In vivo distribution and therapeutic efficacy of a novel amphotericin B poly-aggregated formulation

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Objectives: The purpose of this investigation is the study of toxicity, in vivo distribution and therapeutic activity against candidiasis of poly-aggregated amphotericin B, in two different formulations: not microencapsulated (P-AMB) or incorporated in albumin microspheres (MP-AMB).

Methods: The therapeutic efficacy and toxicity of amphotericin B formulations was studied in an immunocompetent murine model of systemic candidiasis. A pharmacokinetic study was also performed to measure the plasma, kidney, liver and spleen amphotericin B concentrations after administration of the three formulations to mice.

Results: The acute toxicity of P-AMB in mice is lower than that of the conventional amphotericin B reference formulation (D-AMB). The 50% lethal doses were increased at least eight times. Intravenous bolus administration of doses up to 40 mg/kg of body weight of poly-aggregated amphotericin B, either P-AMB or MP-AMB, did not produce acute symptoms of toxicity. Interestingly, in the pharmacokinetic study, significant \( \left( P < 0.05 \right) \) lower plasma and kidney amphotericin B concentrations and higher liver and spleen amphotericin B concentrations were achieved after poly-aggregated amphotericin B formulation (P-AMB and MP-AMB) administration in relation to the reference formulation (D-AMB). At high amphotericin B doses, no significant differences in efficacy \( \left( P > 0.05 \right) \) were observed among the formulations (D-AMB, P-AMB and MP-AMB).

Conclusions: Although the efficacy in the candidiasis treatment was decreased as a consequence of amphotericin B aggregation, it can be compensated by the possibility of increasing the doses with lower nephrotoxicity. Moreover, due to its lower toxicity while maintaining its effectiveness, the poly-aggregated formulations (P-AMB and MP-AMB) have a better therapeutic index than the conventional formulation (D-AMB).

Keywords: aggregation state, candidiasis, therapeutic index, toxicity

Introduction

Amphotericin B remains the reference treatment for invasive fungal infections and the current guidelines for the treatment of haematogenous candidiasis recommend the use of amphotericin B.1 The conventional amphotericin B deoxycholate (Fungizone®) formulation has been available since 1958 and is a colloidal suspension of amphotericin B. This preparation has a wide variety of acute and chronic side effects; nephrotoxicity being the major adverse effect limiting its use.2 Toxicity has limited the daily dose of the deoxycholate formulation of amphotericin B to \( \sim 1 \) mg/kg. This toxicity is partially reduced when a lipid carrier is used, so three lipid preparations of amphotericin B have been licensed and are currently available. Animal studies and clinical use in humans have indicated that liposomal and lipid formulations of amphotericin B are less toxic than Fungizone®,3–5 but their efficacy is not yet clear for Andes et al.6 because these new formulations are not as potent as the conventional amphotericin B on an mg/kg basis. In addition to these marketed formulations, several other carrier systems such as pegylated liposomes,7 microemulsions,8 cochleates,9 water-soluble complexes,10 microspheres,11 nanosuspensions12 and nanospheres13 have been designed to reduce nephrotoxicity and to improve the deoxycholate amphotericin B

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efficacy. In comparison with the conventional Fungizone® formulation, these carrier systems are able to modify the in vivo amphotericin B distribution and so improve the amphotericin B therapeutic index. It is clear that amphotericin B can be considered a model drug for drug targeting vehicles.

In liquid media, amphotericin B molecules can adopt several different organizations. Depending on the medium, excipients and temperature, amphotericin B molecules can remain freely separated in true solutions or assemble to build up macromolecular structures. Some authors have catalogued the amphotericin B molecules in three categories based on their states of aggregation. If a true solution is obtained, amphotericin B is obviously presented in a monomer form. Due to the low aqueous amphotericin B solubility, amphotericin B is usually in an aggregated disposition. In the conventional Fungizone® preparation, amphotericin B is in an oligomer form. Different types of aggregation of oligomers or poly-aggregates can be obtained by heating Fungizone®, by interactions with different excipients, or by variations on the pH conditions during the formula preparation procedure. The different types of aggregates amphotericin B molecules are associated with different stability, toxicity and efficacy profiles. For instance, mild heating of the Fungizone® formulation induces aggregation of amphotericin B molecules. These poly-aggregated forms of amphotericin B are much less toxic to mammalian cells than the small soluble water aggregates (oligomers) present in Fungizone®. This increase in amphotericin B aggregation produces only a slight reduction of in vivo activity against Candida albicans but an increase in its activity against Leishmania donovani.

We have recently reported a method to obtain amphotericin B in a poly-aggregated disposition that has proved to be less toxic than the conventional amphotericin B and more efficacious for the treatment of experimental leishmaniasis. Our aim in the current work was to test these new formulations of amphotericin B against conventional amphotericin B deoxycholate (D-AMB) as regards to compartmental pharmacokinetics, toxicity and efficacy in mice with disseminated Candida infection. Specifically, the two new formulations that we examined were a free form of poly-aggregated amphotericin B (P-AMB) and a form microencapsulated in human albumin microspheres (MP-AMB).

Materials and methods

Materials and chemicals

Amphotericin B was a gift from Squibb Bristol Myers, Barcelona, Spain. High-pressure liquid chromatography (HPLC) grade acetone, nitrile and methanol (MeOH) were purchased from Labscan, Dublin, Ireland. Sodium hydroxide, dibasic sodium phosphate, monobasic sodium phosphate, orthophosphoric acid (85%) and acetic acid were supplied by Panreac S.A., Barcelona, Spain. Sodium deoxycholate was provided by Fluka Chemie A.G., Buchs, Switzerland. Human serum albumin was purchased from Aventis Behring, Barcelona, Spain. Deionized water was obtained from a Milli-Q water purification system (Millipore, USA).

Preparations

Three different formulations were prepared: D-AMB, P-AMB and MP-AMB.

(i) D-AMB had similar qualitative and quantitative composition to the commercialized medicine Fungizone® (reference formulation) and was prepared as follows. Amphotericin B was dispersed in 5 mL of a water solution formed by sodium deoxycholate, dibasic sodium phosphate and monobasic sodium phosphate previously adjusted to pH 12.0 with sodium hydroxide. Once the drug was homogeneously dispersed, it was acidified to pH 7.4 with orthophosphoric acid. Water was added to the resulting mixture up to a final volume of 10 mL.

(ii) P-AMB (free poly-aggregated amphotericin B) was prepared similarly to the D-AMB formulation, but drastic changes in pH were not produced. Simply, amphotericin B was dispersed in 5 mL of a solution containing sodium deoxycholate, dibasic sodium phosphate and monobasic sodium phosphate in water. The resulting homogeneous suspension was also diluted in water up to 10 mL. The amphotericin B molecular organization was analysed by spectrophotometry.

(iii) MP-AMB (microencapsulated poly-aggregated amphotericin B) was prepared by spray-drying of a suspension similar to the P-AMB formulation but mixed with a 20% serum albumin solution. Amphotericin B was dispersed in 5 mL of a water solution formed by sodium deoxycholate, dibasic sodium phosphate and monobasic sodium phosphate. The resulting dispersion was subjected to moderate stirring to achieve a homogeneous suspension. A 20% serum albumin solution (5 mL) was added, and the final mixture was spray-dried using a Büchi B 191 spray-drier to obtain albumin microspheres containing P-AMB. The mean particle size of the microspheres (1.0 ± 0.7 µm), amphotericin B content and molecular aggregation state were assayed as previously reported.

Animals

Albino ICR mice (20–30 g) were housed in groups of six in plastic cages in a 12 h dark–light cycle animal facility with controlled temperature (25°C) and humidity (70%). Water and food were unrestricted throughout the study. All animals were housed and maintained in the Laboratory of Animal Facilities of the University Complutense according to the principles of animal protection amended by Directive 86/609/EU of European Union legislation.

Acute toxicity

Mice were divided into groups containing six animals each. Each group was injected by intracardiac route with one of the formulations of amphotericin B. Doses were administered at 1, 2, 5, 10 or 40 mg/kg amphotericin B until the 50% lethal dose (LD50) was reached. The animals were examined for mortality over the following 48 h.

In vivo distribution study

All preparations for the amphotericin B-distribution study were administered as a bolus into the heart at doses of 1 mg/kg (body weight) of D-AMB formulation and 2 mg/kg (body weight) of P-AMB and MP-AMB formulations. Immediately before heart administration, all preparations were reconstituted with sterile 5% dextrose in water. The intracardiac route instead of the intravenous route was chosen due to its rapidity and capacity for the injection of high doses. Blood samples were taken from the retro-orbital venous sinuses at 5, 30 and 90 min; 6, 18 and 40 h; and 4 and 7 days after drug injection. Mice were then sacrificed by chloroform inhalation,
Poly-aggregated amphotericin B

and the liver, spleen and kidneys were removed immediately. Blood was collected in heparinized tubes and plasma was immediately separated by mild centrifugation. One part of plasma was mixed with four parts of methanol. Kidneys, spleens and livers were weighed and homogenized. Samples of these organs were sonicated in water for 20 min, and four parts of methanol were added to one part of the homogenate. The resulting mixtures obtained from plasma, kidneys, spleen and liver were vortexed and centrifuged (8000 g). Supernatants were stored at –20°C until analysis. Three mice were used for each time point. The level of amphotericin B in the plasma and organs was determined by a validated reverse-phase HPLC technique. Separation was obtained using a Thermo Hypersil BDS C18 250 x 4.6 mm column (5 μm particle size) and an acetonitrile/ acetic acid/water (52:0.4:47.6) mobile phase. An injection volume of 100 μL was used with a flow rate of 1 mL/min. Amphotericin B was eluted at 10.5 min and was detected at 406 nm. Quantification was carried out by measuring the peak area. Concentrations in samples were calculated by interpolation from the calibration curve. Concentration data versus time profiles in plasma, kidneys, spleen and liver were used to estimate the area under curve from 0 to 168 h (AUC0–168). AUC0–168 was calculated by the trapezoidal rule and liver were used to estimate the area under curve from 0 to 168 h.

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Efficacy experiment

Candida strain. C. albicans SC531427 was used in all experiments and was stored at ~80°C in YED broth containing 50% (v/v) glycerol. For the infection studies, the yeast strain that was grown at 30°C in YED broth agar plates (1% yeast extract, 2% dextrose and 2% agar) was harvested, washed twice with phosphate-buffered saline (PBS) and diluted in the same buffer to the desired density of 2 x 10³ cfu/mL.

Infection model for the immunocompetent mice. Disseminated infection with Candida was achieved by injection of 2 x 10⁸ cfu/mL via the lateral tail vein of immunocompetent mice 72 h prior to starting of drug therapy.28 The course of C. albicans infections was monitored by the general condition of the mice, survival and evaluation of fungal load in the most representative organs: brain and kidney.29,30 At the end of the study, mice were sacrificed by chloroform inhalation, and then kidneys and brain from individual mice, randomly selected (five per group), were removed aseptically and placed in a tissue homogenizer with 5 mL of PBS. The number of cfu in the specimens was determined by a plate dilution method, using yeast extract–dextrose–chloramphenicol and counting colonies after 24–48 h of incubation at 37°C. Survival was monitored up to day 24. Mean survival times (MSTs) and standard errors were estimated by the Kaplan–Meier method (Statgraphics Plus V. 5.1, Manugistics, Rockville, MD, USA) and comparisons among groups were performed using the logrank test.

Drug administration. In this study, 0.2 mL of each preparation was administered by injection via the lateral tail vein of infected immunocompetent mice (eight per group) in triplicate. Drug solutions were prepared on the day of study by dissolving them in sterile H2O; subsequent drug dilutions were made with 5% dextrose. Treatment (a single dose) with D-AMB (1 mg/kg amphotericin B), MP-AMB (1, 5 and 10 mg/kg amphotericin B) or P-AMB (10 mg/kg amphotericin B) was initiated 72 h after infection with C. albicans.

Results

Acute toxicity

Results are reported in Table 1. The formulations P-AMB and MP-AMB did not cause acute toxicity even at doses up to 40 mg/kg. However, the formulation D-AMB (conventional formulation) caused mortality at much lower doses than the other two formulations. LD₅₀ of D-AMB was found to be between 2 and 5 mg/kg. Therefore, MP-AMB and P-AMB were at least eight times safer than D-AMB.

Distribution of amphotericin B depending on amphotericin B formulation

Amphotericin B concentrations in plasma. Amphotericin B concentrations in the plasma of mice injected either with 1 mg/kg (body weight) of formulation D-AMB or with 2 mg/kg (body weight) of formulations P-AMB and MP-AMB were measured at different times after injection (Figure 1a). Clearly, D-AMB rendered higher plasma amphotericin B concentrations than formulation P-AMB or MP-AMB, which produced similar levels of plasma amphotericin B.

Amphotericin B concentrations in different organs. Amphotericin B concentrations in kidneys, liver and spleen versus time are shown in Figure 1 (b, c and d, respectively). In kidneys, the highest amphotericin B concentrations were produced by the D-AMB formulation (Figure 1b), a fact that might suggest a higher potential nephrotoxicity than the other two formulations. Amphotericin B concentrations in the kidneys from animals treated with formulation P-AMB were lower than those of the ones treated with D-AMB, but significantly (P < 0.05) higher than the ones obtained with MP-AMB. The bulk of amphotericin B was found in the liver (Figure 1c), where the pattern was different from that seen in kidneys. In this organ, amphotericin B levels were higher in the animals receiving formulations P-AMB and MP-AMB than in those receiving D-AMB, being very similar for both poly-aggregated formulations. High hepatic amphotericin B concentrations were found even 7 days post-treatment with P-AMB and MP-AMB.

Table 1. Mortality in mice during the 48 h after administration of a single dose of amphotericin B (AMB)

<table>
<thead>
<tr>
<th>AMB dose (mg/kg)</th>
<th>No. of animals that died/total no. of animals tested (%)</th>
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</thead>
<tbody>
<tr>
<td>D-AMB</td>
<td>P-AMB</td>
</tr>
<tr>
<td>1</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>2</td>
<td>2/6 (33)</td>
</tr>
<tr>
<td>5</td>
<td>4/6 (67)</td>
</tr>
<tr>
<td>10</td>
<td>0/6 (0)</td>
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<tr>
<td>40</td>
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formulations, which suggests a high affinity for this organ. In the spleen, amphotericin B concentrations showed a similar pattern to that observed in the liver, although the clearing was faster (Figure 1d), since amphotericin B could be detected for up to 4 days in the spleen from animals treated with P-AMB and MP-AMB.

The relative tissue amphotericin B concentrations depending on the formulation (Table 2) reflect the effect of the formulation on the amphotericin B distribution in the body. The values lower than 1 in plasma and kidneys obtained with both P-AMB/D-AMB and MP-AMB/D-AMB ratios indicate that amphotericin B in a poly-aggregated form renders lower concentrations than the less aggregated D-AMB formulation. This effect is even increased by the microencapsulation of poly-aggregated amphotericin B in albumin microspheres (MP-AMB), as can be deduced from the lower $P$ values obtained in the comparison of MP-AMB/D-AMB with respect to the P-AMB/D-AMB ratio. The effect of microencapsulation is significant in the kidneys, where lower values were obtained for MP-AMB/D-AMB than for P-AMB/D-AMB. At the same time, values higher than 1 in liver and spleen indicate that poly-aggregated formulations, specially the microencapsulated one, are targeted to the spleen and the liver, where the high amphotericin B concentrations are maintained during several days.

Comparison of efficacy among D-AMB, P-AMB and MP-AMB

The efficacy of the different formulations was deduced from the survival curves (Figure 2) and the MSTs (Table 3) of the mice infected with a lethal dose of $C. albicans$ blastospores and the subsequent treatment with different doses of the poly-aggregated amphotericin B versus the D-AMB reference formulation. Due to the lower acute toxicity of poly-aggregated amphotericin B, higher doses were administered than with the conventional D-AMB formulation, either as the free P-AMB or microencapsulated MP-AMB. Obviously, all amphotericin B formulations showed efficacy with respect to the untreated control group. It is important to recognize that, at the same dose, MP-AMB formulation is less effective than the conventional D-AMB formulation ($P < 0.05$). Nevertheless, when higher doses of P-AMB and MP-AMB were used, a certain increase in the percentage of survival (from 70% to 85%) was obtained in comparison with the reference D-AMB formulation, although the colonization in kidneys and brains was not statistically different for the three formulations ($P > 0.05$). For the treatments of D-AMB at 1 mg/kg and the poly-aggregated P-AMB and MP-AMB formulations both at 10 mg/kg, the fungal load in kidneys was always higher than $4.5 \log \text{cfu}$ while in brain it was higher than $1.5 \log \text{cfu}$. The same percentage of mice, ~20%, cleared the infection regardless of the treatment.

Interestingly, at the end of the study, when animals were sacrificed and organs were removed for the different assays, unilateral kidney atrophy was observed in most of the mice treated with D-AMB (Figure 3a), whereas most of the mice treated with P-AMB or MP-AMB conserved both kidneys with a normal size.

![Figure 1. Amphotericin B concentrations [average values and standard errors (bars)] versus time in plasma (a), kidneys (b), liver (c) and spleen (d) after the injection of D-AMB at 1 mg/kg, P-AMB at 2 mg/kg and MP-AMB at 2 mg/kg.](https://academic.oup.com/jac/article-abstract/61/5/1125/846277 by guest on 21 November 2018)
and appearance (Figure 3b), although sometimes they were also affected (Figure 3c).

Discussion

The mild heating of Fungizone\textsuperscript{2} induces amphotericin B aggregation and a reduction in its acute toxicity, as shown by several prior studies, in which toxicity was reduced 2-fold compared with unheated Fungizone\textsuperscript{23} or others where lethality was observed at doses higher than 7 mg/kg.\textsuperscript{21} Under our experimental conditions, Table 1 shows that acute lethality has been reduced at least eight times, and doses up to 40 mg/kg have been tolerated without lethal effects. The noteworthy differences between our results and those previously reported\textsuperscript{21,23} could be related to the method used to induce amphotericin B aggregation. Probably, the mild heating of conventional Fungizone\textsuperscript{2} does not induce the same degree of aggregation as our pH-dependent procedure. The reduced toxicity allowed us to administer higher doses of poly-aggregated amphotericin B, either free (P-AMB) or microencapsulated (MP-AMB), and to compare their effects with those of conventional Fungizone\textsuperscript{2} (D-AMB).

The aggregation state of amphotericin B affects its distribution in the body after intravenous administration. The aggregated forms of amphotericin B can be cleared by the reticulo-endothelial system, as has been previously studied in macrophage cultured cell lines.\textsuperscript{31} If the same occurs in vivo, a different body distribution should be obtained in comparison with non-aggregated forms of amphotericin B. In our work, amphotericin B distribution in plasma, liver, spleen and kidneys was selected for examination because they are target organs of infectious agents. Kidneys are recognized as the principal organs where the toxic effect of amphotericin B is expressed and as one of the organs where the evolution of the systemic candidiasis can be studied.\textsuperscript{29,30} Our current study demonstrates that free poly-aggregates (P-AMB) and microencapsulated poly-aggregates (MP-AMB) yielded higher concentrations of amphotericin B in mouse liver and spleen compared with the reference formulation (D-AMB), whereas levels in plasma and kidneys
were lower. Both poly-aggregated formulations showed a similar pattern of distribution, although microencapsulation significantly ($P < 0.05$) decreased kidney uptake (Table 2). Our data suggests that the reticulo-endothelial system constitutes a major pathway for the accumulation of poly-aggregated forms of amphotericin B. This behaviour is similar to that previously reported for other microparticulate delivery systems like liposomes, nanospheres or lipid complex.32–35

The tissue distribution of heated Fungizone® has been previously studied in rabbits by Kwong et al.36 These authors found that the aggregation of amphotericin B induced by the heating treatment significantly decreases plasma concentrations and increases liver concentrations with respect to conventional amphotericin B, in a similar way as seen in our mouse model. Interestingly, in their experiment with heated Fungizone®, the kidney concentrations, although lower than with the reference formulation, were not statistically significant. In our experiments, the reductions were statistically significant (Table 2). The divergence between our results and those reported by Kwong et al.36 may be due either to the different animal models and/or to the differences in the aggregation state, as has been previously mentioned in relation to the acute lethality results.21,23

We performed a comparative study of efficacy with the three formulations (D-AMB, P-AMB and MP-AMB) in a C. albicans murine model of systemic infection. The diagnosis of systemic candidiasis is rarely reached in the first stages of infection and that is why many fungal infections are diagnosed only at autopsy.37 That is why we established 72 h as the time between infection and treatment of mice, proving that our formulations are effective against candidiasis even on an established infection. However, in previous experiments by other authors, the treatment has been administered at earlier times, such as 6 or 16 h of infection.21,23 The non-treated mice and the mice receiving MP-AMB at the dose of 1 mg/kg showed higher mortality than the reference D-AMB formulation (Figure 2). This may be due to the fact that lower amphotericin B concentrations were achieved in plasma and kidneys after poly-aggregated amphotericin B (P-AMB or MP-AMB) administration compared with amphotericin B deoxycholate. However, increasing the doses of either MP-AMB or P-AMB led to an improved survival rate and no significant ($P > 0.05$) differences in efficacy were found in relation to the conventional treatment (D-AMB). Moreover, the decrease in amphotericin B concentration in kidneys did not reduce efficacy in clearing the fungal load in this organ, but the nephrotoxicity was appreciably reduced (Figure 3).

This similarity in efficacy against C. albicans of the three amphotericin B formulations studied correlates with efficacy data on clinical treatment of candidiasis in humans. In clinical practice, different amphotericin B formulations have been compared and although all of them have proved to be equally effective,38 some of them are less toxic and therefore are considered safer. In our experimental conditions, a similar conclusion can be obtained with both poly-aggregated amphotericin B formulations because their lower toxicity permits an increase in the therapeutic index as compared with the amphotericin B conventional formulation, and makes them suitable for the treatment of candidiasis.

In other infections in which the amphotericin B susceptible pathogenic agent is preferentially located in liver or spleen, the new poly-aggregated amphotericin B formulations have proved to be especially effective, as in the case of visceral leishmaniasis, where Leishmania amastigotes are located in the mononuclear phagocyte cells of the reticulo-endothelial system. In this case, the aggregation of amphotericin B molecules, either by heat39 or by pH-dependent procedure,40 clearly improves its activity with respect to conventional amphotericin B.

In conclusion, different pharmacokinetic distribution results in relevant differences in efficacy and toxicity. In our experimental conditions, it has been demonstrated that there is a clear relationship between the aggregation state of amphotericin B and its toxicity and distribution. The poly-aggregated formulations (P-AMB and MP-AMB) have a better therapeutic index than the conventional formulation (D-AMB) due to their lower toxicity, without sacrificing efficacy. This fact should encourage scientists in the search for new preparations of lesser toxicity than the conventional formulation of amphotericin B (Fungizone®) and less expensive than the lipid formulations recently marketed (AmBisome®, Abelcet® and Amphotec®), which are of limited utility due to their high cost.30 Finally, our poly-aggregated amphotericin B formulations may be of significant promise for the improvement in treatment of systemic C. albicans infections and probably for other Candida species.

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Transparency declarations

None to declare.

Supplementary data

A colour version of Figure 3 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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