Low nephrotoxicity of an effective amphotericin B formulation with cationic bilayer fragments

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Objectives: Evaluation of nephrotoxicity of a novel amphotericin B (AMB) formulation with dioctadecyl(dimethylammonium bromide (DODAB) bilayer fragments (DOD/AMB).

Methods: Dose-dependent cytotoxicity of DOD/AMB was evaluated in vitro against cultured kidney epithelial cells in culture. For in vivo experiments, Swiss Webster female mice were injected intraperitoneally for 10 consecutive days with 0.4 mg/kg/day AMB in the form of traditional bile salt desoxycholate (DOC)/AMB or DOD/AMB. Body and spleen weight, and biochemical and histopathological data were obtained at days 11 and 180 after injection.

Results: Nephrotoxicity of the novel formulation was lower than that of Fungizone (DOC/AMB), which is the traditional AMB formulation using DOC. Dose-dependent cytotoxicity of DOD/AMB was lower than that exhibited by DOC/AMB. At day 11, DODAB and DOD/AMB caused loss of body weight and increase in spleen weight, which were not observed for DOC/AMB, although the changes were reversible and weights returned to control values at day 180. Ten days after injection, biochemical parameters for hepatic and renal function remained unaltered. At day 180, renal cortex histopathology revealed leucocytic infiltration and moderate hydropic degeneration of the renal tubules in the DODAB and DOD/AMB groups, in contrast to more severe lesions observed for the DOC/AMB group such as tubular cystic degeneration and glomerular injury, which were absent for the former groups.

Conclusions: The DOD/AMB formulation exhibited differential cytotoxicity and low nephrotoxicity, but there were also important aspects of general toxicity that will require evaluation with full-scale toxicity protocols.

Keywords: dioctadecyldimethylammonium bromide, cytotoxicity, renal function, histopathology

Introduction

A limited spectrum of antymycotic agents is available for the treatment of disseminated fungal infections. Amphotericin B (AMB) is the therapy of choice for most invasive Candida infections.1-3 This broad-spectrum fungicide is a polyene antibiotic that exerts its toxic effect on fungal cells by forming complexes with membrane sterols4,5 that can act as transmembrane channels, allowing leakage of ions and other vital components from the cell.5 Unfortunately, AMB nephrotoxicity is high1,2,4 and has often been related to the occurrence of AMB as large aggregates both in water solution and in the lipid membrane.7,8 The low AMB solubility in water and in many organic solvents is a problem that is not easily solved. In order to formulate AMB, liposomes,9 surfactants,10 oil-in-water emulsions11,12 and cochleates13,14 have been used, and have certainly improved the therapeutic index of the drug. However, Fungizone (DOC/AMB), the very first formulation for the drug using the bile salt desoxycholate (DOC), is still the most frequently used formulation in clinics despite the formation of a very unstable and toxic detergent–micelle complex.8,15,16 The reason for this seems to be related to two major drawbacks of lipid-based formulations:

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(i) AMB is required at higher doses than those in DOC/AMB (owing to competition between carrier lipids and fungus membrane for drug solubilization), and (ii) lipids in the formulations are expensive.

Dioctadecyldimethylammonium bromide (DODAB) is a synthetic and inexpensive cationic lipid that assembles in water solution forming bilayer vesicles or bilayer fragments depending on the dispersion method. DODAB bilayer vesicles have been used as interface agents in several instances such as vaccine adjuvants interacting with different antigens, biocidal agents against bacteria and fungi, and they exhibit differential cytotoxicity. Solubilization of AMB by nanosized, synthetic and charged bilayer fragments electrostatically stabilized in water dispersion has been described; unlike other formulations, this did not make use of entire and closed bilayer vesicles. These results were also extended to include miconazole, a clinically important hydrophobic drug, which was solubilized and/or collooidally stabilized using synthetic bilayer fragments. The AMB solubilization was explained by the very large area of hydrophobic 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 (Figure 1).

Recently, the DODAB bilayer fragment/AMB formulation, DOD/AMB, was compared with the classical deoxycholate formulation mostly in its monomeric form, as indicated by the UV–visible absorption spectra and dynamic light scattering particle sizing. Survival and tissue burden experiments showed that a relatively low dose of AMB (0.4 mg/kg/day given intraperitoneally for 10 consecutive days) in the form of DOD/AMB was as efficient as the same dose in DOC/AMB against systemic candidiasis. In comparison with other lipid formulations, the main advantage of the inexpensive DOD/AMB formulation is a high efficiency at a low AMB dose. However, the nephrotoxicity of this novel formulation required further evaluation, since this is a central issue to be solved when AMB is used in therapy.

**Materials and methods**

**Drug, lipid, microorganisms and animals**

AMB (batch 0008000336) was purchased from Bristol-Myers Squibb (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. Swiss Webster mice 8–12 weeks old weighing 32–40 g were obtained from the University of São Paulo. The animal ethics committee of the Faculty of Pharmaceutical Sciences at the University of São Paulo approved the study. Candida albicans ATCC 90028 was obtained from the ATCC. A clinical isolate of C. albicans, strain HU168, was obtained from the Microbiology Laboratory Universidade de São Paulo Hospital, Brazil.

**Preparation of DOD/AMB formulation**

The DODAB synthetic bilayer fragments were obtained by sonication with a titanium macrotip probe. The method for obtaining large and closed DODAB vesicles was previously described in 1983, and consisted of injecting a chloroformic DODAB solution in a water solution kept at 70°C (above chloroform boiling point and above the mean phase transition temperature for the DODAB bilayer). The method for obtaining open DODAB bilayer fragments based on sonication with a tip was also previously described and discussed in comparison to others. The final DODAB concentration was determined by microtitration and adjusted to 10 g/L. AMB solubilization in the 10 g/L DODAB synthetic bilayer fragments 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 AMB yellow aggregates could be detected in the pellet of centrifuged samples. As a control for complete AMB solubilization in the DODAB synthetic bilayer fragments, increasing concentrations of AMB and 10 g/L DODAB synthetic bilayer fragments were mixed, incubated (25°C, 24 h) and centrifuged (15 000 g 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, 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.
Determination of in vitro activity of DODAB 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. The medium used for diluting cells was 0.264 M d-glucose. 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. Counts for cell suspension were confirmed by plating on SDA plates after adjusting to 2.5 × 10^7 cfu/mL. C. albicans viability in the presence of DODAB formulations was determined by cfu counts in a volume of 0.1 mL after 48 h of incubation at 35°C.

Percentage average weight change and relative spleen weights

Average weight change (%) was calculated by the following equation: 100 × [(W_{daily} – W_{initial})/W_{initial}], where W is weight. At day 11 and 180 after injection, five mice from each group were weighed and euthanized, spleens were excised and weighed, and the relative spleen weight as a percentage of body weight was calculated for each group.

Determination of hepatic and renal function

At day 11 after injection, five mice from each group were weighed and euthanized. Blood was collected without anticoagulant, centrifuged at 2000 g and the serum was frozen at −30°C until analysis. For each experimental condition, serum pools were prepared by mixing equal volumes collected from three mice so that five serum pools were obtained. Alkaline phosphatase (ALP) activity, transaminase activity, and serum urea and creatinine levels were determined in 1 mL of each serum pool using an auto-analyser for clinical chemistry assay (LIASYS Random Access Analyzer; AMS, Roma, Zenica). The auto-analyser was validated for mouse liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST) and ALP] before analytical determinations.

Histopathology of renal cortex

At day 11 and 180 after injection, five mice from each group were weighed and euthanized. The kidneys were carefully dissected and fixed by immersion in a 10% solution of buffered formalin (pH 7.4). Transverse sections were processed according to usual histological techniques for paraffin embedding. Five micrometre sections were taken and stained with haematoxylin and eosin.

Statistical analysis

All data are expressed as means ± s.d. Differences between groups were analysed by ANOVA one-way multiple comparison tests, and the Kruskal–Wallis non-parametric test was used when needed. A P value of ≤ 0.05 was considered significant.

Results and discussion

DODAB, DOD/AMB and DOC/AMB cytotoxicity in vitro

The DOD/AMB formulation based on a single synthetic lipid which forms bilayer fragments upon sonication with a tip is shown schematically in Figure 1. Bilayer fragments are bilayer
discs produced by ultrasonically dispersing certain synthetic lipids in water solution. They have been observed by transmission electron microscopy (TEM) over the last 20 years for single lipid dispersions, such as those of sodium dihexadecylphosphate, or dioctadecyldimethylammonium bromide or chloride. DODAB bilayer fragments are stiff and flat due to their rigid gel state at room temperature. They are composed of open, charged and electrostatically stabilized bilayer pieces obtained from sonication with a tip. This is a high energy input procedure that not only disperses the lipid powder in water producing bilayer vesicles, but also disrupts them, thereby originating bilayer fragments. The main pieces of evidence for their existence are: (i) osmotic non-responsiveness of the dispersion indicative of absence of inner vesicle compartment; (ii) TEM micrographs with electronic staining; (iii) cryo-TEM micrographs; (iv) quasi-elastic light scattering and electron paramagnetic resonance spectroscopy; and (v) solubilization of hydrophobic drugs such as AMB at the borders of DODAB bilayer fragments, which does not occur for DODAB closed vesicles. They differ from the closed vesicles in the AmBisome formulation by providing hydrophobic borders at their edges that are absent in closed bilayer systems such as vesicles or liposomes. The inexpensive DODAB synthetic lipid is a potent bactericide and exhibits cytotoxicity against mammalian cells in culture that is much lower than against prokaryotic or yeast cells. Bacteria are very susceptible to DODAB, with micromolar DODAB concentrations effectively killing four bacteria species of clinical importance. Table 1 shows DODAB concentrations for 0% survival of C. albicans HU168 and C. albicans ATCC 90028: 0.8 and 0.4 mM, respectively. Therefore, DODAB concentrations for killing yeast cells are much higher than those required to kill bacteria. Regarding kidney epithelial cells, the DODAB concentration required for 50% survival of cultured cells was 5.4 mM, i.e. these cells were much less susceptible to DODAB than bacteria or yeast cells. Percentage cytotoxicity against kidney epithelial cells as a function of DODAB and AMB concentrations is shown in Figure 2. Two AMB formulations were tested: (i) DOD/AMB based on DODAB lipid only; and (ii) DOC/AMB based on deoxycholate. At equivalent AMB doses, DOC/AMB cytotoxicity against cultured kidney epithelial cells was higher than DOD/AMB over a range of AMB concentrations (Figure 2a). It
was interesting to note that despite DODAB cytotoxicity (Figure 2b), the DOD/AMB formulation was less toxic than DOC/AMB. Schematically illustrated in Figure 1, the monomeric form of AMB in the DOD/AMB formulation was less toxic against kidney cells than drug aggregates that are extensively present in DOC/AMB. This is in agreement with previous results showing that the aggregated form of AMB is more toxic against human erythrocytes and other cultured mammalian cells than the monomeric form of the drug.

DODAB, DOD/AMB and DOC/AMB toxicity in vivo

At day 11 after injection of each formulation in mice, DODAB, DOD/AMB and DOC/AMB did not affect any of the serum biochemical parameters currently used to evaluate renal and hepatic function (Table 2). Activity of ALT, AST or ALP, as well as creatinine and urea concentrations in serum, were not altered relative to controls after intraperitoneal administration of 80 mg/kg/day DODAB or 80/0.4 mg/kg/day DOD/AMB or 0.4 mg/kg/day AMB in DOC/AMB for 10 consecutive days. In clinics, DOC/AMB is used at doses < 1.0 mg/kg/day because this is the limit for tolerable nephrotoxicity, meaning no significant changes in creatinine serum level. Above this limit, nephrotoxicity and creatinine serum levels rapidly increase within days of treatment. One should note that the present study was conducted at low AMB doses both for DOC/AMB and DOD/AMB owing to the high efficacy of the DOD/AMB formulation. Therefore, increases in creatinine serum levels were not expected even for DOC/AMB. In fact, measured creatinine levels did not increase at day 11 compared with controls for both formulations (Table 2). As a more sensitive marker for nephrotoxicity, renal histopathology examination revealed differences between DOC/AMB and DOD/AMB effects on renal morphology.

In comparison with the usual AMB dose for liposomal AMB, AmBisome, which is 3 mg/kg/day for 10 consecutive days, the DOD/AMB monomeric formulation contained ~10 times less drug and even so yielded high efficacy of treatment in model mice where candidiasis had been developed. Thus, this low dose as employed in this work was previously reported to yield 100% mice survival, among several efficacy parameters. Especially, survival had been defined as one of the primary efficacy variables in the quoted study. DODAB and DOD/AMB formulations reduced the percentage average weight change, whereas DOC/AMB did not (Figure 3). Nevertheless, this effect was reversible and not significant over the long term (see percentage average weight at day 180 in Table 3).

**Table 2. Effect of DODAB, DOD/AMB and DOC/AMB on serum biochemical data at day 11 after injection of each formulation**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>ALP (IU/L)</th>
<th>Creatinine (mg/dL)</th>
<th>Urea (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86 ± 82</td>
<td>91 ± 40</td>
<td>304 ± 58</td>
<td>0.98 ± 0.06</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>DODAB</td>
<td>72 ± 56</td>
<td>118 ± 29</td>
<td>314 ± 77</td>
<td>0.93 ± 0.05</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>DOD/AMB</td>
<td>81 ± 42</td>
<td>108 ± 3</td>
<td>319 ± 16</td>
<td>0.97 ± 0.08</td>
<td>56 ± 19</td>
</tr>
<tr>
<td>DOC/AMB</td>
<td>121 ± 58</td>
<td>122 ± 22</td>
<td>344 ± 51</td>
<td>0.94 ± 0.06</td>
<td>50 ± 8</td>
</tr>
</tbody>
</table>

DOD/AMB and DOC/AMB were used at therapeutic doses (DODAB and AMB doses at 80 and 0.4 mg/kg/day, respectively) for 10 consecutive days. Data are presented as means ± s.d. (n = 5 serum pools for each group).

**Table 3. Effect of DODAB and amphotericin B formulations on relative spleen weight as a percentage of body weight (W)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of evaluation</th>
<th>W (g)</th>
<th>Wspleen (g)</th>
<th>100 × (Wspleen/W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>32 ± 3</td>
<td>0.10 ± 0.00</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>DODAB</td>
<td>11</td>
<td>50 ± 8</td>
<td>0.14 ± 0.02</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>DOD/AMB</td>
<td>11</td>
<td>31 ± 3</td>
<td>0.26 ± 0.02</td>
<td>0.83 ± 0.07*</td>
</tr>
<tr>
<td>DOD/AMB</td>
<td>180</td>
<td>53 ± 11</td>
<td>0.19 ± 0.03</td>
<td>0.38 ± 0.13</td>
</tr>
<tr>
<td>DOC/AMB</td>
<td>180</td>
<td>54 ± 10</td>
<td>0.20 ± 0.02*</td>
<td>0.63 ± 0.10*</td>
</tr>
<tr>
<td>DOC/AMB</td>
<td>180</td>
<td>33 ± 5</td>
<td>0.11 ± 0.01</td>
<td>0.34 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>47 ± 11</td>
<td></td>
<td>0.16 ± 0.04</td>
<td>0.35 ± 0.09</td>
</tr>
</tbody>
</table>

Data are presented as means ± s.d. (n = 5). DOD/AMB and DOC/AMB were used at therapeutic doses (0.4 mg/kg/day, intraperitoneal) for 10 consecutive days. DODAB was used at 80 mg/kg/day. *Significant at P < 0.05.
Figure 3). DODAB has been recognized as an efficient immunostimulator in animals.\(^{21-24}\) On the other hand, the normal pathway of drug clearance from the peritoneum is through direct absorption across the peritoneal membrane and by drainage into the lymphatic system.\(^{50}\) Owing to intraperitoneal administration, the formulations were expected to interact with the spleen, the main lymphatic organ connected to systemic circulation. DODAB, DOD/AMB and DOC/AMB affected relative spleen weight as a percentage of body weight as determined at days 11 and 180 after administration. The spleen responded to DODAB formulations by having significantly increased relative weight at day 11 (Table 3). However, this effect receded at day 180, at which point no significant increase in relative spleen weight was seen (Table 3). In contrast, DOC/AMB, which was not expected to act as an immunostimulator, did not affect relative spleen weight.

When injected subcutaneously as adjuvant to present a variety of antigens, DODAB has been reported to form local depots that lasted up to 60 days in mice, without any evidence of overt toxicity.\(^{22,23}\) In fact, autopsy of euthanized animals in this work revealed an intraperitoneal depot at day 11 that was identified by histopathological examination as chronic necrotizing (N. Lincopan, P. Borelli, R. A. Fock, E. M. Mamizuka and A. M. Carmona-Ribeiro, unpublished results). This lesion receded substantially by day 180.

The histopathology of the mouse kidneys at day 11 is shown in Figure 4 at two magnifications: \(\times 100\) and \(\times 400\). On the left in Figure 4, the control showed cross-sections of intact glomeruli and normal tubuli. In the middle, DOD/AMB treatment caused mild tubular hydropic degeneration, although at \(\times 100\) magnification, the overall aspect of glomeruli and tubuli appeared normal. On the right, DOC/AMB caused early focal lymphocytic infiltrates (indicated by an asterisk) and tubular cystic degeneration filled with haemorrhagic fluid (indicated by an arrow) plus cellular debris in the lumen (indicated by ‘D’).

The histopathology of the mouse kidneys at day 180 is shown in Figure 5 at two magnifications: \(\times 100\) and \(\times 400\). On the left in Figure 5, DODAB caused perivascular mononuclear infiltrate
(indicated by an asterisk) and mild hydropic degeneration. In the middle, DOD/AMB showed a similar picture, also presenting mononuclear infiltrates and mild hydropic degeneration. On the right, DOC/AMB caused much more severe lesions such as irreversible damage in kidney tissues as seen from dense projections of lymphocytic infiltrates (indicated by an asterisk), focal glomerulosclerosis (indicated by ‘G’), hyaline cylinders (indicated by ‘H’) and hyaline mass in sclerotic areas, suggesting protein reabsorption. In the literature, persistent acute tubular toxicity after switch from conventional AMB to liposomal AMB, despite complete preservation of glomeruli, has been previously reported. Tubular cell function was shown to be more sensitive to high doses of liposomal AMB than glomerular cell function. For the DOD/AMB formulation in comparison with AmBisome, tubular integrity remained almost unaffected (Figures 4 and 5).

In summary, this toxicity study comparing DOD/AMB with conventional AMB has shown that DOD/AMB is less nephrotoxic than DOC/AMB both in vitro and in vivo. In clinical trials, early nephrotoxicity is usually controlled by monitoring creatinine levels and tubular abnormalities. In the present study, neither DOD/AMB nor DOC/AMB given at the relatively low dose of 0.4 mg/kg/day AMB, gave rise to any change in serum creatinine, and only DOC/AMB gave rise to abnormal tubuli and glomeruli upon histopathological examination. Nephrotoxicity is usually reduced with all known lipid formulations, but the use of such expensive agents should be highly restricted to those who are intolerant of or refractory to DOC/AMB.

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 <0.1 g/L. Therefore, the DOD/AMB treatment regimen would possibly have to be prolonged to administer small daily AMB doses distributed over a large number of days. Interestingly, the inherent immunostimulatory effect associated with DODAB carrier could be favourable to neutropenic patients, who suffer severe fungal infections. Consistently, we have been determining a DODAB-induced rise in the count of polymorphonuclear neutrophils cells in peripheral blood (N. Lincopan, P. Borelli, R. A. Fock, E. M. Mamizuka and A. M. Carmona-Ribeiro, unpublished results).

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