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In vitro evaluation of antifungal combination against *Cryptococcus neoformans*

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ABSTRACT

This study evaluated combinations of amphotericin B with anidulafungin, caspofungin, and micafungin against 30 clinical isolates of *Cryptococcus neoformans* following the CLSI M27-A3 and the checkerboard microdilution method. The combination amphotericin B + micafungin showed 60% of synergistic effect against *C. neoformans*, while most of the other interactions were indifferent.

Cryptococcus neoformans is an opportunistic pathogen that infects humans through inhalation and is associated with most cases of fungal meningitis in immunocompromised hosts, especially those with acquired immune deficiency syndrome (AIDS) (Liu et al., 2012; Rajasingham et al., 2015; Vu et al., 2013). Cryptococcal meningitis has a global impact with nearly 223,000 new cases each year and results in 181,100 deaths among HIV-infected individuals (Rajasingham et al., 2017). Although there are approved guidelines for treating cryptococcosis with amphotericin B (AMB), with or without flucytosine (5FC) and fluconazole (FLC) (Perfect et al., 2010), high rates of mortality and morbidity are observed. The echinocandins are antifungal agents which have several advantages, including low toxicity, rapid fungicidal activity, and predictable and favorable kinetics, allowing for once-a-day dosing against *Candida* spp., *Aspergillus* spp., *Pneumocystis jiroveci*, but not *C. neoformans* (Glasmacher et al., 2006; Morrison, 2006).

However, the combination of micafungin (MFG) and AMB has synergistic potential *in vitro* (Serena et al., 2005), prophylaxis for cryptococcosis may result in *Candida* resistance to antifungal agents (Apisarnthanarak and Mundy, 2008), and cryptococcosis may occur as a co-infection with *Candida* spp. (Manfredi et al., 2002) and *Aspergillus* spp. (Enoki et al., 2012). Thus, it is important to evaluate the antimicrobial activity of echinocandins in combination with other antifungal drugs, particularly AMB, against *Cryptococcus* spp. In this context, the objective of this work is to evaluate the *in vitro* activity of anidulafungin (AND), caspofungin (CSP), and MFG in combination with AMB against *Cryptococcus neoformans*. A total of 30 C. *neoformans* strains, obtained from the fungi collection of the Mycological Research Laboratory from the Department of Microbiology and Parasitology of the Federal University of Santa Maria, Brazil, were used in this study. The strains were recovered from the cerebrospinal fluid of HIV-positive patients. All these strains were previously genotyped and deposited at the Genbank database (Rossato et al., 2015). The minimal inhibitory concentrations (MICs) for AMB and minimum effective concentrations (MECs) for echinocandins were determined following the Clinical and Laboratory Standards Institute M27-A3 guidelines (Clinical and Laboratory Standards Institute, 2008). The MICs were obtained with the inoculums of 0.5×10^3 to 2.5×10^3 CFU/mL after incubation at 35 °C during 72 h. AMB (Sigma-Aldrich®, St. Louis, USA), ANID (Pfizer®, New York, USA), CSP (Sigma-Aldrich®, St. Louis, USA), and MFG (Astellas®, Chuo, Japan) were obtained as standard

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Table 1
In vitro susceptibility and interactions of anidulafungin (ANID), caspofungin (CASP), and micafungin (MICA) with amphotericin B (AMB) against 30 clinical isolates of *Cryptococcus neoformans*.

MIC range (GM)		Drug combination					
		Drugs	MIC associated range (GM)	FICI mean range (GM)	Interpretation (%)		
					Syn	Ind	Ant
Drugs alone		Combinations					
AMB	0.06–0.25 (0.16)	AMB/ANID	0.03–0.12 (0.06)/0.5–16 (1.35)	0.12–2.04 (0.41)	46.7	53.3	0
ANID	>64	AMB/CASP	0.03–0.25 (0.09)/0.5–16 (0,9)	0.14–4.03 (0.61)	30	66.7	3.3
CASP	>64	AMB/MICA	0.01–0.25 (0.06)/0.5–32 (3.25)	0.18–1.03 (0.43)	60	40	0
MICA	>64						

Syn = synergism; Ind = indifference; Ant = antagonism.

powders. AMB and AND were diluted in dimethyl sulfoxide, whereas CSP and MFG were diluted in distilled water to generate stock solutions. The final concentrations tested ranged from 1 to 0.015 μg/mL (AMB) and 64 to 0.5 μg/mL (AND, CSP, and MFG). For AND, CSP, and MFG, the MEC concentration was determined microscopically as the lowest drug concentration showing abnormal growth compared to the drug-free control. *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019, and *Cryptococcus neoformans* ATCC 90112 were used as quality control strains. The interaction between AMB and the echinocandins drugs was evaluated using the microdilution checkerboard method (Johnson et al., 2004). The interpretation of the combinations was based on the lowest fractional inhibitory concentration index (FICI) as follows: FICI ≤0.5, synergism; FICI >0.5 to ≤4, indifference; FICI >4, antagonism (Johnson et al., 2004). All tests were performed in duplicate and obtained on the 2 different days. Off-scale MICs were converted to the next higher dilution for calculation purposes (*e.g.*, >32 = 64 μg/mL). The results of the *in vitro* MICs/MECs susceptibility tests, FICI values, combinations of drugs, and the percentages of synergism from clinical isolates of *Cryptococcus neoformans* are shown in Table 1. In each batch of broth microdilution tests, the MICs/MECs of the QC strains were within the reference ranges. The MICs for AMB ranged from 0.06 to 0.25 μg/mL, and the MECs for AND, CSP, and MFG were >64 μg/mL. The results revealed that only AMB of the abovementioned drugs was effective *in vitro* when tested against *Cryptococcus* strains alone. The combination AMB plus MFG was synergistic for (60%) *Cryptococcus* strains with FICI geometric mean (GM) 0.41 μg /mL. The result of the combination of AMB plus CSP demonstrated 30% synergism, with FICI-GM 0.61 μg/mL, and the combination of AMB plus AND showed 46.7% synergism with GM 0.41 μg /mL. Antagonism was observed in 1 strain in the combination AMB plus CSP.

Infections caused by the fungus *Cryptococcus neoformans* are the major cause of fungal-related death, with higher mortality in AIDS-related deaths globally, and treatment options are limited mainly to AMB and FLC or 5FC. The synergistic interaction between AMB and MFG might have great clinical significance for management of cryptococcosis. Franzot and Casadevall (1997) observed synergistic interactions between L-743,872, a pneumocandin antifungal drug, and AMB against 18 strains of *Cryptococcus*, including 11 *C. neoformans* and 3 *Cryptococcus gattii*. The combination of AMB and CSP in *in vivo* infections of *Aspergillus fumigatus* was effective in reducing pulmonary fungal burden and increasing survival (Spellberg et al., 2005). The hypothetical mechanism explaining the putative synergy of this combination includes the disruption of β-glucan cross-linking of the cell wall that could enhance polyene delivery to the cell membrane. This synergistic effect has also been observed *in vitro* between CSP and posaconazole against zygomycete strains. In contrast, the same strains were all resistant to CSP alone (Guembe et al., 2007). Therefore, combining a drug that targets cell wall can be used in conjunction with antifungals that target the cell membrane, such as polyenes and echinocandins, a strategy that could be beneficial in critically ill patients, and possible additive effects might be achieved. The results presented here have great clinical value because a decreased required concentration of AMB can confer safety and efficacy during treatment and can improve the activity of echinocandins. Further work is warranted in order to translate this *in vitro* synergistic interaction in animal models or clinical trials with the aim to obtain higher cure rates of cryptococcosis.

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Conflict of interest

This study does not present any conflict of interest for its authors.

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