

In vivo activity of a novel amphotericin B formulation with synthetic cationic bilayer fragments

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Solubilization of amphotericin B (AMB) by dioctadecyldimethylammonium bromide (DODAB) bilayer fragments inspired this evaluation of its *in vivo* activity from survival and tissue burden experiments against systemic candidiasis in a mouse model. AMB (≤ 0.1 g/L) was simply added to a DODAB powder dispersion in water (10 g/L) previously prepared by sonication in the absence of organic solvents. The AMB aggregation state was evaluated from UV–visible light absorption and dynamic light scattering for aggregate sizing. AMB was stabilized by the DODAB bilayer fragments in its monomeric form, mixing of AMB and DODAB dispersion in pure water causing disappearance of large water-insoluble drug aggregates. From survival experiments, both the bilayer, DODAB/AMB, and the traditional deoxycholate/AMB formulation (DOC/AMB) had identical effect when given by the same route at the same dose of 0.4 mg/kg/day given intraperitoneally for 10 days. From spleen and kidneys tissue burden experiments, similar efficacy of both preparations in reducing tissue cfu counts was obtained. In summary, DODAB/AMB was as effective as DOC/AMB for treating systemic candidiasis in a mouse model.

Keywords: drug delivery, amphotericin B, *in vivo*

Introduction

Opportunistic infections caused by fungi are one of the leading causes of death in immunocompromised individuals.^{1–3} In the hospital environment yeast infections account for up to 10% of nosocomial bloodstream infections and are a serious cause of mortality.⁴

Only a limited spectrum of antimycotic agents is available for the treatment of disseminated mycotic infections. Amphotericin B (AMB) is the therapy of choice for most invasive *Candida* infections. This broad-spectrum fungicide is a polyene antibiotic that exerts its toxic effect on fungal cells by forming complexes with membrane sterols⁵ that act as transmembrane channels allowing leakage of ions and other vital components from the cell.⁶ Unfortunately, AMB nephrotoxicity is high^{7,8} and has often been related to the occurrence of AMB as large aggregates both in aqueous solution and in lipid formulations.^{9,10} The low solubility of AMB in water and many organic solvents is a problem that is not easily solved. In order to formulate AMB, various strategies including the use of liposomes,¹¹ surfactants,¹² oil-in-water emulsions^{13,14} and cochleates^{15,16} have been employed, and have certainly improved the therapeutic index of the drug. However, DOC/AMB, the very first formulation for the drug, using the bile salt desoxycholate, is still the most frequently used

formulation in clinics despite the formation of a very unstable detergent–micelle complex, with considerable toxicity.¹⁷ The reason for this seems to be related to two major drawbacks of lipid-based formulations: (i) AMB is required at higher doses than those in DOC/AMB (due to competition between carrier lipids and fungus membrane for drug solubilization);^{10,18} and (ii) lipids in the formulations are expensive.¹⁷

Dioctadecyldimethylammonium bromide (DODAB) is a synthetic and inexpensive cationic lipid that assembles in water solution, forming bilayer vesicles¹⁹ or bilayer fragments²⁰ depending on the dispersion method. DODAB bilayer vesicles have been used as interface agents in several settings,²¹ such as vaccine adjuvants interacting with different antigens,^{22–24} and biocidal agents against bacteria^{25–28} and fungi,¹⁸ and they exhibit differential cytotoxicity.²⁹ Recently, solubilization of AMB by nanosized, synthetic and charged bilayer fragments electrostatically stabilized in water dispersion was described; unlike other formulations this did not make use of entire and closed bilayer vesicles.³⁰ These results were also extended to include miconazole, a clinically important hydrophobic drug that was solubilized and/or colloidally stabilized using synthetic bilayer fragments.³¹ The AMB solubilization was explained by the very large area of hydro-

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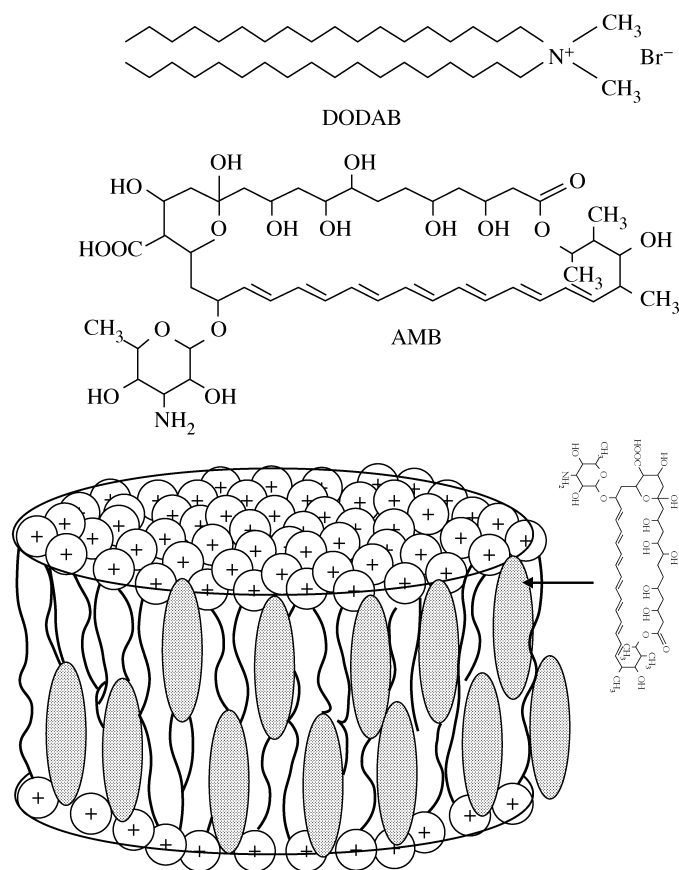


Figure 1. Schematic representation of AMB monomers in the DOD/AMB aqueous formulation (adapted from Vieira & Carmona-Ribeiro³⁰).

phobic nanosurfaces offered by the bilayer fragments, so that the polyenic moiety of the antibiotic interacted with the hydrophobic borders of the bilayer fragments while the hydroxylated moiety of the drug remained in contact with the surrounding water from its solubilization site (see Figure 1).^{30,31}

In this study, the DODAB bilayer fragment/AMB formulation (DOD/AMB) was compared with the classical DOC/AMB formulation, Fungizone, *in vivo* against a mouse model of systemic candidiasis. The drug was present in the DOD/AMB formulation mostly in its monomeric form as indicated by the UV-visible absorption spectra and dynamic light scattering for particle sizing. Survival and tissue burden experiments showed that DOD/AMB was as efficient as DOC/AMB against systemic candidiasis when given by the same route at the same dose.

Materials and methods

Drugs, lipids, microorganism and animals

AMB (batch 0008000336) was purchased from Bristol-Myers Squibb, São Paulo, Brazil. DOC/AMB (AMB clinical preparation; Bristol-Myers Squibb) was purchased from a hospital pharmacy. DODAB, 99.9% pure, was obtained from Sigma Chemical Co. (St Louis, MO, USA). Agar was purchased from Difco. Female Swiss Webster mice 6–8 weeks old weighing 25–30 g were obtained from the University of São Paulo. *Candida albicans* ATCC 90028 was obtained from the ATCC. A clinical isolate of *C. albicans*, strain HU168, was obtained from the Microbiology Laboratory of the Universidade de São Paulo Hospital, Brazil.

Preparation of DOD/AMB formulation

The DODAB synthetic bilayer fragments (SBF) were obtained by sonication with a titanium macrotip probe.¹⁹ The final DODAB concentration was determined by microtitration,³² and adjusted to 10 g/L. AMB solubilization in the 10 g/L DODAB SBF dispersion was performed in the absence of any organic solvent, and the final AMB concentration was 0.05 g/L (DOD/AMB formulation). At final concentrations of AMB ≤ 0.1 g/L (10 g/L DODAB), no yellow AMB aggregates could be detected in the pellet of centrifuged samples. As a control for complete AMB solubilization in the DODAB SBF, increasing concentrations of AMB and 10 g/L DODAB SBF were mixed, incubated (25°C, 24 h) and centrifuged (15 000g for 50 min). No yellow pellet was observed for AMB concentrations < 0.1 g/L; all AMB molecules were solubilized at the hydrophobic rim of the DODAB bilayer fragments,³⁰ whereas the control mixtures (without DODAB) displayed yellow pellets at the bottom of the glass assay tubes. The bilayer fragments, as usual, remained in the supernatant. DOC/AMB was diluted in 5% glucose for parenteral administration, as recommended.³³

Physicochemical characterization of DOD/AMB formulation

Prior to parenteral administration, UV-visible optical spectra were obtained in the double-beam mode of a Hitachi spectrophotometer against a blank of lipid dispersions only (without drug), to subtract light scattered by the lipid dispersions from light absorption spectra for the drug. Routinely, the concentration of AMB in the DOD/AMB formulation was determined by means of spectrophotometry at 412 nm against a proper blank composed of DODAB only. The size distribution for DOD/AMB formulations (mean zeta-average diameter, D_z), was determined by using a ZetaPlus Zeta-Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) equipped with a 570 nm laser and dynamic light scattering at 90° for particle sizing.³⁴ All spectra and size distributions were obtained at room temperature (25°C) just after mixing AMB and DODAB SBF or at 24 h, 72 h, 7 days and 15 days thereafter.

The monomeric form of the drug was stabilized by the DODAB bilayer fragments and remained as such in water solution over at least 15 days at 4°C in contrast to DOC/AMB, a formulation where AMB is preferentially found as large aggregates of the hydrophobic drug.

Determination of *in vitro* activity of DOD/AMB against *C. albicans*

C. albicans ATCC 90028 and HU168 strains were subcultured on Sabouraud dextrose agar (SDA) plates and grown at 35°C for 24 h prior to testing. The macrodilution method was performed according to the recommendations of the NCCLS.³⁵ The medium used for diluting cells was culture medium in the case of DOC/AMB, and pure water for DOD/AMB. The interaction between DODAB and components of the culture medium would completely prevent targeting of the cationic DODAB-drug complex to the oppositely charged *Candida* cells.¹⁸ Counts for cell suspension were confirmed by plating on SDA plates after adjusting to 2.5×10^3 cfu/mL. Minimum lethal concentrations (MLC) for DOD/AMB formulations were determined by cfu counts on a volume of 0.1 mL after a 48 h incubation at 35°C. The endpoint was the lowest drug concentration that killed $\geq 99.9\%$ of the inoculum.³⁶

Development of systemic candidiasis in mouse models

Mouse model systemic candidiasis was developed in accordance with standard procedures, and ethics committee approval was obtained.^{37,38} *C. albicans* isolate HU168 was freshly subcultured (24 h, SDA plates); cells were scraped off, transferred to Sabouraud dextrose broth (SDB) and grown at 37°C overnight. Thereafter, cells were washed three times in saline and adjusted to 70% transmittance at 530 nm yielding a

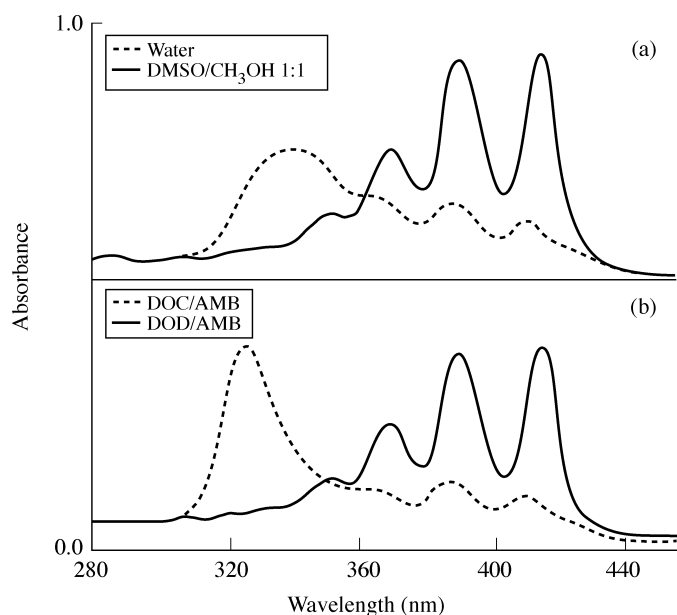


Figure 2. Optical absorption spectra of AMB. (a) AMB in DMSO:methanol (1:1) (unbroken line) or in water (broken line). (b) AMB in DOD/AMB (unbroken line) or in DOC/AMB (broken line). Final AMB and DODAB concentrations are 7 mg/L and 1.4 g/L, respectively.

2.5×10^6 cfu/mL inoculum. Mice were infected by injecting 0.1 mL of the inoculum intravenously (iv) through the tail vein.

Determination of *in vivo* activity of DOD/AMB against systemic candidiasis in mouse models

The *in vivo* activity was evaluated by survival and tissue burden experiments for each group under test.

(i) Survival experiments. At 24 h post-infection (p.i.), groups of 10 female Swiss Webster mice were treated with DOD/AMB, DODAB, DOC/AMB or placebo, either iv or intraperitoneally. Animals were given two different treatments with AMB formulations. In the first, 0.2 mg/kg/day AMB was administered intravenously for 4 days. In the second, 0.4 mg/kg/day AMB was given intraperitoneally for (ip) 10 days. Control group mice received DODAB alone or remained untreated. Animal groups were observed twice daily for death or abnormal behaviour over 35 days p.i.

(ii) Tissue burden study. Seven mice per group were infected as described above and treated 24 h p.i. with AMB 0.2, 0.4, 1.0 and 4.0 mg/kg/day over either 4 or 7 days administered as DOC/AMB or DOD/AMB formulations either iv or ip and compared with control groups of untreated or DODAB-treated mice. The tissue burden was assessed at day 4 and 8, for iv- and ip-treated groups, respectively. Kidneys and spleens were aseptically removed, weighed and each was homogenized in 1 mL of sterile saline. Ten-fold dilutions of the homogenate were plated, incubated (48 h, 37°C) and counted for cfu determinations. Results were expressed as cfu/g tissue.

Statistics

Differences in survival after 35 days of observation were assessed by Kaplan–Meier analysis followed by the Wilcoxon test. Comparisons of colony counts among different treatment groups were performed by the Kruskal–Wallis test. $P < 0.05$ was considered statistically significant. All statistical analysis was performed with the software STATISTICA for Windows (StatSoft, Inc., Tulsa, OK, USA).

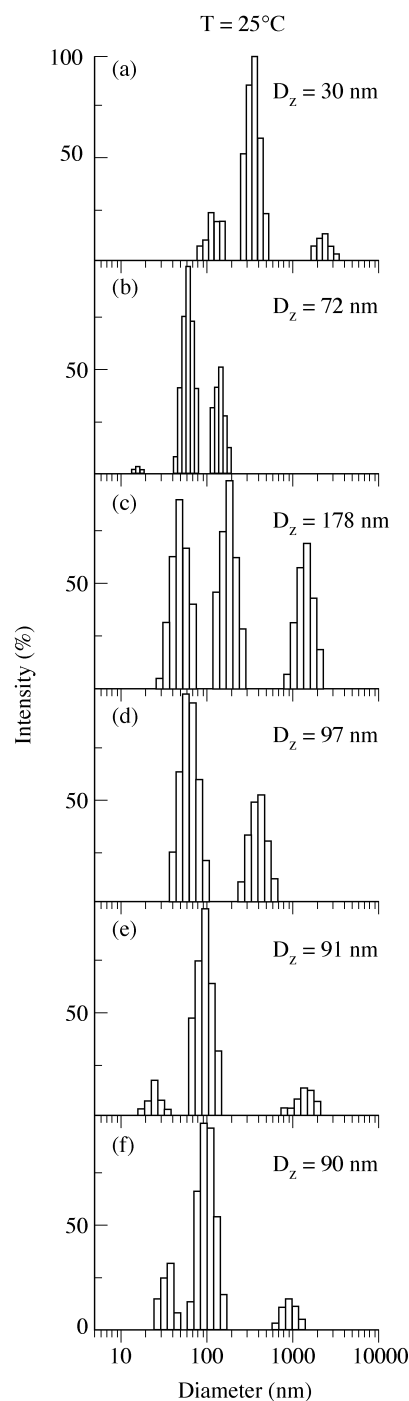


Figure 3. Non-negatively constrained least squares (NNCLS) size distributions and mean D_z for different dispersions in pure water. (a) AMB 30 mg/L; (b) DODAB SBF 10 g/L obtained by sonication with tip. (c–f) DODAB and AMB were mixed to yield the DOD/AMB formulation at 1 h, 24 h, 7 days and 15 days after mixing, respectively. Final AMB and DODAB concentrations were 30 mg/L and 6 g/L, respectively.

Results

Chemical and colloidal stability for monomeric AMB in SBF

Drug, DODAB SBF and mixtures of both were characterized by light absorption spectroscopy and dynamic light scattering.

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Table 1. MLC of DOD/AMB and DOC/AMB for *C. albicans* ATCC 90028 and HU168

Formulation	MLC (AMB mg/L)	
	<i>C. albicans</i> ATCC 90028	<i>C. albicans</i> HU168
Control (AMB/DMSO:methanol, 1:1)	0.125	0.250
DOD/AMB (AMB/DODAB SBF)	0.125	0.250
DOC/AMB (AMB/deoxycholate)	0.250	0.500

The UV–visible spectra of polyenes are very sensitive to conformational changes induced by different molecular interactions, including aggregation. Figure 2(a) illustrates the optical spectra for the drug in its monomeric form [dimethyl sulphoxide (DMSO):methanol, 1:1] and as aggregates (in water). Figure 2(b) shows the optical spectra for DOD/AMB and DOC/AMB formulations. For DOC/AMB, the spectrum resembles the one exhibited by AMB in pure water (compare Figure 2a and b). For DOD/AMB, the AMB spectrum became very similar to the one exhibited by AMB in its best organic solvent (compare Figure 2a and b). In DOC/AMB, the drug was present in the form of large aggregates rather than small mixed micelles with deoxycholate, as previously reported in the literature.³⁹ In contrast to DOC/AMB, AMB addition (in minute volumes of its best solvent) to DODAB SBF solubilized the drug in its monomeric form³⁰ as confirmed in this work also for solvent-free AMB/DODAB SBF mixtures.

Figure 3 shows particle size distribution in water for AMB at 0.03 g/L, DODAB SBF at 10 g/L and DOD/AMB formulation keeping these final concentrations. AMB aggregates of 308 nm mean diameter were solubilized by DODAB SBF, as seen from the progressive decrease of mean sizes with time (Figure 3c–f). This time effect on mean particle size showed that DOD/AMB colloid stability increased as a function of time. In contrast to the drug solubilization and high colloid stability of the DOD/AMB formulation, progressive drug aggregation and low colloid stability was observed for DOC/AMB both at 4 and 25°C; stability was higher at 4°C than at 25°C (Figure 4a–d). Thus, whereas DOC/AMB should be stored in the fridge, DOD/AMB remained stable at 25°C. It was previously reported that AMB solubilization in the DODAB SBF dispersion could be improved by increasing the incubation temperature to 37°C. This condition allowed drug solubilization overnight.³⁰

In order to evaluate AMB chemical stability in both formulations, light absorption spectra were determined as a function of time at two different temperatures (data not shown). AMB spectra in DOD/AMB remained essentially unchanged, indicative of a high AMB stability in the monomeric state at 4°C over 15 days (data not shown). At 25°C, there was a reduction of AMB light absorption peaks from 24 h (data not shown). A similar experiment was performed for DOC/AMB. Extensively aggregated AMB was stable up to 15 days at 4°C and up to 7 days at 25°C (not shown). The drawback in the case of DOC/AMB was the propensity of AMB to form aggregates, as reported previously by others.³⁹

In vitro and in vivo activity of the DOD/AMB formulation

In this work, the MLC of DOD/AMB against *C. albicans* ATCC 90028 and pathogenic *C. albicans* HU168 was determined as 0.125 and 0.250 mg/L, respectively (Table 1). The activity of DOD/AMB *in vitro* was similar to that exhibited by DOC/AMB, since there was

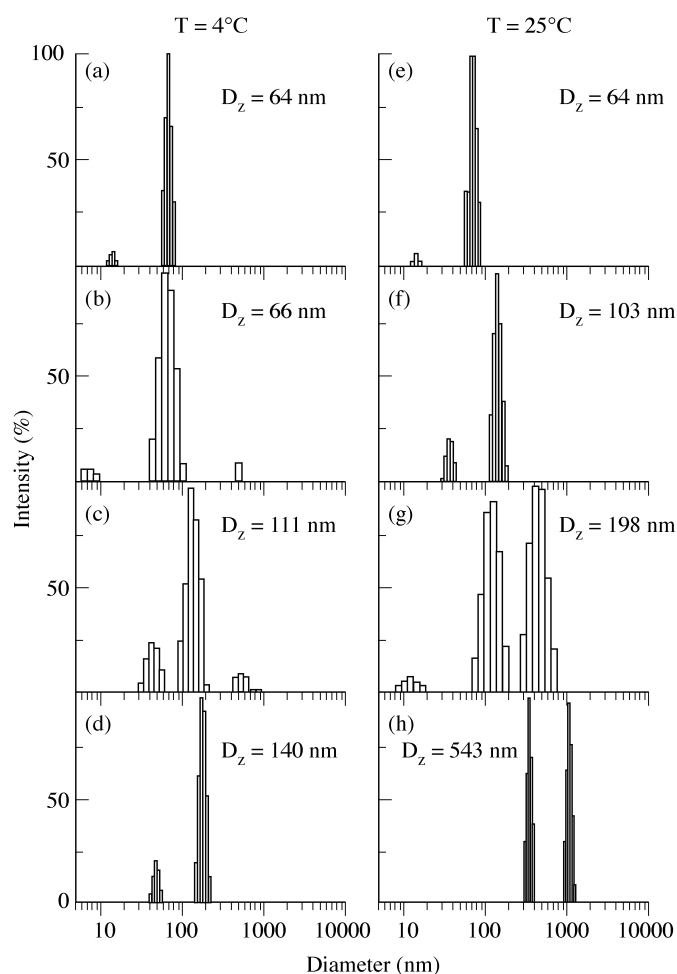


Figure 4. NNCLS size distributions and mean D_z for DOC/AMB dispersions in water as a function of time at two different temperatures. At 4°C (a–d) or 25°C (e–h), size distributions were obtained 1 h (a, e), 24 h (b, f), 7 days (c, g) and 15 days (d, h) after mixing DOC/AMB with water. The final AMB concentration was 30 mg/L.

no significant difference between the mean values (Table 1). It is important to emphasize that the DOD/AMB formulation did lose its fungicidal activity in the culture medium where cationic DODAB SBF interacted with several anionic medium components.

In vivo, survival percentiles for mice in the intraperitoneal DOD/AMB treatment group were similar to those obtained for equivalent AMB concentrations delivered by the DOC/AMB formulation (Figure 5a and b). One hundred per cent survival was achieved at

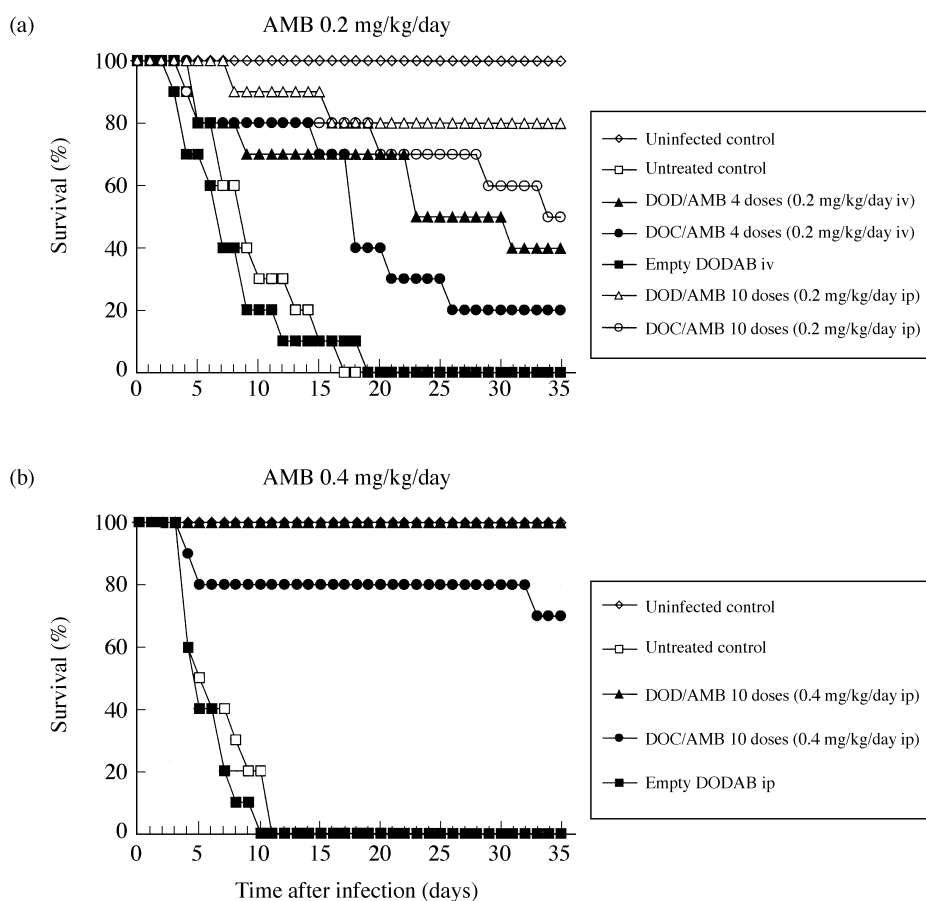


Figure 5. (a) Survival of mice infected with *C. albicans* and treated with either: DOD/AMB at AMB 0.2 mg/kg/day for 4 days iv (closed triangles), or for 10 days intraperitoneally (open triangles); DOC/AMB at AMB 0.2 mg/kg/day for 4 days iv (closed circles), or for 10 days intraperitoneally (open circles); or with empty DODAB SBF iv (filled squares), compared with the untreated (open squares) and uninfected (open diamonds) control. (b) Survival of mice infected with *C. albicans* and treated with either: DOD/AMB at AMB 0.4 mg/kg/day for 10 days intraperitoneally (filled triangles); DOC/AMB at AMB 0.4 mg/kg/day for 10 days intraperitoneally (filled circles); or with empty DODAB SBF intraperitoneally (filled squares), compared with the untreated (open squares) and uninfected (open diamonds) control.

0.4 mg/kg/day given intraperitoneally for 10 days, and this survival could not be considered statistically different from the 70 % survival obtained with the same AMB dose given as DOC/AMB (Figure 5b). DODAB SBF by itself, without drug, did not present any statistically significant *in vivo* activity against the fungus, as expected (Figure 5a, filled squares).

Tissue burden studies were conducted in parallel with the survival studies. The tissue burden experiments showed that DOD/AMB was as efficient as DOC/AMB in reducing cfu/g tissue in spleen (Figure 6a) and kidneys (Figure 6b). For all groups, cfu found in the kidneys and spleens were reduced significantly relative to untreated controls ($P < 0.05$).

Discussion

In vitro, DODAB SBF alone has been described as a potent bactericide^{25–28} and fungistatic agent¹⁸ under conditions of very low ionic strength. The fungistatic DODAB effect *in vitro* was first reported against *C. albicans* in pure water.¹⁸ In order to achieve complete fungicidal action against *C. albicans* *in vitro*, miconazole and AMB were complexed with DODAB. This antifungal activity for DODAB bilayers alone could be explained by their positive charge that drove the bilayer to adsorption onto the oppositely charged

fungal cells. *In vivo*, however, physiological ionic strength (150 mM NaCl) is much higher than that in pure water and the positive charge on the DODAB bilayer is practically zero. Therefore, *in vivo* DODAB cytotoxicity was expected to be negligible, with the observed *in vivo* effects ascribed only to AMB.

Many studies have characterized DODAB self-assembly in aqueous solution.⁴⁰ Depending on the DODAB dispersion method, large or small vesicles or bilayer fragments can be obtained. Using ultrasonic disruption with a tip, which introduces a high energy input in the DODAB powder/water system, DODAB bilayer vesicles are not only formed but also disrupted; bilayer fragments conveniently offer a very large area of hydrophobic nanosurfaces suitable for the solubilization of hydrophobic substances or drugs (Figure 1).^{30,31} Figure 2 confirmed solubilization of AMB using the 10 g/L DODAB dispersion prepared by ultrasonic disruption with a tip. In contrast, aggregation of AMB in the presence of deoxycholate (DOC/AMB) was evident from light absorption spectra (Figure 2). In addition to the advantageous monomeric state for AMB in the DOD/AMB formulation, Figures 3 and 4 also demonstrated its high colloid stability. Whereas the size of DOD/AMB aggregates decreased as a function of time, the opposite occurred for DOC/AMB at 25°C (Figures 3 and 4). Therefore, colloid stability for the new formulation was also better than the one exhibited by DOC/AMB.

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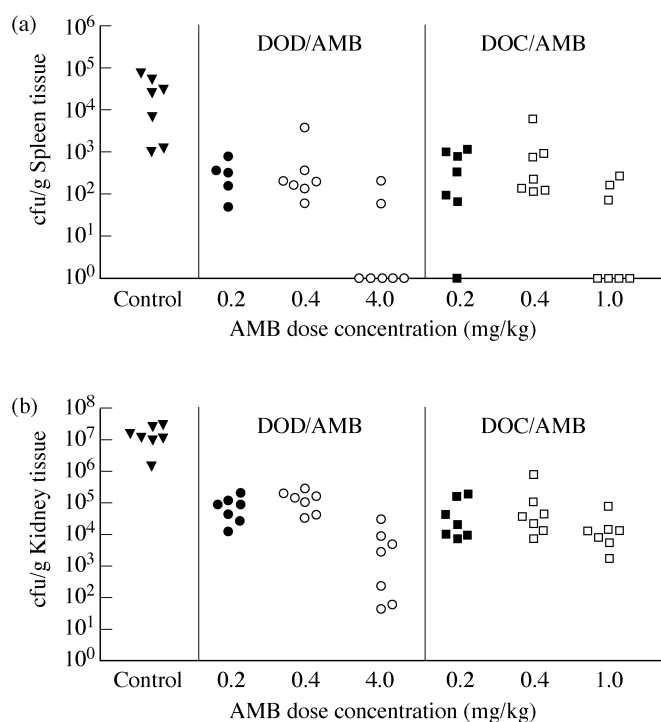


Figure 6. (a) Spleen tissue burden of mice infected with *C. albicans* and treated with either: DOD/AMB iv (filled circles) at AMB 0.2 mg/kg/day for 4 days, or intraperitoneally (open circles), at AMB 0.4 or 4.0 mg/kg/day for 7 days; DOC/AMB iv (filled squares), at AMB 0.2 mg/kg/day for 4 days, or intraperitoneally (open squares) at AMB 0.4 or 1.0 mg/kg/day for 7 days, compared with untreated controls (inverted filled triangles). (b) Kidney tissue burden for the same groups of mice. The effect of treatment was determined at day 4 for the iv group and at day 8 for the intraperitoneal group.

The AMB monomeric state in the DOD/AMB formulation may be responsible for the similar survival percentiles relative to those obtained with DOC/AMB at equivalent AMB doses (Figure 5). Thirty-five days after infection, 0.4 mg/kg/day given intraperitoneally for 10 days resulted in 100% survival for the DOD/AMB formulation; similar survival patterns were obtained with the same dose as DOC/AMB. At this point, since the efficacy of both formulations was similar, one should recognize that there are not yet convincing data to show the real therapeutic advantages of this formulation over Fungizone or other lipid-based formulations that have markedly reduced toxicity relative to Fungizone.⁴¹ In fact, the major problem with Fungizone, its toxicity, is well circumvented by using the lipid-based formulations such as liposomal AMB (AmBisome) or AMB lipid complex (Abelcet). The presence of lipids has improved the therapeutic index by markedly reducing AMB nephrotoxicity. As DODAB is also a lipid preparation, a similar reduction in nephrotoxicity for this novel formulation could be anticipated and toxicity evaluations are currently being undertaken.

The main disadvantage of the DOD/AMB formulation is related to its limited capacity to carry the monomeric form of the drug: 10 g/L DODAB dispersion was able to solubilize AMB over a drug concentration range that had to be lower than 0.1 g/L. Therefore, DOD/AMB treatment regimens would possibly have to be prolonged to administer small daily AMB doses distributed over a larger number of days.

Regarding administration of the novel formulation, there would be no need to wait for longer than overnight at 37°C to administer the

DOD/AMB formulation, since complete AMB solubilization under these conditions has been documented.³⁰

A central question regards the mechanism by which AMB would be delivered to the fungal cell *in vivo*. The drug is expected to be readily available from the bilayer disc border in order to complex with ergosterol present in the fungus membrane. However, contrary to other lipid carriers such as fusogenic liposomes or cochleates, which require fusion of the bilayer carrier with the target cell, the DODAB SBF bilayer is in the rigid gel state and is not expected to fuse with the target cell membrane. Studies have failed to demonstrate leakage of radioactive water-soluble markers in the inner compartment of large DODAB vesicles upon their interaction with bacterial cells or leakage of phosphorylated cytosol components from the bacterial cells upon interaction with DODAB bilayers.²⁷ In contrast, leakage was observed upon cell interaction with surfactants that form micelles instead of bilayers.²⁷ Possibly, since the affinity of DODAB for serum proteins was high *in vitro*,⁴¹ DODAB SBF complexed with AMB could interact with albumin from serum and the stable complex DOD/AMB–albumin might then freely travel in the blood until the ergosterol- or cholesterol-rich cell membrane of the fungus or of the host, respectively, was found and the drug delivered.

In conclusion, DOD/AMB formulation is a monomeric, colloidal and chemically stable, organic solvent-free, inexpensive lipid-based formulation with *in vitro* and *in vivo* activity against *C. albicans*. DOD/AMB was as effective as DOC/AMB against *C. albicans* *in vivo*. Reduced nephrotoxicity relative to Fungizone has been repeatedly reported in the literature for lipid-based AMB formulations.⁴² Therefore, it is to be anticipated that the lipid-based DOD/AMB formulation will also exhibit reduced nephrotoxicity.

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