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Article

Synergistic effect of ibuprofen with itraconazole and fluconazole against *Cryptococcus neoformans*

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The present study investigated the association of the non-steroidal anti-inflammatory drug ibuprofen with itraconazole, fluconazole and amphotericin B against *Cryptococcus neoformans* isolates. The minimal inhibitory concentration (MIC) was found according to M27-A3 protocol and *in vitro* interactions were evaluated using checkerboard microdilution method. Synergism was demonstrated between azoles and ibuprofen for most isolates. However, no synergistic effects were seen when amphotericin B was combined with ibuprofen. Therefore, our results suggest that ibuprofen presents clinical potential when combined with azole drugs in the treatment of cryptococcosis.

Keywords: *Cryptococcus neoformans.* Ibuprofen. Itraconazole. Fluconazole. Synergism.

INTRODUCTION

Opportunistic pathogenic fungi such as *Candida, Aspergillus* and *Cryptococcus* species are responsible for systemic infections affecting mainly immunodeficient patients such as neonates, transplanted and patients with acquired immunodeficiency syndrome (AIDS) (Grimaldi *et al*., 2010). Cryptococcosis is an infection mainly caused by encapsulated yeast fungus such as *Cryptococcus* neoformans. This microorganism is found in bird droppings and contaminated soil with higher prevalence in tropical and subtropical regions (Ramos-e-Silva *et al*., 2012). It has the airway as portal of entry causing pulmonary infection and can be disseminated to the brain resulting in severe meningoencephalitis (Prates *et al*., 2013; Chen *et al*., 2015). It is estimated that cryptococcal meningitis result in 120.000 to 240.000 deaths per year worldwide (Rajasingham *et al*., 2017).

The classical treatment of cryptococcosis is based on amphotericin B alone or combined with 5-fluorocytosine or azoles (Reichert-Lima *et al*. 2016; Rossato *et al*., 2016). However, amphotericin B formulations have restricted use due to nephrotoxicity problems and must be administered by intravenous infusion (Kagan *et al*., 2012; Xie *et al*., 2014; Lai *et al*., 2016); and 5-flucytosine is expensive and is not present in therapeutic protocols in several countries, making this combination difficult to administer, particularly in resource-poor settings (Smith *et al*., 2015; Lai *et al*., 2016). Fluconazole is cheap, safe, and easy to administer and is the drug of choice in maintenance therapy, typically after cerebrospinal fluid cultures are negative (Lai *et al*., 2016). Nonetheless, fluconazole monotherapy is not recommended because it has shown ineffectiveness due development of resistant strains (Gullo *et al*., 2013). Ttriazoles voriconazole and posaconazole are highly

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active *in vitro* but have unpredictable bioavailability and experience with their use for cryptococcosis is still lacking. Furthermore, echinocandins, has no useful activity against *Cryptococcus* (Chen *et al*., 2012). Thus, mortality remains high and there are still considerable rates of permanent neurological sequels including seizures, headache, memory loss, blindness, and personality disorders (Chen *et al*., 2012; Lai *et al*., 2016).

Currently few antifungals are commercially available and the development of new drugs does not accompany the high incidence of the development of resistant strains and the market needs (Liu *et al*., 2014). Combination therapy with two or more antifungals has the potential to reduce antifungal resistance and decrease toxicity of each drug, but its side effects should be evaluated with caution (Hatipoglu, Hatipoglu, 2013). Thus, *in vitro* association studies with non-antifungal agents and antifungal drugs have been performed and are still required to delineate *in vivo* assays and consequent clinical trials (Venturini *et al*., 2011; Hatipoglu, Hatipoglu, 2013). Ibuprofen is a non-steroidal anti-inflammatory drug commonly used for its antipyretic, analgesic, and anti-inflammatory effects (Arai, Sugita, Nishikawa, 2005). Ibuprofen inhibits inflammation by suppressing cyclooxygenase 1 and 2 (COX-1 and COX-2) activity with subsequent inhibition of prostaglandin (PG) synthesis (Matos, Jordan, 2015). Ibuprofen is easily accessible because it is inexpensive and has shown synergistic effect when combined with fluconazole in *Candida* strains (Hatipoglu, Hatipoglu, 2013; Liu *et al*., 2014). So, the present study aims to test the association of ibuprofen with itraconazole, fluconazole and amphotericin B against *C. neoformans* isolates.

MATERIAL AND METHODS

Fungal strains

A total of twenty five clinical isolates of *C. neoformans* isolated from cerebrospinal fluid were included in this study. All isolates were previously confirmed by PCR (Polymerase Chain Reaction) using primers CNa-70S (5'-ATTGCGTCCACCAAGGAGCTC-3') and CNa-70A (5'-ATTGCGTCCATGTTACGTGGC-3'). The isolates were provided by the Clinical Analysis Department of the Federal University of Rio Grande do Sul, Porto Alegre, RS. All isolates were grown on Sabouraud dextrose agar at 35 ° C for 48 h prior to the experiments.

Drugs

The drugs were prepared according to Clinical and Laboratory Standards Institute (CLSI) recommendations. Fluconazole (FLC) stock solution (Metrochem Api Private Limited, India) was prepared in distilled water. Ibuprofen (IBP; Sigma-Aldrich, USA), itraconazole (ITC; MetrochemApi Private Limited), and amphotericin B (AMB; MetrochemApi Private Limited) stock solution were prepared in dimethylsulfoxide (DMSO; Nuclear, Brazil). For the experiments, the compounds were diluted in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma-Aldrich) to obtain a maximum concentration of 2% DMSO.

Antifungal susceptibility testing

Minimum inhibitory concentrations (MICs) of IBP and antifungal agents were determined in duplicate by the broth microdilution method according to M27-A3 protocol (CLSI, 2008). Serial two-fold dilutions were made in RPMI 1640 medium (Sigma-Aldrich) buffered with morpholinepropansulfonic acid (MOPS; Sigma-Aldrich) and concentrations' ranges tested were: 0.0312 - 16 µg/mL of ITC, 0.125 - 64 µg/mL of FLC, 0.0312 - 16 µg/mL of AMB and 1 - 512 µg/mL of IBP. The experiments were carried out in duplicate. MICs values were defined as the lowest concentration of compounds at which the microorganisms tested did not show visible growth (AMB) or reduced 50% of visible growth (FLC, IBP and ITC) in 72 h.

Checkerboard assay

The interaction between IBP and each antifungal was evaluated for eight randomly selected *C. neoformans* isolates using the checkerboard method (Johnson *et al*., 2004). The assay lead to forty nine different concentration combinations between IBP and antifungal agents in concentrations of MIC/8, MIC/4, MIC/2, MIC, MICx2, MICx4 and MICx8. The experiments were conducted in duplicate and incubated at 35°C for 72 h. The effect of the combinations was classified by determining the fractional inhibitory concentration index (FICI) expressed as the sum of the fractional inhibitory concentrations (FIC), as defined by the following equation:

FICA + FICB = MICa in combination MICA tested alone + MICB in combination MICBtested alone

where MICA and MICB are the MICs of ibuprofen and antifungal agent, respectively (Mukherjee *et al*., 2005). Synersgim was defined when FICI ≤ 0.5, indifference when 0.5 < FICI ≤ 4 and antagonism when FICI > 4 (Odds, 2003).

RESULTS AND DISCUSSION

MIC values of each antifungal agents against twenty-five *C. neoformans* isolates were determined. MIC range, Geometric means (GM), MIC50 (MIC value which inhibits 50% of the isolates) and MIC90 (MIC value that inhibits 90% of the isolates) for itraconazole (ITC), fluconazole (FLC), amphotericin B (AMB) and ibuprofen (IBP) are presented in Table I. Standardizations of susceptibility tests for *C. neoformans* are less developed than for *Candida* spp.; so that, there are no breakpoints established. The isolates showed variable susceptibility to antifungal agents and isolates with low sensitivity were found. The isolates showed low MICs range for ITC (0.03125 – 1 µg/mL) compared to FLC (0.25 – 8 µg/mL) and AMB (0.5 – 16 µg/mL). The geometric mean of MIC was also lower for ITC (0.66 µg/mL) than for FLC (1.74 µg/mL) and AMB (6.17 µg/mL). Since IBP is not an antifungal and there is no standardization in relation to the evaluation of its inhibitory effect, we consider as MIC the concentration that reduces 50% fungal growth. Based on the high MICs, the non-antifungal agent showed weak antifungal activity against *C. neoformans.*

Table II presents the effects of antifungal agent combination, which demonstrated synergism or indifference. The combination of azoles (ITC and FLC) with IBP resulted predominantly in synergism, which was detected in 75% of isolates for combination with FLC and in 62% of isolates for combination with ITC. On the other hand, AMB associated with IBP resulted in 100% of indifference against *C. neoformans.* Antagonism was not detected against both groups. MIC for AMB combined with IBP was chosen when fungal growth was reduced in 100%.

The limited efficacy and the difficulty to introduce new antifungal drugs into the market make the drugs association an important therapeutic strategy to treat potentially life-threatening invasive fungal infections (Fuentefria *et al*., 2018). Previous studies have detected synergism between IBP and azole against *C. albicans* (Ricardo *et al*., 2009; Costa-de-Oliveira *et al*., 2015; Sharma *et al*., 2015) and *C. neoformans* (Ogundeji, Pohl, Sebolai, 2016) increasing the susceptibility of the isolates to these antifungal agents, and corroborating with research. Other non-steroidal anti-inflammatory drugs, such as tenoxicam, diclofenac sodium and sodium salicylate have also shown synergistic effect when combined with azoles (Yücesoy, Oktem, Güllay, 2000). However, studies are commonly performed with *Candida* species.

Several mechanisms may be involved in the selection of azole resistant strains, such as mutations causing structural changes in enzyme affinity, overproduction of enzymes and overexpression of efflux pumps (Gullo *et al*., 2013). Efflux pumps are transporter proteins present in the plasma membrane and are involved in the removal of azoles from the cytoplasm. When efflux pumps are overexpressed there is expulsion of the drug out of the cell reducing the drug concentration at the action site. These mechanisms are responsible for *Cryptococcus* resistance against most azoles (Basso Jr *et al*., 2015).

TABLE I - Susceptibility profile of twenty five isolates of *Cryptococcus* neoformans to antifungal agents. Rresults were expressed in ranges of variation of minimum inhibitory concentrations values (MIC ranges), geometric mean (GM) of 25 isolates, MIC50 (MIC value that inhibits 50% of the isolates) and MIC90 (MIC value that inhibits 90% of the isolates)

Agents	MIC range (µg/mL)	GM (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Itraconazole (ITC)	0.03125 - 1	0.66	1	1
Fluconazole (FLC)	0.25 – 8	1.74	2	4
Amphotericin B (AMB)	0.5 – 16	6.17	4	16
Ibuprofen (IBP)	128 - 512	319.57	256	512

TABLE II - *In vitro* susceptibility of *Cryptococcus* neoformans to ibuprofen (IBR) combined with itraconazole (ITC), fluconazole (FLC) and amphotericin B (AMB)

Isolate	MIC (µg/mL)				IBP + FLC MIC combination (µg/mL)				IBP + ITC MIC combination (µg/mL)				IBP + AMB MIC combination (µg/mL)			
	IBP	FLC	ITC	AMB	IBP	FLC	FICI	Interaction	IBP	ITC	FICI	Interaction	IBP	AMB	FICI	Interaction
CN06	512	4	0.25	2	8	1	0.26	Syn	256	0.0625	0.5	Syn	8	2	1.01	Ind
CN07	512	8	0.5	2	128	0.25	0.25	Syn	8	0.25	0.52	Ind	16	2	1.03	Ind
CN10	512	2	0.25	2	64	0.5	0.375	Syn	64	0.03125	0.25	Syn	512	1	1.5	Ind
CN11	512	4	0.5	4	8	2	0.52	Ind	16	0.125	0.28	Syn	256	2	1	Ind
CN17	512	4	0.25	0.5	8	1	0.27	Syn	8	0.125	0.52	Ind	256	0.25	1	Ind
CN19	512	4	0.5	2	64	1	0.38	Syn	8	0.125	0.27	Syn	512	1	1.5	Ind
CN24	256	4	0.25	4	4	1	0.26	Syn	64	0.125	0.75	Ind	512	2	2.5	Ind
CN25	512	2	0.25	2	256	0.5	0.75	Ind	128	0.0625	0.5	Syn	256	0.25	0.63	Ind

Syn = Synergism
Ind = Indifferent

Understanding the resistance mechanisms of azoles and the action of IBP helps to explain our findings of *in vitro* synergy. IBP is an efflux pump blocker and can prevent the output of azole from the fungal cell. Thus, the high susceptibility of cells to IBP + azoles association may be attributed to the increase in intracellular concentration of the antifungal (Pina-Vaz *et al*., 2005). On the other hand, AMB does not require internalization into fungal cells for exerting their antifungal activity and so they escape from efflux systems (Vandeputte, Ferrari, Coste, 2012). This may justify the indifferent effect of IBP + AMB association found in the present study.

Besides, previous studies showed that IBP causes fungal membrane damage and can be considered, depending on the dose, fungicide or fungistatic (Argenta *et al*., 2012; Arai, Sugita, Nishikawa, 2005). Our results corroborate these studies, since IBP alone was able to inhibit cell growth of *C. neoformans* isolates. The anti-inflammatory effect of IBP can also be relevant in the treatment of fungal infections, since prostaglandins may be involved in fungal colonization's and its anti-inflammatory mechanism works mostly by inhibiting cyclooxygenase isoenzymes (Rusu *et al*., 2014).

In addition to the advantageous effects mentioned above, IBP has a good record of efficacy and safety, and thus it is the most commonly used nonsteroidal anti-inflammatory drug. It can be used even for the most vulnerable patient populations. Furthermore, pharmacokinetic studies of ibuprofen showed that it penetrates into the cerebrospinal fluid, which may be advantageous in treatments of cryptococcal meningitis (Bannwarth *et al*., 1995; Kokki *et al*., 2007). Thus, IBP may improve the action of azoles and may still present clinical benefits due to anti-inflammatory action and its favorable pharmacokinetics.

CONCLUSION

The results of this present study suggest that the combination of IBP and azole drugs may be suitable for cryptococcosis therapy since synergism was demonstrated. Further *in vivo* studies in clinical

situations are still required to prove the effects of the combination of ibuprofen and azoles antifungals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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