Fluconazole resistance mechanisms in *Candida krusei*: The contribution of efflux-pumps

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The main resistance mechanism for fluconazole in *Candida krusei* is the diminished sensitivity of the target enzyme cytochrome P450 sterol 14 α-demethylase (CYP51) to inhibition by azole agents. An alternative mechanism of resistance, efflux-pump activity, has been proposed. The aim of our study was to find out the possible contribution of efflux-pumps in conferring resistance to fluconazole in 33 *C. krusei* isolates from different clinical sources. The activity of efflux-pumps was checked using the inhibitor CCCP (carbonyl cyanide 3-chloro-phenylhydrazone), which decreases the minimum inhibitory concentration (MIC) when resistance is attributed. We established a concentration of 0.5 μg/ml of CCCP. The susceptibility patterns of our isolates for five antifungal drugs (amphotericin B, fluconazole, itraconazole, flucytosine and voriconazole) were determined according to an NCCLS M27-A2 protocol modification (Sensititre Yeast One). We tested all the strains before and after adding CCCP to the RPMI medium. The MICs and ranges of the drugs were identical before and after addition of CCCP. The MIC for fluconazole was higher than for the other antifungals. The new triazoles were active and the MICs were lower, although this should be interpreted carefully as the drugs showed different cut-offs. Only one isolate showed a two-fold decrease in MIC to fluconazole when CCCP was added. We did not find any multi-resistant strains. According to our study with *C. krusei*, CCCP-inhibited efflux-pumps do not play a significant role in resistance to fluconazole.

**Keywords** fluconazole, resistance, mechanisms, *C. krusei*, efflux-pumps

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**Introduction**

Recent years have seen an increase in invasive fungal infections, particularly those caused by *Candida* spp. [1–5]. The increase is accompanied by a shift towards a higher proportion of species other than *Candida albicans*, which are frequently resistant to fluconazole. *Candida krusei* has an inherited resistance to fluconazole and infects mainly patients suffering from hematologic malignancies and other immunocompromised patients [6,7].

Fluconazole resistance in *Candida* spp. is mainly due to a diminished sensitivity of the target enzyme cytochrome P450 sterol 14 α-demethylase (CYP51) to inhibition by azole agents. In the case of *C. krusei*, one study using two multi-resistant strains suggests an alternative mechanism of resistance based on efflux-pumps [8].

The aim of our study was to find out the possible contribution of efflux pumps in conferring resistance to
fluconazole in a large series of unselected C. krusei isolates.

Materials and methods
All suspected Candida spp. specimens were first cultured in fungal media and the strains were identified by conventional methods [9]. We tested 32 clinical isolates from urine (4), mucous membranes (7), respiratory tract (2), blood (11), catheters (3), sterile fluids (4), and wound (1). We used two control strains: ATCC 6258 strain and the azole-resistant CA17 which over-expresses the multidrug resistance gene, provided by Spencer Redding et al. MDR1 [10].

We determined the minimum inhibitory concentration (MIC) of each C. krusei isolate against five antifungal drugs (amphotericin B, fluconazole, itraconazole, flucytosine and voriconazole) according to an NCCLS M27-A2 protocol modification (Sensititre Yeast One ™). The plates were incubated in air for 48 h at 35°C, the azoles were read at 24 h and amphotericin B at 48 h. MICs were interpreted as those dilutions which showed a 50% decrease in growth compared with the control well.

The activity of efflux-pumps was checked using the inhibitor CCCP (carbonyl cyanide 3-chloro-phenylhydrazone), which decreased the MIC when resistance was correlated to this mechanism [11]. CCCP was dissolved in DMSO and a specified volume was transferred into the culture medium. We established a final concentration of 0.5 μg/ml of CCCP, and verified that this concentration did not kill the yeast. We tested all the strains before and after adding CCCP to the RPMI 1640 medium.

Results
The activity of the antifungal drugs studied did not change with the addition of CCCP (Table 1 and Fig. 1). We did not observe any changes in the susceptibility of the control strain CA17 to fluconazole before and after the addition of CCCP, which may indicate that CCCP does not inhibit the efflux-pumps in C. albicans. We found that all the strains were susceptible to amphotericin B, itraconazole, and voriconazole. Seven strains were intermediate to flucytosine (MICs ≥ 8 μg/ml). We observed that the MIC of C. krusei for fluconazole was higher than for the other antibiotics due to its natural resistance to this drug; seven strains showed an MIC ≤ 8 μg/ml, 21 strains showed by a MIC of 16–32 μg/ml and five strains by a MIC > 64 μg/ml. The new triazoles were active and the MICs were lower. We did not find any multi-resistant strains. Only one of the 22 strains showed a significant reduction in the MIC for fluconazole (more than one dilution) after treatment with CCCP.

Discussion
Reports on resistance to azole antifungal agents were rare until the late 1980s. The wide use of azole derivatives has increased fungal resistance to these drugs. Several mechanisms are involved in the resistance of Candida spp. to azoles and alterations in the affinity of azoles to CYP51 have been described in different yeasts [12]. In other cases, mutations causing overexpression of this target may occur [13]. Other extra-enzymatic mechanisms related to altered drug transportation suggest that active efflux is an important mechanism of resistance to azole antifungals. Recent studies indicate that species of Candida other than C. krusei possess at least two efflux systems: proteins belonging to the major facilitator superfamily (MFS) and ATP-binding cassette (ABC) superfamily proteins [14].

As far as C. krusei is concerned, different mechanisms conferring resistance to fluconazole have been proposed. The diminished sensitivity of the target (CYP51) to inhibition by azole agents has been proposed as the main resistance mechanism for fluconazole [13,15,16]. Alternatively, mutations causing overexpression of this target may occur [13]. Reduced permeability of the membrane to antifungal drugs and the potential role of efflux-pumps in the extrusion of antifungal molecules have also been considered [13,17].

The presence of energy-dependent efflux-pumps conferring resistance to fluconazole was demonstrated in two strains of C. krusei in which multidrug resistance

<table>
<thead>
<tr>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (μg/ml)</th>
<th>Amphotericin B</th>
<th>Fluconazole</th>
<th>Itraconazole</th>
<th>Voriconazole</th>
<th>Flucytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>0.25</td>
<td>0.125</td>
<td>(0.06–0.25)</td>
<td>8</td>
</tr>
<tr>
<td>(0.25–1)</td>
<td>(2–64)</td>
<td>(0.03–0.5)</td>
<td>(0.06–0.25)</td>
<td>(0.016–16)</td>
<td></td>
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</table>
related to ABC transporter genes was found [8]. The role of the efflux-pumps in *C. krusei* in conferring resistance to fluconazole is not well established and the literature does not answer this question [15,18,19].

CCCP is a non specific inhibitor of the molecules transported across the cell membrane. The inhibition of the efflux pumps in yeasts such as *Saccharomyces cerevisiae* by this substance has also been assayed [11]. The role of CCCP in interfering with the normal transport of fluconazole across the cell membrane may be scarce in *C. krusei* and in the strain of *C. albicans* carrying efflux-pumps.

Our study aimed to examine the contribution of efflux-pumps to resistance to fluconazole. Our hypothesis was that, if resistance was mainly conferred by efflux-pumps, we would observe a decrease in MICs when the pump inhibitor was present. We determined the MIC with and without inhibitor and observed that there were very few differences between both MICs for fluconazole. Only in one isolate were we able to obtain a two-fold decrease in the MIC (from 32 μg/ml to 8 μg/ml). Further studies could be carried out in clinical *C. krusei* strains to determine the intracellular accumulation of fluconazole with and without CCCP and other efflux-pump inhibitors. In our study, we did not determine the intracellular levels of fluconazole but, with the addition of CCCP, we would be able to interfere with the ‘potential’ normal transport of fluconazole in *C. krusei* isolates with fluconazole-pumping ability. We should add that CCCP was unable to interfere with the susceptibility of fluconazole against a strain of *C. albicans* carrying an efflux-pump. These findings suggest that, if present, the contribution of the efflux pumps that could be inhibited by CCCP in conferring resistance to fluconazole in *C. krusei* is scarce, but other cell-transport inhibitors should be studied. They also support the explanation that the diminished sensitivity of the target (CYP51) to inhibition by azole agents has been proposed as the main resistance mechanism for fluconazole in *C. krusei*.

**Acknowledgements**

This study was partially financed by grants from Red Española de Investigación en Patología Infecciosa C/03/14 (REIPI: Estrategias para las políticas de antibióticos, control de resistencias microbianas para el tratamiento de enfermedades complejas). Jesús Guinea Pharm D. PhD receives a pre-doctoral grant from Universidad Complutense de Madrid.

We thank Spencer Redding and his colleagues for control strain CA17. We would also like to thank Thomas O’Boyle for his help in the translation of the help in the translation of the article.

This study does not present any conflict of interest for any of its authors.

**References**

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