Original Article

Analysis of *Candida glabrata* Strains with Reduced Sensitivity to Micafungin In Vitro Isolated from a Patient with Persistent Candidemia

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SUMMARY: We report the appearance of *Candida glabrata* strains with reduced sensitivity during treatment with the echinocandin drug micafungin (MCF). Four *C. glabrata* strains were isolated from sputum, gastric juice, and blood taken from a patient during hospitalization. Two of these strains, one of which was obtained after treatment with MCF for suspected *Candida* pneumonia and the other of which was obtained during MCF treatment for candidemia, were isolated from blood and found to have a reduced susceptibility to MCF. These two clinical isolates showed a high minimum inhibitory concentration (MIC) for MCF, with this change in MIC being unique for MCF among established antifungal drugs. To further investigate the mechanism underlying this reduced sensitivity, an in vivo mouse infection model and in vitro enzymatic analysis were performed. MCF had little effect in the mouse disseminated infection model and enzymatic analysis showed the low affinity of MCF to the 1,3-β-D-glucan synthase of the clinical isolates, although the enzymes of both clinical isolates and control strain were noncompetitively inhibited by MCF. Taken together, this low affinity of MCF for the enzymes is likely to cause the reduced sensitivities. These data further indicate that MCF could induce acquired MCF-resistant strains during clinical use.

INTRODUCTION

Micafungin (MCF) is a member of the echinocandins, a new class of antifungal agents which inhibit the synthesis of the fungal cell wall (1). It is active against both *Candida* and *Aspergillus* spp. (2) and also has important antifungal activity against fluconazole-resistant *Candida* spp., including non-albicans *Candida* (2,3). Non-albicans *Candida* spp. have become more widely recognized as significant agents in candidiasis (4–8), and MCF is now considered to be one of the most useful antifungal drugs against candidiasis due to its clinical efficacy and excellent safety and tolerability. Indeed, it has been approved by the FDA for treatment of candidemia and candidiasis and prophylaxis of candida infection (9–12).

Echinocandins, including MCF, are thought to inhibit the synthesis of 1,3-β-D-glucan, which forms a key component of the cell wall of the medically important fungi described above (13). Echinocandins noncompetitively inhibit 1,3-β-D-glucan synthase in the cell membrane, and exposure of the cell wall to these drugs results in a notable loss of rigidity, incomplete morphogenesis, and/or bursting and cell death (14,15). A recent report indicated that strains of *Candida albicans* with reduced sensitivities to caspofungin (CSF) were recovered after clinical treatment (16,17). These strains displayed a point mutation in highly conserved and narrow regions (echinocandin resistant region; hot spot 1 and 2) of the *FKSI* gene or its homolog, the *FKS2* gene, which codes for 1,3-β-D-glucan synthase (1,16,18). Furthermore, these mutant strains showed reduced sensitivities to all the clinically launched echinocandins (CSF, MCF, and amikafungin) (19). *Candida glabrata* strains with reduced sensitivity were reported previously, and clinical failure, high MIC, and mutation analysis focused on the narrow hot-spot region of the *FKS* gene of *C. glabrata* showed that these strains were interrelated (17,20–24).

Although the glucan synthase from those strains with elevated MIC and mutant *FKS* gene showed reduced sensitivity to echinocandins, mutations in glucan synthase influence high MIC in different ways (19,20,24,25). Thus, an *Aspergillus fumigatus* strain with reduced MIC but no *FKSI* mutation was isolated recently and elevated expression of the gene observed...
In light of these observations, we believe that the sensitivity of glucan synthase should be evaluated as a first step when investigating the mechanism of reduced sensitivity in isolated strains.

We report here a case of candidemia caused by *C. glabrata* with reduced sensitivity to MCF and analysis of the mechanism of this reduced sensitivity at the target enzyme level.

**MATERIALS AND METHODS**

**Case:** A 70-year-old Japanese woman, diagnosed with peritonitis carcinomatosa of unknown primary, was admitted to our hospital for further examination for fever with nausea and vomiting. She was managed with a peripherally inserted central catheter (PICC) and treated with antimicrobial agents and other drugs, as indicated in Fig. 1. Informed consent was obtained from the patient. Four strains of *C. glabrata* were isolated from sputum, gastric juice, and blood during hospitalization; no other significant organisms were not isolated. MCF (150 mg/day) was administered for suspected *Candida* pneumonia and candidiasis. The underlying disease progressed, her condition gradually deteriorated, and she died on day 72.

**Strains:** The clinically isolated *C. glabrata* strains were seeded onto Sabouraud dextrose agar (Difco, Detroit, Mich., USA), incubated for 48 h at 35°C, and stored in liquid N₂. The strains isolated on days 31 and 58 were designated as 4872 and 4897, respectively. These strains were identified as *C. glabrata* using the API32C testing kit (bioMerieux SA, Marcy-l’Etoile, France) and PCR analysis of a specific region of DNA topoisomerase II (27). *C. glabrata* ATCC90030 was used as the control strain. The isolates from sputum and gastric juice were not stored and their MICs are therefore not known.

**Antifungal susceptibility testing:** A broth microdilu-

![Fig. 1. Clinical course of the patient. The isolates from blood culture investigated in the test are marked with triangles. MCF, micafungin; FCZ, fluconazole; PICC, peripherally inserted central catheter; ABPC/SBT, ampicillin-/sulbactam; MEMP, meropenem; MINO, minocycline; CLDM, clindamycin; BIPM, biapenem; PSL, prednisolone.](image)

**Table 1. Susceptibility of strains isolated from the patients and enzymatic profiles in vitro compared to control strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg/L)¹</th>
<th>Glucan synthesis²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF</td>
<td>AMPH-B</td>
</tr>
<tr>
<td>4872</td>
<td>2.0</td>
<td>1</td>
</tr>
<tr>
<td>4897</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>ATCC90030</td>
<td>0.0156</td>
<td>0.5</td>
</tr>
</tbody>
</table>

¹: Tests were carried out according to the CLSI M27-A3 protocol (28). For MCF, MIC was also measured in the presence of serum as described (29) and the results were shown in parentheses.

²: Enzymatic parameters were determined as described in Materials and Methods. Km (Michaelis-Menten constant), concentration of substrate that gives half-maximal activity of the enzyme. The parameter shows the affinity of the substrate for the enzyme. IC₅₀, concentration of inhibitor at which 50% inhibition of vehicle treated enzyme activity is achieved. Ki (dissociation constant for binding of inhibitor to the enzyme): If the Ki value is low (the affinity is high), the IC₅₀ will be low.

4872, *C. glabrata* strain isolated on the 31st day after admission. 4897, *C. glabrata* strain isolated on the 58th day after admission. ATCC 90030, *C. glabrata* ATCC90030 (control strain). MCF, micafungin; AMPH-B, amphotericin B; 5-FC, 5-flucytosine; FCZ, fluconazole; MCZ, miconazole; ICZ, itraconazole; VCZ, voriconazole.
tion assay was performed for all antifungals, following the Clinical and Laboratory Standards Institution (CLSI) guidelines, as indicated in Table 1 (28). A serum-MIC assay was also performed, as described previously, for MCF only (29). Briefly, the strains were inoculated in buffered mice serum and incubated at 37°C under 5% CO₂. After 14 h, serum-MIC was determined microscopically and defined as the lowest drug concentration giving rise to substantial inhibition of hyphal elongation.

**MCF susceptibility in the mouse disseminated candidemia model:** Antifungal activities of MCF for the strains in the mouse disseminated candidiasis infection model were determined according to a previous study (30), with the following modification. Four-week-old male ICR mice were purchased from Nippon SLC (Shizuoka, Japan). Immunosuppression in mice was induced by intraperitoneal administration with cyclophosphamide (200 mg/kg; Sigma-Aldrich, St. Louis, Mo., USA) 4 days before and 1 day after infection and subcutaneous administration with hydrocortisone (100 mg/kg; Nacalai Tesque, Kyoto, Japan), and 1 day before and 3 h, 1, and 2 days after infection. Disseminated candidiasis was induced by intravenous infection with 9.8 × 10⁵ cells of *C. glabrata* 4872 in 0.2 ml suspension. MCF (10 mg/kg) was administrated intravenously once daily for 4 days starting 1 h after infection. Survival was compared with the control group by the log-rank test and a *P* value of less than 0.05 was considered significant.

**Membrane fractionation:** Membrane fractions of *C. glabrata* strains were prepared as 1,3-β-D-glucan synthase sources according to a previous report (31). Briefly, *C. glabrata* strains were grown with vigorous shaking at 30°C to early stationary phase in YPD medium, and the cells collected by centrifugation. Washed cell pellets were disrupted with 0.6-mm glass beads in buffer A (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM 2-mercaptoethanol [2-ME], 1 M sucrose). An equal volume of buffer B (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM 2-ME, and 25 μM GTP) was added to the lysate, and cell debris and glass beads were removed by centrifugation at ×3,000 g. The membrane fraction containing glucan synthase activity was isolated twice by sedimentation at ×100,000 g for 1 h. Membranes were suspended in buffer C (add 0.5 volume of glycerol to buffer B) at a protein concentration of 10 mg/ml then stored at −80°C for further analysis.

**1,3-β-D-glucan synthase assay:** The enzymatic profile of 1,3-β-D-glucan synthase was obtained as described previously (31). To determine the Michaelis-Menten constant (Km) values, which are a measure of enzyme-substrate binding affinity, glucan synthase activity was assayed as a function of increasing substrate concentration and the data fitted to the Michaelis-Menten equation. IC₅₀, which represents 50% inhibitory concentration of MCF against 1,3-β-D-glucan synthase activity, was determined from the reaction inhibitory curve using the XLfit curve-fitting software for Microsoft Excel (ID Business Solutions, Guildford, UK).

A Dixon plot, in other words a plot of the reciprocal of the velocity against inhibitor concentration, was produced and the inhibitor constants, Ki, a large value of which indicates a low affinity for the target (31,32), calculated by varying the MCF concentration at different fixed substrate concentrations (see Fig. 3). The inhibitory mechanism of MCF against the enzyme was also determined.

**RESULTS**

The MICs of the clinical isolates and the control strain for the antifungal agents tested in this study are shown in Table 1. The sensitivities of the clinical MCF isolates (≥64-fold) were reduced 64- to 128-fold with respect to the susceptible control strain, whereas the MICs for the other tested drugs were only 2- to 4-fold higher than those for the control strain, suggesting an MCF-specific MIC increase. The MICs for the isolates in the presence of serum were also much higher than for
the control strain (Table 1). MCF (10 mg/kg) showed a modestly improved survival, although there was no significant difference between MCF-treated and drug-free control groups ($P = 0.6$, Fig. 2). Although the experiments were not conducted simultaneously, as a previous report showed that as low as 0.3 mg/kg of MCF had a significant survival effect on a sensitive strain of *C. glabrata* in a disseminated mouse infection model (30), the clinical isolates were found to have drastically reduced sensitivity to MCF in vivo. The change in vitro MIC also supported the reduced in vivo potency (Table 1).

In order to investigate the mechanism underlying this reduced sensitivity, the enzymatic parameters for glucan synthase in these strains were compared to those of the control strain. The parameters which are most indicative of enzymatic activity, namely the $K_m$ values, were found to be similar between the clinical isolates and the control strain (Table 1), whereas the 50% inhibitory concentrations ($IC_{50}$) and affinity (Ki) of MCF for 1,3-$\beta$-D-glucan synthase were much higher (more than several hundred fold) than for the control strain (Table 1). The changes in inhibitory activity at the enzyme level reflected the higher MIC and lower in vivo potency. Kinetic inhibition using Dixon plots showed that the enzymes were inhibited by MCF in a noncompetitive manner, as also seen for the control strain (Fig. 3). Taken together, the enzymes of the clinical strains with reduced sensitivity toward MCF show a similar mechanism in terms of anti-enzyme activity to that of the control strain. The high MICs were caused by a reduced inhibitory activity of MCF for the enzyme as a result of decreased affinity. Furthermore, the above results suggest that the 1,3-$\beta$-D-glucan synthases from the clinical isolates show a reduced affinity for MCF but no loss of their enzymatic activity, thus leading to the reduced sensitivity of these strains toward this agent.

**DISCUSSION**

Very few cases of *Candida* spp. with reduced susceptibility to echinocandins (CSF, MCF, etc.) upon treatment with these agents have been isolated, although most of the cases reported involve *C. albicans*. Although there have been some reports of MCF-resistant *C. glabrata* strains (17,22) upon treatment with CSF, to the best of our knowledge, this is the first report of *C. glabrata* strains with reduced sensitivity to MCF isolated from a patient under treatment with MCF in Japan. In a previous report (22), the MICs of MCF-resistant *C. glabrata* strain were measured in the presence and absence of serum (0.25 and 4, respectively), although the MICs are even higher in the present study (1 or 2, and >32, respectively). Although the effect of serum on the activity of MCF is not fully understood (22), similar shifts of MIC in the presence of serum were also observed in the present study.

It was suspected that the candidemia in this case was due to microbial translocation, because the *C. glabrata* strain was isolated from gastric juice prior to isolation from blood. Unfortunately, this hypothesis was not proven as the isolates from gastric juice and blood were not compared, therefore the exact route must remain unknown.

The strains with reduced sensitivity to MCF were considered medically important because of their pathogenicities, drug resistance, refractoriness, and intractability to MCF in a mouse infection model. Although the two clinical isolates could be defined as being susceptible toward MCF in accordance with the CLSI breakpoint categorization (28,33), the nonclinical and clinical data showed that treatment for these strains may require special attention. Indeed, Garcia-Effron et al. reported that the breakpoint for MCF and anidulafungin should be considered to be 0.5 mg/ml, similar to the MIC value for *C. albicans* (19). From our observations, we initially attempted to clarify the reduced sensitivity in the mouse infection model and the catalytic profiles as regards 1,3-
β-D-glucan synthase for the clinical isolates. Further investigation of the mechanism underlying this reduced sensitivity is, however, still required.

The mechanism underlying the reduced MCF sensitivity could not be completely elucidated in this case, although it has been suggested previously that the MCF binding site of C. albicans glucan synthase is independent of the substrate binding site (19). This would mean that mutation at the MCF binding site has little or no effect on substrate binding. Indeed, our data indicate there is no change in Km between reduced-sensitive strains and the control strain, whereas an increase in the Ki value is associated with reduced MCF sensitivity. Reduced-sensitive strains also exhibit a similar growth rate (data not shown) to the control strain in YPD broth and exhibit pathogenicity in immunocompromised mice. These data appear to be linked to the lack of change in Km. Consequently, reduced MCF-sensitive strains may simply have a higher MIC than the control strain.

The increases in MCF MIC for the clinical isolates and the control strain (64- to 128-fold) are lower than those for Ki and IC_{50} (more than several hundred fold), as shown in this study (Table 1) and a previous one (19). We speculate that these differences between the shifts in MIC and the enzyme profiles can be explained as follows. The whole cell assay (MIC) is likely to reflect the MCF sensitivities of total Fks proteins. In fact, Fks1p and Fks2p dominate the total mRNA expression level of FKS genes and these two genes are expressed in various ratios in different strains (17,24). In contrast, the MCF inhibition curve prepared from the results of the in vitro enzyme assay seems to show a single kinetic species (data not shown). The membrane fractions prepared according to our method may therefore contain only Fks1p, as suggested previously for C. albicans (19,34).

This hypothetical difference in Fksp’s observed between the MIC test and the enzyme assay may result in the difference between the shifts in MIC and IC_{50}. It would therefore be necessary to determine whether 1,3-β-D-glucan synthase from the membrane fraction reflects the total sum of activity of Fks proteins, which is often problematic due to the difficulty in isolating each individual Fksp complex.

This study has shown that a mutation in the pathogen which results in reduced sensitivity to MCF does not affect its pathogenicity or viability, therefore switching from MCF to other antifungal agents or combination therapy should be considered if clinical isolates with a high MIC for echinocandins emerge during prolonged treatment.

In this study, we isolated C. glabrata strains with reduced MCF sensitivity during treatment with this agent and analyzed their enzymatic profiles. MCF appears to induce acquired MCF-resistant strains during clinical use.

REFERENCES

3783–3785.