IN VITRO AND IN VIVO STUDY OF ANTIFUNGAL FORMULATION AMPHOTERICIN B-7-DEHYDROCHOLESTEROL COMPLEX: TOXICITY AND ACTION ON EXPERIMENTAL PARACOCCIDIOIDOMYCOSIS

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the fungus *Paracoccidioides brasiliensis* that, when not diagnosed and treated early, can progress to severe and lethal disseminated forms. Amphotericin B deoxycholate (AMB-D) is the main option for the intravenous treatment of severe cases of the disease; however, its use is limited due to high nephrotoxicity. This study aimed to evaluate the *in vitro* and *in vivo toxicity* of the AMB-7DC complex—a water-soluble and thermostable formulation resulting from the binding of AMB-D to the 7-dehydrocholesterol radical. This modification reduces its reactivity with cholesterol, which can minimize side effects. In addition, the in vivo fungicidal action against *P. brasiliensis was evaluated*. In *vitro* toxicity was analyzed by hemolysis test in sheep erythrocytes, while *in vivo* toxicity was determined by the maximum tolerated dose in BALBc mice. The antifungal efficacy was evaluated by quantifying the fungal load (CFU) in BALB/c mice infected with *P. brasiliensis* (1.2×10^6 cells/mL) and treated or not with antifungals. The study was approved by the Ethics Committee on Animal Experimentation of the State University of Londrina (CEEA nº 38/05, process nº 23944/05). The results indicated that AMB-7DC showed lower toxicity compared to AMB-D, both in *vitro* and *in vivo* assays. Regarding the fungal load, a reduction was observed in the treated groups compared to the infected control group ($p < 0.05$), but without significant difference between the AMB-D *and* AMB-7DC groups ($p > 0.05$). Based on these preliminary findings, it is concluded that AMB-7DC maintains its antifungal activity against *P. brasiliensis*, presenting lower toxicity compared to AMB-D. Thus, the AMB-7DC complex demonstrates potential for the treatment of PCM, and additional studies are needed for further evaluation.

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INTRODUCTION

Paracoccidioidomycosis (PCM), first described in Brazil by Adolpho Lutz in 1908, is the most prevalent systemic mycosis in Latin America, being frequently diagnosed in Brazil, Venezuela, Colombia, Ecuador and Argentina (BRUMMER et al., 1993). The etiological agents of the disease are *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* (BRUMMER et al., 1993; TEIXEIRA et al., 2013).

PCM presents a wide spectrum of clinical manifestations. The chronic form, the most frequent, can be classified as unifocal or multifocal, depending on the number of sites affected by the lesions (SHIKANAI-YASSUDA et al., 2017). Lesions can affect the lungs, lymph nodes, mucous membranes, upper airways, skin, adrenal glands, gastrointestinal tract, bones, joints, central nervous system, eyes, urogenital tract, thyroid and other organs (MENDES, 1994; SHIKANAI-YASSUDA et al., 2017). The severity of the disease depends on both the virulence of the fungus and host factors (BURGER et al., 1996, CEZAR-dos-SANTOS et al., 2020; ASSOLINE et al., 2021, KAMINAMI et al., 2024).

In severe cases, amphotericin B deoxycholate (AMB-D) is the main therapeutic option. However, despite its efficacy, its use is limited by high nephrotoxicity and the need for long periods of hospitalization for intravenous administration (CAMPOS et al., 1984; BERNARD et al., 1995; NETO et al., 1998). To increase the specificity and reduce the toxicity of amphotericin B, several lipid formulations have been developed and tested, including liposomes (e.g., AmBisome), lipid complexes (e.g., ABLC), and emulsions. Although these formulations allowed the use of higher doses with lower toxicity, side effects, such as ototoxicity, fever, renal dysfunction, and anaphylactic reactions, attributed to lipid components, have still been reported. In addition, therapeutic failures have been documented, such as in the treatment of juvenile PCM with colloidal amphotericin B (LAING et al., 1994, DAS et al, 2014, MOEN et. Al, 2009).

In order to minimize the interaction of amphotericin B with cholesterol in mammalian cell membranes and, consequently, reduce its side effects, the amphotericin B-7-dehydrocholesterol complex (AMB-7DC) was developed. This compound results from the binding of commercial amphotericin B (AMB-D) to the 7-dehydrocholesterol radical (patent no. 2915296; Nipro Co. Ltd, Japan). In *vitro tests* have shown that AMB-7DC presents lower toxicity without compromising its antifungal activity against *Candida albicans*, *C. parapsilosis*, *C. glabrata*, *C. brucei*, *C. tropicalis*, *C. stellatoidea*, *C. guilliermondii* and *Cryptococcus neoformans* (UNO et al., 2001). Unlike conventional AMB, this complex proved to be highly water-soluble and resistant to light and thermal variations. This study

aimed to evaluate the toxicity and efficacy of AMB-7DC in the antifungal activity against the fungus *P. brasiliensis* using an experimental PCM model.

METHODOLOGY

DRUGS TESTED

The drugs used in the in vivo and in vitro tests were AMB-D (Fungizona, Bristol-Myers K.K., Tokyo, Japan) and AMB-7DC (Nipro Co. Ltda., Japan).

IN *VITRO TOXICITY ASSESSMENT*

In vitro toxicity was evaluated following the protocol of LARABI et al. (2003), with some modifications. Sheep erythrocytes (ECs) were suspended in phosphate saline buffer (PBS, 5% v/v), washed twice in the same buffer and centrifuged at 3,000 × g for 10 minutes. The hemolysis reaction was performed in tubes containing 0.1 mL of AMB-D (AMB-D Group) or AMB-7DC (AMB-7DC Group), in addition to the control group, containing only the suspension of ECs. The concentrations tested ranged from 2 mg/mL to 0.0019 mg/mL, in serial 1:2 dilution, in a suspension of 0.9 mL of 2% ECs (approximately 2×10^8 cells/mL). The results were evaluated by spectrophotometry at 550 nm (Multiskan® EX, Thermo Scientific) after one hour of incubation at 37°C in a water bath.

IN VIVO TOXICITY ASSESSMENT

Male BALB/c mice (~30 g) were injected intravenously with different concentrations of AMB-D and AMB-7DC (single dose). The survival of the animals that received the maximum tolerated dose (DMT) was monitored for 7 days.

EXPERIMENTAL MODEL OF PARACOCCIDIOIDOMICOSE (PCM)

BALB/c mice were inoculated with 100 µL of a suspension containing *P. brasiliensis* (Pb18 isolate) at a concentration of 1.2×10^6 cells/mL. The animals were divided into five groups (n = 5 per group):

- C- (Negative control): uninfected and untreated.
- C+ (Positive control): infected and untreated.
- AMB-D: infected and treated with AMB-D (1 mg/kg body weight/day).
- AMB-7DC: infected and treated with AMB-7DC (1 mg/kg body weight/day).

Treatments were initiated 72 hours after infection, administered in a single daily dose, on alternate days, totaling nine doses. After the last dose, 48 hours were waited for the euthanasia of the animals.

The lung was removed, weighed and processed for fungal load quantification (CFU), conducted on BHI agar medium supplemented with 4% horse serum and 5% P*. brasiliensis* filtrate, as described by Singer-Vermes et al. (1992). The CFU evaluation was performed after 14 days of incubation.

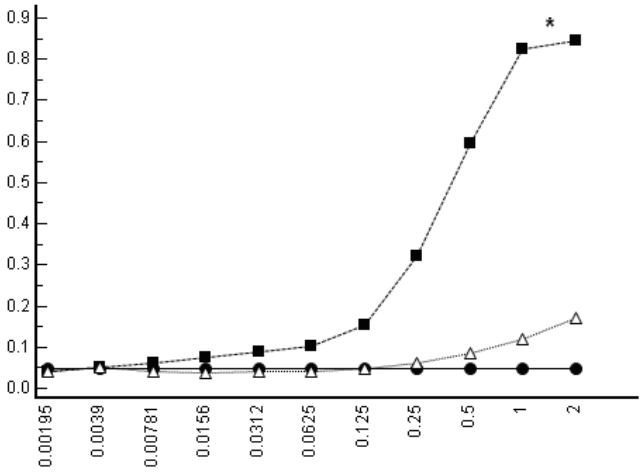
All procedures involving animals were conducted in accordance with the guidelines of the Ethics Committee on Animal Experimentation of the State University of Londrina (CEEA No. 38/05, process No. 23944/05). The results were expressed as the number of viable CFUs of *P. brasiliensis* per mg of tissue.

RESULTS

IN *VITRO TOXICITY ASSESSMENT*

In vitro *toxicity* was determined by erythrocyte hemolysis (ECs). For the analysis, the Kruskal-Wallis test was used in the SPSS software, version 15.0. A significant difference ($p \leq 0.05$) was observed between the AMB group (0.287 ± 0.316) and the AMB-7DC group (0.067 ± 0.043), with the AMB group being the most hemolytic (Figure 1).

FIGURE 1. Determination of *in vitro* toxicity by hemolysis of sheep erythrocytes: AMB group (■), AMB-7DC group (∆), Control (●)



IN VIVO TOXICITY ASSESSMENT

The results presented in Table 1 indicate that the AMB-7DC complex has lower acute toxicity than AMB-D. The maximum tolerated dose (MTD) for AMB-D was 4.0 mg/kg, while for AMB-7DC it was 25 mg/kg body weight.

TABLE 1. Acute toxicity of AMB-D complex and AMB-7DC in BALB/c mice

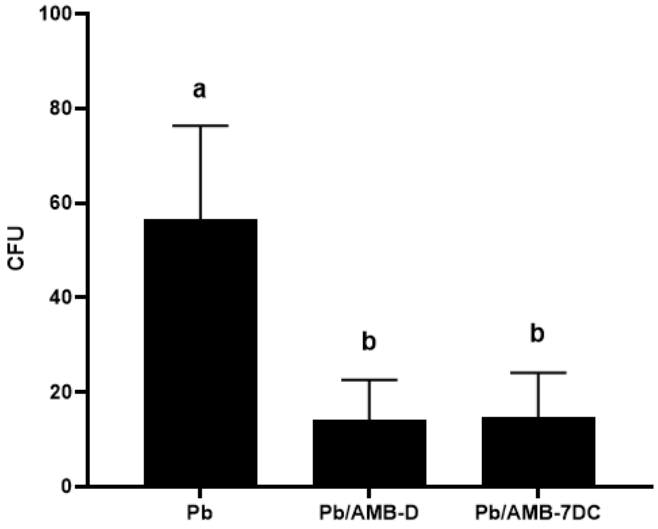
Drug	Dose (mg)	N0 animals	Distribution of death Time Days 1						Percentage survival (%)
			1	1	2	3	7		
AMB-D	2.0	5	0	0	0	0	0		100
	4.0*	5	0	0	0	0	0		100
	8.0	5	1	3	-	-	-		20
AMB-7DC	8.0	5	0	0	0	0	0		100
	25* 50	5 5	0 1	0 2	0 0	0 0	0 0		100 40

* DMT, dose maximal tolerada. AMB-D=Fungizone, complexoAMD-7DC Anfotericina B-7-dehidrocolesterol

EVALUATION OF THE EFFECT OF AMB-D AND AMB-7DC DRUGS ON EXPERIMENTAL PCM

To evaluate the effect of drugs on PCM, the number of colony-forming units (CFU) in the lungs of mice infected and treated with AMB-D or AMB-7DC was used as a parameter, as well as a control group inoculated with sterile PBS. The lung fractions (0.2 g of tissue/ml) of all groups were homogenized, seeded on BHI agar culture medium, and incubated at 37ºC for 14 days. CFUs were counted and results expressed as CFUs per mg of tissue. In the control group, there was no fungal growth. In the treated groups, only two plaques showed zero growth (one in the AMB-7DC1 group and one in the AMB-7DC group). In the other groups, viable fungi were found, indicating that there was no complete eradication of the fungus. Statistical analysis using the Shapiro-Wilk normality test, followed by one-way ANOVA and Tukey's multiple comparison test, revealed a significant difference between the infected group and the treated groups ($p < 0.05$), but there was no significant difference between the treated groups ($p > 0.05$) (Figure 2).

Figure 2 - P. brasiliensis *colony-forming unit* (CFU) in the lungs of groups of mice infected with *P. brasiliensis* (Pb), infected with *P. brasiliensis* and treated with AMB-D (Pb/AMB-D) and infected with *P. brasiliensis* and treated with AMB-7DC (Pb/AMB-7DC). The results are expressed as the number of viable CFUs of *P. brasiliensis* per mg of tissue after 14 days of incubation on BHI agar medium.



DISCUSSION

Fungi, as eukaryotic organisms like humans, have physiological and biochemical similarities that impose limitations on drug therapy. Amphotericin B (AMB), the first commercially significant antifungal, has been available for more than 60 years and has the broadest spectrum of action among antifungals, and is especially indicated for invasive fungal infections. Its activity covers fungi such as *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Candida* spp., *Aspergillus fumigatus*, as well as protozoa such as *Leishmania donovani* and *Leishmania brasiliensis* (GALLIS et al., 1990; GALLIS et al., 1995; DUPONT, 2002).

However, AMB has significant toxicity, requiring strict care in clinical application (DUPONT, 2002; CHAPMAN et al., 2008, LOUÇÃO et al., 2018). But with the increase in cases of immunocompromised patients and the increasing burden of systemic fungal infections, conventional AMB has been widely used due to its broad spectrum of action. To reduce its side effects, several new antifungal agents derived from AMB have been developed in recent years (PATRICK & SHALAL 2009.

It is known that the lipid formulations of AMB maintained or significantly improved the therapeutic index (GALLIS, 1996; DUPONT, 2002; DILLON, 1986, PATRICK & SHALAL 2009). However, its clinical use is still limited due to its high cost, restricting access for patients who depend on the public health system. In addition, there have been reports of anaphylaxis following administration of AmBisome in patients without a history of AMB-D allergy (LAING et al., 1994).

In view of this scenario, a new formulation, AMB-7DC, emerges as a promising alternative for study, aiming to establish a safe and effective dosage for the treatment of different pathologies. In addition, we seek to evaluate its suitability for immunocompromised patients, who represent the main indication for AMB-D.

In this study, as expected, AMB-7DC showed lower toxicity compared to AMB-D, both in vitro and in vivo tests. These results become even more relevant when considering that AMB-7DC is water-soluble and thermally more stable than conventional AMB (UNO et al., 2001). This characteristic allows it to be heated, reducing the care required for transport and storage. The lower toxicity of AMB-7DC may enable the use of higher doses in in vivo studies, prolonging the circulation of the drug, favoring greater accumulation in the affected tissues and extending the treatment time, which requires further investigations.

AMB-7DC demonstrated antifungal activity against *Candida albicans*, *C. parapsilosis*, *C. glabrata*, *C. brucei*, *C. tropicalis*, *C. stellatoidea*, *C. guilliermondii,* and *Cryptococcus neoformans* (UNO et al., 2001). In the present study, we showed that its antifungal activity is also maintained against *P. brasiliensis*, as demonstrated by the analysis of the colony-forming unit (CFU) count in the lungs of infected mice.

Under the conditions of this study, it was not possible to eradicate *P. brasiliensis* (Pb18), a highly virulent strain (BURGER et al., 1996) under the conditions of the experiment. Thus, investigations with longer treatment periods would be pertinent. It is worth noting that, even in human PCM, AMB is used as an attack therapy in severe cases, without the initial objective of eradicating the fungus, but rather to promote the recovery of cellular immunity, later allowing the transition to prolonged maintenance treatment with another medication (MACKINNON, 1958; SHIKANAI-YASUDA et al., 2017).

CONCLUSION

Based on these preliminary findings, we conclude that AMB-7DC maintains its antifungal activity against *P. brasiliensis* and presents lower toxicity compared to AMB-D, with the advantage of being more thermostable. Thus, this complex demonstrates potential for the treatment of PCM, and additional studies are needed for further evaluation.

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