ANTIMICROBIAL AGENTS

Antifungal activity of ibuprofen alone and in combination with fluconazole against Candida species

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Ibuprofen, a non-steroidal anti-inflammatory drug, exhibited antifungal activity against Candida albicans and non-albicans strains. At 10 μg/ml, ibuprofen showed a rapid cidal activity against exponential growth phase C. albicans, accompanied by rapid and extensive leakage of intracellular K+, permeation to propidium iodide, lysis of spheroplasts and severe membrane ultrastructural alterations. These results indicate that the killing of Candida cells is due to direct damage to the cytoplasmic membrane. At 5 μg/ml, ibuprofen inhibited growth; however, it did not kill the yeasts and did not directly affect the cytoplasmic membrane. Evaluation of yeast metabolic vitality with the fluorescent probe FUN-1 showed that growth inhibition induced by the fungistatic drug concentration was due to metabolic alterations. The combination of ibuprofen with fluconazole resulted in synergic activity with eight of the 12 Candida strains studied, including four of the five fluconazole-resistant strains. The MICs of fluconazole for the fluconazole-resistant strains decreased 2–128-fold when the drug was associated with ibuprofen. When in combination with fluconazole, MICs for ibuprofen decreased by up to 64-fold for all the 12 strains studied. These results point to the practicability of using ibuprofen, alone or in combination with azoles, in the treatment of candidosis, particularly when applied topically, taking advantage of the drug’s antifungal and anti-inflammatory properties.

Introduction

Candida species, mainly C. albicans, are the commonest causative agents of human fungal infections [1–3]. The increase in the rate of opportunistic infections by Candida has resulted from the growing use of broad-spectrum antibacterial agents, as well as from an increasing number of immunocompromised patients due to the use of aggressive chemotherapy for cancer and of immunosuppressive drugs in organ transplantation. The progress of infection by the human immunodeficiency virus (HIV) is another important reason for immunocompromise [4, 5]. Antifungal drugs are used increasingly both as prophylactic and curative agents [6], which, in turn, has led to the widespread emergence of resistant strains [5–7]. This situation has prompted the search for alternative anti-Candida therapeutic agents. Furthermore, many currently used drug regimens are highly costly and lead to serious side-effects.

A number of registered non-antibiotic drugs possess an antimicrobial effect that has generally been regarded as a side-effect, as is the case with antidiuretic, antidiabetic, β-blocker, psychotherapeutic and non-steroidal anti-inflammatory molecules [9]. Among the latter compounds, ibuprofen has been reported to possess antifungal activity [10–12]. The present report extends the previous limited information about the antifungal activity of ibuprofen and reports on its activity against C. albicans and non-albicans strains.

Materials and methods

Organisms

Twelve Candida strains were used. Apart from the two American Type Culture Collection (ATCC) strains, all
were recent clinical isolates (Table 1). The yeasts were stored at −70°C in Brain-Heart Broth (Difco Laboratories) with glycerol 5% until tested. For each experiment, the yeasts were subcultured twice on Sabouraud agar (Difco Laboratories) for 24 h at 35°C and resuspended in saline to a cell density of (1 – 5) × 10^6 cells/ml (cells in the stationary phase). Some assays were also performed with yeast cells in the exponential phase in Sabouraud broth.

**Antifungal drugs**

Ibuprofen (alpha-methyl-4-2-methylpropiolbenzene-acetic acid) was purchased from Sigma and fluconazole from Pfizer (Groton, CT, USA).

**Incubation of yeast cells with ibuprofen**

Suspensions in saline of yeast cells in the stationary phase were resuspended in 10 mM sodium HEPES buffer (pH 7.2) supplemented with 2% glucose (GH solution), with and without increasing serial concentrations of ibuprofen. The number of total cells and the number of viable yeasts in GH were determined in a Neubauer haemocytometer and by counting the number of cfu, respectively. As indicated in the legends to Figs, 1 and 2, ibuprofen was tested for several exposure periods and at various concentrations, ranging from 5 to 25 mg/ml. Incubations were at 35°C, with shaking at 200 strokes/min. At the end of the incubation period, the suspensions were centrifuged for 10 min at 1800 g and the antifungal activity was assayed by viable counts and flow cytometry analyses after incubation with the fluorescent probe propidium iodide (PI, Sigma), as described in detail below. To test the influence of pH on the activity of ibuprofen, yeasts suspended in GH solution at a pH of 4.0 were used. The influence of the age of Candida cells on the antifungal activity of ibuprofen was evaluated by testing yeast cells in their exponential growth phase.

**Determination of MICs**

The MIC of ibuprofen was determined with a macro-dilution test (M27-A protocol), according to the reference method of the National Committee for Clinical Laboratory Standards (NCCLS) [13]. Readings were taken after incubation for 48 h at 35°C. The MICs for combinations of ibuprofen and fluconazole were determined after incubation for 24 h at 35°C in 96-well plates by a micro-dilution procedure, with 80% growth inhibition (measured spectrophotometrically at 500 nm) as the end-point [12]; these MICs will be referred to as MIC80. RPMI 1640 medium (Difco) was used in both the macro- and micro-dilution tests [13]. Possible interaction between ibuprofen and fluconazole was determined by a checkerboard method [12] based on the fractional inhibitory concentration (FIC) and fractional inhibitory index (FIX); synergistic, indifferent and antagonistic interactions were defined by a FIX of < 0.5, 0.5–4.0 or > 4.0, respectively [12].

**Viability counts**

Viability is defined here as the capacity of the yeast cells to proliferate in culture medium. CFU of Candida treated by the test drugs and untreated (controls) were counted after plating serial dilutions of suspensions in saline on Sabouraud agar. Colonies were counted after incubation for 48 h at 35°C.

**Evaluation of membrane lesion with PI**

Incubation with PI was used to detect membrane damage induced by ibuprofen. Preliminary experiments were performed to optimise flow cytometric conditions for the present experiments. Experiments varying the inoculum size from 10^3 to 10^7 yeasts/ml, the PI concentration from 1 to 5 μg/ml and the incubation time from 5 to 180 min were performed. Optimal results were obtained with 10 μg/ml stained for 30 min with PI 1 μg/ml in 0.05 M sodium HEPES buffer, pH 7.2, at room temperature, in the dark. All results with PI-treated yeasts were obtained with this protocol. Yeasts were analysed by flow cytometry as described in detail below. Incubation with PI 1 μg/ml had no toxic effect on the Candida cells as indicated by viable counts.

**Flow cytometry**

A Beckman Coulter XL-MCL flow-cytometer equipped with a 15 mV argon laser was used. Yeast suspensions were analysed at 620 nm (FL3), after incubation with and without PI for measurement of any autoflourescence. The percentage of PI-positive cells was determined for each sample. Drug concentrations producing 50% PI-positive yeast cells were calculated according to a linear regression equation. As the assay confirmed PI staining to be an adequate indicator of cell death (see also Results), drug concentrations that produced 50% PI-positive yeast cells were considered to represent LC50.

**Evaluation of cell metabolic vitality with FUN-1**

FUN-1 staining was used to detect non-lethal metabolic alterations in the yeast cells. Normal, biochemically active cells stained with this fluorescent membrane-permeant dye exhibit orange/red cylindrical intravacuolar structures (CIVS) as detected by fluorescence microscopy, whereas viable cells with impaired metabolism, or non-viable cells, do not show any CIVS [14]. Both untreated (control) and treated yeast suspensions in GH solution were incubated in the dark with 0.5 μM FUN-1 (Molecular Probes Europe BV, Leiden, The Netherlands) for 30 min at 30°C. For each cell suspension, the percentage of yeasts with CIVS was determined after observation of 200 cells with a
Leitz Laborlux K epifluorescence microscope, fitted with a mercury 50-W lamp, a BP 450–490-nm excitation filter and an LP 515-nm emission filter. For fluorescence microscopy, the FUN-1-stained cells were mounted on microscope glass slides with the antifading Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA).

**Assay of K⁺ efflux**

Leakage of potassium from yeast cells was assayed as a means to study membrane damage inflicted by ibuprofen. Because the intracellular K⁺ accumulation is higher in yeasts in the exponential growth phase [15], Candida cells grown at 35°C in Sabouraud broth supplemented with K₂HPO₄ 0.5% were harvested in the middle of the exponential phase. The yeasts were washed twice with saline and exposed to ibuprofen for 10 min at 35°C, at the concentrations described above. At intervals, treated and untreated (control) suspensions were filtered through 0.45-µm Millipore filters and the filtrates were assayed for K⁺ with a K⁺-sensitive glass electrode connected to a Spotlyte analyser (Menarini Diagnostics). The results are represented as percentage of K⁺ leaked by cells boiled for 30 min [16, 17]. The K⁺ efflux was also analysed for Candida cells treated with 10 mM sodium azide for 5–30 min. Viable counts and PI staining were determined in parallel in the samples used for the K⁺ efflux assays.

**Effect of ibuprofen on spheroplasts**

Spheroplasts of C. albicans ATCC strain 10231 were obtained by enzymic digestion of cell walls with Lyticase (Boehringer Mannheim, Cat. no. 1372464) [18]. Because preliminary studies showed that the cell walls of Candida in their exponential growth phase were more susceptible to Lyticase digestion than stationary phase cells, middle exponential phase cells (c. 1 × 10⁷ cells/ml) that had been grown at 35°C in YEPD medium (yeast extract 1%, Bacto peptone 2%, glucose 2%) were used. The yeasts were collected by centrifugation at 1800 g for 10 min, and washed once each with water and 1.4 M sorbitol. The pellets were resuspended to a concentration of (1–5) × 10⁷ cells/ml in the spheroplast mixture which consisted of 0.04 M HEPES buffer (pH 7.4), 0.5 mM MgCl₂ and mercaptoethanol (Sigma) 0.5% in 1.4 M sorbitol. Lyticase was added to the suspensions at a concentration of 10 units/10⁷ cells. The suspensions were incubated at 30°C with gentle, occasional shaking. The progress in spheroplast lysis was followed by evaluating spheroplast lysis after mixing 10 µl of the yeast suspension with 10 µl of sodium dodecyl sulphate (SDS) 5% on a glass slide and observation by phase contrast microscopy. SDS did not lyse yeasts not exposed to Lyticase. When most Candida cells had converted into spheroplasts, the suspension was centrifuged at 1800 g for 10 min and the pellet resuspended in GH medium supplemented with 1.4 M sorbitol and with ibuprofen 12.5 mg/ml or without the drug (controls). The turbidity of the suspensions was followed at 600 nm for 30 min in a Shimadzu spectrophotometer, model UV-160A. Samples of treated spheroplasts and controls were viewed by phase contrast microscopy and processed for electron microscopy (see below).

**Transmission electron microscopy**

Samples of intact cells and spheroplasts of C. albicans ATCC strain 10231, either treated with ibuprofen or untreated (controls), were processed for ultrastructural analysis. Procedures for fixation were adapted either for intact cells or for spheroplasts. As yeast cells with unaltered cell walls are difficult to fix by conventional methods, they were fixed with glutaraldehyde 2.5% in 0.1 M cacodylate buffer, pH 7.2, followed by washing in the same buffer and fixation with potassium permanganate 1.5% in water for 1 h [19]. These steps were followed by washing and post-fixation with uranyl acetate 1% in water for 30 min [20]. Spheroplasts were prefixed with glutaraldehyde 2.5% by adding 1 vol of a 25% stock solution to 9 vol of spheroplast suspension. After incubation for at least 4 h at room temperature, the samples were washed with 0.1 M cacodylate buffer, pH 7.2, and fixed overnight at room temperature with OsO₄ 1% in 0.1 M acetate-Veronal buffer, pH 7.0, supplemented with 10 mM Ca²⁺ [21]. After washing with water, suspensions were post-fixed with aqueous uranyl acetate 1% for 30 min at room temperature. Intact cells and spheroplasts were dehydrated in ethanol after which the samples were embedded in Epon [21]. Ultra-thin sections were cut with an LKB Ultratome III microtome and contrasted with uranyl acetate followed by lead citrate [21]. Observations and micrographs were done with a Zeiss EM 10C electron microscope.

**Statistical analysis**

Correlation coefficients (r) were calculated with the Anova program.

**Results**

**Fungistatic activity of ibuprofen**

Ibuprofen inhibited the growth of the 12 Candida strains studied, with MICs of 1–3 mg/ml (Table 1). After exposure of C. albicans ATCC strain 10231 for 5–30 min to ibuprofen 5 mg/ml, i.e., a concentration slightly above the MIC for this strain, only a very small proportion of the cells was killed (Figs. 1a and 2a). With this treatment, most cells remained impermeable to PI (Figs. 1b and 2b) and the K⁺ efflux was minimal (Fig. 2c). However, under these conditions, the drug significantly impaired the metabolic vitality of the yeasts, as indicated by the substantial reduction in the percentage of cells with CIVS (Table 2).
Table 1. MICs of ibuprofen and fluconazole and LC50 of ibuprofen for the 12 Candida strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Isolated from</th>
<th>MICa (μg/ml)</th>
<th>MICb (μg/ml)</th>
<th>LC50b (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans 10231</td>
<td>ATCC</td>
<td>2</td>
<td>1</td>
<td>16.11</td>
</tr>
<tr>
<td>C. albicans H37</td>
<td>Bronchial wash</td>
<td>2 &gt; 64</td>
<td>11.89</td>
<td></td>
</tr>
<tr>
<td>C. glabrata H30</td>
<td>Vagina</td>
<td>3 &gt; 64</td>
<td>11.99</td>
<td></td>
</tr>
<tr>
<td>C. glabrata H16</td>
<td>Vagina</td>
<td>3 &gt; 64</td>
<td>15.35</td>
<td></td>
</tr>
<tr>
<td>C. krusei H9</td>
<td>Blood</td>
<td>1 &gt; 64</td>
<td>8.44</td>
<td></td>
</tr>
<tr>
<td>C. krusei H32</td>
<td>Blood</td>
<td>3 &gt; 64</td>
<td>10.78</td>
<td></td>
</tr>
<tr>
<td>C. tropicalis 13803</td>
<td>ATCC</td>
<td>3</td>
<td>4</td>
<td>19.28</td>
</tr>
<tr>
<td>C. tropicalis H18</td>
<td>Blood</td>
<td>3</td>
<td>4</td>
<td>16.17</td>
</tr>
<tr>
<td>C. guilliermondii MAT23</td>
<td>Vagina</td>
<td>1</td>
<td>4</td>
<td>16.27</td>
</tr>
<tr>
<td>C. guilliermondii MAT23</td>
<td>Vagina</td>
<td>2</td>
<td>2</td>
<td>11.5</td>
</tr>
<tr>
<td>C. haastiae H22</td>
<td>Vagina</td>
<td>3</td>
<td>1</td>
<td>19.99</td>
</tr>
<tr>
<td>C. haastiae H54</td>
<td>Bronchial wash</td>
<td>2</td>
<td>1</td>
<td>13.67</td>
</tr>
</tbody>
</table>

*MICs were determined by a macro-dilution method for ibuprofen (Ibu) and fluconazole (Flu).

LC50: ibuprofen concentrations resulting in 50% propidium iodide-positive (PI+) yeasts.

Fig. 1. Percentage of (a) non-viable and (b) propidium iodide-positive (PI+) cells after exposure of C. albicans strain ATCC 10231 to ibuprofen for 30 min (●, 5 mg/ml; ■, 12.5 mg/ml; ▲, 20 mg/ml; ×, 25 mg/ml). Stationary cells were used.

Fungicidal activity of ibuprofen

Treatment of stationary (Fig. 1a) or exponential growth phase cells (Fig. 2a) of C. albicans ATCC strain 10231 with ibuprofen 20–25 or 10–20 mg/ml, respectively, resulted in a marked cidal effect. An increased susceptibility of exponential as compared with stationary phase cells was also seen with C. albicans strain H9 (not shown). The results of staining yeast cells with PI in the experiment presented in Figs. 1a and 2a are shown in Figs. 1b and 2b. The number of PI+ cells and of non-viable yeast cells correlated well. Because staining with PI accurately measured cell death, the study relied on the flow cytometric analyses of PI-stained cells to further analyse the antifungal activity of ibuprofen on the clinical Candida isolates. Fig. 3 shows the effect of exposure for 60 min of stationary phase cells to increasing concentrations of ibuprofen at pH 7.0. From these results, LC50 were calculated for the 12 strains studied (Table 1). The correlation between LC50 and MIC values for ibuprofen for the 12 strains was poor (r = 0.255). At pH 4.0, LC50 of ibuprofen for C. albicans ATCC strains 10231 and H9 was 1.521 and 0.479 mg/ml, respectively, that is substantially lower than the LC50 values obtained at pH 7.0 (Table 1).

The efflux of K+ from the cells exposed to different concentrations of the drug for 5 and 10 min further indicates that ibuprofen causes cell membrane damage. At concentrations that resulted in extensive killing (Fig. 2a) and permeability to PI (Fig. 2b), ibuprofen induced a quick and extensive K+ efflux in ATCC strain 10231 (Fig. 2c). Treatment of this strain with 10 mM sodium azide for 10 min produced only a slight K+ efflux (Fig. 2c); about the same low cation efflux was found after treatment for 30 min (data not shown).

Transmission electron microscopy showed that exposure of intact C. albicans ATCC strain 10231 stationary phase cells to ibuprofen 20 mg/ml for 10 min completely solubilised the cytoplasmic membrane (Fig. 4). Treatment of exponential phase cells of ATCC strain 10231 with Lyticase under the conditions described resulted in detergent-sensitive structures, due to a partial lysis of the yeast cell wall. Therefore, this report refers to the resulting structures as spheroplasts instead of protoplasts. The fact that the cell wall was not completely removed in most cells was concluded by observations made by phase contrast microscopy.
that revealed ovate cell shapes and by the transmission electron micrographs, showing loose cell-wall remnants (Fig. 5a); only occasional completely free protoplasts were found. Ibuprofen, at fungicidal concentration, quickly lysed the spheroplasts, as evaluated by absorbance readings at 600 nm, by phase contrast microscopy (not shown) and by electron microscopy (Fig. 5b). Spheroplasts not exposed to the drug remained intact and showed numerous ribosomes, normal intracellular organelles, i.e., a nucleus, mitochondria (Fig. 5a) and intact cell membrane with a continuous triple-layered profile (Fig. 6a). Conversely, most spheroplasts treated with ibuprofen 12.5 mg/ml for 10 min had lysed (Fig. 5b) and showed severely damaged cell membranes with gross fractures or extensive solubilisation (Fig. 6b).

**Interaction between ibuprofen and fluconazole**

The checkerboard technique was used to determine the effect of different combinations of ibuprofen and fluconazole on growth of the 12 Candida strains. Table 2 shows the MIC80s for the drugs individually and in combination. The correlation between the MIC80 of each drug was poor for all strains tested ($r = 0.09$), indicating different patterns of susceptibility to the two drugs. Based on the FIX values, combinations of the drugs had a synergistic effect on eight of the 12 strains studied, including four of the five strains that were fluconazole-resistant (Table 3). The association of the two drugs had an indifferent effect for the other four strains (Table 3). When the drugs were used in combination, the MIC80s of fluconazole for the fluconazole-resistant strains, i.e., *C. albicans* H37, *C. glabrata* H30 and H16, *C. krusei* H9 and H32, decreased 2–128-fold. For all 12 strains, MIC80s for ibuprofen decreased by 6–64 times when used in combination with fluconazole.

As fluconazole is known to have fungistatic activity on *Candida* through interaction with metabolic pathways [22], the anti-*Candida* activity of this drug was analysed alone and in combination with ibuprofen, by determining the percentage of yeasts with CIVS after exposure to the drugs for 1 h and staining with FUN-1. Table 2 shows the reduction in the percentage of cells with CIVS after treatment with fluconazole at a concentration corresponding to the MIC. Table 2 also shows the synergy between the two drugs against *C. tropicalis* ATCC strain 13803.

**Discussion**

The need for novel antifungal regimens prompted this study of the activity of ibuprofen, a non-steroidal anti-inflamatory drug which has previously been shown to have such an activity [10–12], against *C. albicans* and non-* albicans* species, as well as the synergistic effect of ibuprofen in association with fluconazole. Ibuprofen

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**Table 2. Reduction in metabolic vitality of *C. tropicalis* ATCC 13803 cells exposed to fluconazole (Flu) and ibuprofen (Ibu) alone or in combination**

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Percent of cells with CIVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.0</td>
</tr>
<tr>
<td>Flu 4 µg/ml</td>
<td>13.5</td>
</tr>
<tr>
<td>Ibu 3 mg/ml</td>
<td>14.2</td>
</tr>
<tr>
<td>Flu 1 mg/ml</td>
<td>88.0</td>
</tr>
<tr>
<td>Ibu 0.0625 mg/ml</td>
<td>87.1</td>
</tr>
<tr>
<td>Flu 1 µg/ml + Ibu 0.0625 mg/ml</td>
<td>26.5</td>
</tr>
</tbody>
</table>

*The drug concentrations used correspond to the MIC80 of the individual drug or of their combination (see Table 3).*

*Percentage of cells with CIVS after staining with the probe FUN-1 was used as a measure of metabolic vitality.*

**Fig. 2.** Exponential phase *C. albicans* ATCC 10231 cells were exposed to ibuprofen for 10 min (a, 5 mg/ml; b, 10 mg/ml; c, 20 mg/ml). In the same suspensions three parameters were determined: (a) the percentage of non-viable cells (determined by cfu counts); (b) the percentage of PI+ cells; (c) K+ efflux (as percentage of the total intracellular K+). K+ efflux was also analysed in yeasts treated with 10 mM sodium azide (X; control).
Fig. 3. Percentage of PI+ cells in suspensions of Candida strains exposed for 60 min to different concentrations of ibuprofen. Stationary phase cells were used. ■, ATCC alb; □, H37; △, H30; +, H16; ●, H9; ○, H32. - - - - - , ATCC trop: - - - - - H18; - - - - - , MAT23; ○, MAT24; □, H22, ▲, H54.

was found to have a marked effect on C. albicans and non-albicans strains. Previously reported MIC values are similar to those found in the present study. Moreover, the present study confirmed the observation by Sanyal et al. [11] of greater activity of the drug at pH 4.0 than at pH 7.0. However, the mechanism of the antifungal activity of ibuprofen was not reported in the previous studies. The anti-Candida effects found in the present study included metabolic impairment, growth inhibition, lost of viability, membrane permeability changes, spheroplast lysis and ultrastructural alterations.

The activity of ibuprofen against Candida can be either fungistatic or fungicidal, depending on the drug concentration used. Ibuprofen, at a concentration of 5 mg/ml, inhibited growth of C. albicans ATCC strain 10231 without killing the yeast, whereas concentrations of 10–20 mg/ml had a rapid cidal action. The cidal effect of ibuprofen on Candida was found to be due to damage to the cytoplasmic membrane, as Candida cells exposed to cidal concentrations of the drug quickly become permeable to PI. This membrane-impermeable fluorochrome [23] has been used widely to evaluate membrane permeability in diverse cell types, including yeasts. It is considered to be a good marker for cell death associated with membrane alterations [23, 24].

Quantification of K+ efflux from micro-organisms, both from bacteria [17] and yeasts [25–28], has been used to evaluate membrane-damaging actions by different compounds. Yeast cells in the exponential phase of growth are known to accumulate intracellularly K+ to levels as high as 220 mM [15]. Loss of selective membrane permeability in microbes due to membrane damage results in rapid leakage of that
Fig. 5. (a) Untreated spheroplasts of *C. albicans* strain ATCC 10231. Note the loose cell walls (W) and the intact protoplast with nucleus (N), mitochondria (M) and vacuoles (V) (×13200). Section stained with uranyl-lead. (b) Spheroplasts from the same preparation as in (a) but exposed to ibuprofen 12.5 mg/ml for 10 min (×13200). Note several lysed spheroplasts (*) with collapsed cell walls. The non-lysed spheroplasts have an altered ultrastructure. Section stained with uranyl-lead. Bar = 1.0 μm.
Fig. 6. (a) High magnification of a control spheroplast showing the continuous, triple-layered cytoplasmic membrane (M). W, almost completely digested cell wall (<×71 400). Section stained with uranyl-lead. (b) As in (a), but spheroplast exposed to ibuprofen 12.5 mg/ml for 10 min (<×71 400). Note the serious structural alterations of the cytoplasmic membrane, which is extensively solubilised, with only small remnants left (arrows). W, partially digested cell wall. Section stained with uranyl-lead. Bar = 0.25 μm.

Table 3. Checkerboard assay of the effect of ibuprofen (Ibu) and fluconazole (Flu) alone and in combination (comb) on *Candida* strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC Ibu (mg/ml)</th>
<th>MIC Ibu comb (mg/ml)</th>
<th>MIC Flu (μg/ml)</th>
<th>MIC Flu comb (μg/ml)</th>
<th>FIC Ibu</th>
<th>FIC Flu</th>
<th>FIX</th>
<th>Outcome</th>
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<tbody>
<tr>
<td><em>C. albicans</em> 10231</td>
<td>2</td>
<td>0.0625</td>
<td>1</td>
<td>0.031</td>
<td>1</td>
<td>1.031</td>
<td></td>
<td>Indifference</td>
</tr>
<tr>
<td><em>C. albicans</em> H37</td>
<td>3</td>
<td>0.500</td>
<td>256</td>
<td>2</td>
<td>0.170</td>
<td>0.086</td>
<td>0.178</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>C. glabrata</em> H30</td>
<td>3</td>
<td>0.0625</td>
<td>128</td>
<td>16</td>
<td>0.021</td>
<td>0.125</td>
<td>0.042</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>C. glabrata</em> H16</td>
<td>1</td>
<td>0.0625</td>
<td>64</td>
<td>2</td>
<td>0.021</td>
<td>0.031</td>
<td>0.052</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>C. krusei</em> H9</td>
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<td>0.0625</td>
<td>64</td>
<td>16</td>
<td>0.062</td>
<td>0.25</td>
<td>0.312</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>C. krusei</em> H12</td>
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<td>0.0625</td>
<td>64</td>
<td>32</td>
<td>0.021</td>
<td>0.5</td>
<td>0.521</td>
<td>Indifference</td>
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<tr>
<td><em>C. tropicalis</em> 13803</td>
<td>3</td>
<td>0.0625</td>
<td>4</td>
<td>1</td>
<td>0.023</td>
<td>0.25</td>
<td>0.271</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>C. tropicalis</em> H18</td>
<td>3</td>
<td>0.250</td>
<td>4</td>
<td>1</td>
<td>0.080</td>
<td>0.25</td>
<td>0.33</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> MAT23</td>
<td>1</td>
<td>0.0625</td>
<td>2</td>
<td>2</td>
<td>0.063</td>
<td>0.5</td>
<td>0.563</td>
<td>Indifference</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> MAT24</td>
<td>3</td>
<td>0.0625</td>
<td>1</td>
<td>2</td>
<td>0.021</td>
<td>2</td>
<td>2.021</td>
<td>Indifference</td>
</tr>
<tr>
<td>*C. lactantue H22</td>
<td>4</td>
<td>0.0625</td>
<td>1</td>
<td>0.25</td>
<td>0.016</td>
<td>0.25</td>
<td>0.266</td>
<td>Synergy</td>
</tr>
<tr>
<td>*C. lactantue H54</td>
<td>3</td>
<td>0.0625</td>
<td>1</td>
<td>0.125</td>
<td>0.021</td>
<td>0.125</td>
<td>0.146</td>
<td>Synergy</td>
</tr>
</tbody>
</table>

MICs were determined by a micro-dilution technique on the basis of an 80% reduction in growth. FIC, fractional inhibitory concentration; FIX, fractional inhibitory index.
cation to the extracellular milieu [17, 25–28]. Concentrations of ibuprofen that quickly kill Candida cells induced a quick and extensive K⁺ efflux. Thus, >90% of the intracellular cation content of exponential growth phase cells was lost in the initial 5 min of exposure to ibuprofen 20 mg/ml. This indicates that killing of Candida by ibuprofen is caused by direct damage to the cell membrane. In fact, the rate of K⁺ efflux from Candida cells exposed to cidal concentrations of ibuprofen was much higher than would be expected if the drug acted only as a metabolic inhibitor, with secondary effects on membrane function. In support of this assumption, a high concentration of the metabolic inhibitor sodium azide induced K⁺ leakage at a much slower rate than ibuprofen. The results of the present study regarding the K⁺ efflux induced by ibuprofen do not necessarily mean that the loss of intracellular K⁺ per se is responsible for the cidal action of the drug, as membrane disorganisation may result in multiple perturbations that would eventually lead to a lethal outcome [16].

Additional support for the interpretation that killing of Candida cells by ibuprofen is due to a direct membrane damaging action of fungicidal concentrations of ibuprofen is provided by the rapid lysis of the spheroplasts, when exposed in an osmotically protective medium to concentrations of the drug that quickly kill the yeasts. Bacterial protoplasts are quickly lysed by treatment with molecules that directly disorganise the cell membrane, e.g., by tetrazolium salts or local anaesthetics [17].

Finally, the severe cell membrane alterations, with fracturing and solubilisation, observed by electron microscopy as soon as 10 min after exposure of C. albicans to fungicidal concentrations of ibuprofen, also indicate a direct membrane-damaging action of the molecule.

The mechanism proposed for the fungicidal activity of ibuprofen is in conformity with its lipophilic character, as indicated by the high octanol/water partition coefficient of the drug [29].

High doses of ibuprofen were fungicidal, whereas low doses were fungistatic. The fact that high doses of ibuprofen kill Candida by direct membrane damage does not preclude the possibility that low concentrations may inhibit growth by another mode of action. Several lipophilic antimicrobial agents can exert their activity either through direct damage of the cell membrane or by interfering with metabolic pathways. This is the case, for example, with the lipophilic azoles [30, 31], butenafine [32] and phenothiazines [33, 34]. In those studies, the concentrations needed to produce direct membrane damage were higher than those producing metabolic imbalance. The poor correlation coefficient between the MIC (indicator of fungistatic activity) and LC50 (fungicidal activity) of ibuprofen for the 12 Candida strains in the present study suggests that the mechanisms for the fungistatic and fungicidal activities differ. Our interpretation is that the fungistatic action of ibuprofen is due to metabolic alterations, rather than resulting from a direct membrane-damaging action. This interpretation is suggested by the observations that ibuprofen, at fungistatic concentrations, neither induces any marked leakage of intracellular K⁺ nor makes the yeasts permeable to PI, but leads to a substantial reduction of metabolic vitality.

The concentrations of ibuprofen in the blood after administration of standard anti-inflammatory doses [35] are below those the present study found to be necessary for an anti-Candida action, when the drug is used alone. However, these results indicate that synergy can be achieved if ibuprofen is administered together with fluconazole, which was demonstrated for eight of the 12 Candida strains tested. The significant reduction in the MIC of ibuprofen when used in combination with fluconazole indicates that antifungal plasma concentrations can be achieved following standard medication when used in association with fluconazole. Furthermore, the concentration of ibuprofen in urine of patients under the conventional standard systemic regimen [36] reaches values above those shown to possess an antifungal activity, pointing to the potential usefulness of ibuprofen for treatment of urinary tract candidiasis. It should be noted that urinary tract infections caused by Candida species have increased in frequency and represented c. 10% of such infections in a series of hospitalised patients [2]. Ibuprofen could also be used topically to treat mucosal or cutaneous candidiasis, as concentrations of the molecule that are capable of killing the fungi rapidly in vitro are easily obtained in topically available formulations. In fact, cream and spray solutions in current commercial use contain ibuprofen 5%, i.e., more than 10 times the concentrations found to quickly kill Candida cells. Thus, both formulae would provide locally active concentrations of ibuprofen. The anti-inflammatory and analgesic properties of ibuprofen may represent an additional advantage for its use in the management of infections with Candida.

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