Introduction

*Candida* species produce a broad range of serious illnesses especially in immunocompromised hosts,1 and such infections are clearly on the rise.2 *Candida albicans* is an important aetiological agent, but frequencies of candidaemia due to *non-albicans* species have increased significantly in recent years.3 As noted by Pfaller et al.,4 the frequencies of bloodstream infections of *Candida* species over a 2 year period were 54.3% due to *C. albicans*, 16.4% due to *Candida glabrata*, 14.9% due to *Candida parapsilosis*, 8.2% due to *Candida tropicalis*, 1.6% due to *Candida krusei* and 4.6% due to other *Candida* species. However, the distribution of species varied markedly between the countries contributing data to the study.3

Therapy of these infections is difficult due to the limited number of systemically active antifungal drugs.3,6 Although amphotericin B (AMB) is the most effective antifungal drug,7 its use is associated with many toxic effects.8 Azole antifungal agents, especially fluconazole, have been used extensively to treat a wide range of *Candida* infections particularly in AIDS patients.9 Among the different species of *Candida*, high-level resistance to fluconazole has been observed with *C. glabrata* and *C. krusei*.10,11

Several lipid-based formulations of AMB such as liposomal AMB (Ambisome), AMB colloidal dispersion (Amphocil) and AMB–lipid complex (Abelcet) have been introduced into clinical use over recent years.12 The lipid formulations have increased therapeutic indices in comparison with AMB,13 but their higher costs are a major limitation in clinical practice.

In our previous *in vitro* study14 we showed that admixtures of AMB and Intralipid (AMB-IL) obtained by vigorous and prolonged agitation are stable, inexpensive and can be standardized. These preparations exhibited *in vitro* activity against various *Candida* species and had significantly lower toxicity.

Furthermore, promising results were obtained in a recent study *in vivo*, in an experimental model of systemic candidosis in naive mice.15 The data indicated that AMB-IL...
is significantly more effective than conventional AMB. Thus, in the present study we focused on the efficacy of the AMB-IL admixtures in cyclophosphamide (CY)-compromised animals inoculated iv with *C. albicans* and the non-*albicans* species *C. glabrata* and *C. tropicalis*. This model is analogous to clinical situations, which are associated with an increased risk of development of systemic candidosis caused by *C. albicans* and non-*albicans* species.

### Materials and methods

**Induction of a compromised state**

Four-week-old female ICR mice were used in all experiments. A compromised state was induced as described in previous studies, by ip injection of the immunosuppressive agent CY at a dosage of 200 mg/kg. Animals were monitored on various days post-treatment by determination of total blood leucocyte count and by body weight. A decrease in the number of white blood cells (WBCs) and reduction in the body weight were indicative of a compromised state. Permission for the animal experiments described in this study was granted by the ethics committee of the Faculty of Medicine, Tel Aviv University.

**Organisms**

Several strains of *Candida* were used throughout the study. *C. albicans* CBS 562 (ATCC 18804), which is the type strain of the species, was obtained from the Centraalbureau of Schimmelcultures, Delft, The Netherlands; *C. glabrata* was obtained from Hy Laboratories Ltd (Rehovot, Israel) and *C. tropicalis* 75-043 was obtained from Dr F. Odds’ collection, UK.

**Induction of experimental candidosis**

Experimental systemic candidosis was induced in CY-compromised mice by iv inoculation of 0.2 mL of a suspension of *C. albicans* and the non-*albicans* species *C. glabrata* and *C. tropicalis* into the tail vein, following a procedure used previously in naive mice. *C. albicans* and the non-*albicans* species were injected at different concentrations for each species, as determined to be optimal for causing systemic candidosis leading to death within 5–17 days.

Animals were inoculated on the day that the debilitated state was demonstrated by the most prominent decrease in the number of WBCs and loss of body weight (generally, day 4 after pretreatment with CY). Evaluation of systemic candidosis was performed for a follow-up period of 42 days as in the naive animal model. Specifically, criteria included assessment of percentage mortality and the mean survival time (MST). Morbidity was assessed by determination of fungal colonization in kidneys by macroscopic observation and by microscopic examination of kidney tissue sections and homogenates.

### Antifungal agents

A stock solution of conventional AMB (Fungizone; Bristol-Myers Squibb Pharmaceuticals Ltd, Dublin, Eire) (5 mg/mL) was prepared in 5% dextrose. AMB-IL was prepared as described previously, by a 25-fold dilution of conventional AMB in the lipid emulsion Intralipid 20% (Kabi Pharmacia, Stockholm, Sweden) to an AMB final concentration of 0.2 mg/mL and then agitated vigorously at 24°C for 18 h on a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Edison, NJ, USA) at 300 rpm.

**Treatment of candidosis with AMB-IL admixtures or conventional AMB in compromised mice**

Infected mice were treated iv (0.2 mL) with either conventional AMB (as control) or AMB-IL at different doses (range from 0.4 to 2 mg/kg/day). Treatment began 48 h after fungal inoculation and consisted of five consecutive daily injections of AMB (conventional AMB or AMB-IL). A control group treated with placebo [phosphate-buffered saline (PBS)] was included. Survival was assessed for 42 days. Assessment of activity of the AMB-IL was based on the parameters described above for characterization of the systemic candidosis model and evaluated by mortality in comparison with untreated animals and with animals treated with conventional AMB (expressed as survival rate and MST).

### Statistical analysis

The MST data of each group of treated mice were compared with those from untreated controls using one-way analysis of variance (ANOVA). The data of the end point survival rates (day 42) were analysed by the $\chi^2$ test. Significance level was defined as $P < 0.05$.

### Results

#### Compromised state

As shown in Figure 1a, treatment of mice with CY at a dosage of 200 mg/kg resulted in a decrease in the number of WBCs. The lowest level was noted on day 4 post-treatment with a reduction from $8 \times 10^6$/mL to $9.1 \times 10^5$/mL. After day 4 the WBC number gradually increased. In addition, the most marked reduction in body weight from 26.2 to 21.6 g was also observed on day 4 post-treatment (Figure 1b). Hence, day 4 post-CY treatment was chosen as a model of a compromised state.
Amphotericin B–Intralipid in compromised mice

Systemic candidosis in compromised mice

Compromised animals were inoculated iv with C. albicans and the non-albicans species on day 4 after pretreatment with CY.

As can be seen from Table I, CY pretreatment increased the mortality rate elicited by C. albicans. For example, an inoculum of $10^4$ blastospores per mouse caused systemic candidosis that led to death in compromised animals within a 4 day period. The optimal inoculum for C. albicans was determined to be $10^4$ blastospores per mouse. This concentration caused 100% mortality within 10–16 days (MST was 9.36 ± 0.4) and the model was more suitable for the drug evaluation experiments.

In addition to C. albicans infection, we attempted to induce murine systemic candidosis by inoculating mice with the non-albicans species C. glabrata and C. tropicalis. However, as shown in Table I, all our attempts to elicit systemic candidosis with non-albicans spp. in naïve mice failed. Only in CY-compromised animals did we succeed in attempts to induce systemic candidosis with the non-albicans spp. that resulted in 100% mortality within 5–17 days. The experiments revealed that $5 \times 10^5$ blastospores/mouse of C. tropicalis and $5 \times 10^6$ blastospores/mouse of C. glabrata were suitable inocula for induction of systemic candidosis in CY-treated mice.

Macroscopic observation of the kidneys of dead mice infected with C. albicans and non-albicans spp. revealed numerous microabscesses, which upon microscopic observation demonstrated hyphae and yeasts.

Figure 1. Effect of CY pretreatment on mice. Mice were injected ip with 200 mg/kg of CY. (a) WBC count and (b) body weight measured on various days post-CY injection.

Treatment of C. albicans infection in CY-compromised mice with low doses of AMB

The experiments involved CY-compromised ICR mice infected iv with $1 \times 10^7$ blastospores of C. albicans and treated 48 h later with either conventional AMB or AMB-IL at a concentration of AMB of 0.4 mg/kg for 5 days. This concentration was also used in the model of systemic candidosis in naïve mice. Data were obtained from four experiments with 94 mice (34 placebo treated, eight or nine per experiment; 30 treated with conventional AMB, seven or eight per experiment; and 30 with AMB-IL, seven or eight per experiment). The data show (Figure 2) that both preparations (AMB and AMB-IL) increased significantly ($P < 0.001$) the survival rate of infected mice as compared with the untreated control group. The percentage survival at day 42 was 0, 13.3 ± 2.4 and 43.3 ± 1.48 for the control, AMB and AMB-IL-treated groups, respectively. The follow-up of the course of infection indicates that the MST was 9.36 ± 0.49, 18.73 ± 2.45 and 26.9 ± 0.68 for the control, AMB and AMB-IL groups, respectively (Table II). Hence, these preparations increased significantly ($P < 0.001$) the survival time of the treated mice.

These data demonstrate that even at low doses, AMB-IL admixtures were more effective than conventional AMB.

Treatment of C. albicans infection in CY-compromised mice with high doses of AMB

Although treatment with low doses of AMB-IL preparations was more effective than conventional AMB, only 43.3% of treated mice survived. We therefore decided to increase the doses of AMB. We performed two additional experiments involving 79 mice using similar animal and treatment models, which included four experimental groups: control (34, as in the low-dose model); conventional AMB 1 mg/kg (15 mice, seven or eight per experiment); AMB-IL 1 mg/kg (15 mice, seven or eight per experiment) and AMB-IL 2 mg/kg (15 mice, seven or eight per experiment). The treatment was administered over five consecutive days by iv injection. The data in Figure 2d show that all AMB formulations at high doses increased significantly ($P < 0.001$) the survival of the mice. Furthermore, AMB-IL at the higher concentration of 2 mg/kg/day for 5 days was very effective: 100% of treated mice survived.

Hence, data obtained to date show that AMB-IL admixtures at a high dose are more efficient in the treatment of experimental C. albicans systemic infection in CY-compromised mice, as was the case in the non-compromised animals.

Treatment of C. glabrata and C. tropicalis infections in CY-compromised mice with AMB preparations

Following infection by non-albicans species in CY-compromised mice we initiated treatment experiments. A total
Table I. Murine systemic candidosis in naive and compromised animals

<table>
<thead>
<tr>
<th>Candida species and infecting inoculum (blastospores/mouse)</th>
<th>Normal (naive) mice</th>
<th>CY-compromised mice</th>
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<tbody>
<tr>
<td></td>
<td>% mortality&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MST (days)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. albicans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0 (0/10)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42</td>
</tr>
<tr>
<td>1 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>70 (7/10)</td>
<td>23.4</td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>100 (36/36)</td>
<td>7.4</td>
</tr>
<tr>
<td>C. glabrata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0 (0/5)</td>
<td>42</td>
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<tr>
<td>1 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>0 (0/5)</td>
<td>42</td>
</tr>
<tr>
<td>2 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>100&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>C. tropicalis</td>
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<td></td>
</tr>
<tr>
<td>5 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0 (0/5)</td>
<td>42</td>
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<tr>
<td>1 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>2 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>100&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
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<sup>a</sup>Percentage mortality at day 42.
<sup>b</sup>Mean survival time: follow-up of 42 days.
<sup>c</sup>Number in parentheses indicates number of dead mice/total.
<sup>d</sup>ND, not done.
<sup>e</sup>Immediate death (within 1 h).

Table II. Mean survival time (days) of CY-compromised mice infected with Candida spp. and treated with AMB formulations (observation period 42 days)

<table>
<thead>
<tr>
<th>Group</th>
<th>C. albicans&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C. tropicalis&lt;sup&gt;b&lt;/sup&gt;</th>
<th>C. glabrata&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.36 ± 0.49</td>
<td>10.29 ± 1.91</td>
<td>10 ± 2.09</td>
</tr>
<tr>
<td>AMB 0.4 mg/kg</td>
<td>18.73 ± 2.45</td>
<td>17.73 ± 4.83</td>
<td>28.3 ± 4.96</td>
</tr>
<tr>
<td>AMB-IL 0.4 mg/kg</td>
<td>26.9 ± 0.68</td>
<td>26.75 ± 4.50</td>
<td>34.8 ± 3.81</td>
</tr>
<tr>
<td>AMB 1 mg/kg</td>
<td>25.85 ± 0.55</td>
<td>28.23 ± 2.65</td>
<td>32.2 ± 2.53</td>
</tr>
<tr>
<td>AMB-IL 1 mg/kg</td>
<td>29.85 ± 1.85</td>
<td>31.76 ± 2.49</td>
<td>37.2 ± 2.01</td>
</tr>
<tr>
<td>AMB-IL 2 mg/kg</td>
<td>42 ± 0</td>
<td>37.06 ± 1.71</td>
<td>40.73 ± 0.89</td>
</tr>
</tbody>
</table>

<sup>a</sup>Injected dose 1 × 10<sup>8</sup> blastospores/mouse.
<sup>b</sup>Injected dose 5 × 10<sup>3</sup> blastospores/mouse.
<sup>c</sup>Injected dose 5 × 10<sup>6</sup> blastospores/mouse.
Figure 2. Course of murine infection with various *Candida* species treated with AMB formulations. Mice were inoculated iv on day 4 post-CY treatment with various *Candida* species: (a and d) *C. albicans*, (b and e) *C. tropicalis* and (c and f) *C. glabrata*. Forty-eight hours post-*Candida* inoculations mice were injected iv with AMB formulations for 5 days with I: low doses: (■) control, untreated; (●) AMB 0.4 mg/kg; (▲) AMB-IL 0.4 mg/kg; II: high doses: (■) control, untreated; (●) AMB 1 mg/kg; (▲) AMB-IL 1 mg/kg; (▼) AMB-IL 2 mg/kg.
of 127 CY-compromised mice were injected with $5 \times 10^5$ blastospores of C. tropicalis and treatment included six experimental groups: control (14 mice, four or five per experiment), conventional AMB 0.4 mg/kg (11 mice, five or six per experiment), AMB-IL 0.4 mg/kg (12 mice, six per experiment), conventional AMB 1 mg/kg (30 mice, 10 per experiment); AMB-IL 1 mg/kg (30 mice, 10 per experiment) and AMB-IL 2 mg/kg (30 mice, 10 per experiment). In addition, a total of 130 CY mice were injected with $5 \times 10^6$ blastospores of C. glabrata and were divided into six experimental groups: control (16 mice, five or six per experiment), conventional AMB 0.4 mg/kg (12 mice, six per experiment); AMB-IL 0.4 mg/kg (12 mice, six per experiment), conventional AMB 1 mg/kg (30 mice, 10 per experiment); AMB-IL 1 mg/kg (30 mice, 10 per experiment) and AMB-IL 2 mg/kg (30 mice, 10 per experiment). For the treatment of infections caused by non-albicans species we used similar treatment protocols as for C. albicans.

The data presented in Figure 2 (b, c, e and f) and Table II, which summarize the results of experiments with C. tropicalis and C. glabrata-infected mice, show that treatment of candidosis in CY-compromised mice with AMB-IL admixtures was more effective than with conventional AMB. Specifically, treatment with low doses of AMB-IL preparations (Figure 2b and c) resulted in significant increases in survival rates from 0 to 41.7% in the case of C. tropicalis ($P = 0.032$) and from 0 to 75% for C. glabrata ($P < 0.001$).

As shown in Figure 2 (e and f), the high doses of AMB were more effective in the treatment of candidosis in CY-compromised mice. Specifically, the survival rate of C. tropicalis-infected mice was about 80% and over 90% for animals infected with C. glabrata.

**Discussion**

The main goal of this study was to compare the antifungal efficacy of AMB-IL with that of conventional AMB. The data presented in this study show that in a compromised animal model obtained by CY pretreatment, the AMB-IL admixtures were more effective than conventional AMB in treatment of systemic experimental candidosis. This was true not only for C. albicans infection, but also for non-albicans candidosis induced by C. glabrata and C. tropicalis, which were only able to establish infection in compromised animals. These experiments showed that while all control animals died, the survival rate of the AMB-treated mice ranged between 13 and 65% (depending on dose/species), whilst that of the AMB-IL-treated mice was in the range 30–100% (depending on dose/species). The follow-up of the course of infection showed that AMB-IL admixtures increased the survival time of the treated mice. The MST was significantly higher for the mice treated with AMB-IL in comparison with those treated with conventional AMB, especially in the groups treated with high doses of the drug.

It is of interest that AMB-IL admixtures were more effective in the treatment of C. glabrata than C. tropicalis infection. While treatment of C. glabrata-infected mice with the high doses of AMB-IL led to a 93.3% survival rate, the survival rate of C. tropicalis-infected animals was only 76.7%.

Our data indicate that AMB-IL preparations were effective in compromised mice for treatment of systemic candidosis caused by various Candida spp., especially when high doses of AMB-IL admixtures were used. These findings are of particular relevance as the compromised state is analogous to clinical situations that carry a high risk for development of candidosis. Thus, it may be stated that Intralipid preparations seem to be very promising as alternatives for the treatment of systemic candidosis.

The mechanism by which the AMB-IL admixtures reduce the toxicity of AMB without alteration of its antifungal activity is not clear. Nevertheless, experiments both in vitro and in vivo, and clinical observations in patients confirm this observation. It is possible that AMB, during overnight agitation in Intralipid, binds to oil components of the lipid emulsion, resulting in reduced delivery of AMB from AMB-IL admixtures.

In conclusion, from our data obtained in a model of candidosis induced by C. albicans, C. glabrata or C. tropicalis in CY-compromised mice, we demonstrated that AMB-IL admixtures were very effective. An added advantage is that this preparation is inexpensive.

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**References**

Amphotericin B–Intralipid in compromised mice


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