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Study of the antifungal activity of ibuprofen and its association with amphotericin B or ketoconazole against *Candida* spp.

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Summary

The objective was evaluating the antifungal activity of ibuprofen alone and when associated with amphotericin B or ketoconazole against *Candida* species. Strains of *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei* and *C. parapsilosis* were used. The minimum inhibitory concentration (MIC) was determined by the microdilution method and the association study performed through the checkerboard assay. The concentration of 512 μg/mL inhibited approximately 65% of the tested strains, while against 35% of the strains presented MIC values above 2048 μg/mL. Associations of ibuprofen with amphotericin B against *C. tropicalis* and ibuprofen with ketoconazole against *C. krusei* showed synergistic effect. Antagonistic effects were evidenced in the combination of ibuprofen with amphotericin B against *C. guilliermondii* and *C. albicans*, as well as in the association of ibuprofen with ketoconazole against *C. albicans* and *C. tropicalis*. Through the experiments, it was found that ibuprofen showed antifungal activity against most of the *Candida* species tested. The combinations of ibuprofen and antifungals had synergistic effects. However, antagonistic results were evidenced in the association with ibuprofen, which would make clinical applicability difficult. Therefore, studies of this combined activity should be investigated, considering that this association may be positive for antifungal therapy.

Key words: *Candida*, ibuprofen, antifungals, association.

Resumen

Estudio de la actividad antifúngica del ibuprofeno y su asociación con anfotericina B o ketoconazol contra *Candida* spp.

El objetivo fue evaluar la actividad antifúngica del ibuprofeno solo y asociado con anfotericina B o ketoconazol contra especies de *Candida*. Se utilizaron cepas de *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei* y *C. parapsilosis*. La concentración inhibitoria mínima (MIC) se determinó mediante el método de microdilución y el estudio de asociación fue realizado a través del ensayo de checkboard. La concentración de 512 μg/mL inhibió aproximadamente el 65% de las cepas analizadas, mientras 35% de las cepas presentaron valores de MIC superiores a 2048 μg/mL. Las asociaciones de ibuprofeno con anfotericina B contra *C. tropicalis* e ibuprofeno con ketoconazol contra *C. krusei* mostraron un efecto sinérgico. Se evidenciaron efectos antagonistas en la combinación de ibuprofeno con anfotericina B contra *C. guilliermondii* y *C. albicans*, así como en la asociación de ibuprofeno con ketoconazol contra *C. albicans* y *C. tropicalis*. Se descubrió, a través de los experimentos, que el ibuprofeno mostró actividad antifúngica contra la mayoría de las especies de *Candida* probadas. Las combinaciones de ibuprofeno y antifúngicos tuvieron efectos sinérgicos. Sin embargo, se evidenciaron resultados antagónicos en la asociación con ibuprofeno, lo que dificultaría la aplicabilidad clínica. Por lo tanto, los estudios de esta actividad combinada deben investigarse, considerando que esta asociación puede ser positiva para la terapia antimicótica.

Palabras clave: *Candida*, ibuprofeno, antifúngicos, asociación.

Resumo

Estudo da atividade antifúngica do ibuprofeno e sua associação com anfotericina B ou cetoconazol contra *Candida* spp.

O objetivo do estudo foi avaliar a atividade antifúngica do ibuprofeno sozinho e quando associado com anfotericina B ou cetoconazol contra espécies de Candida. Foram utilizadas cepas fúngicas de *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei* e *C. parapsilosis*, entre isolados clínicos e cepas padrão. A concentração inibitória mínima (CIM) foi determinada pela técnica de microdiluição e o estudo de associação realizado

através do ensaio checkerboard. A concentração de 512 μg/mL foi capaz de inibir, aproximadamente, 65% das cepas ensaiadas, enquanto que 35% das estirpes apresentaram valores da CIM acima de 2048 μg/mL. Associações do ibuprofeno com anfotericina B contra *C. tropicalis* e ibuprofeno mais cetoconazol contra *C. krusei* mostraram efeito sinérgico. Efeitos antagônicos foram evidenciados na combinação do ibuprofeno com anfotericina B contra *C. guilliermondii* e *C. albicans*, como também na associação do ibuprofeno com cetoconazol contra *C. albicans* e *C. tropicalis*. Por meio dos experimentos, pôde-se afirmar que o ibuprofeno exerceu atividade antifúngica contra a maioria das espécies de *Candida* ensaiadas. Os efeitos das combinações entre o ibuprofeno e os antifúngicos promoveram efeitos sinérgicos. No entanto, resultados antagônicos foram evidenciados na associação com o ibuprofeno, o que dificultaria aplicabilidade clínica. Logo, estudos dessa atividade combinada devem ser investigados, pois esta associação pode traçar pontos positivos na terapia antifúngica.

Palavras-chave: *Candida*, ibuprofeno, antifúngicos, associação.

Introduction

Candida species are commensal fungi that live on the skin and the oral, vaginal and intestinal mucous membranes of the human body. The genus Candida is related to a wide range of clinical manifestations, mainly when the immune defense mechanisms of the individual are compromised by several risk factors, including the use of corticosteroids, systemic antibiotics, internal medical devices, total parenteral nutrition, surgeries and others [1-3]. Although the most prevalent species of this genus involved in invasive fungal infections is *C. albicans*, infections caused by non-*albicans* species have increased significantly, further raising a worrying scenario because such infections are often more severe, rapidly progressive, treatment-refractory and associated the highest mortality and morbidity [1, 4]. Antifungals available to treat infections caused by *Candida* spp. include topical or systemic drugs, showing fungistatic or fungicidal action [5]. However, the low number of antifungal drugs available, the high rates of resistant microorganisms, as well as the inherent toxicity of these drugs have underlined the importance for researching new strategies that lead to effective treatments for the control of fungal infections [6, 7]. With this propose, recent studies have focused on the association between conventional non-antifungal pharmacological agents and conventional antifungal agents [8-12].

Ibuprofen is a non-steroidal anti-inflammatory inhibitor of cyclooxygenase (COX-1 and COX-2) isoenzymes, which specifically blocks mammalian prostaglandin biosynthesis [13]. This anti-inflammatory is classically used due to its antipyretic,

analgesic, and anti-inflammatory effects [14]. The antimicrobial potency of ibuprofen has been demonstrated in its ability to reverse resistance related to efflux pump activity in *C. albicans* [15]. Recently, ibuprofen showed *in vitro* antifungal activity against *Cryptococcus* [16]. Based on this, the present study aimed to evaluate the antifungal activity of ibuprofen alone and when associated with amphotericin B or ketoconazole against *Candida* species.

Materials and methods

Strains

For this study, 14 *Candida* strains were used, including clinical isolates (LM) and standard strains (American Type Culture Collection - ATCC). Amongst them, *C. albicans* (LM-13, LM-410, LM-178, LM-703, ATCC 76485, ATCC 40042); *C. tropicalis* (LM-10, ATCC 13803); *C. guilliermondii* (LM-703, LM-103); *C. krusei* (LM-120, LM-13); *C. parapsilosis* (ATCC 22019, ATCC 20019). All strains were provided by the Mycology Laboratory of the Federal University of Paraíba, João Pessoa-PB, Brazil.

Substances

The substances to which antifungal activity was performed were ibuprofen, amphotericin B and ketoconazole. In addition, sabouraud dextrose agar (SDA) and RPMI-1640 broth were purchased from Difco laboratories and Inlab, respectively. All substance solutions were prepared only at the time of testing by dissolving them in sterile distilled water with the addition of 50 µL dimethylsulfoxide (DMSO). DMSO controls were tested at the same concentrations.

Inoculum

Suspensions were prepared from fresh *Candida* fungal cultures, kept in SDA, and incubated at 37 °C for 24-48h. After this period, colonies of these cultures were suspended in 4 mL of sterile saline (0.85%). Finally, these suspensions were homogenized, and the turbidity was adjusted to 0.5 McFarland scale. Thus, the final inoculum concentration obtained was 1-5 x 10^6 CFU/mL [17, 18].

Minimum inhibitory concentration (MIC)

MIC determination of ibuprofen and antifungals was performed by the 96-well plate microdilution technique. Initially, 100 µL of double concentrated RPMI-1640 was added to the wells of the plate. Then 100 µL of the substance was distributed in the first-row wells of the plate. Through a serial dilution in the ratio of 2, concentrations

ranged from 2048 to 8 μg/mL for ibuprofen and from 512 to 0.0625 μg/mL for antifungals. Then 10 μL of the inoculum was added to each well. Finally, the plates were incubated at 37 °C and read after 24-48 h, observing the presence or absence of visible fungal growth [17-20]. Then 20 μL of 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich®) was added to each well of the plate to prove fungal growth and the plate incubated for a further 12 h [21]. The MIC of the tested drugs was defined as the lowest concentration capable of producing visible inhibition of fungal growth, as indicated by TTC. The following controls were tested: negative controls (RPMI-1640 only) and positive controls (RPMI-1640 and microorganism) to evaluate medium sterility and inoculum viability, respectively. All assays were performed in triplicate [17-20].

Drug association

The association assay between ibuprofen and antifungals was conducted using the checkerboard method [22]. Initially, 100 μL of double concentrated RPMI-1640 was added to the wells of the plate. Then 50 μL of ibuprofen at different concentrations (MICx8, MICx4, MICx2, MIC, MIC/2, MIC/4 and MIC/8) were added horizontally and 50 μL amphotericin B or ketoconazole, also at different concentrations (MICx8, MICx4, MICx2, MIC, MIC/2, MIC/4 and MIC/8) were added vertically to the plate. Thus, different ibuprofen concentrations were tested in the presence of various antifungal concentrations individually. Subsequently, 20 μL of the corresponding inoculum, previously adjusted to 0.5 McFarland scale, were added. The plates were incubated at 37 °C and read after 24-48 h to observe the presence or absence of visible fungal growth [22], as indicated after 12 h of the addition of 20 μL of 1% TTC [21]. All assays were performed in triplicate and the negative (RPMI-1640 only) and positive (RPMI-1640 and microorganism) controls tested.

The effect produced between the combination of anti-inflammatory and amphotericin B or ketoconazole was determined by the fractional inhibitory concentration index (FICI). This index was calculated by the sum of fractional inhibitory concentrations (FIC), where FIC_A= (MIC of substance A in combination)/(MIC of substance A alone) and FIC_B= (MIC of substance B in combination)/(MIC of substance B alone), thus FICI= FIC_A + FIC_B. The association was defined as synergistic for FICI ≤ 0.5, as additive for 0.5 <FICI<1, as indifferent for 1 ≤ FICI < 4, and as antagonistic for FICI ≥ 4 [23, 24].

Results and discussion

The MIC values of ibuprofen against *Candida* species are shown in table 1.

Table 1. MIC (μg/mL) of ibuprofen against *Candida* spp.

Strains	Ibuprofen
C. albicans	
LM-13	32
LM-410	128
LM-178	128
LM-703	256
ATCC 76485	32
ATCC 40042	>2.048
C. tropicalis	
LM-10	128
ATCC 13803	>2.048
C. guilliermondii	
LM-703	512
LM-103	>2.048
C. krusei	
LM-120	>2.048
LM-13	>2.048
C. parapsilosis	
ATCC 22019	128
ATCC 20019	512

>: MIC higher than the concentrations tested.

The results were quite variable, showing a higher antifungal activity against *C. albicans* strains, different from that observed for *C. krusei* where ibuprofen showed lower activity. The concentration of 512 μg/mL inhibited approximately 65% of the tested strains, whereas against 35% of the strains the MIC values were above 2048 μg/mL.

Table 2 presents the MIC for antifungals against the various strains tested.

Amphotericin B showed the best activity, where the concentration of 2 μg/mL was able to inhibit 100% of the strains. Ketoconazole presented MIC ranging from 0.125 to 64 μg/mL. From the individual antifungal MIC, it was possible to make the

Table 2. MIC (μg/mL) antifungals against *Candida* spp.

Strains	Amphotericin B	Ketoconazole
C. albicans		
LM-13	0.5	64
C. tropicalis		
ATCC 13803	2	0.5
C. guilliermondii		
LM-703	0.5	0.125
C. krusei		
LM-120	2	64
C. parapsilosis		
ATCC 20019	2	0.5

associations with ibuprofen. The results of the combination of ibuprofen and amphotericin B against *Candida* strains are shown in table 3.

Table 3. Minimum inhibitory concentration (MIC) in the combination; fractional inhibitory concentration (FIC); and fractional inhibitory concentration index (FICI) of the association between ibuprofen and amphotericin B against *Candida* spp.

Strains	MIC (μg/mL) in combination		FIC of drugs		FICI	Result
	Ibuprofen	Amphotericin B	Ibuprofen	Amphotericin B		
C. albicans LM-13	256	0.25	8	0.5	8.5	Antagonism
C. tropicalis ATCC 13803	512	0.25	0.25	0.125	0.375	Synergism
C. guilliermondii LM-703	4096	4	8	8	16	Antagonism
C. krusei LM-120	512	1	0.25	0.5	0.75	Additivity
C. parapsilosis ATCC 20019	256	0.25	0.5	0.125	0.625	Additivity

The association of ibuprofen with amphotericin B against *C. tropicalis* ATCC 13803 showed a synergistic effect. Additivity was observed in the combinations against *C. krusei* LM-20 and *C. parapsilosis* ATCC 20019. Antagonism was evidenced in 40% of the combinations.

Table 4 shows the effects of the combination of ibuprofen and ketoconazole against *Candida* spp.

Table 4. Minimum inhibitory concentration (MIC) in the combination; fractional inhibitory concentration (FIC); and fractional inhibitory concentration index (FICI) of the association between ibuprofen and ketoconazole against *Candida* spp.

Strains	MIC (μg/mL) in combination		FIC of drugs		FICI	Result
	Ibuprofen	Amphotericin B	Ibuprofen	Amphotericin B		
C. albicans LM-13	128	8	4	0.125	4.125	Antagonism
C. tropicalis ATCC 13803	256	4	0.125	8	8.125	Antagonism
C. guilliermondii LM-703	64	0.125	0.125	1	1.125	Indifference
C. krusei LM-120	256	8	0.125	0.125	0.25	Synergism
C. parapsilosis ATCC 20019	256	0.25	0.5	0.5	1	Indifference

Different forms of interactions between anti-inflammatory and ketoconazole were observed, among them: synergism in 20% of the associations, indifference in 40% and, finally, 40% of the combinations had an antagonistic effect. Non-steroidal anti-inflammatory drugs (ibuprofen, indomethacin, diclofenac sodium and acetylsalicylic acid) are therapeutic options for *Candida*-related infections by inhibiting COX-1 and/or COX-2 that are involved in prostaglandin E_2 biosynthesis, which is a virulence factor in promoting fungal colonization and chronic infections [25].

Studies conducted to evaluate the antimicrobial activity of ibuprofen have shown antibacterial action against methicillin-resistant *Staphylococcus aureus* (MIC 2500 μg/mL), *Salmonella choleraesuis*, *Pseudomonas aeroginosa*, *Klebsiella pneumoniae*, *Escherichia coli* (MIC> 5000 μg/mL) [26], and antifungal activity against *Trichosporon asahii* (MIC 500 to 2000 μg/mL) [9], besides inhibiting the growth of 10 clinical isolates of *Candida*, among them *C. albicans*, *C. krusei*, *C. tropicalis* and *C. guilliermondii*, with MIC value similar to that found in this study [27].

The effect of the association with ibuprofen has been investigated in several studies, including synergism in 43.5% of the combinations of ibuprofen and amphotericin B against *Fusarium* spp. strains [28], this same association showed indifferent results against *Aspergillus* spp. [29]. Recently, this association showed synergistic effects in 86.67% and indifferent effects in 13.33% of the associations against clinical isolates

of *Trichosporon asahii* [9]. Amphotericin B leads to rapid death of fungal cells by causing plasma membrane damage when interacting with ergosterol, resulting in pore formation, surface adsorption and ergosterol extraction from the fungal membrane [30]. Ketoconazole interferes with ergosterol synthesis, which prevents the conversion of lanosterol to ergosterol by inhibiting 14α-demethylase enzyme of cytochrome P450 [31]. In addition to a major problem in the eradication of nosocomial infections, resistance to these drugs is multifactorial and causes several complications in therapy [32]. For this reason, it could be useful to increase the effectiveness of these drugs through combinations with non-antifungal medicines.

Conclusions

This study showed that ibuprofen exerted antifungal activity against most *Candida* species tested, and this information provides more enlightened expectations for future studies that detail the mechanisms of action and resistance involved to ensure its clinical applicability in the treatment of fungal infections caused by *Candida* spp. The combinations of ibuprofen and antifungals promoted synergistic effects. However, antagonistic results were evidenced too, which would hinder its clinical applicability in this case. Therefore, studies of this combined activity should be investigated, as the use of these combinations would bring positive points in antifungal therapy.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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