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In Vitro Antifungal Susceptibility of *Candida glabrata* to Caspofungin and the Presence of *FKS* Mutations Correlate with Treatment Response in an Immunocompromised Murine Model of Invasive Infection

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It has been argued that the *in vitro* activity of caspofungin (CSP) is not a good predictor of the outcome of echinocandin treatment *in vivo*. We evaluated the *in vitro* activity of CSP and the presence of *FKS* mutations in the hot spot 1 (HS1) region of the *FKS1* and *FKS2* genes in 17 *Candida glabrata* strains with a wide range of MICs. The efficacy of CSP against systemic infections from each of the 17 strains was evaluated in a murine model. No HS1 mutations were found in the eight strains showing MICs for CSP of ≤0.5 μg/ml, but they were present in eight of the nine strains with MICs of ≥1 μg/ml, i.e., three in the *FKS1* gene and five in the *FKS2* gene. CSP was effective for treating mice infected with strains with MICs of ≤0.5 μg/ml, showed variable efficacy in animals challenged with strains with MICs of 1 μg/ml, and did not work in those with strains with MICs of >1 μg/ml. In addition, mutations, including one reported for the first time, were found outside the HS1 region in the *FKS2* gene of six strains with different MICs, but their presence did not influence drug efficacy. The *in vitro* activity of CSP was compared with that of another echinocandin, anidulafungin, suggesting that the MICs of both drugs, as well as mutations in the HS1 regions of the *FKS1* and *FKS2* genes, are predictive of outcome.

Candida glabrata is a common agent of invasive candidiasis (IC) and the most prevalent species after *Candida albicans* that causes it (1–3). Azoles and the lipid formulation of amphotericin B are commonly used to treat IC, but for *C. glabrata* strains with decreased azole susceptibility, echinocandins are the preferred front-line therapy (4, 5). Caspofungin (CSP) has been successfully used in the treatment of esophageal candidiasis and IC (including candidemia) (4, 6). Although *in vitro* CSP resistance among *C. glabrata* strains is rare, infections with poor or no response to treatment have been reported (7–13), with therapeutic failure being associated with the presence of mutations in two hot spot (HS) regions of the *FKS* genes (14). These genes encode the major subunit of the (1,3)-β-D-glucan synthase complex, which is involved in the synthesis of (1,3)-β-D-glucan, the major cell wall component (6, 15–17). EUCAST has abstained from setting CSP breakpoints because of unacceptable variation in the MIC ranges obtained over time and between centers; therefore, EUCAST recommends in the meantime that anidulafungin (AFG) or micafungin be used as a marker for CSP susceptibility (18). Recently, a similar approach was proposed by Espinel-Ingroff et al. (19). To detect reduced echinocandin susceptibility and predict clinical failure, epidemiological cutoff values (ECVs) and clinical breakpoints (CBP) were established based on clinical, molecular, and microbiological data. The proposed EUCAST CBP of AFG for *C. glabrata* are ≤0.06 μg/ml for susceptibility and >0.06 μg/ml for resistance (18). The ECV of CSP proposed by the CLSI for *C. glabrata* is 0.12 μg/ml, while the CBP are set at ≤0.12 μg/ml for susceptibility, 0.25 μg/ml for intermediate susceptibility, and ≥0.5 μg/ml for resistance (19). The aim of this study was to determine, using a murine model of disseminated infection by *C. glabrata* treated with CSP, whether MIC values and the presence of *FKS* mutations in such a fungus are predictive of *in vivo* outcome.

MATERIALS AND METHODS

Strains. Seventeen clinical *C. glabrata* strains representing a wide range of MICs for CSP and AFG (0.06 to 16 μg/ml and <0.03 to 4 μg/ml, respectively) were included in this study (Table 1). MICs were determined using a microdilution approach, according to CLSI standards (20).

DNA sequence analysis of *FKS* genes. *C. glabrata* strains were grown at 37°C overnight on Sabouraud dextrose agar (SDA). DNA was extracted and purified as previously described (21). The HS1 regions of the *FKS1* and *FKS2* genes were amplified and sequenced using previously described primers to detect the presence of possible mutations (22). The sequence quality was checked, the alignments were made, and mutations were detected using the BioNumerics software version 6.6. The translation of the nucleic acid sequence into an amino acid sequence was performed using the European Bioinformatics Institute (EBI) Transeq tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/), and amino acid alignments were made using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Animals. Male OF1 mice (Charles River, Criffa S.A., Barcelona, Spain), weighing 30 g, were used. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. The mice were housed under standard conditions and immunosuppressed 1 day before infection by a single intraperitoneal

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TABLE 1 Isolates of *C. glabrata*, *in vitro* activity of caspofungin, mutations on *FKS* genes, mean survival times, and fungal loads in kidneys

C. glabrata strain	MIC (μg/ml) for[a]:		Mutation(s) in gene:		MST (95% CI) (days) in[b]:			Log$_{10}$ CFU/g of kidney tissue (mean ±SD) for:		
	AFG	CSP	*FKS1*	*FKS2*	Controls	Treated group	*P*	Controls	Treated group	*P*
FMR 11381	<0.03	0.06			18.1 (4.56–31.78)	22.17 (9.43–34.91)	0.050	6.367 ± 0.333	5.397 ± 0.227	0.034
UTHSC 08-134	<0.03	0.06		L707S[c]	10.5 (0.42–20.58)	18.67 (5.63–31.70)	0.052	4.762 ± 0.226	1.623 ± 0.110	0.019
FMR 8489	<0.03	0.12		L707S[c]	18.1 (4.56–31.78)	30.00 (30.00–30.00)	0.004	8.318 ± 0.393	6.005 ± 0.262	0.042
FMR 8498	<0.03	0.12		L707S[c]	18.5 (5.26–31.70)	19.00 (6.34–31.66)	0.326	6.968 ± 0.567	4.030 ± 0.549	0.015
UTHSC 11-149	0.03	0.25			13.8 (0.67–27.00)	30.00 (30.00–30.00)	0.004	6.827 ± 0.371	5.685 ± 0.101	0.039
UTHSC 11-68	0.03	0.25			10.6 (0.67–20.66)	25.17 (17.30–33.03)	0.002	7.018 ± 0.383	5.712 ± 0.156	0.023
UTHSC 073662	0.03	0.5			14.0 (0.97–27.02)	30.00 (30.00–30.00)	0.004	7.427 ± 0.548	4.732 ± 0.304	0.014
UTHSC 10461	0.03	0.5		L707S[c]	17.6 (3.48–31.85)	18.5 (5.263–31.74)	0.186	6.377 ± 0.368	5.152 ± 0.076	0.028
JMI-2092	0.5	1		L707S[c]	15.6 (3.85–27.48)	22.17 (9.43–34.91)[c]	0.037	4.955 ± 0.656	3.665 ± 0.136	0.038
JMI-206	1	1		F659S[d]	16.3 (4.87–27.80)	23.00 (11.62–37.38)	0.212	7.174 ± 0.094	7.044 ± 0.416	0.061
JMI-211	1	1		S663P[d]	7.1 (5.02–9.30)	13.83 (0.66–27.00)	0.174	6.711 ± 0.587	6.391 ± 0.179	0.055
JMI-297	1	1	S629P[d], R631S[d], A1037T[c]		15.0 (2.71–27.29)	21.83 (8.55–35.11)	0.008	5.436 ± 0.269	3.558 ± 0.061	0.029
JMI-760	1	1		S663P[d]	8.0 (6.67–9.33)	16.00 (4.14–27.85)	0.062	6.706 ± 0.539	6.782 ± 0.364	0.064
JMI-10956	1	2		F659V[d], L707S[c]	18.3 (4.85–31.82)	19.67 (7.78–31.55)	0,192	5.34 ± 0.155	4.882 ± 0.340	0.078
JMI-14378	2	4	S629P[d]		7.5 (5.53–9.46)	12.00 (9.79–14.20)	0.073	7.669 ± 0.428	7.046 ± 0.546	0.063
JMI-127	2	16	S629P[d]		14.8 (2.32–27.35)	7.16 (5.62–8.71)	0.432	5.00 ± 0.528	5.587 ± 0.387	0.455
JMI-729	4	>16		F663P[d]	6.66 (4.95–8.38)	9.33 (8.47–10.19)	0.331	5.599 ± 0.170	5.381 ± 0.171	0.052

[a] The anidulafungin (AFG) MIC is given for comparison with the caspofungin (CSP) MIC, as recommended by Arendrup et al. in a EUCAST technical note (18).
[b] MST, mean survival time; CI, confidence interval.
[c] Mutations outside of the hot spot 1 (HS1) region of the *FKS1* or *FKS2* gene (as indicated).
[d] Mutations in the hot spot 1 (HS1) region of the *FKS1* or *FKS2* gene (as indicated).

(i.p.) injection of 200 mg/kg of body weight of cyclophosphamide (Genoxal; Laboratories Funk S.A., Barcelona, Spain) and a single intravenous (i.v.) injection of 150 mg/kg of 5-fluorouracil (Fluorouracilo; Ferrer Farma S.A., Barcelona, Spain) (23).

Infection. All isolates were grown on SDA for 48 h. Next, the cultures were suspended in sterile saline and adjusted to the desired concentration by hemocytometer counts and serial plating on SDA to confirm viability. For all the strains tested, the mice were infected with 2×10^8 CFU in 0.2 ml of sterile saline injected via the lateral tail vein (24).

Treatment. CSP (Cancidas; Merck and Co., Inc., Whitehouse Station, NJ, USA) was administered at 1 mg/kg/day i.p., a dose based on previous pharmacokinetic studies (24–26). The treatment was started 24 h after infection and lasted for 7 days. In addition, all animals received 5 mg/kg/day of ceftazidime administered subcutaneously to prevent bacterial infection. The efficacy of therapy was evaluated through prolonging survival time and reducing the fungal tissue burden. For the survival studies, groups of six mice were randomly established for each strain and checked daily for 30 days after infection. For the tissue burden studies, groups of six mice were also used, and each animal was euthanized 5 days postinfection in order to compare the results with those of the control group, which started to die on that day. The kidneys were aseptically removed, weighed, and mechanically homogenized in 1.0 ml of sterile saline. Serial 10-fold dilutions of the homogenates were placed on SDA and incubated for 48 h at 35°C to determine the CFU per g of tissue.

Statistics. The mean survival time was estimated by the Kaplan-Meier method and compared between groups using the log rank test. The colony counts in the kidneys were analyzed using the Mann-Whitney U test. A *P* value of ≤0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Table 1 shows the MICs of the strains tested, the results of the survival and fungal load studies, and the *FKS* mutations. Thirteen strains showed mutations in one of the two genes explored, although HS1 mutations were present only in those strains with both AFG and CSP MICs of ≥1 μg/ml, with the exception of *C. glabrata* strain JMI-2092 for CSP. One mutation outside the HS1 in the *FKS2* gene (L707S), which was not previously reported, was detected. This mutation was present in 6 (46%) strains, which showed MICs from 0.06 to 2 μg/ml for CSP and <0.03 to 1 μg/ml for AFG, but all strains that had only that mutation responded to CSP treatment.

Although the same inoculum size was used for all the fungal strains tested, which might be a limitation of the study, acute infection was achieved in all cases, showing survival rates from 60% to 100% (data not shown). However, strain-by-strain inoculum adjustments to obtain similar survival curves would increase enormously the number of animals used, thus transgressing ethical issues. In any case, variability was less for fungal load than that observed for survival. The tissue burden study results correlated better with either MICs or with the presence of HS1 *FKS* mutations than those in survival studies, i.e., none of the strains with MICs for CSP or AFG of <1 μg/ml showed HS1 mutations, and CSP treatment reduced the fungal load in all cases. Strains with MICs for both drugs of >1 μg/ml showed HS1 mutations, and the outcome was always negative; all strains with MICs of 1 μg/ml, with the exception of one for CSP, showed HS1 mutations, and the treatment response was positive in only 1 of the 5 cases. Interestingly, this case with a favorable outcome might be explained by the strain used (*C. glabrata* JMI-297), which showed additional mutations on *FKS1*, one inside the HS1 and the other outside the hot spot. Those mutations may have a compensatory effect in the gene, leading to differences in the quaternary structure of the protein or differences in permeability that cause such a variation in the MIC (27).

Antifungal susceptibility testing for echinocandins has been standardized by the CLSI and EUCAST and has proven to be useful in detecting resistance in *Candida* spp. (28). However, only the CLSI has set up the CBP for CSP, since EUCAST has shown significant interlaboratory variation, with remarkably wide MIC ranges, truncated dilutions, and bimodal MIC distributions (18, 19, 28, 29). This variability might be caused by many factors, such as the CSP powder source, stock solution solvent, powder storage time length and temperature, and MIC determination testing pa-

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rameters (29, 30). For that reason, EUCAST has established CBP for only AFG and micafungin and recommends these echinocandins for susceptibility testing instead of CSP (18, 28). In the present study, no significant variations in the CSP MICs were found, despite the *in vitro* susceptibility testing being carried out in three different laboratories, and a correlation was found between the MIC ranges for both AFG and CSP, the presence of HS1 mutations, and *in vivo* outcome.

The generally good response of *C. glabrata* infections to CSP is well known, and previous animal studies have shown a high efficacy of CSP in reducing the fungal load in the kidneys at doses as low as 0.3 mg/kg (24, 31–33). In our study, we chose CSP at a dose of 1 mg/kg because previous pharmacodynamic studies, in a neutropenic murine model of invasive infection by *C. glabrata*, demonstrated that this dose can simulate a serum drug exposure in mice comparable to that in humans (24, 25, 34). There have been few previous studies that attempted to correlate CSP susceptibility and *FKS* mutations with the *in vivo* outcomes of invasive infection by *C. glabrata*, and they have yielded contradictory results (35, 36). Shields et al. (35) demonstrated in patients with IC that the presence of *FKS* mutations has a higher predictive value for echinocandin treatment failure than MICs, but using a murine model of invasive *C. glabrata* infection, Lepak et al. (36) showed that CSP efficacy was closely linked to the *in vitro* MIC rather than to the presence of *FKS* mutations. Our results show that MICs for AFG of $\leq$0.5 μg/ml, which coincided with the absence of *FKS* mutations, were predictive of positive therapeutic response, and mice infected with strains with MICs of $>$1 μg/ml, which coincided with the presence of *FKS* mutations, did not respond to CSP treatment. The mutation L707S, located outside the HS region in the *FKS*2 gene, elevated the MICs for AFG within some isolates above even the ECV but did not influence echinocandin efficacy. Similarly, Castanheira et al. (37) reported that strains carrying amino acid substitutions outside the defined HS exhibit MICs greater than the ECV. However, further studies are necessary to ascertain if they can confer resistance to AFG or micafungin.

The presence of mutations related to resistance to echinocandins is not a rare phenomenon in *C. glabrata* (38). It was demonstrated that different resistance mechanisms can evolve in a very short period during treatment with the drug. Singh-Babak et al. (39) sequenced the whole genome of a susceptible isolate recovered before CSP treatment and the last resistant isolate from a patient that received multiple rounds of echinocandin treatment for recurrent candidemia. The results revealed that in $<$1 year, 9 nonsynonymous mutations had evolved in the patient. One was in the *FKS2* gene, and the others were in genes not previously involved in echinocandin resistance, providing a novel resistance mechanism.

Although studies with more strains are needed, our results suggest that both AFG MICs and *FKS* HS mutations, as well as compensatory mutations, are involved in the efficacy of the echinocandin treatment, but not *FKS* mutations outside the known HS regions; this information seems useful for predicting, at least with our experimental model, the therapeutic outcome.

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