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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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Abstract

It has been argued that in vitro activity of caspofungin (CSP) is not a good predictor of the in vivo outcome of echinocandins treatment. We evaluated the in vitro activity of CSP and the presence of *FKS* mutations in the hot spot 1 (HS1) region of *FKS*1 and *FKS*2 genes of 17 C*andida glabrata* strains with a wide MICs range. The efficacy of CSP against systemic infections by all those strains was evaluated in a murine model. No HS1 mutations were found in the eight strains showing MICs of CSP ≤ 0.5 µg/ml, but they were present in eight of the nine strains with MICs ≥ 1 µg/ml, i.e. three in the *FKS*1 and five in the *FKS*2 genes. CSP was effective to treat mice infected with strains with MICs ≤ 0.5µg/ml, showed variable efficacy in animals challenged with strains with MICs = 1µg/ml and did not work in those with strains with MICs > 1µg/ml. In addition, mutations outside the HS1 region were found in the *FKS* 2 gene of six strains with different MICs, including a first time reported mutation, but their presence did not influence the drug efficacy. In vitro activity of CSP was compared with other echinocandin i.e., anidulafungin suggesting that MICs of both drugs as well as mutations in the HS1 regions of *FKS*1 or *FKS*2 genes are predictive of the outcome.

Introduction

Candida glabrata is a common agent of invasive candidiasis (IC) and the most prevalent species after *C. albicans* (1-3). Azoles and the lipid formulation of amphotericin B are commonly used for the treatment of IC, but for *Candida glabrata* with decreased azoles-susceptibility, echinocandins are the preferred front line therapy (4, 5). Caspofungin (CSP) has been successfully used in the treatment of oesophageal 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), 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 MIC ranges obtained over time and among centers and therefore recommends in the meantime that anidulafungin (AFG) or micafungin are 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 to predict clinical failure, epidemiological cut-off values (ECVs) and clinical breakpoints (CBP) were established based on clinical, molecular, and microbiological data. Thereof, 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 proposed ECV of CSP by 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 at ≥ 0.5 µg/ml for

resistance (19). The aim of this study was to determine, using a murine model of disseminate infection by *C. glabrata* treated with CSP, whether MIC values and presence of *FKS* mutations in such fungus are predictive of *in vivo* outcome.

Material and Methods

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

DNA sequence analysis of *FKS* genes

Candida glabrata strains were grown at 37°C overnight on Sabouraud dextrose agar (SDA). DNA was extracted and purified as previously described (21). The HS1 region, of the *FKS*1 and *FKS*2 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 detected using the BioNumerics Software V 6.6. Translation of nucleic acid sequence into amino acid sequence was performed using EBI Transeq (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. Mice were housed under standard conditions and immunosuppressed one day before the infection by a single intraperitoneal (i.p.) injection of 200 mg/kg of cyclophosphamide (Genoxal;

Laboratories Funk S.A., Barcelona, Spain) and a single intravenous (i.v.) injection of 150 mg/kg of 5-fluorouracil (Fluorouracilo; FerrerFarma S.A., Barcelona, Spain) (23).

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

Treatment. CSP (Cancidas, Merck and Co., Inc., Whitehouse Station, N.J, USA) was administered at 1 mg/kg/d i.p., based on previous pharmacokinetic studies (24-26). The treatment was started 24 h after infection and lasted for seven days. In addition all animals received 5 mg/kg/d of ceftazidime subcutaneously to prevent bacterial infection. The therapy efficacy was evaluated through prolonging survival time and fungal tissue burden reduction. 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, the animals being euthanatized five days post infection in order to compare the results with the control group, which started to die at this day**.** 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 ºC to determine CFUs per gram of tissue.

Statistics. Mean survival time was estimated by the Kaplan-Meier method and compared among groups using the Log Rank test. Colony counts in kidneys were analyzed using the Mann-Whitney U test. A P value ≤ .0.05 was considered statistically significant.

Results and Discussion

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

Although the same inoculum size was used for all the fungal strains tested, which could be a possible limitation of the study, an acute infection was achieved in all cases, showing a survival rate from 60% to 100% (data no shown). However, inocula adjustment strain by strain to obtain similar survival curves would increase enormously the number of animals used, thus transgressing ethical issues. In any case, variability was less in terms of fungal load than was observed in survival. Tissue burden study results correlated better with either MICs or with the presence of HS1 *FKS* mutations than survival studies, i.e. none of the strains with MICs of CSP or AFG <1 µg/ml showed HS1 mutations and CSP treatment reduced fungal load in all cases. Strains with MICs of both drugs >1 µg/ml showed HS1 mutations and the outcome was always negative; all the strains with MICs = 1 µg/ml, with the exception of one for CSP, showed HS1 mutations and the treatment response was positive only in 1 of the 5 cases. Interestingly this case of favourable outcome might be explained due by the strain (JMI-297) showed additional mutations on FKS1, one inside of 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 the detection of resistance in *Candida* spp. (28). However, only the CLSI has set up the CBP for CSP since EUCAST has shown significant inter-laboratory variations 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 CSP powder source, stock solutions solvent, powder storage time, length and temperature, and MIC determination testing parameters, may be the cause of such variability (29, 30). For that reason EUCAST has only established CBP for AFG, and micafungin and recommends these echinocandins for susceptibility testing instead CSP (18, 28). In the present study, no significant variations on the CSP MICs were found, despite the *in vitro* susceptibility testing being carried out in three different laboratories, and correlation among MICs ranges for both AFG and CSP, presence of HS1 mutations and *in vivo* outcome was found.

The in generally good response of *C. glabrata* infections to CSP is well known and previous animal studies have shown a high efficacy of that drug in reducing the fungal load in kidney at doses as low as 0.3 mg/kg (24, 31-33). In our study, we have chosen CSP at doses 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 have 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 incasive *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 of AFG ≤ 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 >1 µg/ml, which coincided with the presence of *FKS* mutations did not respond to the CSP treatment. The mutation L707S, located outside of the HS regions in the *FKS*2 gene, elevated the MICs of AFG within some isolates above even the ECV but did not influence the echinocandin efficacy. Similarly, Casthaneira *et al*., (37), who reported that strains carrying amino acid substitutions outside the defined HS exhibit MICs > ECV. However, further studies are necessary to ascertain if they can confer resistance to AFG or micafungin.

The presence of mutations related with 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 the treatment with the drug. Singh-Babak *et. al.,* (39) sequencing the whole genome of a susceptible isolate recovered before to CSP treatment and the last resistant isolate from a patient that received multiple round of echinocandin treatment for recurrent candidemia revealed that in less than one year 9 non-synonymous mutations were accumulated during evolution in the patient. One was in *FKS* 2 gene and the others in genes not previously involved in echinocandin resistance providing novel resistance mechanism.

Although studies with more strains are needed, our results suggest that both AFG MICs and *FKS* HS mutations, if not compensatory mutations are involved, but not *FKS* mutations outside the known HS regions, seems useful for predicting, at least with our experimental model, the therapeutic outcome.

Table 1 Isolates of *Candida glabrata*, *in vitro* activity of caspofungin (CSP), mutations on *FKS* genes and mean survival time (MST) and fungal load in kidney.

Strains	MIC[a] (µg /ml)		Mutation		MST[b] (95%CI)			Mean (±standard deviation) log_{10} CFU/g of kidney tissue		
	AFG	CSP	*FKS*1	*FKS*2	Controls	Treated group	P value	Control	Treated group	P value
FMR 11381	<0.03	0.06	-	-	18.1 (4.56-31.78)	22.17 (9.43-34.91)[c]	0.050	6.367(±0.333)	5.397±0.227[c]	0.034
UTHSC 08-134	<0.03	0.06	-	L707S[d]	10.5 (0.42-20.58)	18.67 (5.63-31.70)	0.052	4.762±0.226	1.623±0.110[c]	0.019
FMR 8489	<0.03	0.12	-	L707S[d]	18.1 (4.56-31.78)	30.00 (30.00-30.00)[c]	0.004	8.318±0.393	6.005±0.262[c]	0.042
FMR 8498	<0.03	0.12	-	L707S[d]	18.5 (5.26-31.70)	19.00 (6.34-31.66)	0.326	6.968±0.567	4.030±0.549[c]	0.015
UTHSC 11-149	0.03	0.25	-	-	13.8 (0.67-27.00)	30.00 (30.00-30.00)[c]	0.004	6.827±0.371	5.685±0.101[c]	0.039
UTHSC 11-68	0.03	0.25	-	-	10.6 (0.67-20.66)	25.17 (17.30-33.03)[c]	0.002	7.018±0.383	5.712±0.156[c]	0.023
UTHSC 073662	0.03	0.5	-	-	14.0 (0.97-27.02)	30.00 (30.00-30.00)[c]	0.004	7.427±0.548	4.732±0.304[c]	0.014
UTHSC 10461	0.03	0.5	-	L707S[d]	17.6 (3.48-31.85)	18.5 (5.263-31.74)	0.186	6.377±0.368	5.152±0.076[c]	0.028
JMI-2092	0.5	1	-	L707S[d]	15.6 (3.85-27.48)	22.17 (9.43-34.91)[c]	0.037	4.955±0.656	3.665±0.136[c]	0.038
JMI-206	1	1	-	F659S[e]	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[e]	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[e], R631S[e], A1037T[d]	-	15.0 (2.71-27.29)	21.83 (8.55-35.11)[c]	0.008	5.436±0.269	3.558±0.061[c]	0.029
JMI-760	1	1	-	S663P[e]	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[e], L707S[d]	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[e]	-	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[e]	-	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[e]	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], Minimal inhibitory concentration (MIC) of caspofungin (CSP) and anidulafungin (AFG). The last given for comparison as recommended by Arendrup MC. *et al.* in EUCAST technical note (18)

[b], MST, mean survival time in days

[c], *P* value < 0.05 in comparison to the respective control group

[d], Mutations outside of the hot spot 1(HS1) region of the *FKS*1 and *FKS*2 genes

205 [e,] Mutations in the hot spot 1(HS1) region of the *FKS*1 and *FKS*2 genes.

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