Potent synergic effect between ibuprofen and azoles on *Candida* resulting from blockade of efflux pumps as determined by FUN-1 staining and flow cytometry

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**Objectives:** Resistance to antifungals often relates to efflux pumps exporting drugs; several modulators may block them, reverting resistance. Verapamil, oestradiol and progesterone, known efflux pump inhibitors of human neoplastic cells, and ibuprofen were tested as potential modulators of resistance of *Candida* spp.

**Methods:** Forty-two clinical isolates of *Candida* (38 fluconazole-resistant), two ATCC type strains and two *C. albicans* strains with known mechanisms of fluconazole resistance were incubated with subinhibitory concentrations of the modulators. After exposure, MICs of fluconazole, itraconazole and voriconazole were re-determined. Simultaneously, yeasts exposed to modulators were stained with FUN-1 and analysed by flow cytometry. ³H-labelled itraconazole was also used to study efflux in the presence and absence of modulators.

**Results:** Fluconazole MICs decreased in most strains after exposure to modulators, including control strains with documented efflux overexpression. No significant MIC variation was noticed for: all *C. krusei* strains tested, for the resistant strain by target change, for susceptible strains, and for a very few other clinical isolates. Reverted resistant phenotypes showed cross-resistance to itraconazole and to voriconazole, which was also reverted by the modulators. For these strains, an increase in FUN-1 staining and increased accumulation of ³H-labelled itraconazole were noticed after incubation with modulators.

**Conclusions:** Resistance related to overexpression of efflux pumps was common among clinical isolates and could be reverted by the assayed modulators, particularly ibuprofen. The mechanism of resistance in all tested *C. krusei* and in a few other strains seems, however, to be of a different nature. Ibuprofen is a promising compound in association with azoles, deserving future clinical trials. FUN-1 proved to be a good marker of efflux in *Candida*.

Keywords: yeasts, antifungal susceptibility, mechanisms of resistance, antifungals

**Introduction**

There are a limited number of antifungals available, most just providing fungistatic but not fungicidal effects. Frequent cross-resistance also stresses the need for developing new therapeutic alternatives. Resistance has been classified as primary or intrinsic when occurring prior to drug exposure and as secondary whenever it develops after exposure to antimicrobial drugs.¹ ² Fluconazole is one of the most widely used antifungal agents, both for prophylaxis and therapy of *Candida* infection. Several mechanisms ofazole resistance in *Candida albicans*, including reduced accumulation of the drugs through active efflux (overexpression of genes CDR1, CDR2 and MDR1), alteration or overexpression of the target enzyme (14α-sterol-demethylase, encoded by ERG11) and loss-of-function downstream mutation in the ergosterol pathway (defective Δ5,6-desaturase encoded by ERG3), have already...
Reversion of resistance by blocking efflux pumps

been described. The most prevalent mechanism seems to be active efflux, although the relative frequency of resistance mechanisms is difficult to ascertain. Additionally, some strains express multiple genetic alterations. Antifungal susceptibility testing, as recommended by the Clinical Laboratory Standards Institute (formerly NCCLS) is laborious and time consuming, giving no information regarding the mechanism of resistance. Flow cytometry provides consistent results concerning the susceptibility of Candida to fluconazole in a few hours. Using the fluorescent stain FUN-1, it is possible to classify Candida strains as susceptible, susceptible dose-dependent or resistant to fluconazole. This approach also allows clarification of the mode of action of some non-antifungals, such as local anaesthetics, benzylamine and ibuprofen. The main objective of our investigation regards the mechanisms of resistance reversion. In eukaryotic neoplastic cells, drug efflux is common and confers multiresistance to antineoplastic drugs. The use of blockers of efflux pumps such as verapamil or sex hormones, allows reversion of resistance. Thus, we decided to assay such compounds in the reversion of resistance of Candida to azoles such as fluconazole, itraconazole and voriconazole, and included ibuprofen, which at low concentrations has a synergic effect with fluconazole and has an unrelated fungicidal effect at higher concentrations. Reversion of resistance to azoles occurred in the majority of the resistant strains. An objective method, based on radioactive measurement of the uptake of itraconazole in yeast cells in the presence or absence of the modulators, proved our hypothesis of reversion of efflux. The results strongly suggest that ibuprofen, a potent anti-inflammatory and analgesic drug, might have a future role in therapy of candidosis, in association with a classical antifungal drug like fluconazole.

Materials and methods

Strains

Forty-six isolates of Candida rated as resistant (R, 40 strains), susceptible dose-dependent (S-DD, four strains) and susceptible (S, two strains) to fluconazole, as determined by the reference microdilution test-protocol M27-A2 of CLSI (details shown in Table 1) were assayed. Clinical isolates were obtained from the respiratory tract, vaginal fluids, blood or urine. C. albicans strain 90028, and C. krusei strain 6258, both from American Type Culture Collection (ATCC), recommended as quality control for determining MICs of azole compounds, were included in each experiment. Two Candida strains whose dominant mechanism of resistance is well characterized, were also included, i.e. C. albicans strain 95-68, with overexpression of CDR1 and CDR2, and C. albicans strain 12-99, with overexpression of CDR1 and CDR2 but also ERG11 and MDR1 (gift from Dr Ted White). Until testing, the yeasts were kept frozen in Brain-Heart broth (Difco Laboratories, Detroit, MI, USA) with 5% glycerol. For each experiment, the strains were subcultured twice on Sabouraud agar (Difco) for 24 h at 35°C. Suspensions of blastoconidia with a cell density of 1–5 × 10⁴ cells/mL were prepared in sterile saline.

Chemicals

Fluconazole and voriconazole were supplied by Pfizer (Groton, CT, USA), and itraconazole and [³H]itraconazole were obtained from Janssen (Beere, Belgium) and maintained in stock solutions at −70°C until use. Verapamil, β-oestradiol, progesterone and ibuprofen were purchased from Sigma (St. Louis, MO, USA) and FUN-1 from Molecular Probes (Europe BV, Leiden, Holland).

Determination of MICs of the different azoles

MIC values of fluconazole, itraconazole and voriconazole were determined according to the CLSI M27-A2 protocol. Phenotypes of fluconazole and itraconazole were defined accordingly. For fluconazole: MIC ≤ 8 mg/L, susceptible (S); MIC between 16 and 32 mg/L, susceptible-dose-dependent (S-DD); and MIC ≥ 64 mg/L, resistant (R). For itraconazole: MIC ≤ 0.125 mg/L, S; MIC between 0.25 and 0.5 mg/L, S-DD; and MIC ≥ 1 mg/L, R. For voriconazole: MIC ≤ 1 mg/L, S; MIC of 2 mg/L, S-DD; and MIC ≥ 4 mg/L, R (newly established tentative breakpoints).

Selection of subinhibitory concentrations of the different modulators

Several concentrations of each potential modulator used were incubated with yeast cells according to the NCCLS M27-A2 protocol in order to select a subinhibitory concentration for each compound: verapamil, β-oestradiol and progesterone were tested from 50 to 500 μM; ibuprofen from 100 to 5000 mg/L.

Effect of subinhibitory concentrations of modulators on cellular viability

Following determination and selection of a subinhibitory concentration for each modulator, i.e. 100 μM verapamil, 50 μM β-oestradiol, 50 μM progesterone and 100 mg/L ibuprofen, the viability of each strain was determined by enumeration of the number of cfu/mL. Additionally, an aliquot from each yeast suspension was stained with 1 μM FUN-1 and analysed with a Leitz Laborlux K epifluorescence microscope (Leica, NY, USA) equipped with a mercury 50 W lamp, a BP 450-490 excitation filter and an LP 515 nm emission filter in order to enumerate the number of cells showing cylindrical intravacuolar structures (CIVS). FUN-1 is converted by yeasts sustaining membrane integrity and full metabolic capabilities into CIVS. Metabolically active cells show CIVS in a light yellow-green cytoplasm. Conversely, metabolically impaired cells, although not yet dead, exhibit a marked diffuse yellow-green cytoplasmic fluorescence, without CIVS.

Determination of MICs of azoles associated with modulators

The MICs of fluconazole, voriconazole and itraconazole were determined in the presence of the selected subinhibitory concentrations of each modulator, according to the CLSI M27-A2 protocol, for all strains.

Flow cytometry analysis

Yeast cells were incubated at 150 rpm overnight (16 h) in Sabouraud broth (Difco) at 37°C. A yeast suspension was prepared in PBS supplemented with 2% glucose, the final concentration adjusted to 1 × 10⁶ cells/mL and later divided into aliquots of 1 mL. The yeast cells were then incubated for 90 min at 35°C with shaking, with or without the different modulators at concentrations that had shown a synergic effect with fluconazole. The cells were washed and resuspended in 10 mM HEPES supplemented with 2% glucose (GH solution) and stained with 0.5 μM FUN-1. A Beckman Coulter XL-MCL flow cytometer (Beckman-Coulter Corp., Hialeah, FL, USA) equipped with a 15 nm argon laser was used. From each yeast suspension, 30 000–50 000 cells were analysed. The intensity of fluorescence shown by cells incubated with the modulators was determined at FL2 (575 nm) and compared with non-treated cells.
Table 1. MICs (mg/L) and phenotypes of strains of *Candida* to fluconazole (FLC) and voriconazole (VRC) alone and in combination with subinhibitory concentrations of verapamil (Ver) (100 µM), β-oestradiol (β-estra) (50 µM), progesterone (Prog) (50 µM) and ibuprofen (Ibu) (100 mg/L).

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<th>MIC FLC + β-estra/phenotype</th>
<th>MIC FLC + Prog/phenotype</th>
<th>MIC FLC + Ibu/phenotype</th>
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<th>MIC VRC + Ver/phenotype</th>
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| Phenotype defined according to NCCLS. MICs were determined as described in the Materials and methods section. S, susceptible; R, resistant; S-DD, susceptible-dose-dependent; –, not done.
Accumulation of intracellular $^3$H-labelled itraconazole

*Candida* cells were initially incubated under similar conditions as described for flow cytometry assays. The cells were then harvested by centrifugation at 3120 rpm for 10 min at 4°C, and washed twice with 20 mL of phosphate-buffered saline (PBS). The yeast cells were resuspended in Sabouraud broth, at a final concentration of 2.5 x 10^8 cells/mL. Each strain was incubated with the different modulators at 37°C, with continuous shaking at 300 rpm for 30 min. For each strain, drug-free suspensions were used. $[^3]$H-Itraconazole (specific activity, 7.98 GBq/mmol; Johnson and Johnson Pharmaceutical Research and Development, PCPK-synthesis group, Beerse, Belgium) was added to all yeast suspensions at a final concentration of 3 μM. The cells were incubated in 10 mL glass tubes at 37°C, with continuous shaking at 300 rpm, in an orbital shaker for 60 min. The cells were harvested by centrifugation at 5000 rpm for 10 min and washed three times with 3 mL of ice-cold culture medium containing 10 μM unlabelled itraconazole. The pellets were later resuspended in 500 μL of PBS. The radioactivity of cells was determined by adding a scintillation cocktail (Optiphase ‘Hisafe3’, Perkin-Elmer). The radioactivity was measured in a liquid scintillation counter (LKB Wallac, 1209 RackBeta).

Statistical analysis

Each experiment was repeated at least three times. The coefficients of correlation ($r$) between the effects of the different modulators were calculated using the ANOVA statistic, in SPSS 11.5 for Windows.

Results

Effect of modulators on susceptibility to azoles

Susceptibility profiles for fluconazole and itraconazole were identical. Most *C. albicans* strains resistant to fluconazole were also resistant to voriconazole. However, most *C. tropicalis*, *C. glabrata* and all *C. krusei* strains, known to be intrinsically resistant to fluconazole, were susceptible to voriconazole (Table 1). In all strains showing cross-resistance to azoles, a reversion of the R phenotype was observed in the presence of modulators (Table 1). Most of the strains that remained resistant in the presence of one modulator also remained resistant to the other modulators. The reversion of resistance to fluconazole was not observed in all *C. krusei*, in two of 24 clinical isolates of *C. albicans* and in three of five tested *C. glabrata*. The *C. albicans* 95–68 strain known to be resistant by efflux showed a reversion of resistance to azoles, but not the strain 12–99 with the target changed beyond efflux. Considering the strains which were already susceptible to azoles prior to exposure to the modulators, only minor variations were found in MICs of all azoles after exposure to modulators (Table 1).

Comparing the reduction in MICs of fluconazole and itraconazole by the different modulators, extensive correlation coefficients were found: $r = 0.993$ between the two hormones; $r = 0.920$ between progesterone and verapamil; $r = 0.915$ between β-oestradiol and verapamil; $r = 0.858$ between ibuprofen and progesterone; $r = 0.863$ between ibuprofen and β-oestradiol; and $r = 0.853$ between ibuprofen and verapamil, all these correlations being significant ($P < 0.001$).

Comparing the reduction in MICs of voriconazole in the presence of different modulators resulted in correlation coefficients of: $r = 1$ between β-oestradiol and verapamil, between progesterone and verapamil and between the two hormones; and $r = 0.879$ between ibuprofen and verapamil, between progesterone and ibuprofen, and between β-oestradiol and ibuprofen.

Effect of modulators on viability

The different modulators did not affect the cellular viability (determined by cfu counting) or the processing of FUN-1, confirmed by the fact that over 90% of the blastoconidia exposed to subinhibitory concentrations of the modulators showed CIVS.

Effect of modulators on FUN-1 staining

In strains with reverting resistant phenotypes, FUN-1 staining was examined by cfu counting or the processing of FUN-1, confirmed by the fact that over 90% of the blastoconidia exposed to subinhibitory concentrations of the modulators showed CIVS.

**Figure 1.** Histograms obtained by flow cytometry analysis at FL2 of *C. albicans*, strain H37. Fluorescence of cells incubated with different modulators for 90 min: 100 μM verapamil (A); 50 μM β-oestradiol (representative of the hormones) (B); and 100 mg/L ibuprofen (C). af, autofluorescence; V, viable cells stained with FUN-1.
by an increase in FUN-1 staining. In resistant strains that did not revert with the modulators, no changes in intensity of fluorescence were noticed.

Effect of modulators on \(^{3}H\)-labelled itraconazole

Azole-susceptible strains accumulated \(^{3}H\)itraconazole in higher amounts than the resistant strains (Figure 3). After incubation with modulators, the resistant strains with reverting phenotype accumulated more radioactive drug (Figure 3). In strain \(C.\ albicans\) 95–68, with a well-documented efflux mechanism, an increase in radioactive itraconazole was detected after exposure to modulators, but not in strain \(C.\ albicans\) 12–99, with additional resistance mechanisms.

Discussion

Antifungal resistance by an efflux mechanism had been suspected as common among \(Candida\) species, with the exception of \(C.\ krusei\).\(^{7,14}\) As efflux pumps are non-specific transporters, they provide a plausible explanation for cross-resistance between different azoles. It is highly probable that the fluorescent marker FUN-1 is exported in a similar fashion. This could explain the decrease in intensity of fluorescence seen in R strains, similarly to what happens with rhodamine.\(^{14}\) Incubation of \(Candida\) strains with sodium azide, at concentrations able to block selectively the active efflux in eukaryotic cells,\(^{7}\) showed an increase in the staining by FUN-1. A reversion of resistance to all azoles by the modulators used supports our hypothesis of efflux mechanisms. The existence of efflux pumps in human eukaryotic cells, i.e. in tumour cells, explains resistance to cytostatic drugs.\(^{10,15,16}\) The blockade of such proteins with compounds designated as modulators or chemosensitisers\(^{10}\) reversed resistance, an important issue in the treatment of neoplastic disease. Many substances have been tested with this objective, e.g. phenothiazines,\(^{16,17}\) anti-malaria drugs,\(^{18}\) anti-arrhythmics,\(^{19}\) hormonal steroids\(^{20}\) and ciclosporins.\(^{21}\) The mechanisms by which such diverse compounds produce blockade of efflux pumps remain unknown. It might result from distinct compound–molecule interactions or be related to drug hydrophobicity. The latter may induce non-specific cellular membrane alterations, leading to intracellular accumulation of cytostatics.\(^{10}\)

Considerable physiological similarities have been described between the eukaryotic yeast cell and the human cell.\(^{22}\) Thus, we proceeded to test the ability of different modulators to induce the blockade of efflux pumps in \(Candida\) strains resistant to fluconazole, with the objective of achieving reversion of resistance. Such an approach has already been achieved in agriculture and biotechnology with promising results.\(^{23}\) Although resistance may be multifactorial, our results strongly suggest that the resistance to fluconazole in the majority of \(Candida\) strains results from over-expression of efflux pump activity. Whenever the blockade of the pumps was achieved, a concomitant reversion of resistance was registered, with a decrease in the MIC values of fluconazole and itraconazole. In most cases, cross-resistance with voriconazole was also observed which was reverted by the modulators. This also supports our hypothesis of efflux being the explanation of common resistance to azoles. However, the modulators assayed were unable to revert the resistance of \(C.\ krusei\), known as being 'intrinsically' resistant to azoles, as was also the case in a few strains of \(C.\ albicans\), in which other mechanisms may be involved. We also showed by flow cytometry that, in \(C.\ albicans\), the same modulators block the transport of FUN-1, resulting in increased

Figure 2. Histograms obtained by flow cytometry analysis at FL2 of \(C.\ glabrata\) strain H30. a, autofluorescence; V, viable cells stained with FUN-1; A, fluorescence of cells treated with 100 \(\mu\)M verapamil for 90 min (shown as a typical modulator).

Figure 3. Effects of the energy inhibitor, sodium azide (NaN3), and modulators on \(^{3}H\)itraconazole accumulation in azole-susceptible strain \(C.\ albicans\) ATCC 90028, azole-resistant strain \(C.\ albicans\) I8 and \(C.\ krusei\) H9 strain. Accumulation of itraconazole was measured in the absence and presence of: 0.1 mM NaN3; 50 \(\mu\)M \(\beta\)-oestradiol; 100 mg/L ibuprofen; 50 \(\mu\)M progesterone; and 100 \(\mu\)M verapamil.

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intensity of fluorescence (despite the extensive number of cells forming CIVS, showing that the yeasts were metabolically undisturbed). For other probes, like rhodamine 123, it was also shown that this transport is not specific. However, none of the modulators impaired the efflux of FUN-1 in C. glabrata, although it blocked the efflux of fluconazole. A plausible explanation for this discrepancy might be that different efflux pumps are involved in this species, a concept in accordance with the observation by Clark et al. regarding transport of rhodamine 123 and fluconazole. In C. glabrata, they seem to be distinct and competitive. The high correlation found between the effect of the different modulators assayed suggests a common target, i.e. efflux pumps. Verapamil is a known inhibitor of human glycoprotein-P (P-gp), which has been utilized in reversion of resistance to cytostatic drugs. In spite of great homology between the amino acid sequence of P-gp and the fungal ‘ABC’ (ATP binding cassette) protein transporters, great variability in sensitivity of yeast ABC proteins has been described. In the fungal strains we studied, verapamil proved to be extremely efficient in reversing resistance to fluconazole. However, the concentration required to produce this effect in vivo might cause significant side effects, such as cardiovascular ones, which make its clinical usefulness doubtful. Progesterone and -oestradiol may be more useful in this respect.

We have previously shown that ibuprofen possesses a potent fungicidal activity by causing membrane lesions, while being fungistatic at lower concentrations. However, a tenuous correlation was found between MIC and the minimal lethal concentration, indicating that the fungistatic and fungicidal activities resulted from distinct mechanisms. Ibuprofen is a lipophilic compound (a characteristic common to the modulators we used) and, owing to its synergic activity with fluconazole, we decided to test the hypothesis that ibuprofen, at low concentrations, blocks efflux pumps. This could, in fact be confirmed by our study. Other authors have shown a synergic effect between ibuprofen and fluconazole and econazole, but without elucidating the mechanism of action.

Ibuprofen may possess a marked therapeutic potential, particularly due to its ability to revert resistance to fluconazole. The serum concentration of ibuprofen needed to achieve an anti-inflammatory effect is lower than the concentration needed to obtain an antifungal effect. This concentration is thus sufficient to induce the blockade of efflux pumps and to revert resistance to azoles. The anti-inflammatory and analgesic properties of ibuprofen might also represent an additional advantage. Additionally, our study opens new perspectives for treatment of candidiasis by rehabilitating an important drug like fluconazole.

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References


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