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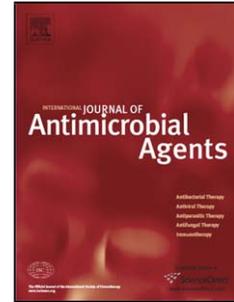
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Efficacy of a new formulation of amphotericin B in murine disseminated infections by *Candida glabrata* or *Candida tropicalis*

Marçal Mariné ^a, Raquel Espada ^b, Juan Torrado ^b, F. Javier Pastor ^{a,*}, Josep Guarro ^a

^a *Unitat de Microbiologia, Facultat de Medicina, IISPV, Universitat Rovira i Virgili, Carrer Sant Llorenç 21, 43201 Reus, Spain*

^b *Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad Complutense, Madrid, Spain*

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* Corresponding author. Tel.: +34 977 759 359; fax: +34 977 759 322.

E-mail address: franciscojavier.pastor@urv.cat (F.J. Pastor).

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ABSTRACT

Amphotericin B microspheres (M-AMB) are a new, inexpensive formulation of amphotericin B. In this study, we tested the efficacy of this new formulation for treating murine disseminated infections by *Candida glabrata* or *Candida tropicalis*. M-AMB showed a similar efficacy to that of amphotericin B deoxycholate and liposomal amphotericin B in the treatment of both disseminated murine infections. Its low toxicity and inexpensive production costs make this formulation potentially attractive for the treatment of fungal infections.

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1. Introduction

Amphotericin B has been the drug of choice for the treatment of a wide range of fungal infections for almost 50 years. This drug is active against many fungal species that cause life-threatening infections, and increased resistance rates over time are rarely reported. The major flaw of amphotericin B treatment is its nephrotoxicity, which greatly limits its use. To palliate its adverse effects, various formulations such as emulsions [1], liposomes [2] and nanoparticles [3] have been developed. Currently, lipid formulations are accepted as a less toxic alternative to the traditional colloidal dispersion. However, high production costs of these new formulations limit their use.

A new formulation of amphotericin B has been developed that consists of drug-containing albumin microspheres, elaborated by a spray-drying process [4]. This new formulation has proven to be active against leishmaniasis and to have a lower toxicity profile than amphotericin B deoxycholate in animal models [5,6]. The new microsphere formulation can be produced more cheaply than the lipid amphotericin B formulations already marketed and this may allow wider usage of this drug. Recently, this new microspheres formulation of amphotericin B has shown efficacy against *Candida albicans* in a murine model of candidiasis comparable with that of the deoxycholate formulation [7,8].

Infections caused by *Candida* spp. are some of the most common among the fungal infections, with amphotericin B being a recommended treatment for systemic infections [9]. Amphotericin B microsphere formulation is an interesting option for improving current therapies against *Candida* infections. In the present

study, we tested the efficacy of this new formulation in murine models of *Candida glabrata* and *Candida tropicalis* infection, comparing it with the traditional deoxycholate and liposomal formulations.

2. Materials and methods

2.1. Fungal strains

Two clinical isolates of *C. glabrata* (FMR 8497 and FMR 8489) and one of *C. tropicalis* (FMR 9727) were used in this study. The isolates were subcultured on Sabouraud dextrose agar (SDA) (Laboratorios Conda Sa, Madrid, Spain) plates and incubated at 35 °C for 24 h. In vitro susceptibilities of the three strains to amphotericin B were determined using the Clinical and Laboratory Standards Institute broth microdilution reference method [10]. All three strains showed identical in vitro susceptibility to amphotericin B, i.e. a minimal inhibitory concentration (MIC) of 1 mg/L.

2.2. Animals

Male OF1 mice were immunosuppressed by a single intraperitoneal injection of 200 mg/kg cyclophosphamide plus a single intravenous injection of 150 mg/kg 5-fluorouracil on the day of infection [11].

2.3. Compounds

Amphotericin B deoxycholate (ABD) was purchased as FungizoneTM (Squibb Industria Farmacéutica S.A., Barcelona, Spain) and liposomal amphotericin B

(L-AMB) was provided as AmBisome (Gilead Sciences S.A., Madrid, Spain). Amphotericin B as raw material was provided by Bristol-Myers Squibb (Barcelona, Spain). Amphotericin B microspheres (M-AMB) were prepared as follows: amphotericin B was dispersed in 5 mL of a water solution formed by sodium deoxycholate, dibasic sodium phosphate and monobasic sodium phosphate. The resulting dispersion was stirred moderately to achieve a homogeneous suspension. A 20% serum human albumin (Grifols, Barcelona, Spain) solution (5 mL) was added and the final mixture was spray-dried using a Büchi B-191 spray-dryer (Büchi, Flawil, Switzerland) [5]. Albumin microspheres of $1.0 \pm 0.7 \mu\text{m}$ with a content of 4.0% (w/w) amphotericin B in the final product were obtained. Before drug injection, M-AMB were manually dispersed in a 5% glucose–water solution. The stability of the new formulation has been described in previous studies [6].

2.4. Experimental design

The efficacy of the different drugs was evaluated by prolongation of survival and by reduction of fungal tissue burden of mice challenged with the abovementioned strains. For the survival and tissue burden studies, groups of ten mice were randomly established for each strain, one group for each treatment and another as control. Mice were challenged with 2×10^8 colony-forming units (CFU) of *C. glabrata* or 1×10^5 CFU of *C. tropicalis* in 0.2 mL into the lateral tail vein. The different groups were treated as follows: L-AMB (10 mg/kg body weight/day) given intravenously [12]; ABD (1.5 mg/kg/day) given intraperitoneally [11]; and M-AMB (20 mg/kg) given intravenously [5]. Both commercial formulation treatments began 24 h after challenge and the therapy

lasted for 5 days. M-AMB were administered in three different regimens, i.e. either as a single dose on Day 1 post infection, two doses at Days 1 and 3 post infection or three doses at Days 1, 3 and 5 post infection.

For survival studies, mice were checked daily for 15 days. For tissue burden studies, five of the surviving mice were randomly chosen and were sacrificed 1 day after the end of treatment. Spleen and kidneys were aseptically removed and the entire organs were homogenised in 1 mL of sterile saline. Serial 10-fold dilutions of the homogenates were plated on SDA, incubated at 35 °C and examined daily for 3 days, and the numbers of CFU/g of tissue were calculated.

2.5. Statistical analysis

Mean survival time was estimated by the Kaplan–Meier method and was compared among groups using the log-rank test. Colony counts in tissue burden studies were analysed using the Mann–Whitney *U*-test. Calculations were made using SPSS 15.0 (SPSS Inc., Chicago, IL) and GraphPad 4.0 for Windows (GraphPad Software Inc., La Jolla, CA).

3. Results

Fig. 1 shows the results of survival studies obtained with the three strains tested. In the case of *C. glabrata*, the results were contradictory. Whilst all the treatments prolonged the survival of mice infected with strain FMR 8489, none of them significantly prolonged the survival of mice infected with strain FMR 8497. For the *C. tropicalis* strain (FMR 9727), all the formulations and doses

except ABD significantly prolonged the survival of mice with respect to the control group. No statistical differences were observed between the three amphotericin B formulations for either *C. glabrata* strain, but microspheres and liposomal formulations both significantly improved the survival with respect to the deoxycholate formulation for the *C. tropicalis* strain.

Tissue burden results are shown in Fig. 2. L-AMB and ABD significantly reduced the fungal load in kidneys and spleen for all the strains ($P < 0.05$). All doses of microspheres reduced the tissue fungal burden for all strains and organs studied, with the exception of the kidney in mice infected with strain FMR 8489 treated with the lowest dose of this formulation as well as the kidney in mice infected with strain FMR 8497 treated with any of the three doses of microspheres. L-AMB and ABD were more effective in reducing the fungal load with respect to the microspheres formulation for all the strains and organs, except for strain FMR 8489 in spleen for the deoxycholate formulation.

No statistical differences were observed between microsphere dosage regimens for the survival or tissue burden studies, except for the *C. tropicalis* strain fungal burden in the spleen where higher doses of M-AMB significantly reduced the CFU counts with respect to the 20 mg/kg dose.

4. Discussion

Candida glabrata and *C. tropicalis* are two of the most common *Candida* spp. causing human infections. Poorer outcomes have been reported for patients with fungaemia caused by these two species than patients infected by other

Candida spp. [13]; however, this has not been widely observed [14]. In terms of drug resistance, higher MICs, delayed killing kinetics or no fungicidal activity have been reported for the commonest non-*albicans* species with the new azoles such as voriconazole and posaconazole [15–17] in addition to the increased resistance rates to fluconazole reported for these species [18]. In contrast to the abovementioned reports on the azole efficacy for *C. glabrata* and *C. tropicalis*, resistance to amphotericin B is rarely reported and amphotericin B remains one of the recommended first-line treatments against both *Candida* spp. [19]. In addition, we previously demonstrated that amphotericin B showed higher efficacy than representatives of other antifungal classes such as the echinocandins and azoles in a murine model of disseminated infection by *C. glabrata* [11]. The good results obtained by Espada et al. [7,8] with the microsphere formulation against *C. albicans* encouraged us to test such a new formulation against other *Candida* spp.

Acute toxicity of the new M-AMB formulation has been determined in previous studies in hamsters and mice [5,7], where they were reported as being less toxic than the traditional deoxycholate formulation and similar to the liposomal formulation. The dosage of M-AMB is based on the existing data; however, instead of daily administration, subsequent doses of microspheres were delayed to avoid an immune response to the human albumin used to prepare this formulation.

Although in the tissue burden studies microspheres reduced the CFU counts for the *C. tropicalis* strain and one of the *C. glabrata* strains in kidneys, the

commercial formulations were more effective in this organ. A lower distribution of microspheres in kidneys, which is associated with the lower toxicity profile of this formulation [7], could explain why the microspheres are less effective than the commercial formulations. In the spleen, where the microsphere formulation distribution is higher than in the kidneys, a significant reduction in CFU counts was observed for all the strains tested, even attaining a fungal burden reduction equal to that obtained with the deoxycholate formulation in one of the *C. glabrata* strains tested.

Despite the modest results observed in reducing tissue burden, similar or even better results were obtained in the survival studies with the new microsphere formulation of amphotericin B with respect to the commercial formulations. Similar results were obtained with the microspheres and the L-AMB formulation; however, two of the microspheres dosage regimens accounted for a lesser total dose compared with the total 50 mg/kg L-AMB administered in the course of the 5-day treatments. In addition, the fact that higher survival rates were attained with the new formulation with respect to the deoxycholate formulation is very encouraging and makes the new formulation worthy of further investigation. This new microsphere formulation that can be administered at escalated or much larger doses than previous formulations with apparently less toxicity [8] appears to have great potential for the treatment of *Candida* infections.

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Competing interests: None declared.

Ethical approval: The procedure for the immunosuppression of mice followed the standards approved by the Animal Welfare Committee of the Universitat Rovira i Virgili (Spain).

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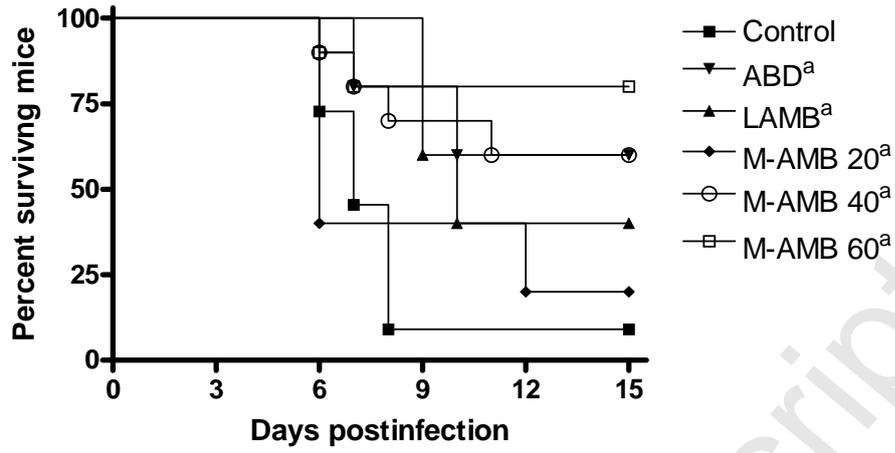
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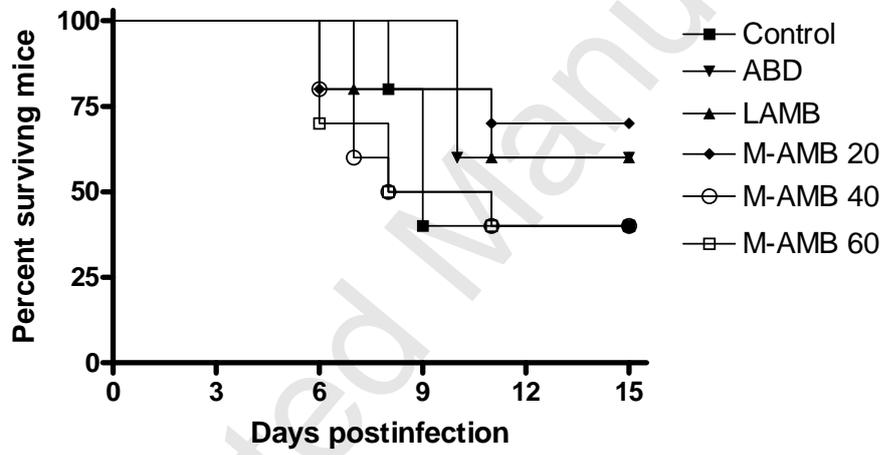
Fig.1. Cumulative mortality of mice infected with (A) *Candida glabrata* FMR 8489, (B) *C. glabrata* FMR 8497 (B) and (C) *Candida tropicalis* FMR 9727. ABD, amphotericin B deoxycholate 1.5 mg/kg/day \times 5 for a total dose of 7.5 mg/kg; LAMB, liposomal amphotericin B 10 mg/kg/day \times 5 for a total dose of 50 mg/kg; and M-AMB 20, M-AMB 40 and M-AMB 60, amphotericin B microspheres at 20 mg/kg administered once, twice or three times every other day for total doses of 20, 40 or 60 mg/kg. ^a $P < 0.05$ versus control; ^b, $P < 0.01$ versus control and $P < 0.05$ versus amphotericin B deoxycholate.

Fig. 2. Effects of antifungal treatments on tissue burden of (A) *Candida glabrata* FMR 8489, (B) *C. glabrata* FMR 8497 and (C) *Candida tropicalis* FMR 9727 in kidneys and spleen of mice. ABD, amphotericin B deoxycholate 1.5 mg/kg/day \times 5 for a total dose of 7.5 mg/kg; LAMB, liposomal amphotericin B 10 mg/kg/day \times 5 for a total dose of 50 mg/kg; and M-AMB 20, M-AMB 40 and M-AMB 60, amphotericin B microspheres at 20 mg/kg administered once, twice or three times every other day for total doses of 20, 40 or 60 mg/kg. ^a $P < 0.05$ versus control; ^b $P < 0.01$ versus control and $P < 0.05$ versus microspheres. Horizontal lines of scatter plots indicate mean values.

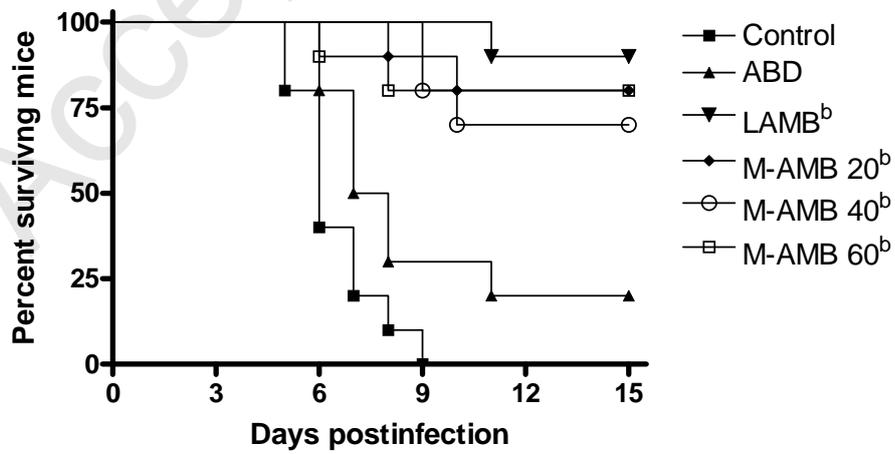
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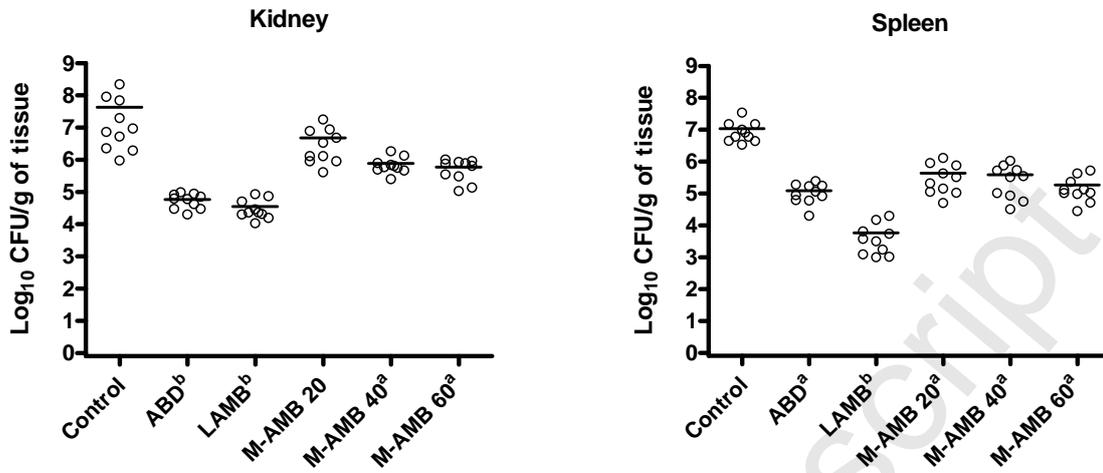
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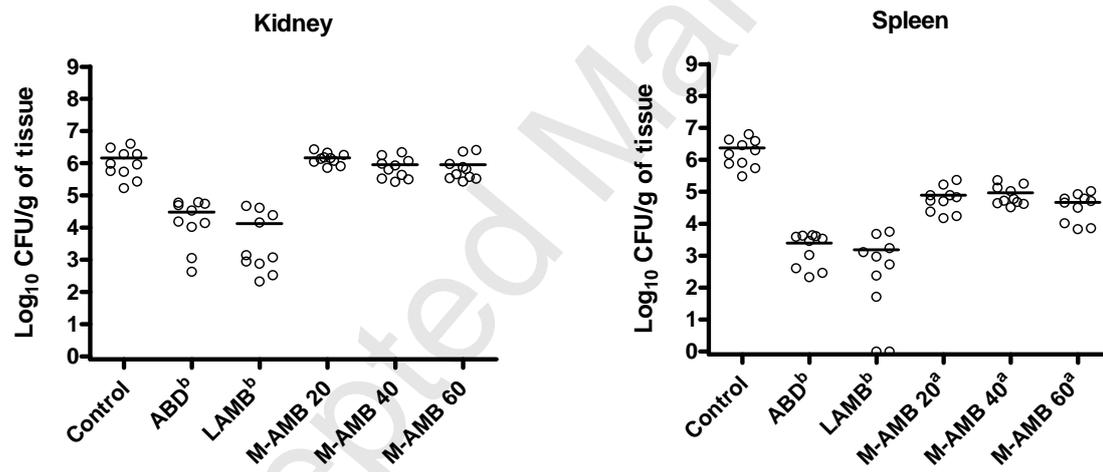
(C)



(A)



(B)



(C)

