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Caspofungin activity against clinical isolates of azole cross-resistant *Candida glabrata* overexpressing efflux pump genes

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***Objectives*: Several studies have documented the potent *in vitro* activity of caspofungin against *Candida* spp. This is of special concern for *Candida glabrata* infections that are often resistant to many azole antifungal agents and, consequently, difficult to treat. The aim of the present study was to expand the data on the *in vitro* activity of caspofungin against azole-resistant isolates of *C. glabrata*.**

***Methods*: A total of 50 clinical isolates of *C. glabrata* were tested for susceptibility to caspofungin. The isolates were cross-resistant to multiple azoles, including fluconazole, itraconazole, ketoconazole and voriconazole. Expression of the resistance-related *CgCDR1* and *CgCDR2* genes was evaluated by quantitative RT–PCR analysis. The MICs of caspofungin were determined by using the National Committee for Clinical Laboratory Standards M27-A2 reference method.**

***Results*: *C. glabrata* isolates exhibited increased expression of the CDR efflux pump(s), and this was in accordance with their high-level azole resistance. In contrast, all the isolates were highly susceptible to caspofungin (100% of isolates were inhibited at ≤1 mg/L).**

***Conclusions*: Our results represent further evidence for the excellent antifungal potency of caspofungin, particularly against *C. glabrata* isolates expressing cross-resistance to azoles.**

Keywords: antifungals, susceptibility testing, *CgCDR* genes

Introduction

Candida glabrata infections have markedly increased in the past decades and, when invasive, are associated with a high mortality rate, especially in immunocompromised patients such as intensive-care, post-surgical and neutropenic patients.[1] This, in part, has been attributed to the reduced susceptibility of this pathogen to antifungal agents, especially the azoles.[2] Recent studies revealed that the MICs of extended-spectrum triazoles (fluconazole, itraconazole and voriconazole) for *C. glabrata* were higher than those seen for most *Candida albicans* isolates.[3–6] Fluconazole-resistant isolates have been found not only in AIDS patients with oropharyngeal candidiasis, but also in fungaemic patients and among vaginal isolates as a consequence of widespread and often indiscriminate use of the drug.[7] *C. glabrata* is able to rapidly develop azole resistance, which is probably related to the haploid state of the microorganism.[2] In this context, studying the resistance mechanisms of this pathogenic yeast may provide answers in developing new antifungal agents.[6,8]

Caspofungin is an echinocandin with potent fungicidal activity against many *Candida* species[3,9] and could be particularly useful to treat *C. glabrata* infections, which often develop resistance to azoles. To further demonstrate the *in vitro* efficacy of this promising new antifungal agent in *C. glabrata*, we report here caspofungin susceptibility of a large collection of clinical isolates which were cross-resistant to multiple azoles. Molecular studies showed that azole resistance in these isolates is associated with the up-regulation of the ABC transporter genes *CgCDR1* and *CgCDR2*.

Materials and methods

Yeast isolates and antifungal susceptibility testing

A total of 50 clinical isolates of azole-resistant *C. glabrata* were tested: 30 isolates were recovered from blood (4 isolates), respiratory tract (10), vagina (3), mouth (1), urine (10), skin (1) and drainage fluid (1) of patients hospitalized between January 2004 and June

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Table 1. *In vitro* activity of caspofungin and azole antifungal agents against 80 clinical isolates of *C. glabrata* stratified by fluconazole susceptibility

Fluconazole susceptibility category[a]	No. tested	Antifungal agent	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
S	15	caspofungin	0.03–0.125	0.06	0.125
		itraconazole	0.125–0.5	0.25	0.5
		ketoconazole	0.06–0.25	0.125	0.25
		voriconazole	0.06–0.25	0.125	0.125
S-DD	15	caspofungin	0.03–0.25	0.125	0.25
		itraconazole	0.125–2	0.5	1
		ketoconazole	0.06–0.5	0.125	0.5
		voriconazole	0.125–1	0.25	0.5
R	50	caspofungin	0.06–1	0.25	1
		itraconazole	2–>8	8	>8
		ketoconazole	1–8	4	4
		voriconazole	2–8	4	8

[a]S, MIC ≤ 8 mg/L; S-DD, MIC = 16–32 mg/L; R, MIC ≥ 64 mg/L.

2005, and 20 other isolates, cultured from various patient specimens (6 blood, 5 urine, 3 vagina, 2 respiratory tract, 2 mouth, 1 skin and 1 drainage fluid) before 2004, had been explored for molecular mechanisms of antifungal resistance in a previous study.[6] Each isolate represented an individual infectious episode. The isolates showed high MICs of multiple azoles, as determined by the broth microdilution method (Table 1). A panel of 30 fluconazole-susceptible or fluconazole-susceptible dose-dependent (S-DD) isolates was also included. All isolates were identified to the species level by standard methods[10] and stored as 20% glycerol stocks until use. Prior to testing, each isolate was subcultured onto Sabouraud dextrose agar. *C. glabrata* ATCC 36909 reference strain and two well-characterized *C. glabrata* isolates, DSY562 (a susceptible isolate) and DSY565 (a resistant isolate),[11] were included as controls. Antifungal susceptibility testing was performed by the broth microdilution method in RPMI with 2% glucose according to the M27-A2 document of the Clinical and Laboratory Standards Institute (CLSI, formerly the NCCLS).[12] The inoculum suspensions were adjusted spectrophotometrically at 530 nm to match the concentration of $1.5 \pm 1.0 \times 10^3$ cells/mL. Stock solutions of caspofungin (Merck & Co., Whitehouse Station, PA, USA), fluconazole (Pfizer, Inc., New York, NJ, USA), itraconazole (Janssen, Beerse, Belgium), ketoconazole (Sigma Aldrich, Milan, Italy) and voriconazole (Pfizer) were prepared in water (caspofungin and fluconazole) or dimethyl sulphoxide (itraconazole, ketoconazole and voriconazole). The final concentrations of the antifungal agents were 0.008–8 mg/L for caspofungin, itraconazole, ketoconazole and voriconazole and 0.125–128 mg/L for fluconazole. The trays were incubated at 35°C, and MIC end points were read at 48 h. All tests were carried out in duplicate. For caspofungin, the MIC end point was defined as the lowest concentration determining complete inhibition of growth. This end point was chosen based on the fungicidal activity of caspofungin as already described.[9] For azoles, the MIC end point was defined as the lowest concentration that produced a prominent decrease in turbidity (80% inhibition) compared with that of the growth control. The interpretive criteria used for fluconazole, itraconazole and voriconazole were those published by the CLSI.[12,13] Interpretive breakpoints have not yet been established for either ketoconazole or voriconazole, but we used susceptibility breakpoints of ≤0.125 and ≤1 mg/L, respectively.[6] The CLSI quality control strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were tested and MICs were within the expected range.

Rhodamine 6G accumulation assay

The rhodamine 6G accumulation assay was performed as described previously.[11,14] Following growth overnight in YEPD (1% yeast extract, 2% peptone, 2% glucose) liquid medium at 30°C, yeast cells were diluted 100-fold in 5 mL of the same medium. The culture was grown to reach a cell density of $\sim 10^7$ cells/mL, then placed at 4°C and divided into 1 mL aliquots. Labelling of the cells (10^7) with 10 μM rhodamine 6G (Società Italiana Chimici, Rome, Italy) was performed under constant shaking for 30 min at 30°C, then stopped by placing reaction tubes on ice. The labelled cells were diluted 40-fold in ice-cold 0.1 M phosphate-buffered saline (PBS) at pH 7.0 and then subjected to flow cytometry using a Coulter EPICS XL instrument (Beckman Coulter, Brea, CA, USA). Experiments were repeated two times.

Expression levels of ABC transporter genes

Quantitative expression of the *CgCDR1* and *CgCDR2* genes was performed by real-time RT–PCR analysis with an i-Cycler iQ system (Bio-Rad Laboratories, Hercules, CA, USA), following a previously described protocol.[6] Relative quantification of the target gene was carried out by using a primer pair and a Taqman probe in combination with primers and a probe specific for the *URA3* reference gene. Each reaction was performed in triplicate. For each isolate, fold increases in gene expression were calculated from the mean normalized expression relative to the mean normalized expression of the DSY562 isolate, used as a susceptible control.[15] Genes with a ΔC_T value, calculated as C_T [test gene]–C_T [*URA3*], that fell over the 3-SD range measured in the azole-susceptible isolates were considered overexpressed, as reported previously.[16]

Results and discussion

Table 1 summarizes the *in vitro* susceptibility of 80 *C. glabrata* clinical isolates to caspofungin, itraconazole, ketoconazole and voriconazole stratified by fluconazole susceptibility category. With respect to fluconazole MIC, the isolates fell into the following groups: susceptible (15 isolates), S-DD (15 isolates) and resistant (50 isolates). All fluconazole-resistant isolates were also cross-resistant to the other azoles tested (Table 1), and this phenotype was found to be associated with the overexpression of drug efflux pump-encoding *CgCDR* genes. The expression of *CgCDR1* and *CgCDR2* was strongly increased in the majority of the 50 azole-resistant isolates compared with that of the susceptible control isolate DSY562, but not in the 15 azole-susceptible isolates studied (Figure 1). Of the resistant isolates 46 exhibited 12.4- to 483.0-fold up-regulation of the ABC transporter gene *CgCDR1*. Notably, 27 of these 46 also exhibited 3.9- to 70.6-fold up-regulation of *CgCDR2*, with the remaining 4 isolates showing 26.1- to 61.2-fold up-regulation of this second ABC transporter gene. The 15 fluconazole-S-DD isolates up-regulated *CgCDR1* or *CgCDR2* (Figure 1), but the levels of up-regulation were significantly less than those by the resistant isolates studied (109.9- to 5.5-fold for *CgCDR1* and 17.4- to 2.6-fold for *CgCDR2*). Furthermore, the increased expression of efflux pumps in the fluconazole-resistant and fluconazole-S-DD isolates was also accompanied by decreased accumulation of rhodamine 6G, as shown by their mean fluorescence values, which differed from

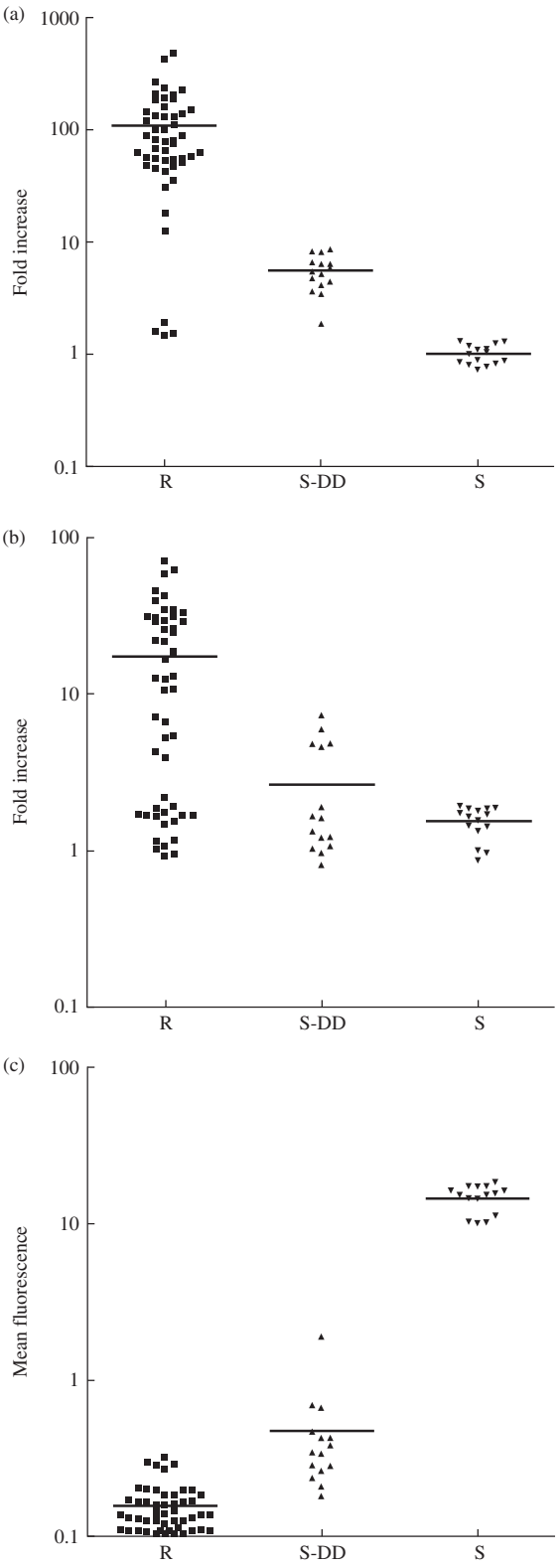

Figure 1. Expression of ABC transporters *CgCDR1* (a) and *CgCDR2* (b) and rhodamine 6G accumulation (c) in *C. glabrata* clinical isolates. With respect to fluconazole, isolates were defined as resistant (R), susceptible-dose dependent (S-DD) or susceptible (S). Fold increases in gene expression were determined by RT–PCR analysis relative to DSY562. Fluorescence values were determined by flow cytometry. The mean value (a and b) or the geometric mean value (c) for each group is indicated by a dark solid horizontal line.

those of the susceptible isolates by 96.1- and 37.0-fold, respectively (Figure 1). In contrast, caspofungin was very active *in vitro* against all the fluconazole-resistant and fluconazole-S-DD isolates and, as expected, against the fluconazole-susceptible isolates. MIC_{90} (MIC at which 90% of the isolates were inhibited) of fluconazole-resistant isolates was 1 mg/L. MIC_{90}s of fluconazole-S-DD isolates and fluconazole-susceptible isolates were 0.25 and 0.125 mg/L, respectively. For all the isolates, the MIC of caspofungin was ≤1 mg/L. Low MICs (0.06 mg/L) of caspofungin were also obtained for the *C. glabrata* reference strain, ATCC 36909, and two well-characterized *C. glabrata* isolates, DSY562 and DSY565.

Cross-resistance within a class of antimicrobial agents is an important issue, especially if therapeutic options are limited by a poor number of available compounds as for the antifungal agents. Although the new systemic antifungal triazoles (ravuconazole and voriconazole) are more active *in vitro* than fluconazole and itraconazole against *Candida* spp.,[17] cross-resistance between fluconazole and ravuconazole has been observed, so that fluconazole was recently proposed as a surrogate marker to predict the susceptibility of *Candida* spp. to ravuconazole.[18] The cross-resistance involving ravuconazole,[18] like that involving voriconazole,[4,6] applies most directly to fluconazole-resistant *C. glabrata*. As stated by Sobel,[7] *C. glabrata* can be considered the Achilles heel of all available azoles, thus *C. glabrata* infections treated with these drugs are difficult to eradicate.

Our results showed that caspofungin had excellent activity against fluconazole (multiazole)-resistant isolates, as well as against isolates susceptible and dose-dependent susceptible to fluconazole. This is not surprising considering that the echinocandin caspofungin belongs to a class of antifungal agents that act by inhibiting the cell wall glucan synthesis. Interestingly, fluconazole-resistant isolates showed a higher MIC_{90} value compared with fluconazole-S-DD or fluconazole-susceptible isolates. This difference indicates that azole resistance can contribute to an increase in caspofungin MIC. The basis for such a difference is still unknown.

In conclusion, the present study confirms the efficacy of caspofungin against clinical isolates of *C. glabrata*, but, for the first time, the potent activity of this drug was assessed with a large number of *C. glabrata* isolates whose cross-azole resistance was well documented by molecular methods. Furthermore, these findings support the use of caspofungin to treat those *C. glabrata* infections for which amphotericin B represents the sole therapeutic choice.

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Transparency declarations

None to declare.

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