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Antifungal and immunomodulatory activity of a novel cochleate for amphotericin B delivery against *Sporothrix schenckii*

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ABSTRACT

Introduction: Sporotrichosis is an emergent subcutaneous mycoses caused by species of the *Sporothrix schenckii* complex. Amphotericin B (AmB) remains the main antifungal drug for the treatment of systemic infections, but its use is limited by toxicity reasons. AFCo3 is a novel cochleate containing detoxified LPS, which exhibits drug delivery and immunomodulating properties. Here, AFCo3 was used as the vehicle for AmB to evaluate the immunomodulatory and antifungal efficacy against *S. schenckii in vitro* and *in vivo*. Methods and results: The minimum inhibitory concentrations of AFCo3-AmB and AmB were 0.25 and 1 μg/mL respectively. The minimum fungicidal concentration was 0.5 μg/mL for AFCo3-AmB and 2 μg/mL for AmB. AFCo3-AmB was less cytotoxic than AmB for peritoneal macrophages, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and reduced the AmB-induced hemolysis in murine erythrocytes. AFCo3-AmB improved the intracellular killing of phagocytized yeast and it enhanced the *in vitro* production of IL-1β, TNF-α and NO in peritoneal macrophages. Moreover, AFCo3-AmB was more effective than AmB in reducing spleen and liver fungal burden after repeated (five days) intraperitoneal administration of 5 mg/kg of AmB, in a Balb/c model of systemic infection, associated to a significant induction of Th1/Th17 response. Finally, blood chemistry revealed that AFCo3-AmB did not cause changes suggestive of nephrotoxicity, such as increases in total proteins, albumin, creatinine and blood urea nitrogen that were caused by free AmB. Conclusions: AFCo3-AmB exhibited a significant immunomodulator action, reduced toxicity and improved antifungal action against *S. schenckii*, suggesting a potential use as AmB delivery for systemic sporotrichosis treatment.

1. Introduction

Sporotrichosis is an emergent subcutaneous mycosis with worldwide distribution and alarming increasing incidence, especially in tropical and subtropical regions [1,2]. Human and animal sporotrichosis are caused by several species of the *Sporothrix schenckii* complex including: *Sporothrix schenckii sensu stricto*, *Sporothrix brasiliensis* and *Sporothrix globosa*, the species of greatest clinical and global epidemiological importance [3]. The disease is generally acquired by the traumatic inoculation with soil, plants, and organic matter contaminated with the fungus or, more rarely, by inhalation of conidias. Recently the zoonotic transmission, especially from cat scratches, is gaining relevance, mostly in Brazil [4]. Clinical manifestations of sporotrichosis depend mainly on host immune competence. Localized subcutaneous forms are observed in immunocompetent individuals, while immunocompromised patients are predominantly affected by disseminated and systemic forms, especially in patients with HIV infection [5,6]. In this way, several studies have demonstrated the importance of the innate and specific immune system for the control of the infection [7–13].

Itraconazol and terbinafine are the antifungal drugs used for the treatment of uncomplicated cutaneous sporotrichosis in humans, but several fungal isolates have shown resistance [14]. On the other hand, amphotericin B (AmB) which was introduced in the 1950s, still remains the most valuable drug reserved for systemic sporotrichosis [15]. AmB binds preferentially to ergosterol in fungal plasma membranes, although it also interacts with sterols, such as cholesterol, in host cells, which largely explains its known toxicity. Thus, application of AmB is hampered by the long-lasting therapy associated with severe adverse effects, including: nephrotoxicity, neurotoxicity and hepatotoxicity [16]. Accordingly, many efforts have been made to develop more effective and less toxic formulations of AmB, including the use of target delivery, such as it encapsulation in liposomes [17,18] and cochleates

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[19–22]. Despite the existence of diverse experimental AmB formulations, to our knowledge, there are no studies evaluating the effect of these new formulations against *S. schenckii* complex species. In addition, the combined antifungal and immunomodulation therapy has been proposed as a promissory alternative against sporotrichosis, but this strategy has not been sufficiently studied [23].

Cochleates are a novel lipid-based system with a unique multi-layered structure consisting of a large, continuous, solid lipid bi-layer sheet rolled into a spiral, with no internal aqueous space [21]. Adjuvant Finlay Cochleates 3 (AFCo3) is a cochleate containing purified and non-toxic LPS derived from *Neisseria meningitidis* B, which has the properties of a vaccine adjuvant and drug carrier. The presence of detoxified LPS as pathogen-associated molecular patterns (PAMPs) in their composition, confers immunomodulating properties to this structure. This novel cochlear microparticle can be formulated with antibiotics/chemotherapeutic agents with the aim of using them to improve the treatment of several veterinary and human infectious diseases [24,25]. In this study was evaluated the *in vitro* and *in vivo* antifungal activity of AFCo3-AmB against *S. schenckii sensu stricto*, including the toxicity and the immunomodulatory effects in comparison with free AmB.

2. Materials and methods

2.1. Preparation of AFCo3-AmB formulations

AFCo3-AmB was prepared according to the methodology described for AFCo3 production [24], adapted for the formulation with AmB. Briefly, the LPS from *N. meningitidis serogroup B*, previously purified by an industrial process in the Finlay Institute (Havana, Cuba) under strict Good Manufacturing Practices (GMP), was detoxified (LPSd) in sodium hydroxide (NaOH) 0.7 N and placed into an oven at 100 °C for 4 h. The pH was neutralized with hydrochloric acid 1:2 (*v*/*v*), and the concentration of LPSd was adjusted to 40 mg/mL in a re-suspension solution (Tris 30 mM, 1% sodium deoxycholate (DOC)). The solution that was thereby obtained, was filtered through a filter with a pore size of 0.2 mm. Through a process of continuous agitation (330 rpm) and slow dripping (2.2 mL/min), the formation solution ($CaCl_2$ 10 mM, NaCl 100 mM) was added. In this moment AmB (Amphotericin B dissolved in sodium deoxycholate, Sigma) was added and adjusted at a concentration of 0.5 mg/mL. The formation of cochlear structures was evidenced by the presence of a white precipitate and subsequent observation with an optic microscope. Once the cochlear structures were formed, the process continued to the wash step. Wash solution (Tris 10 mM, $CaCl_2$ 5 mM) was added under a process of continuous agitation (300 rpm) and a third volume of medium was dropped at 5 mL/min. After 30 min of rest without agitation, the process continued with the step of centrifugation at 1500 rpm for 15 min at 4 °C. During the processing of the cochlear structure sediment, the supernatant was completely discarded and the pellet slowly re-suspended in Tris 10 mM, DOC 0.25%, and $CaCl_2$ 2.5 mM at the same volume of the formation. All of these processes were performed in a closed-loop cycle under completely sterile conditions and the AFCo3-AmB suspension was conserved in sterile hermetically closed vials (20 mL). The stability of cochleates was evaluated after incubation for at least 3 months at 4 °C and for 7 days at 37 °C by microscopic observation, quantification of LPS and AmB in the supernatant of the wash and in the precipitate after centrifugation.

2.1.1. Quantification of LPS

The content of LPS was evaluated in the final formulation through the determination of 2-keto-3-deoxyoctonoic acid (KDO) using the thiobarbituric acid (TBA) method described by Osborn. The percentage of incorporation of LPS in AFCo3 (B) was determined using the calculation (I × 100) / T wherein "I" is the concentration of LPS in the precipitate and "T" is the concentration of LPS in total [24].

2.1.2. Quantification of AmB and incorporation percentage

The determination of AmB was carried out by high-performance liquid chromatography [26]. The content of AmB was quantified in the supernatant of the wash and in the precipitate upon centrifuging containing the AFCo3-AmB cochleates. The percentage of incorporation of AmB in AFCo3 was determined using the calculation (I × 100) / T wherein "I" is the concentration of AmB in the precipitate and "T" is the concentration of AmB in total.

2.2. Microorganism and culture conditions

Sporothrix schenckii ATCC 16345, originally obtained from a human case of diffuse lung infection (Baltimore, MD) and kindly provided by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), was used for all experiments. For mice infection, a piece of the fungal mycelium grown on Mycosel (BD Biosciences) agar tubes was transferred to an Erlenmeyer flask containing 100 mL of brain-heart infusion broth (Difco) and then cultured for 5 days at 37 °C with constant shaking at 150 rpm. After that, an aliquot containing 2×10^7 yeast cells was transferred to a new medium and cultured for 7 days more at the same conditions in order to achieve a virtually 100% mycelium-to-yeast conversion in a logarithmically growing culture.

2.3. Antifungal susceptibility testing

2.3.1. Minimum inhibitory concentration (MIC)

Susceptibility tests were performed using microdilution techniques based on the Clinical and Laboratory Standards Institute (CLSI) protocols M27-A3 (for the yeast form) [27]. AmB and AFCo3-AmB were tested over a final concentration range of 16 to 0.078 μg/mL. One hundred microliters of the prepared cell suspension (1×10^3 to 5×10^3 CFU/mL) was added to each well of 96-well microtiter plates containing 100 μL of previously prepared antifungal drugs in RPMI 1640 to bring the drug dilutions and inoculums to the final desired test concentrations. After this step, the final concentration of DMSO in test wells became 1%. Growth and sterility controls were included for each isolate tested (growth control, RPMI medium with DMSO and organisms but with no drug added; sterility control, RPMI medium only, with no organisms or drug added). The inoculated plates were incubated at 35 °C for 5 days. The MIC was defined as the lowest concentration at which there was complete inhibition of growth. Susceptibility test was performed in triplicate on three different days (independent experiments).

2.3.2. Determination of fungal viability by microplate Alamar Blue assay (MABA)

The MABA was employed according to the manufacturer's instructions (Invitrogen), adding 20 μL to the well of Alamar Blue at 72 h and the plates were incubated for an additional 24 h, totaling 4 days for the MIC final reading. The lowest antifungal agent concentration that substantially inhibited the growth of the organism was visually determined at the point at which there was no change in the original blue colour of the reagent [28].

2.3.3. Determination of the minimum fungicidal concentration (MFC)

MFC was determined after 5 days of incubation by removing 10 μL of the contents from all wells showing no visible growth and spreading them onto Sabouraud dextrose agar plates. The plates were incubated at 35 °C for 72 h. The lowest antibiotic concentration in which no cell colonies were observed after 4 days of incubation at 30 °C was defined as the MFC.

2.4. Preparation of peritoneal exudate cells (PECs)

Thioglycollate-elicited PECs were harvested from male Balb/c mice of six weeks 3 days after i.p. inoculation with 3% sodium-thioglycollate,