Study of the toxicity of a new lipid complex formulation of amphotericin B

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Objectives: The aim of this study was to evaluate the toxicity of a new lipid complex formulation of amphotericin B (LC-AmB) produced by a simple process.

Methods: Toxicity was evaluated after daily administration for 21 consecutive days in female CD1 mice. Doses of LC-AmB up to 20 mg/kg were used, and compared with Fungizone at 0.5 mg/kg and Abelcet at 10 mg/kg. Acute toxicity after a single bolus injection was also determined, as well as the haemolytic activity and toxicity to mouse macrophages in vitro.

Results: LC-AmB reduced both the haemolytic activity of amphotericin B and its toxicity towards mouse peritoneal macrophages. Its acute toxicity (LD50 > 200 mg/kg in CD1 mice) was similar to that in the literature for the least toxic lipid formulations of amphotericin B. The relative liver weight increased slightly in mice treated daily with a dose of 20 mg/kg LC-AmB, as did the kidney weight in this group and the group treated with Fungizone. There was also a dose-dependent decrease in the haematocrit with all formulations. All treatments caused significant increases in transaminase levels. Total hepatic CYP 450 was slightly but not significantly increased in the groups treated with 20 mg/kg LC-AmB, Abelcet and Fungizone. However, expression of some isoforms of CYP 450 was reduced, the most marked being the hepatic CYP 3A1 after treatment with 20 mg/kg LC-AmB, Abelcet and Fungizone. The effects on hepatic function are probably related to accumulation in organs rich in phagocytic cells.

Conclusion: LC-AmB did not induce any new toxicity compared with Abelcet and Fungizone.

Keywords: LD50, lipid complex, cytochrome P450, antifungal

Introduction

Invasive fungal infections are important causes of morbidity and mortality among immunodeficient patients (transplants, chemotherapy, AIDS). The polyene macrolide amphotericin B (AmB) is the most effective antibiotic for the treatment of most systemic and visceral fungal infections in humans.1,2 Its usefulness is nevertheless limited by its pronounced side effects, both immediate (chills, fever, nausea, headache) and delayed, in particular dose-limiting nephrotoxicity. Up to 80% of patients treated with amphotericin B develop some degree of renal impairment.3-5 This includes increases in serum creatinine, hypokalaemia, hypomagnesaemia and finally nephrotoxicity. Renal impairment may warrant a reduction in dosage or even cessation of treatment, worsening the overall efficacy of therapy.3-5 In addition, because of its high affinity for biological membranes and for lipoproteins,6 amphotericin B may accumulate in tissues, especially in the liver6 and may induce dysfunction in this organ.6,11 Amphotericin B has been reported to decrease bile flow and bile acid secretion of perfused rat liver12 and to alter hepatic metabolic function in the rat.13 Amphotericin B may also induce reversible haematological alterations including normochromic anaemia, thrombocytopenia and agranulocytosis.

To overcome the severe side effects of conventional amphotericin B dosage forms, other formulations have been developed, in which this poorly water-soluble drug is associated with lipids in the form of liposomes or complexes.14,15 Different lipid formulations of amphotericin B have been developed and commercialized in recent years (AmBisome, Abelcet and Amphocil). These formulations constitute an important therapeutic advance by providing a less nephrotoxic alternative to conventional amphotericin B. For example, Abelcet consists of amphotericin B complexed with two phospholipids:
dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG), yielding a final diameter of 1–6 µm. The therapeutic index of Abelcet is greater than that of Fungizone (the conventional formulation of amphotericin B with deoxycholate) used in perfusion. Abelcet does reduce renal toxicity at the recommended doses (1.0–5.0 mg/kg per day), but shows some toxic effects on organs rich in phagocytic cells (liver, spleen, etc.) at higher doses. In fact, this formulation reduces the doses of amphotericin B delivered to the kidneys but is rapidly removed from the blood and accumulates in organs of the mononuclear phagocyte system.

Materials and methods

Materials

Chemicals. Amphotericin B was purchased from Sigma (Saint-Quentin-Fallavier, France). Fungizone (mixed micelles with deoxycholate) was obtained from Bristol-Myers Squibb, France (Neuilly, France). Abelcet was kindly provided by the Liposome Company Ltd (London, UK). AmBisome was a kind gift from Nexstar Pharmaceuticals (now Gilead Sciences, Foster City, CA, USA). Lipids (DMPC and DMPG) were purchased from Avanti Polar Lipids Inc (Alabaster, AL, USA). Solvents and other reagents were obtained from Carlo Erba reagenti (Val de Reuil, France). Fungizone (mixed micelles with deoxycholate) was supplied by the Liposome Company Ltd (London, UK). A control stock solution was prepared by extemporaneous solubilization of amphotericin B in DMSO at a concentration of 10 mg/mL. Polymyxin B sulphate was obtained from Fluka (Mulhouse, France).

Preparation of a lipid complex of amphotericin B (LC-AmB)

LC-amphotericin B with the same composition as Abelcet was prepared by nanoprecipitation as previously described. The amphotericin B powder (3.5 mg) was dissolved in methanol (15 mL) with DMPC (3.5 mg) and DMPG (1.5 mg) and this organic phase was added to aqueous phase (pure water, 15 mL). The volume was reduced by low-pressure evaporation to 5 mL. The mean particle diameter, measured by laser light scattering on a typical preparation (Nanosizer N4, Coultronics, Margency, France) was 250 ± 50 nm (mean ± S.D. for three runs), with a polydispersity index of 0.12. For in vitro and in vivo toxicity, the final preparation was respectively 5 mL at 1 mg/mL and 20 mL at 5, 10, 15 and 20 mg/mL. For the second preparation, the initial volumes of the two phases were increased accordingly. A fresh preparation was made every day and was stored at 4°C, after size and spectral analysis control. The following morning, this stock was diluted in isotonic sterile water to allow the appropriate dose to be injected in a volume of 200 or 300 µL for 150 and 200 mg/kg.

Electron microscopy

Freeze fracture. A drop of the suspension containing 30% glycerol as a cryoprotectant was deposited on a thin copper planchet and rapidly frozen in liquid propane. Fracturing and shadowing using Pt-C were carried out in a Balzers BAF 310 freeze-etch unit. The replicas were examined with a Philips 410 electron microscope.

Air drying. The sample was deposited on a freshly cleaved mica plate, dried at room temperature and shadowed in the Balzers unit. The shadowing using Pt-C was carried out in a Balzers BAF 310 freeze-etch unit. The replicas were examined with a Philips 410 electron microscope.

In vitro toxicity

Macrophages. Thioglycollate-elicited mouse peritoneal macrophages were harvested from 20–25 g female CD1 mice (Charles River Ltd, France), as described previously. The macrophages were plated in 96-well plates at 10^5 cells/well. After adherence, the medium was removed and replaced by medium containing the different formulations of amphotericin B. The plates were incubated for 4, 24, 48 and 72 h at 37°C in a humidified 5% CO₂ incubator. Control cells were incubated with culture medium alone. Cell viability was determined by a colorimetric assay using the tetrazolium salt MTT. Each concentration was tested on three wells, and three independent experiments were carried out. The mean absorbances were expressed as a percentage of that in control wells and the IC₅₀ values were calculated graphically. In parallel experiments, the protein content of the macrophage monolayers was determined after the same incubation times after washing twice in warmed phosphate-buffered saline followed by lysis in 0.1% (w/v) Triton X-100, using the Bio-Rad detergent-compatible assay (Lowry method, Bio-Rad, Ivory-sur-Seine, France) and bovine serum albumin as a standard. Finally, to ensure that the toxic effect of the amphotericin B formulations was not the result of contamination with traces of bacterial lipopolysaccharide (LPS), experiments were carried out in which the formulations were pre-incubated with polymyxin B (2 mg/L) for 15 min at 37°C before addition to the cells.

Measurement of antibiotic-induced haemoglobin (Hb) release from erythrocytes. Amphotericin B formulations were dispersed in PBS at different concentrations (0.1–100 mg/L) and incubated for 5 min at 37°C. Freshly isolated human erythrocytes (RBC) were then added to a final haematocrit of 2% (approximately 2 × 10^6 cells/mL) and incubated at the same temperature for 30 min. After the centrifugation (1500g, 5 min at 4°C) the supernatant was removed and the RBC pellet was lysed with sterile water. The haemoglobin remaining in the pellet was estimated from its absorption at 560 nm recorded with a spectrophotometer. Control RBCs incubated with PBS alone in the same experiment were used to estimate the total Hb content after lysis. The percentage of haemolysis was calculated from the difference between the Hb remaining in the test samples and the control. The experiment was carried out three times with each concentration in triplicate. The results were expressed as Hb₅₀ calculated from the mean of all determinations.

In vivo toxicity

Animals. CD1 mice (Charles River, France) 6–8 weeks old weighing 25–30 g were supplied by the Central Animal Laboratory. They were housed in groups not exceeding six per cage and maintained under standard conditions. Food and tap water were available ad libitum. Room temperature was maintained at 21 ± 1°C with a dark/light cycle of 12:12 h. Animal experiments were carried out according to the guidelines for...
Reduction of toxicity by a new carrier of AmB

(a) (b)

Figure 1. Electron microscopy of LC-AmB. Samples were prepared with DMPC/DMPG/amphotericin B in the ratio of 7:3:5, with freeze-fracture (a) or without freeze-fracture (b) (Bar = 200 nm) as described in Materials and methods.

Acute toxicity. Various doses as amphotericin B of different formulations (Fungizone, Abelcet, LC-AmB) were given into the retroorbital sinus to groups of 10 male mice. Dead mice were counted daily for 30 days and the LD₅₀ was determined by the method of Litchfield & Wilcoxon.²⁸

Toxicity after repeated administration. This was measured in eight groups of six female mice each as follows: Five groups received various doses (1, 2, 5, 10 and 20 mg/kg) of LC-AmB. One group received Abelcet (10 mg/kg). One group received Fungizone (0.5 mg/kg). One group (control) received saline alone. The different amphotericin B formulations were injected intravenously in a constant volume of 200 μL daily for 3 weeks. Twelve hours after the last treatment, mice were weighed and euthanized. Blood was collected over heparin, centrifuged at 2000 rpm, and plasma urea and creatinine were determined using an automatic Hitachi 912 apparatus.

Blood biochemistry determinations. The haematocrit was measured after centrifugation in a capillary tube. Transaminase activities, plasma urea and creatinine were determined using an automatic Hitachi 912 apparatus (Roche Diagnostics Corporation, Indianapolis, IN, USA).

Characterization of mouse hepatic and renal cytochrome P450

Microsomal suspensions were prepared by successive centrifugations of liver and kidney homogenates. Aliquots were stored at –80°C until analysis. The protein concentration (mg/mL) of each microsomal preparation was measured with Bradford's microassay (Bio-Rad) using a Labsystem Multiscan MS microplate reader, in duplicate, using gamma-globulins as standard.

The total CYP content of the liver homogenate was determined by a modification of the method of Omura & Sato.²⁹ A difference spectrum between reduced carbon monoxide (CO) and oxidized samples was obtained and an extinction coefficient (Ɛ₅₄₅ nm) of 106 mM⁻¹·cm⁻¹ was used to calculate total CYP 450 concentration. Specific CYP hepatic and renal CYP isoenzymes were detected by western blot analysis. Microsomal proteins (5 μg) were separated by SDS–PAGE using 10% acrylamide gels, then electro-transferred to Hybond ECL nitrocellulose membranes. After blocking, membranes were incubated for 1 h at room temperature with rabbit polyclonal anti-rat CYP antibodies specific for CYP 1A1, 2B1, and 2E1 or 3A1. These isoforms were chosen as being the most important for drug metabolism in rodents. The specific bands were revealed with a horseradish peroxidase-conjugated secondary antibody and visualized by chemiluminescence using ECL detection reagents (Amersham Life Science) followed by a brief exposure to Kodak XAR scientific imaging film. Light signals were digitized and quantified with NIH Image software.

Statistical analysis

Results are expressed as the mean ± S.E.M. of at least four determinations. The data were analysed using analysis of variance followed by Dunnett’s multiple comparison tests.

Results

Structure of the formulation

Electron microscopy of LC-AmB after freeze-fracture showed a very thin (a few nm) dumb-bell-like structure with a length of about 250 nm (Figure 1a). In contrast, electron microscopy of the complex performed without fracture by just drying and shadowing showed a thin disc-like structure of around 250 nm in diameter. The thickness of the disc was evaluated from the angle of shadowing and length of the shadow and found to be about 29 Å (Figure 1b). If the formulation had been spherical liposome-like particles, both freeze-fracture and shadowing electron microscopy would have shown round or ellipsoid objects. We therefore conclude that the preparation is in disc form. The thickness, and the lack of fusion between two discs in the centre of Figure 1(b) suggest that the lipids are interdigitated rather than in a bilayer arrangement. These structures are only formed when amphotericin B is present, indicating that it is capable of binding to and organizing the lipids. Since reduction in AmB toxicity is related to its binding to lipids in formulations, it was interesting to evaluate this formulation. LC-AmB was stable in solution at 4°C for 1 year, as determined by particle size and the spectral properties of AmB.

Toxicity towards mouse peritoneal macrophages

Table 1 shows the results obtained using the MTT conversion test. Protein assays to determine the relative number of macrophages that remained adherent yielded similar results. AmBIsome and the new formulation, LC-AmB, were the least toxic with an IC₅₀ above 100 mg/L after 24 h exposure (Table 1). The toxicity increased with the time of exposure for all formulations (after a 48 h exposure the IC₅₀ of LC-AmB was 86 mg/L, data not shown). Very similar results were obtained in the presence of polymyxin B (data not shown), eliminating the possibility that the toxicity was the result of contamination of the formulations with LPS.
10 mg/mL of amphotericin B, corresponding to 80 mg/kg. Although this new formulation, the mice that received this dose behaved the injection, three mice in this group died a few days later. Therefore, all the mice given 200 mg/kg of amphotericin B as LC-AmB survived the injection, leading to an increase in viscosity at concentrations above.

In vivo toxicity for repeated doses

Animals and organ weights (Table 2). A 3 week exposure to amphotericin B did not lead to any overt signs of toxicity whatever the formulation. However, three of the 40 mice died during this period: one receiving 1 mg/kg and one receiving 20 mg/kg of LC-AmB, and one receiving 0.5 mg/kg of Fungizone. In the surviving mice, the body weights were similar in all the groups, whether treated or control (Table 2). Kidney weights were not significantly altered, although a tendency to increase could be observed in mice treated with the highest dose of LC-AmB (20 mg/kg) or with Fungizone (0.5 mg/kg) (Table 2). No difference appeared between the left and right kidney weights in any mouse (data not shown). The relative liver weight increased significantly in mice treated with 20 mg/kg (0.5 mg/kg) (Table 2). No difference appeared between the left and right kidney weights in any mouse (data not shown). The relative liver weight increased significantly in mice treated with 20 mg/kg LC-AmB but not in those receiving Fungizone or Abelcet.

Blood biochemistry analysis. Exposure to LC-AmB resulted in a dose-dependent decrease in the haematocrit which became significant at 10 mg/kg (P < 0.05). A significant decrease was also seen in all the treated groups. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) increased

### Table 1. *In vitro* and *in vivo* toxicity of amphotericin B formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>IC₅₀ (mg/L of amphotericin B)</th>
<th>LD₅₀ (mg/kg of amphotericin B)</th>
<th>LD₅₀ (mg/kg of amphotericin B) in the literature (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungizone</td>
<td>4.5</td>
<td>3.5</td>
<td>2.5–5</td>
</tr>
<tr>
<td>LC-AmB</td>
<td>&gt;100</td>
<td>&gt;200</td>
<td>ND</td>
</tr>
<tr>
<td>Abelcet</td>
<td>84</td>
<td>40</td>
<td>50–70</td>
</tr>
<tr>
<td>AmBisome</td>
<td>&gt;100</td>
<td>ND</td>
<td>&gt;175</td>
</tr>
</tbody>
</table>

ND, not done.

*In vitro* toxicity of different formulations of amphotericin B towards peritoneal murine macrophages after 24 h of incubation (IC₅₀) using the MTT test.

*In vivo* toxicity (acute toxicity LD₅₀) of different formulations of amphotericin B in CD1 male mice after a single bolus injection; values are calculated from the number of mice surviving the injection.

### Table 2. Effect of amphotericin B formulations on body weight, relative liver and kidney weights and haematocrit

<table>
<thead>
<tr>
<th>Amphotericin B formulations</th>
<th>Dose (mg/kg)</th>
<th>Body weight (g)</th>
<th>Liver weight (g) as % of body weight</th>
<th>Kidney weight (g) as % of body weight</th>
<th>Haematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>28.8 ± 1.3</td>
<td>5.37 ± 0.77</td>
<td>0.167 ± 0.009</td>
<td>50.3 ± 4.0</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>32.2 ± 3.1</td>
<td>5.18 ± 0.72</td>
<td>0.161 ± 0.026</td>
<td>47.0 ± 2.2</td>
</tr>
<tr>
<td>Fungizone</td>
<td>20</td>
<td>30.9 ± 2.2</td>
<td>5.83 ± 0.85</td>
<td>0.165 ± 0.008</td>
<td>45.3 ± 1.5</td>
</tr>
<tr>
<td>Fungizone</td>
<td>20</td>
<td>29.6 ± 1.5</td>
<td>5.34 ± 0.33</td>
<td>0.161 ± 0.012</td>
<td>42.0 ± 2.1*</td>
</tr>
<tr>
<td>Fungizone</td>
<td>20</td>
<td>30.0 ± 3.4</td>
<td>6.07 ± 1.20*</td>
<td>0.182 ± 0.009</td>
<td>38.5 ± 2.4*</td>
</tr>
<tr>
<td>Fungizone</td>
<td>10</td>
<td>30.3 ± 0.3</td>
<td>4.83 ± 0.08</td>
<td>0.177 ± 0.019</td>
<td>41.0 ± 0.0*</td>
</tr>
<tr>
<td>Fungizone</td>
<td>4.5</td>
<td>30.4 ± 1.8</td>
<td>4.89 ± 0.45</td>
<td>0.168 ± 0.028</td>
<td>41.5 ± 2.2*</td>
</tr>
</tbody>
</table>

The two commercial formulations, Fungizone and Abelcet, were used at therapeutic doses (0.5 and 10 mg/kg, respectively). The new formulation (LC-AmB) was used in a range of concentrations (1–20 mg/kg). Mean ± S.E.M. (n = 5).

*Significant at P < 0.05, Dunnett’s multiple comparison tests.

Haemolysis

The amphotericin B powder solubilized in DMSO provoked 50% haemolysis of human erythrocytes at 3.5 mg/L of amphotericin B. Fungizone and amphotericin B prepared by the same process as LC-AmB but without lipids were slightly less toxic (Hb₅₀ 5 mg/L). All the lipid formulations caused less than 50% haemolysis at the highest concentration tested (100 mg/L).

*In vivo* acute toxicity

The acute toxicity observed for Fungizone and Abelcet in this study was in accordance with the data reported in the literature.\(^b\) LC-AmB was less toxic than Abelcet. The concentrations of LC-AmB necessary to determine the LD₅₀ without increasing the injection volume were higher than those which could be obtained by the process as described in Materials and methods, and further concentration had to be carried out, leading to an increase in viscosity at concentrations above 10 mg/mL of amphotericin B, corresponding to 80 mg/kg. Although all the mice given 200 mg/kg of amphotericin B as LC-AmB survived the injection, three mice in this group died a few days later. Therefore, the maximum tolerated dose was 100 mg/kg of amphotericin B for this new formulation. The mice that received this dose behaved normally and no visible organ anomalies were found at autopsy.

### Table 3. LD₅₀ (mg/kg of amphotericin B) in the literature

<table>
<thead>
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<td>AmBisome</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

ND, not done.
Reduction of toxicity by a new carrier of AmB

Table 3. Effect of amphotericin B formulations on hepatic and renal parameters

<table>
<thead>
<tr>
<th>Amphotericin B formulations</th>
<th>Hepatic microsomal cytochrome P450 (nmol/mg protein)</th>
<th>ALT a (IU/L)</th>
<th>AST a (IU/L)</th>
<th>Uraemia (mmol/L)</th>
<th>Creatininaemia (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.70 ± 0.05</td>
<td>99.8 ± 14.8</td>
<td>12.0 ± 8.4</td>
<td>5.94 ± 2.68</td>
<td>36.75 ± 2.22</td>
</tr>
<tr>
<td>LC-AmB</td>
<td>1.08 ± 0.03</td>
<td>278.3 ± 122.8*</td>
<td>49.3 ± 11.9*</td>
<td>5.88 ± 0.33</td>
<td>38.75 ± 0.96</td>
</tr>
<tr>
<td>Fungizone</td>
<td>0.71 ± 0.03</td>
<td>261.2 ± 80.4*</td>
<td>39.3 ± 14.7*</td>
<td>6.12 ± 1.92</td>
<td>40.00 ± 1.87</td>
</tr>
<tr>
<td>Abelcet</td>
<td>0.71 ± 0.04</td>
<td>232.2 ± 79.2*</td>
<td>38.8 ± 14.5*</td>
<td>6.15 ± 1.12</td>
<td>36.61 ± 1.52</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.71 ± 0.03</td>
<td>384.0 ± 19.4*</td>
<td>40.6 ± 7.6*</td>
<td>5.83 ± 2.06</td>
<td>38.50 ± 1.00</td>
</tr>
<tr>
<td>Fungizone</td>
<td>0.77 ± 0.05</td>
<td>385.0 ± 78.4*</td>
<td>37.8 ± 15.7*</td>
<td>5.28 ± 2.53</td>
<td>32.50 ± 12.49</td>
</tr>
<tr>
<td>Abelcet</td>
<td>0.74 ± 0.04</td>
<td>404.0 ± 127.6*</td>
<td>54.0 ± 7.2*</td>
<td>5.72 ± 0.20</td>
<td>38.75 ± 2.22</td>
</tr>
<tr>
<td>LC-AmB 10 mg/kg</td>
<td>0.82 ± 0.08</td>
<td>405.2 ± 104.3*</td>
<td>45.8 ± 22.8*</td>
<td>5.87 ± 1.00</td>
<td>36.33 ± 3.14</td>
</tr>
</tbody>
</table>

The two commercial formulations of Amphotericin B, Fungizone and Abelcet, were used at therapeutic doses (0.5 and 10 mg/kg, respectively). Mean ± S.E.M. (n = 5).

aSignificant at P < 0.05, Dunnett’s multiple comparison tests.

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Figure 2. Cytochrome P450 isoform expression in hepatic and renal microsomes from mice treated with various Amphotericin B formulations. Mice were given the different formulations at the doses indicated for 21 consecutive days by i.v. injection. Control mice received saline. After the last dose, the mice were killed and their organs removed and weighed. (a) CYP 2B1 expression in hepatic microsomes; (b) CYP 2E1 expression in hepatic microsomes; (c) CYP 3A1 expression in hepatic microsomes; (d) CYP 3A1 expression in renal microsomes. For all the blots: Lanes 1 and 2: control group; Lanes 3–7: 1, 2, 5, 10 and 20 mg/kg LC-AmB; Lane 8: Abelcet 10 mg/kg; Lane 9: Fungizone 0.5 mg/kg.

by three- to four-fold after treatment with LC-AmB irrespective of the dose. A similar increase was observed in the Fungizone- and Abelcet-treated groups (Table 3).

Evaluation of hepatic and renal cytochrome P450. After treatment with Abelcet (10 mg/kg) or LC-AmB (20 mg/kg), a slight but non-significant increase in hepatic microsomal cytochrome P450 content was observed (Table 3). The total content of CYP 450 in kidney microsomes could not be measured owing to the small amount of tissue available. The levels of expression of specific isoforms of CYP 450 in hepatic and renal microsomal proteins were also assessed using western blotting. CYP 1A1 was not detected in liver microsomes in controls or after treatment with amphotericin B, whatever the formulation used and the dose administered; nor were CYP 1A1, 2B1 or 2E1 detected in renal microsomes. In contrast, CYP 2B1 and 2E1 were detected in liver microsomes and CYP 3A1 in both liver and renal microsomes (Figure 2a-d). A significant decrease in the amount of CYP 2B1 protein was observed in samples from mice exposed to the highest dose (20 mg/kg) of LC-AmB (−30%), to 10 mg/kg Abelcet (−30%) and to 0.5 mg/kg Fungizone (−28%) (Figure 2a). CYP 2E1 protein decreased slightly in mice treated with LC-AmB 20 mg/kg (−20%) and Abelcet (−12%) (Figure 2b). Finally, the most dramatic decrease in enzyme expression was seen for hepatic CYP 3A1 (−50%) at the highest dose of LC-AmB (20 mg/kg), whereas it was less marked (around −20%) for all other LC-AmB doses and in Fungizone- and Abelcet-treated groups (Figure 2c).

Renal CYP 3A1 expression also decreased (−40%) in the group of mice that received the highest dose of LC-AmB (20 mg/kg) and in the group that received Abelcet (10 mg/kg). In the group treated with LC-AmB at 10 mg/kg, the decrease was less severe (−30%) and it was limited (−5 to −10%) in all the other treated groups.

Discussion

Lipid formulations of amphotericin B have been developed to avoid the severe side effects seen with the conventional formulation, Fungizone, which must be used at restricted doses (0.6–1.2 mg/kg per day). One such formulation, the lipid complex Abelcet, allows the dose in humans to be increased to 10 mg/kg per day. 30 However, the large particle size (1–6 µm) may limit its penetration into tissues and hence its efficacy against disseminated mycosis and leishmaniasis. The new amphotericin B formulation proposed in the present work, LC-AmB, has a small particle diameter while maintaining a strong
association between amphotericin B and was designed to increase the drug efficiency by facilitating tissue uptake thus enhancing the antibiotic concentration while maintaining low toxicity.

As far as in vitro toxicity was concerned, the new lipid complex LC-AmB reduced the haemolytic activity of amphotericin B in a similar way to the other lipid formulations, compared with free drug or Fungizone. On the other hand, toxicity towards mouse peritoneal macrophages varied from one lipid formulation to another. The toxicity induced by amphotericin B is now believed to involve mechanisms other than changes in the cell membrane permeability as a result of complexes formed between the antibiotic and cholesterol. In the presence of oxygen, amphotericin B undergoes auto-oxidation and also catalyses the oxidation of membrane lipids. This lipid peroxidation renders the membrane more fragile, thereby making the cell more susceptible to osmotic shock. One factor which could influence the toxicity of the different lipid formulations is the rate at which they release amphotericin B and the form of this amphotericin B (mononeric or aggregated, reviewed by Bratburg & Bolard, Hartel & Bolard). The strong interactions between amphotericin B and the phospholipids in LC-AmB and Abelcet would prevent the release of amphotericin B in the aggregated form and hence reduce its toxicity to mammalian cells. A second factor which could determine the toxicity of these formulations towards macrophages is the rate at which they are taken up by the cells. In previous work, we compared the association of different amphotericin B formulations with mouse peritoneal macrophages. The association of LC-AmB was less than that of Abelcet but greater than that of Fungizone. Therefore, toxicity does not seem to be directly related to the total amount of amphotericin B delivered to the cells. The larger particle size of Abelcet would lead to more rapid sedimentation in culture medium and therefore facilitate its phagocytosis; however, this study did not distinguish between particles which were taken up intact by the cells and amphotericin B which was released into the medium and associated with the cells as the free drug. The stability of the LC-AmB complex in biological fluids, resulting from strong interactions between the amphotericin B and phospholipids, and its small size, which reduces its uptake by phagocytosis, may be assumed to combine to give this formulation low toxicity.

The lipid formulations all reduced the acute toxicity of amphotericin B in mice (Table 2). Again, the formulations were not equivalent. The toxicity of the new complex, LC-AmB, was comparable with that reported in the literature for AmBisome and Abelcet. Although Abelcet and LC-AmB share the same lipid composition, there is a large difference in their acute toxicity. This might be explained by the differences in their distribution, which would be expected from the difference in size and morphology. Abelcet particles have been reported to have a short plasma half-life and are rapidly concentrated in the lungs, liver and spleen.

For the repeated dose toxicity study, LC-AmB was tested in a range of doses. For comparison, Fungizone and Abelcet were used at therapeutic doses (0.5 and 10 mg/kg per day, respectively). During the experiment, the mice showed normal behaviour and body weight gain was similar in control and treated groups. The small number of deaths observed could be attributed to technical problems linked to the repeated injections and not to the drug itself since one mouse died in the group treated with the lowest dose of LC-AmB and one in the group receiving the highest dose, but none in the intermediate groups. This result is not surprising as far as the commercial formulations were concerned, since the doses were chosen to be within the non-toxic, therapeutic range. However, a decrease in the haematocrit occurred with all the formulations. This could have been the result of a direct haemolytic effect of amphotericin B on erythrocytes. However, in vitro experiments with human blood showed that although a concentration of about 5 mg/L of amphotericin B as Fungizone was sufficient to provoke 50% haemoglobin release, this haemolytic effect was not reached with 100 mg/L of amphotericin B included in a variety of lipid formulations, including LC-AmB. Therefore, this result probably reflects the effects of amphotericin B on bone marrow, either by direct toxicity or suppression of erythropoietin production.

In this study, renal function was not impaired by any of the amphotericin B formulations tested. The relative kidney weight tended to increase but there were no signs of functional nephrotoxicity. This result is surprising since a transient moderate azotaemia is currently reported in man after 3 weeks of treatment with Fungizone, although this is reduced with Abelcet. However, the doses chosen were those which, from the acute toxicity data, we expected to be well tolerated by mice. Harbarth et al. showed amphotericin B-related nephrotoxicity is an important dose-dependent and duration-dependent toxicity that is accentuated by certain nephrotoxic drugs and patient characteristics. The renal toxicity of Fungizone is worsened in the presence of increased circulating low density lipoprotein (LDL) levels, since free amphotericin B has a high affinity for this lipoprotein; however, the toxicity of Abelcet was independent of LDL concentration, suggesting that strong binding of the antibiotic to the lipids reduces its transfer to LDL and thereby its uptake by renal epithelial cells in this form.

The most noticeable alteration observed with LC-AmB was a slight increase in the relative liver weight, which was only significant at the highest dose. This was accompanied by a large increase in the AST and ALT transaminase activities which was not dose-dependent. The lack of a dose-dependent effect is probably attributable to individual sensitivities (since CD mice are not inbred) together with the small number of mice per group. The hepatotoxic effect of LC-AmB and Abelcet can be attributed to uptake by liver macrophages (Kupffer cells). Whatever the formulation, a proportion of lipid-associated AmB would be expected to accumulate in the liver.

CD1 mice seem very susceptible to amphotericin B since only limited increases in transaminases were reported after treatment with high doses of Fungizone and AmBisome in rats, AmBisome in rabbits and Amphotec (Liposome Technology, USA) in dogs. Furthermore, female rats seem to be more sensitive than males, since significant changes were seen in transaminase levels in female mice treated with 9 or 20 mg/kg per day of amphotericin B in the form of AmBisome for 30 days, whereas in male mice, enzyme levels were in the normal range. A dose of 2.5 mg/kg of Abelcet could be administered for 42 consecutive days to children with hepatosplenic candidiasis without any elevation of transaminase levels.

On the other hand, administration of AmBisome to neutropenic patients resulted in no net increases in serum transaminase levels. Since amphotericin B is frequently used in combination with other drugs, it seemed important to study the effects of amphotericin B on the drug-metabolizing CYP 450 isoenzymes. The slight increase in the total amount of hepatic cytochrome P450 observed in the groups treated with 10 mg/kg Abelcet and 20 mg/kg LC-AmB seems to be in conflict with the fact that evaluation of the levels of individual isoforms showed only decreases in expression. This may reflect a concomitant increase in other CYP isoforms which were not studied in the present work. In contrast, Inselmann et al. reported a decrease in total CYP 450 concentrations with conventional amphotericin B treatment in rats and the lack of effect of a liposomal formulation. In this study, we measured the hepatic and renal concentrations of four major isoforms involved in the metabolism of xenobiotics, CYP 1A1,
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2B1, 2E1 and 3A1. The use of anti-rat antibodies was possible as a result of the close homologies of amino acid sequences between the two species; the corresponding proteins in mice are CYP 1A1, CYP 2B9, CYP 2E1 and CYP 3A11, respectively.44 CYP 1A1 could not be detected in either liver or kidney, in accordance with the literature.44 CYP 2B1 protein levels were unchanged in liver microsomes up to 5 mg/kg of LC-AmB. At the two highest doses (10 and 20 mg/kg), they decreased in a rather similar way (−25 to −35%) to that observed with 10 mg/kg Abelcet or 0.5 mg/kg Fungizone. Consequently, the new formulation did not exert a novel effect with respect to this isoform.

CYP2E1 protein levels decreased slightly after 20 mg/kg LC-AmB and to a lesser extent after 10 mg/kg Abelcet, whereas they did not vary in the Fungizone-treated group.

Finally, CYP 3A1 protein levels decreased significantly in both hepatic and renal microsomes after treatment with the highest (20 mg/kg) dose of LC-AmB and in the kidney only with Abelcet (10 mg/kg). No change appeared with 0.5 mg/kg Fungizone. Again, this result is probably linked to the uptake of the lipid complexes of amphotericin B by phagocytic cells, and by accumulation of the drug in tissue. The CYP 3A subfamily is the most abundant of all of the CYP isoforms; with a broad substrate specificity which renders it important for drug metabolism, although amphotericin B itself is not a substrate. Estimates based primarily on in vitro studies suggest that the metabolism of perhaps 40–50% of drugs used in humans involves CYP 3A-mediated oxidation to some extent. Consequently the two-fold decrease observed might be of pharmacological and toxicological concern especially when drugs are used in combination. For example, since dematiaceous moulds may be particularly refractory to amphotericin B, a combination of amphotericin B and an antifungal triazole is an attractive therapeutic option.1,21,45 However, these azole antifungal agents may also inhibit CYP 3A,46 so the choice of the associated molecule must be made with care.

Conclusion

The LC-AmB is less toxic than Abelcet in acute studies in mice. Although its in vivo distribution has not yet been studied, its colloidal nature suggests that it would accumulate to some extent in organs of the mononuclear phagocyte system, as has been observed for other lipid formulations of amphotericin B. This study is now a priority, at least, in the form of a pharmacokinetic study, to establish an upper safe dose in the rat. This is an important question, since dematiaceous moulds may be particularly refractory to amphotericin B, a combination of amphotericin B and an antifungal triazole is an attractive therapeutic option.1,21,45 However, these azole antifungal agents may also inhibit CYP 3A,46 so the choice of the associated molecule must be made with care.

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