UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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

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Running headline: Synergism of ibuprofen and azoles

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Significance and Impact of the Study

The high incidence of resistance development in *Cryptococcus neoformans* isolates drives the search for new strategies of treatment such as the association of drugs acting synergistically. Non-antifungal drugs such as ibuprofen have been the subject of research because of the low cost and good knowledge of its adverse and therapeutic effects. Ibuprofen has been explored for its potential ability to increase susceptibility of strains to antifungal and to help combat the inflammatory symptoms that fungal diseases may cause.

Abstract

The present study investigated the association of the non-steroidal anti-inflammatory drug ibuprofen with itraconazole, fluconazole and amphotericin B against *Cryptococcus neformans* isolates. The minimal inhibitory concentration (MIC) was found according to M27-A3 protocol and the *in vitro* interactions were evaluated using a 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 gold standard treatment for cryptococcosis is the combination of amphotericin B and 5-flucytosine (Reichert-Lima *et al*. 2016). However, due to its high cost, 5-flucytosine is not present in therapeutic protocols in several countries giving place to azole drugs (Smith *et al*. 2015). Nevertheless, azoles have shown ineffectiveness in several studies due to the development of strains resistant (Gullo *et al*. 2013). In addition, although the polyene resistance is considered to be rare and has a good spectrum of activity, this drug has restricted use due to nephrotoxicity problems. (Kagan *et al* 2012; Xie *et al.* 2014).

Currently few antifungals are commercially available and the development of new drugs does not accompany the high incidence of the development of resistant strains (Liu *et al*. 2013). 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 and Hatipoglu 2013). Thus, *in vitro* association studies with non-antifungal agents in order to at the potentiation of antifungal drugs have been performed and are still required to delineate *in vivo* assays and consequent clinical trials (Venturini *et al.* 2011; Hatipoglu and Hatipoglu 2013). Ibuprofen is a non-steroidal anti-inflammatory drug easily accessible because it is inexpensive and has shown a synergistic effect when combined with fluconazole in *Candida* strains (Hatipoglu and Hatipoglu 2013; Liu *et al*. 2013). So, the present study aims to test the association of ibuprofen with itraconazole, fluconazole and amphotericin B against *C. neoformans* isolates.

Results and Discussion

The MIC values of each antifungal agents against twenty-five *C. neoformans* isolates were determined. MIC range, Geometric means (GM), MIC_{50} (MIC value which inhibits 50% of the isolates) and MIC_{90} (MIC value that inhibits 90% of the isolates) for itraconazole (ITC), fluconazole (FLC), amphotericin B (AMB) and ibuprofen (IBP) are presented in Table 1. It can be observed that isolates with low sensitivity to the antifungal agents were found. The use of IBP alone resulted in 50% growth reduction. 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 a weak antifungal activity against *C. neoformans.*

Table 2 prsent the effects of antifungal agent combination, which demonstrated synergistic or indifference. The combination of azoles (ITC and FLC) with IBP resulted predominantly in synergism, which was detected in 75% of the isolates for combination with FLC and in 62% of the 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. 2017). Previous studies have detected synergism between IBP and azole agains *Candida albicans* and *Cryptococcus neoformans* increasing the susceptibility of the isolates to these antifungal agents (Ricardo *et al* 2009; Ogundeji *et al* 2016), corroborating with our research. 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 genes that encode proteins that cause drug efflux (Gullo *et al*. 2013). The efflux bombs are plasma membrane transport proteins. AFR1, MDR1 and AFR2 genes plays an important role in encoding of these proteins in *C. neoformans* and *C. gatti*. When these genes are overexpressed occurs to expulsion of the azoles out of the cell contributing to the decrease of the concentration of drug at action site and explains part of the resistance to azole drugs (Basso Jr *et al.* 2015).

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 do not require internalization into fungal cells for exert their antifungal activity and so they escape from efflux systems (Vandeputte *et al.* 2012). This may justify the indifferent effect of the IBP+AMB association found in the present study.

In addition, 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 *et al.* 2005). Our results are in agreement since IBP alone was able to inhibit the cell growth of *C. neoformans* isolates.

An additional benefit in using a nonsteroidal anti-inflammatory drugs such as ibuprofen should be considered since prostaglandins may be involved in fungal colonizations and its anti-inflammatory mechanism works mostly by inhibiting cyclooxygenase isoenzymes (Rusu *et al.* 2014). Thus, in addition to synergism, IBP has advantages because of the clinical manifestations of the disease, which, besides classic pulmonary and central nervous system manifestations, also causes infections of the skin, prostate, eyes and other parts of the body (Maziarz and Perfect 2016).

The results of this present study suggest that the combination between 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.

Material and methods

Fungal Strains

A total of twenty five clinical isolates of *C. neoformans* were included in this study. All isolates had PCR-confirmed molecular identification through primers CNa-70S (5'-ATTGCGTCCACCAAGGAGCTC-3') and CNa-70A (5'-ATTGCGTCCATGTTACGTGGC-3'). The isolates were provided by the Clinical Analysis Departmentof 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

According to CLSI recommendations, FLC stock solution (Metrochem Api Private Limited, India) was prepared in distilled water. IBP (Sigma-Aldrich, USA), ITC (Metrochem Api Private Limited), and AMB (Metrochem Api Private Limited) stock solution were prepared in DMSO (Nuclear, Brazil). For the experiments, the compounds were diluted in RPMI 1640 medium (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 broth microdilution method according to M27-A3 protocol (CLSI, 2008). Serial two-fold dilutions were made in RPMI 1640 medium (Sigma-Aldrich) buffered with MOPS (Sigma-Aldrich) and the concentrations ranges tested were: 16 - 0.0312 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 growth (FLC, IBP and ITC) in 72 h.

Checkerboard assay

The interaction between IBP and each antifungal was evaluated for eight *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 the 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:

$$\mathrm{FICI} = \mathrm{FIC_A} + \mathrm{FIC_B} = \frac{\mathrm{MIC}_A \text{ in combination}}{\mathrm{MIC_A} \text{ tested alone}} + \frac{\mathrm{MIC_B} \text{ in combination}}{\mathrm{MIC_B} \text{ tested alone}}$$

where $\mathrm{MIC_A}$ and $\mathrm{MIC_B}$ are the MICs of ibuprofen and antifungal agent, respectively (Mukherjee *et al*, 2005). Sinergism was defined when FICI ≤ 0.5, indifference when 0.5 <FICI ≤ 4 and antagonism when FICI > 4 (Odds 2003).

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Conflict of Interest

The authors declare no conflict of interest.

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Tables

Table 1. Susceptibility profile (µg/ml) of twenty five isolates of *Cryptococcus neoformans* to antifungal agents expressed in ranges of variation of minimum and maximum MIC values (MIC ranges), geometric mean (GM), MIC_{50} (MIC value that inhibits 50% of the isolates) and MIC_{90} (MIC value that inhibits 90% of the isolates).

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

Table 2. *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