association with nanospheres (AmB-NP) decreased the size of AmB aggregates (Table 1) and also prevented progressive aggregation[34] and precipitation of free drug (data not shown).

In vitro toxicity: effects on RBC membranes

K^+ and haemoglobin leakage from RBC has been extensively used as an index of polyene toxicity.[18] As estimated from the experimental data, the AmB concentration required to trigger 50% K^+ leakage was about 1 μM for AmB, AmB-MM and AmB-NP. This value was higher than the 0.3 μM previously reported by Gaboriau *et al.*[31] The association of AmB with polymers resulted in a large decrease in their haemolytic activity efficiency. Whereas 50% haemolysis was observed with 5 μM AmB, hardly any haemoglobin leakage was observed with the different AmB formulations assayed over the whole range of concentrations tested (0.1–100 μM).

Cytotoxicity and drug uptake by J774.2 macrophages and LLC-PK1 renal cells

Since nephrotoxicity is the most important dose-limiting side effect of AmB therapy and macrophages act as a reservoir of the drug, a comparison was made between the toxicity of AmB and AmB polymeric formulations in the murine macrophage J774.2 and the pig kidney epithelial renal LLC-PK1 cell lines. Cell metabolism was measured by reduction of dimethylthiazole diphenyltetrazolium bromide (MTT) by mitochondria. The cytotoxicity induced by AmB and AmB polymeric formulations as a function of drug concentration is shown in Figure 2.

For J774.2 macrophages (Figure 2a), AmB showed a steep slope and 10 μg/mL of drug induced 70% toxicity compared with the control. On the other hand, AmB-MM exhibited a low, dose-dependent toxicity which reached only 20% at the maximum concentration tested (100 μg/mL). However, AmB-NP toxicity was not concentration-dependent and was found to be about 60% over the whole range of drug concentrations tested (0.1–100 μg/mL). Unloaded nanoparticles exhibited similar toxicity to AmB-NP, indicating that the mitochondrial toxicity of these formulations was probably due to the poly(ε-caprolactone) polymer without any effect of AmB.

Compared with macrophages, the cytotoxicity of free AmB against renal cells as revealed by MTT conversion was greatly decreased (Figure 2b) and toxicity was completely absent when AmB was associated with polymeric colloids (less than 20%) for both nanoparticle and mixed micelle formulations.

The differences in AmB toxicity between both types of cell line and also the decreased toxicity of AmB polymeric formulations were well correlated with the higher amount of drug taken up by macrophages (phagocytic cells) compared with renal cells (non-phagocytic cells), as shown in Figure 3(a and b), respectively. For each cell line, the association of AmB with polymers greatly decreased the uptake of the drug, without notable differences between AmB-MM or AmB-NP.

In vitro activity: extracellular Candida albicans

The MICs of AmB against seven isolates of *C. albicans* ranged from 0.63 to 1.25 mg/L. MICs of AmB-MM for the same isolates ranged from 0.02 to 0.08 mg/L and of AmB-NP from 0.08 to 0.16 mg/L. AmB-free poloxamer micelles and unloaded nanospheres were inactive up to 1250 mg/L.

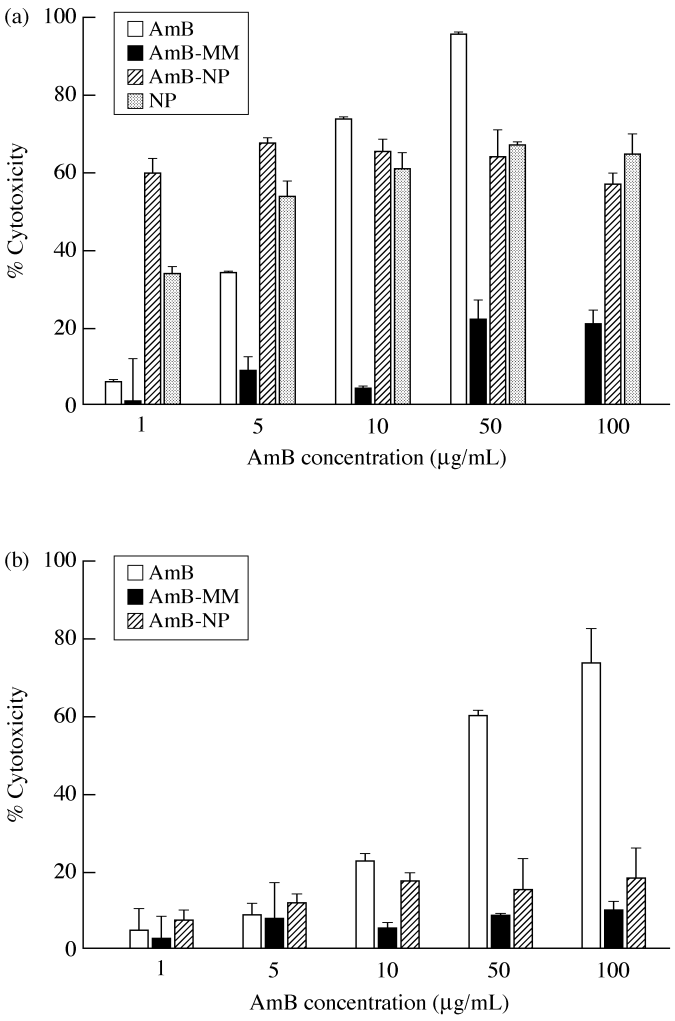

Figure 2. Mitochondrial toxicity (MTT test) of AmB, AmB-MM, AmB-NP and unloaded nanoparticles against macrophages J774.2 (a) and renal cells LLC-PK1 (b) as a function of drug concentration, after 18 h incubation at 37°C (5% CO_2) at 2×10^5 cells/mL in DMEM or Medium 199, respectively supplemented with 5% FCS.

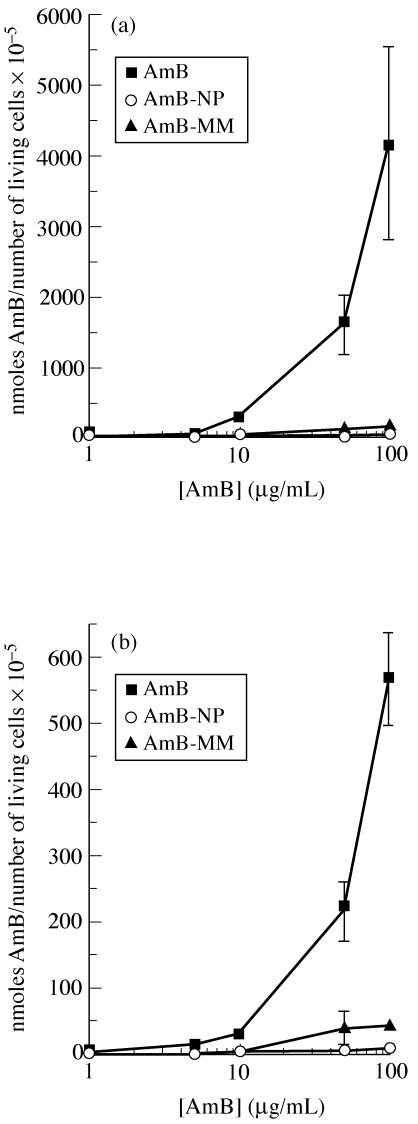

Figure 3. Uptake by J774.2 cells (a) and LLC-PK1 cells (b) of the different AmB formulations as a function of the antibiotic concentration, after 18 h incubation, at 37°C. Symbol explanation: AmB (filled squares), AmB-MM (filled triangles) and AmB-NP (open circles).

Activity against Candida albicans inside J774.2 macrophages

Figure 4 shows the antifungal activities of AmB formulations against intracellular *C. albicans* after a 24 h incubation. Antifungal activity was evaluated in terms of eradication of *C. albicans* from infected macrophages determined by sub-culturing on Sabouraud plates.

It should be noted that no complete eradication of *C. albicans* was observed up to a drug concentration of 2 mg/L. We did not study higher AmB concentrations because of AmB toxicity towards the host cells. The intracellular activity of free AmB (AmB) was slightly higher than that of AmB-MM, and much higher than that of AmB-NP. Thus, in spite of their increased direct antifungal activity, the association of AmB with polymeric colloids only maintained or even decreased the activity of the free drug against intracellular *C. albicans*. Moreover, because a direct interaction of AmB with the yeast inside the macrophage is necessary for drug activity,[10–13] we determined the effect of the carrier on AmB uptake by infected macrophages. The uptake of AmB and AmB-polymeric formulations was concentration-dependent and much higher for the free drug (AmB). So, when cells were incubated with 1 and 2 mg/mL of AmB, 7×10^{-5} and 38×10^{-5} nmol of drug, respectively, were detected inside a living cell; but this dropped to 1×10^{-5} nmol and $2–3 \times 10^{-5}$ nmol (per living cell), when cells were incubated with 1 and 2 mg/L of AmB-NP or AmB-MM. At 2 mg/L, it is also noteworthy that the extent of AmB uptake was higher in infected than in non-infected cells (Figure 3). When the macrophages were incubated with AmB concentrations lower than 1 mg/L, the drug was undetectable inside macrophages.

In vivo experiments: acute toxicity

The dose–toxicity relationship observed on day 7 in non-infected Swiss mice receiving increasing doses of AmB-deoxycholate, AmB-MM and AmB-NP is shown in Figure 5. The LD_{50} calculated from these data was 4, 9.80 and 18.60 mg/kg for AmB-deoxycholate, AmB-MM and AmB-NP, respectively. The maximum tolerated dosage (MTD) was defined as the maximum dose that did not result

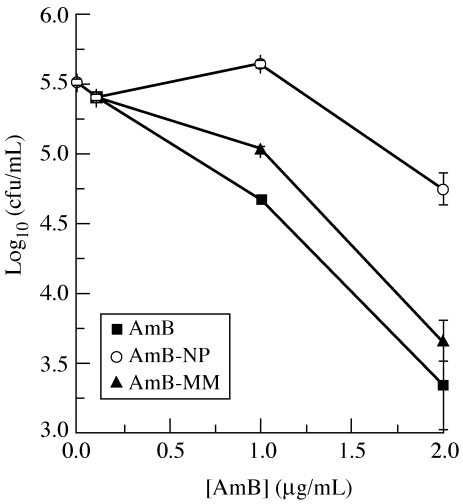

Figure 4. Antifungal activity (log_{10} cfu/mL) of the different AmB polymeric formulations against *C. albicans* inside J774.2 macrophages after a 24 h incubation. Symbol explanation: AmB (filled squares), AmB-MM (filled triangles) and AmB-NP (open circles).

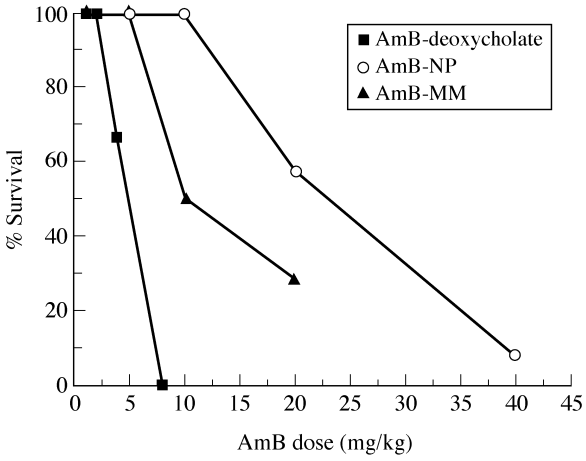

Figure 5. Amphotericin B toxicity after a single bolus administration in the tail vein (0.2 mL) of male Swiss mice (20–25 g). The percentage survival at day 7 is shown. Symbol explanation: AmB-deoxycholate (filled squares), AmB-MM (filled triangles) and AmB-NP (open circles).

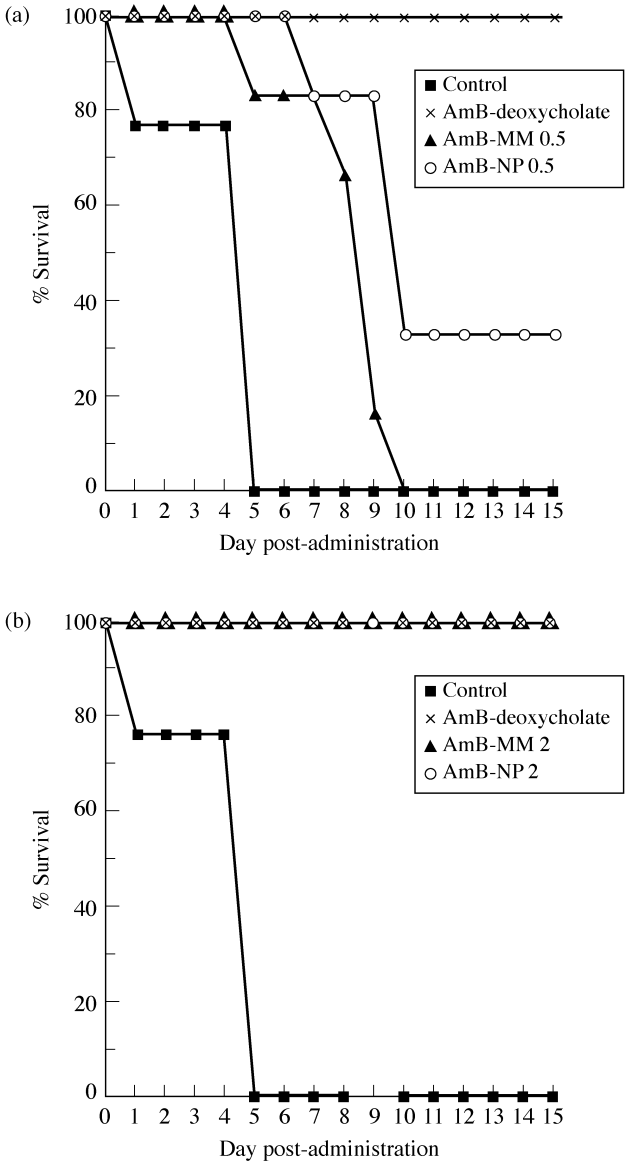

Figure 6. Survival of *Candida*-infected, neutropenic Swiss mice after treatment with different AmB formulations: (a) AmB dose was 0.5 mg/kg; (b) AmB dose was 2 mg/kg for AmB-MM and AmB-NP. Mice were made neutropenic by 5-FU and inoculated with *C. albicans* as described in Materials and methods. Survival was monitored over 25 days. Symbol explanation: Control (filled squares), AmB-deoxycholate (crosses), AmB-MM (filled triangles) and AmB-NP (open circles).

in any deaths. For AmB, the MTD was 2 mg/kg; it was 5 mg/kg for AmB-MM and 10 mg/kg for AmB-NP. When AmB was given at doses greater than 4 mg/kg, all mice died immediately, probably by pulmonary embolism. In contrast, AmB dispersions induced a delayed mortality at the highest doses tested, suggesting other causes of death.

In vivo experiments: activity in experimental candidiasis

For studies of efficacy, groups of 12–15 neutropenic mice were infected with 10^6 cfu of *C. albicans* per mouse and 24 h later they were treated iv with AmB-deoxycholate at 0.5 mg/kg and AmB formulations at 0.5 (Figure 6a) or 2 mg/kg (Figure 6b).

Untreated mice died between 1 and 3 days after infection. Administration of 1 mg/kg AmB-deoxycholate led to the immediate death of the mice.

At 0.5 mg/kg, 100% of the mice treated with AmB-deoxycholate survived, whereas for AmB-MM at this dose, no mice survived after day 12 post-infection. At the end of experiment, only 33% of mice treated with AmB-NP at 0.5 mg/kg survived. However, the efficacy was dose-dependent and with 2 mg/kg of the formulations, 100% of the infected mice survived longer than 20 days post-infection (Figure 6b).

Organ cultures

Organ burden for the liver and kidney was analysed. *Candida* accumulates particularly in kidneys. *Candida* was below detectable levels

Table 2. Organ burdens in liver and kidneys post-*C. albicans* infection and treatment

Treatment	Dose (mg/kg)	cfu/g liver ($\times 10^3$)		cfu/g kidney ($\times 10^3$)	
		3 days	10 days	3 days	10 days
Control	–	13.7 ± 0.5	no survivals	120 ± 2.3	no survivals
AmB-deoxycholate	0.5	0.5 ± 0.9	[a]	0.5 ± 0.5	0.3 ± 0.3
AmB-MM	0.5	4.3 ± 4.2	no survivals	14.9 ± 3.6	no survivals
	2	9.0 ± 7.8	0.1 ± 0.1	6.9 ± 4.5	0.7 ± 0.8
AmB-NP	0.5	6.1 ± 8.7	<0.1[a]	7.0 ± 5.8	0.9 ± 1.5
	2	[a]	1.4 ± 2.4	0.4 ± 0.4	2.2 ± 2.9

Data are expressed as means ± S.D.; limit of detection was >100 cfu/organ.
[a]cfu below detectable levels.

Table 3. AmB concentration in liver and kidneys 24 h after the last treatment

Treatment	Dose (mg/kg)	μg AmB/g of tissue	
		liver	kidneys
AmB-deoxycholate	0.5	9.0 ± 4.3	4.3 ± 0.3
AmB-MM	0.5	4.1 ± 1.2[a]	1.7 ± 0.1[a]
	2	28.3 ± 9.1	5.1 ± 0.2
AmB-NP	0.5	2.2 ± 0.9[a,b]	1.8 ± 0.4[a]
	2	28.4 ± 6.7[b]	5.9 ± 1.4[b]

The drug was extracted and determined by HPLC as described in Materials and methods. Data are expressed as means ± S.D.
[a]$P < 0.05$ versus AmB-deoxycholate treatment.
[b]$P < 0.05$ versus AmB-MM treatment.

(<100 cfu per organ) from the liver and kidneys of surviving mice treated with AmB-deoxycholate 0.5 mg/kg and AmB-NP 2 mg/kg at day 3 after the last treatment (Table 3). However, the yeast could be detected in the liver of those animals treated with AmB-MM at 0.5 and 2 mg/kg and AmB-NP at 0.5 mg/kg. At day 10, *Candida* was undetectable from the liver and kidneys for all treatments, even for AmB-NP 0.5 mg/kg for which the percentage survival was greatly decreased.

Determination of AmB accumulation in organs

The amounts of AmB recovered in liver and kidneys were obtained after administration of two doses of AmB 24 h after the last administration of each of the treatments (Table 3). Following the 0.5 mg/kg dose, AmB-MM and AmB-NP accumulation in liver and kidneys was significantly lower than for AmB-deoxycholate, with no differences between the two polymeric formulations. When doses of 2 mg/kg of AmB-polymeric formulations were administered, the levels of the drug in kidneys were approximately 10–20% of those measured in the liver. AmB concentrations in the kidneys increased in proportion with the dose, which was not the case in the liver. The AmB concentrations found in the liver of mice treated with polymeric formulations (AmB-MM or AmB-NP) at 2 mg/kg were higher than expected based on the results at 0.5 mg/kg, suggesting the existence of an additional uptake mechanism for these formulations in this organ.

Discussion

Toxicity

The AmB polymeric formulations described here displayed a lower *in vivo* toxicity than AmB-deoxycholate (Figure 5). This fact can be associated with a reduction in AmB accumulation in kidneys and in the liver of mice treated with AmB-polymeric formulations, compared with the same dose of AmB-deoxycholate (0.5 mg/kg, Table 3). AmB nephrotoxicity (related to the level of the drug in the kidneys[14,35]) and liver dysfunction are toxic effects mainly found after chronic administration of sublethal doses.[36] Furthermore, at 2 mg/kg, the observed increase in liver concentration exceeding dose proportionality is consistent with a significant uptake of the drug by macrophages.[37–39] Moreover, an increase in the time of circulation of AmB in blood could explain the low AmB amount found in the liver, compared with AmB-deoxycholate (0.5 mg/kg), in spite of the major contribution of this organ to AmB elimination.[39] A decrease in opsonization by plasma components and rapid MPS uptake have been previously described for colloids composed of poloxamer.[40–42]

On the other hand, the low toxicity induced by our formulations at the cellular level, as revealed by the lack of haemolysis in human erythrocytes and by the results of MTT in macrophages (Figure 3a) and renal cells (Figure 3b), were noticed and other factors implied in the results of mice toxicity. In fact, polymeric AmB formulations strongly decreased drug-induced haemolysis efficiency, but they produced the same membrane permeability as free AmB measured by K^+ leakage. It is assumed that the permeabilizing effects (K^+ leakage) are due to the ability of AmB to form transmembrane channels,[43] whereas AmB must also induce oxidation of membrane lipid to cause cell death (Hb leakage) at higher doses.[44] K^+ leakage from human red blood cells has been previously related to the stability of association between AmB and carrier compounds.[16,45] All AmB dispersions exhibited quite similar stability upon dilution (Figure 1) and, in fact, no differences were found between them in their ability to affect membrane permeability. The proportion of free drug was enough to produce K^+ release, the earliest toxic event at low drug concentration.[19] On the other hand, conformational changes in the drug self-aggregates (Table 1) induced by the association of the drug with nanospheres or poloxamer 188 could help to prevent lipid oxidation.[30]

Also, a direct blockage of phagocytosis by poloxamer 188[46] could be the origin of the observed decreased AmB uptake by J774.2 macrophages and LLC-PK1 renal cells, avoiding toxic accumulation of the antibiotic in mitochondrial vacuoles,[47] and decreasing *in vitro* mitochondrial toxicity against these cells (Figure 2).

Activity

Owing to the reduced toxicity of these polymeric formulations, high doses (2 mg/kg) of the AmB polymeric formulations presented here were administrated in a model of systemic candidiasis in neutropenic mice (Figure 5). These doses were required to achieve the same therapeutic effect as AmB-deoxycholate at 0.5 mg/kg (Figure 6) as also described for AmB lipidic formulations.[8,10,11]

The activity of the formulations against candidiasis, was found to depend on both (i) organ accumulation of AmB polymeric formulations, which was not that of the organism in a disseminated infection, and (ii) uptake of drug by infected cells in a way able to damage the fungal cells.[9–13]

(a) Since the activity of a drug in a model of disseminated candidosis after iv administration of inoculum required high drug concentrations in liver and especially in kidneys, the polymeric formulations decreased the activity of AmB against *C. albicans*.

(b) Drugs associated with colloidal carriers are mainly targeted to macrophages. Inside these cells, a direct interaction of AmB with the yeast is necessary for intracellular drug activity. Moreover, macrophages can act as a reservoir. They release the drug and then, it can attack microorganisms disseminated outside cells. Extracellular *C. albicans* were eight-fold more sensitive to AmB-MM and AmB-NP than to free drug. However, AmB-NP and AmB-MM exhibited, respectively, lower or the same activity as the free drug against less accessible *C. albicans* inside macrophages (Figure 4). Like AmBisome,[9–13] the large decrease in the uptake of AmB by cells found when the drug was associated with polymeric colloids was not consistent with the fully retained antifungal activity of some of our formulations (AmB-MM). We suggest that the reduced uptake is compensated by a synergic interaction found between AmB and poloxamer (not so strongly associated as the drug with AmBisome compounds[9,12]) at the fungal membrane,[23] resulting in a level of activity against intracellular *C. albicans* similar to that of AmB-deoxycholate. A similar explanation has been put forward for the activity of PEG-AmB-LIP.[10,11]

In summary, AmB polymeric formulations decreased the *in vivo* antifungal activity of free drug and higher concentrations are necessary to obtain a similar therapeutic effect. However, these doses could be administered because the formulations also reduced the toxicity of AmB. In addition, the formulations could be marketed at a reasonable price. However, further experiments, especially related to chronic toxicity, must be addressed to tackle the proposed AmB formulations presented here as an intermediate alternative between the high efficacy of AmB-deoxycholate and the low toxicity of AmBisome.

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