Mild Heating of Amphotericin B-Desoxycholate: Effects on Ultrastructure, In Vitro Activity and Toxicity, and Therapeutic Efficacy in Severe Candidiasis in Leukopenic Mice

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Heated (20 min at 70°C) amphotericin B-desoxycholate (hAMB-DOC) was further characterized, as was another formulation obtained after centrifugation (60 min, 3000 × g), hAMB-DOC. Conventional AMB-DOC consisted of individual micelles (approximately 4 nm in diameter) and threadlike aggregated micelles, as revealed by cryo-transmission electron microscopy. For both hAMB-DOC and hcAMB-DOC, pleomorphic cobweb structures were observed with a mean particle size of approximately 300 nm as determined by laser diffraction. The potent antifungal activity of AMB-DOC against Candida albicans is not reduced by heating. Effective killing of C. albicans (>99.9% within 6 h) was obtained at 0.1 mg/liter with each of the AMB formulations. For AMB-DOC, hAMB-DOC, and hcAMB-DOC, cation release (86Rb+) from C. albicans of ≥50% was observed at 0.8, 0.4, and 0.4 mg/liter, respectively. After heating of AMB-DOC, toxicity was reduced 16-fold as determined by red blood cell (RBC) lysis. For AMB-DOC, hAMB-DOC, and hcAMB-DOC, hemolysis of ≥50% was observed at 6.4, 102.4, and 102.4 mg/liter, respectively. In contrast, AMB-DOC and its derivates showed similar toxicities in terms of cation release from RBC. For AMB-DOC, hAMB-DOC, and hcAMB-DOC, cation release (86Rb+) of ≥50% was observed at 1.6, 0.8, and 0.8 mg/liter, respectively. In persistently leukopenic mice with severe invasive candidiasis, higher dosages of both hAMB-DOC and hcAMB-DOC were tolerated than those of conventional AMB-DOC (3 versus 0.8 mg/kg of body weight, respectively), resulting in significantly improved therapeutic efficacy. In conclusion, this new approach of heating AMB-DOC may be of great value for further optimizing the treatment of severe fungal infections.

As the overall prognosis for immunocompromised patients with severe invasive fungal infections remains poor, there is an urgent need for therapeutic advances. Amphotericin B-desoxycholate (AMB-DOC) remains the therapy of choice for most invasive fungal infections, but its use is significantly limited by toxic side effects. Lipid formulations of AMB have been developed by the pharmaceutical industry, with the primary aim to reduce AMB’s toxicity. It is now clear from a number of clinical studies that the three industrially produced AMB-lipid formulations (Abelcet, Amphotec/Amphocil, and AmBisome) are substantially less toxic than AMB-DOC (3, 10). Studies with animal models of invasive fungal infections have clearly demonstrated that often high dosages of AMB-lipid formulations are needed for treatment to be effective (5). Questions regarding the optimal dosing and duration of treatment in human patients are still unanswered for each of the AMB-lipid formulations. Therefore, and also because treatment with AMB-lipid formulations is very expensive, many patients with severe invasive fungal infections are still undergoing aggressive treatment with conventional AMB-DOC.

It was recently shown by others (1, 2, 8, 9) that when AMB-DOC is heated for 20 min at 70°C (hAMB-DOC), its toxicity is greatly reduced without any effect on its potent antifungal activity. In different models of invasive fungal infections in immunocompetent mice, viz., systemic candidiasis, cryptococcal pneumonia, and cryptococcal meningoencephalitis, higher dosages of hAMB-DOC were tolerated than those of conventional AMB-DOC, resulting in significantly improved therapeutic efficacy. Although the results of these first studies are very encouraging, there are still many questions which need to be answered.

In the present study, hAMB-DOC was further characterized. Ultrastructure and mean particle size were determined. The in vitro toxicity and influence of storage temperature on in vitro toxicity were investigated. Furthermore, studies on the in vitro antifungal activity and therapeutic efficacy in a model of severe invasive candidiasis in persistently leukopenic mice were performed. Additionally, it was investigated whether the hAMB-DOC formulation could be further improved by centrifugation (hcAMB-DOC), yielding a preparation with relatively increased numbers of AMB aggregates.

Materials and Methods

Materials. Dimethyl sulfoxide (DMSO) was from Janssen Chimica (Tilburg, The Netherlands). 86RbCl (specific activity, 1.5 to 2.2 mCi/mg at the reference date) was from Amersham Pharmacia Biotech UK Limited (Little Chalfont, Buckinghamshire, United Kingdom). Methanol and NaHCO3 were from Merck (Darmstadt, Germany). Cyclophosphamide and DOC were from Sigma (St. Louis, Mo.). Saboraud dextrose agar was from Unipath Ltd. (Basingstoke, United Kingdom). AMB-DOC (Fungizone) and pure AMB were kindly provided by Bristol Myers-Squibb (Woerden, The Netherlands).

AMB-DOC and its derivatives. Stock AMB-DOC was prepared by reconstitution of a vial of Fungizone (50 mg of AMB and 41 mg of DOC) with 10 ml of sterile water for injection. Stock hAMB-DOC was prepared as described by Gaboriau et al. (2). First, a stock of AMB-DOC was prepared as described above. Subsequently, the vial was heated for 20 min at 70°C. Stock hAMB-DOC was prepared as follows. First, a stock of hAMB-DOC was prepared as described above. Subsequently, the total volume (10 ml) of hAMB-DOC was centrifuged (60 min at 3000 × g), after which the supernatant was discarded and the pellet was resuspended in 10 ml of 5% (wt/vol) dextrose in sterile water (5-DW) for injection.

The AMB concentration of the stock of either AMB-DOC, hAMB-DOC, or hcAMB-DOC was determined spectrophotometrically at 405 nm after dilution of
the stock 300-, 400-, and 500-fold in DMSO-methanol (1/1, vol/vol). Calibration standards of pure AMB in DMSO-methanol (1/1, vol/vol) were used, ranging from 2 to 20 mg/liter. For each AMB formulation, complete monomerization of AMB was obtained after incubation of the stock for 8 days of storage in DMSO-methanol (1/1, vol/vol), as similar absorption spectra were obtained for AMB-DOC, hAMB-DOC, and pure AMB at a concentration of 10 mg/liter. After determination of the AMB concentration of the stock (approximately 5 g/liter for both AMB-DOC and hAMB-DOC and approximately 4.7 g/liter for hAMB-DOC), further dilutions were made in aqueous solution as indicated in the descriptions of the various experiments.

Candida strain. Candida albicans ATCC 44888 was used in all of the experiments. It was stored at −80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 10% (vol/vol) glycerol.

RBC. Red blood cells (RBC) were obtained from male rat strain albino rats (specific pathogen free, 18 to 25 weeks old, 185 to 225 g, Harlan CPB, Austerlitz, The Netherlands).

Antibiotic Medium No. 3. During incubation the numbers of viable pellet and supernatant and the 86Rb to 102.4 mg of AMB/liter). RBC were pelleted by centrifugation, and the supernatant was recovered by careful aspiration. Radioactivity counts of 86Rb were measured by gamma counter (Minaxi 107 CFU/liter during 6 h of incubation were determined in three separate experiments as described previously (11, 12). AMB-DOC or its derivatives were each administered i.v. as a single dose either 6 or 16 h after C. albicans inoculation at dosages corresponding to their MTDs. hAMB-DOC and hAMB-DOC were also administered at a dosage equivalent to the MTD of AMB-DOC. Controls were treated with 5-DW, just before treatment and 7 days after C. albicans inoculation, the surviving mice were sacrificed. In the present study, only the number of viable C. albicans organisms in the kidney was determined, as it was previously shown (11) that in this animal model the kidney is the most severely infected organ. Survival rates of mice and reduction in the numbers of viable C. albicans organisms in the kidneys were used as parameters to assess the efficacy of treatment (10 mice per group).

Statistical analysis. Results of cation fluxes and hemolysis were expressed as mean ± standard deviation. The logarithm of the survival data was fitted to the sigmoidal logistic regression model (Fig. 1A). Statistical significance of changes in survival rates of invasive candidiasis were expressed as the geometric means ± SD. Differences in viable C. albicans counts between the various treatment groups were analyzed by using the Mann-Whitney test. P values of ≤0.05 were considered significant in these analyses.

RESULTS

Characterization of AMB-DOC and its derivatives. AMB-DOC consisted of individual micelles (approximately 4 nm in diameter) and threadlike, aggregated micelles, as revealed by cryo-transmission electron microscopy (Fig. 1A). For both hAMB-DOC and hAMB-DOC, pleomorphic cobweb structures were observed (Fig. 1B). C. albicans was exposed during 6 h or 16 h after incubation in DOC alone never exceeded that of the controls (data not shown). After heating of AMB-DOC, toxemic effects were observed (Fig. 1D). C. albicans was exposed during 6 h or 16 h after incubation in DOC alone never exceeded that of the controls (data not shown). After heating of AMB-DOC, toxemic effects were observed (Fig. 1D).
hcAMB-DOC, hemolysis of $\geq 50\%$ was observed at 6.4, 102.4, and 102.4 mg/liter, respectively.

Toxicity in terms of hemolysis of RBC remained low for hAMB-DOC when stored at $280^\circ\text{C}$ for up to 8 days. In contrast, toxicity of hAMB-DOC slowly increased during 8 days of storage at 4°C, indicating that hAMB-DOC is unstable at 4°C. hcAMB-DOC remained stable for 8 days at either 4 or $-80^\circ\text{C}$.

MTD in uninfected mice. The MTDs of AMB-DOC and derivates that were determined 24 h after single-dose treatment are presented in Table 1. Similar results were observed 14 days after treatment (data not shown). For AMB-DOC the MTD was 0.8 mg/kg, restricted by mortality after further increase of the dose. For both hAMB-DOC and hcAMB-DOC the MTD was 3 mg/kg; further increase of the dose resulted in impaired liver function, whereas mortality of mice was observed at dosages above 7 mg/kg.

Efficacies of AMB-DOC and its derivates in leukopenic mice infected with *C. albicans*. The effects of early or delayed treatment with AMB-DOC and its derivates on the survival of leukopenic mice and growth of *C. albicans* in the kidney are presented in Fig. 3 and Table 2. By increasing the delay between *C. albicans* inoculation and the time of treatment, the efficacy of treatment in relation to the severity of infection could be investigated. This was reflected in the number of *C. albicans* CFU in the kidney at the time of treatment (Table 2). Placebo-treated mice die within 3 days of *C. albicans* inoculation. AMB-DOC administered i.v. as a single dose of the MTD, 0.8 mg/kg, 6 h after *C. albicans* inoculation was only partially effective; all mice survived up to 7 days after fungal inoculation ($P \leq 0.001$ versus controls) (Fig. 3A). However, numbers of viable *C. albicans* organisms in the kidney were higher than at the time of treatment (Table 2). AMB-DOC administered i.v. as a single dose of the MTD, 0.8 mg/kg, 6 h after inoculation was only partially effective; all mice survived up to 7 days after fungal inoculation ($P \leq 0.001$ versus controls) (Fig. 3A). However, numbers of viable *C. albicans* organisms in the kidney were higher than at the time of treatment (Table 2).

FIG. 1. Cryo-transmission electron micrographs of small threadlike micelles of AMB-DOC (arrows) (A), pleiomorphic cobweb structures of hAMB-DOC (arrow) (B), and hcAMB-DOC, which has an appearance similar to that of hAMB-DOC (arrow) (C). Regions of higher density near hole edges indicate thicker ice. Bars = 50 nm (A and C) and 60 nm (B).
controls ($P \leq 0.001$) and compared to AMB-DOC treatment at 0.1 mg/kg ($P \leq 0.001$). Survival after hAMB-DOC treatment was more prolonged than after hcAMB-DOC treatment ($P \leq 0.05$) (Fig. 3B). For both derivates, numbers of viable *C. albicans* organisms in the kidney were higher than at the time of treatment (Table 2).

**DISCUSSION**

In the present study, hAMB-DOC was prepared by heating (20 min, 70°C) a stock of conventional AMB-DOC (5 g/liter), suspended in the original vial according to the manufacturer’s instructions. In a previous study on hAMB-DOC (2), the stock AMB-DOC was first diluted to 92 mg/liter ($10^2$ M) before heating. In the present study, spectroscopic measurements were performed for both conventional AMB-DOC and hAMB-DOC after dilution of the stock in aqueous solution (5-DW) to a concentration of 10 mg/liter. Absorption spectra were similar to those described previously by Gaboriau et al. (2), showing an absorption maximum at 327 nm for conventional AMB-DOC and at 322 nm for hAMB-DOC (data not shown). Therefore, it was concluded that hAMB-DOC could

**TABLE 1. MTDs of AMB-DOC, hAMB-DOC, and hcAMB-DOC in uninfected mice**

<table>
<thead>
<tr>
<th>Parameter for toxicity$^b$</th>
<th>MTD (mg of AMB/kg)</th>
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<tbody>
<tr>
<td></td>
<td>AMB-DOC</td>
</tr>
<tr>
<td>Death after treatment</td>
<td>0.8</td>
</tr>
<tr>
<td>Impaired renal function</td>
<td>$&gt;0.8$</td>
</tr>
<tr>
<td>Impaired liver function</td>
<td>$&gt;0.8$</td>
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$^a$ Mice were treated i.v. with a single dose. AMB dosages ranged from 0.1 to 1.0 mg/kg in steps of 0.1 mg/kg and from 1 to 9 mg/kg in steps of 1 mg/kg.

$^b$ Toxicity was determined as causing death after treatment or $>3$-fold increases in the indices for renal function (blood urea nitrogen and serum creatinine) and liver function (aspartate aminotransferase and alanine aminotransferase) compared with those for untreated mice determined 24 h after treatment.
immediate death was observed at dosages almost 10-fold higher (>7 mg/kg). However, liver toxicity was observed at much lower dosages (>3 mg/kg). Apparently, if treated with hAMB-DOC or hcAMB-DOC, mice are protected from the acute toxic effects seen with conventional AMB-DOC. The observed liver toxicity might be the result of increased AMB concentrations in the liver after administration of hAMB-DOC or hcAMB-DOC. The biodistribution in mice of AMB after administration of hAMB-DOC or AMB-DOC is presently under investigation. Next to organ distribution, it is important to further determine the cellular distribution of AMB, e.g., in the liver. It is assumed that the particles in hAMB-DOC will be primarily cleared from the circulation by the cells of the mononuclear phagocyte system in liver and spleen. It is important to investigate whether this might result in toxicity towards liver macrophages in terms of impaired phagocytic functions (13). It is also conceivable that during circulation in the blood, AMB is transferred from hAMB-DOC to lipoproteins (14), by which specific transport of AMB to hepatocytes might occur.

Therapeutic efficacy of AMB-DOC and its derivates was determined in a model of severe invasive candidiasis in persistently leukopenic mice. Previously, improved therapeutic efficacy of hAMB-DOC was demonstrated, compared to efficacy of conventional AMB-DOC, in a model of invasive candidiasis in immunocompetent mice (8). In that previous study, efficacy of treatment was investigated in terms of survival of mice only; quantitative cultures of viable C. albicans in the infected organs were not presented. In the present study, the experimental setup was deliberately chosen, as we wanted to investigate the potential of hAMB-DOC or hcAMB-DOC under very severe circumstances. It was clearly demonstrated that for both hAMB-DOC and hcAMB-DOC, higher dosages were tolerated than those for conventional AMB-DOC, resulting in significantly improved therapeutic efficacy. Furthermore, it became evident that the hAMB-DOC formulation is not further improved by centrifugation.

In our opinion this new approach of heating AMB-DOC may be of great value for further optimizing the treatment of severe fungal infections. At present, the therapeutic efficacy of hAMB-DOC is compared with that of conventional AMB-
DOC in another clinically relevant model of severe invasive fungal infection, viz., pulmonary aspergillosis in persistently leukopenic rats (6).

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REFERENCES