Mycopathologia (2017) 182:819–828
DOI 10.1007/s11046-017-0141-9

Activity of Combined Antifungal Agents Against Multidrug-Resistant *Candida glabrata* Strains

Laura Bedin Denardi · Jéssica Tairine Keller · Vanessa Oliveira · Débora Alves Nunes Mario · Janio Morais Santurio · Sydney Hartz Alves

Received: 8 September 2016 / Accepted: 4 May 2017 / Published online: 10 May 2017

Abstract In this study, we evaluated the in vitro activity of echinocandins, azoles, and amphotericin B alone and in combination against echinocandin/azole-sensitive and echinocandin/azole-resistant *Candida glabrata* isolates. Susceptibility tests were performed using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute document M27-A3. The checkerboard method was used to evaluate the fractional inhibitory concentration index of the interactions. Cross-resistance was observed among echinocandins; 15% of the isolates resistant to caspofungin were also resistant to anidulafungin and micafungin. Synergistic activity was observed in 70% of resistant *C. glabrata* when anidulafungin was combined with voriconazole or posaconazole. Higher (85%) synergism was found in the combination of caspofungin and voriconazole. The combinations of caspofungin with fluconazole, posaconazole and amphotericin B, micafungin with fluconazole, posaconazole and voriconazole, and anidulafungin with amphotericin B showed indifferent activities for the majority of the isolates. Anidulafungin combined with fluconazole showed the same percentage of synergism and indifference (45%). Antagonism was detected in 50% of isolates when micafungin was combined with amphotericin B. Combinations of echinocandins and antifungal azoles have great potential for in vivo assays which are required to evaluate the efficacy of these combinations against multidrug-resistant *C. glabrata* strains.

Keywords *Candida glabrata* · Echinocandins · Azoles · Cross-resistance · Combination therapy

L. B. Denardi (✉) · S. H. Alves
Programa de Pós-Graduação em Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal de Santa Maria (UFSM), Campus UFSM, Prédio 20, sala 4139, 97105-900 Santa Maria, RS, Brazil
e-mail: laura-denardi@hotmail.com

J. M. Santurio
Programa de Pós-Graduação em Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil

D. A. N. Mario
Faculdade Meridional-Imed, Escola de Saúde, Passo Fundo, RS, Brazil

J. T. Keller · V. Oliveira
Curso de Farmácia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil

Introduction

Candida glabrata accounts for approximately 15% of systemic infections related to the *Candida* genus. This species represents a serious clinical problem, as infection is associated with high rates of mortality [1, 2]. Since this species has few virulence factors, the large number of infections by *C. glabrata* is result

from its inherently low susceptibility to azole antifungals, leading to development of resistance [3, 4].

Currently, echinocandins have been recommended as the first-line treatment by the Infectious Diseases Society of America (IDSA) for *C. glabrata* infections, especially in patients with previous exposure to azoles. In addition to a broad-spectrum activity against *Candida* species, echinocandins also have a favorable safety profile [5]. However, evidence of therapeutic failure in treatment with echinocandins has been reported. Reduced susceptibility of *C. glabrata* to caspofungin during prolonged therapy has also been reported [6]. Furthermore, a decrease in in vitro and in vivo susceptibility or resistance of *C. glabrata* to other echinocandins has been shown in other studies [7–11].

In order to study the profile of *C. glabrata* resistance to echinocandins, we evaluated the in vitro susceptibility of fluconazole/caspofungin-sensitive and -resistant *C. glabrata* clinical isolates against antifungal agents. Furthermore, we evaluated in vitro associations between antifungals that could demonstrate a synergistic action against resistant isolates.

Materials and Methods

Microorganisms

A total of 20 clinical isolates of *C. glabrata* obtained from oral lesions of patients with HIV infection were studied. The isolates were identified by the API®/ID32 yeast identification systems (bioMérieux, Marcy I' Etoile, France) supplemented with conventional methods as needed. Four groups were formed from the first wild-type group: *C. glabrata* wild-type (*Cg*-Wt), *C. glabrata* fluconazole-resistant (*Cg*-FR), *C. glabrata* caspofungin-resistant (*Cg*-CR), and *C. glabrata* fluconazole/caspofungin-resistant (*Cg*-FCR). The groups *Cg*-FR, *Cg*-CR and *Cg*-FCR were obtained by exposure to sublethal concentrations of fluconazole and/or caspofungin by using the Fekéte-Forgács et al. [12] method. The isolates were stored in saline solutions until they were used. The reference strains *C. albicans* ATCC 14053, *C. glabrata* ATCC 90030, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were used as quality controls for tests.

Antifungal Agents

Standard posaconazole, voriconazole, fluconazole, and amphotericin B powders were obtained from Sigma-Aldrich, São Paulo, SP, Brazil. Stock solutions of posaconazole, voriconazole, and amphotericin B were prepared in dimethyl sulfoxide (DMSO). Stock solution of fluconazole was prepared in sterile distilled water. Anidulafungin (Pharmacia & Upjohn Co. Kalamazoo, MI, USA), caspofungin (Laboratoires Merck Sharp & Dohme-Chibret, Clermont Ferrand, France), and micafungin (Astellas Pharma Tech Co., Takaoka, Toyama, Japan) were obtained from the manufacturer and prepared in sterile distilled water. The solutions were sealed and frozen at -70 °C until they were used. Final dilutions were made in RPMI 1640 medium (Sigma-Aldrich, São Paulo, SP, Brazil).

Antifungal Susceptibility Testing

All the isolates were tested for in vitro susceptibility to fluconazole, voriconazole, posaconazole, amphotericin B, caspofungin, micafungin, and anidulafungin according to the CLSI broth microdilution method (M27-A3) [13]. The inoculum density ranged from 0.5×10^3 to 2.5×10^3 cells mL^{-1}. Minimum inhibitory concentrations (MICs) for the agents were read after incubation for 24 or 48 h. The MIC results for each agent were determined visually as specified in the CLSI documents M27-A3 [7] and M27-S4 [14]. The isolates were classified as sensitive (S), intermediate (I), or resistant (R) based on the CLSI interpretative breakpoints M27-A3 [13] and M27-S4 [14] and the definition by Pfaller et al. [15] and Pfaller et al. [16]. For anidulafungin and caspofungin MIC values $\geq$0.5, 0.25 and $\leq$0.12 μg mL^{-1} were considered to indicate R, I, and S, respectively. For micafungin MIC values $\geq$0.25, 0.12, $\leq$0.06 μg mL^{-1}, were considered to indicate R, I, and S, respectively. For fluconazole MIC results of 32.00 and $\geq$64.00 μg mL^{-1} were categorized as sensitive dose-dependent and resistant, respectively. For voriconazole and posaconazole, MIC results $\geq$4.00, 2.00, and $\leq$1.00 μg mL^{-1} were categorized as R, I, and S, respectively.

Antifungal Interactions Assays

Interactions between antifungal agents were assessed by checkerboard assays. A modified version of the microdilution methodology from the CLSI document M27-A3 [13] was used. The FIC of each antifungal agent was read visually as the lowest drug concentration that resulted in ≥50% inhibition of the growth as compared to the positive control in azole-echinocandin association and 100% of inhibition in amphotericin B-echinocandin association. The reading was performed after 24 h of incubation at 37 °C for all antifungals. The fractional inhibitory concentration index (FICI) was obtained by summation of the combined effects of antifungal FICs; the obtained FICI values were interpreted as follows: FICI $\leq$ 0.5, synergistic; 0.5 < FICI < 4, indifferent; FICI $\geq$ 4, antagonistic [17].

Statistical Analysis

The in vitro susceptibility data were compared by analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple group comparisons and Student's *t* test followed by the Mann–Whitney *U* test for independent samples. All tests were performed using the GraphPad Prism Program (version 5.0, GraphPad Software, San Diego, CA). A *P* value of <0.05 was assumed for the statistical significant differences.

Table 1 Geometric mean MIC, MIC range, MIC_{50}, and MIC_{90} (μg mL^{-1}) of fluconazole, voriconazole, posaconazole, amphotericin B, caspofungin, anidulafungin, and micafungin for *Candida glabrata* isolates

Agents	Group of isolates	Geometric mean MIC	MIC range	MIC_{50}	MIC_{90}
Fluconazole	*Cg*-WT	4.438	1.00–32.00	4.000	16.00
	Cg-FR	64.00	64.00–128.00	64.00	>64.00
	Cg-CR	7.210	0.500–32.00	8.000	16.00
	Cg-FCR	64.00	64.00–128.00	64.00	>64.00
Voriconazole	*Cg*-WT	0.202	0.030–1.00	0.125	1.000
	Cg-FR	1.795	0.060–8.00	2.000	8.000
	Cg-CR	0.208	0.030–1.00	0.250	0.500
	Cg-FCR	0.569	0.060–8.00	1.000	4.000
Posaconazole	*Cg*-WT	0.266	0.008–1.00	0.500	1.000
	Cg-FR	0.998	0.060–4.00	1.000	2.000
	Cg-CR	0.275	0.030–1.00	0.250	1.000
	Cg-FCR	0.729	0.060–4.00	1.000	2.000
Amphotericin B	*Cg*-WT	0.059	0.015–0.250	0.060	0.125
	Cg-FR	0.164	0.03–0.500	0.250	0.250
	Cg-CR	0.128	0.030–0.500	0.125	0.250
	Cg-FCR	0.126	0.060–0.500	0.125	0.250
Caspofungin	*Cg*-WT	0.068	0.008–0.125	0.125	0.125
	Cg-FR	0.051	0.015–0.125	0.060	0.125
	Cg-CR	0.555	0.500–1.00	0.500	1.000
	Cg-FCR	0.555	0.500–1.00	0.500	1.000
Anidulafungin	*Cg*-WT	0.014	0.002–0.060	0.015	0.030
	Cg-FR	0.011	0.002–0.030	0.015	0.030
	Cg-CR	0.063	0.030–1.00	0.030	0.500
	Cg-FCR	0.066	0.008–0.500	0.030	0.250
Micafungin	*Cg*-WT	0.004	0.002–0.030	0.004	0.008
	Cg-FR	0.004	0.002–0.015	0.004	0.008
	Cg-CR	0.016	0.002–1.00	0.008	0.500
	Cg-FCR	0.004	0.001–0.500	0.002	0.030

Cg-Wt, *Candida glabrata* wild-type ($n = 20$); *Cg*-FR, *Candida glabrata* fluconazole-resistant ($n = 20$); *Cg*-CR, *Candida glabrata* caspofungin-resistant ($n = 20$); *Cg*-FCR, *Candida glabrata* fluconazole/caspofungin-resistant ($n = 20$); MIC_{50}, Minimal Inhibitory Concentration which inhibits 50% of the strains (μg mL^{-1}); MIC_{90}, Minimal Inhibitory Concentration which inhibits 90% of the strains (μg mL^{-1})

Results

Table 1 summarizes the in vitro susceptibilities of all groups of isolates to azoles, amphotericin B and echinocandins. All antifungal agents exhibited good activity against the *Cg*-Wt group. Voriconazole and posaconazole MICs increased significantly ($P < 0.0001$) against the *Cg*-FR and *Cg*-CFR groups compared to those against *Cg*-Wt; 5 *Cg*-FR isolates were resistant to posaconazole, and 10 *Cg*-FR isolates were resistant to voriconazole. This observation demonstrated the expected cross-resistance among azole antifungals (Table 2). The *Cg*-CR group did not show resistance to azole antifungals. Amphotericin B MICs were higher in the *Cg*-FR and *Cg*-CFR groups compared to Cg-Wt (Fig. 1).

Table 2 Frequency of antifungal resistance among fluconazole-resistant, caspofungin-resistant, and fluconazole/caspofungin-resistant *Candida glabrata* isolates ($n = 20$)

Candida glabrata	Antifungal Agents	No. (%) of isolates[a]	
		I	R
Cg-FR	VRC	0	10 (50)
	PCZ	11 (55)	5 (25)
	FLC	0	20 (100)
	CAS	0	0
	ANF	0	0
	MFG	0	0
	AMB	0	0
Cg-CR	VRC	8 (40)	0
	PCZ	7 (35)	0
	FLC	1 (5)	0
	CAS	0	20 (100)
	ANF	5 (25)	3 (15)
	MFG	0	3 (15)
	AMB	0	0
Cg-FCR	VRC	3 (15)	11 (55)
	PCZ	8 (40)	5 (25)
	FLC	0	20 (100)
	CAS	0	20 (100)
	ANF	6 (30)	2 (10)
	MFG	0	1 (5)
	AMB	0	0

Cg-FR, *Candida glabrata* fluconazole-resistant; *Cg*-CR, *Candida glabrata* caspofungin-resistant; *Cg*-FC, *Candida glabrata* fluconazole/caspofungin-resistant; *VRC* voriconazole; *PCZ* posaconazole; *FLC* fluconazole; *CAS* caspofungin; *ANF* anidulafungin; *MFG* micafungin; *AMB* amphotericin B

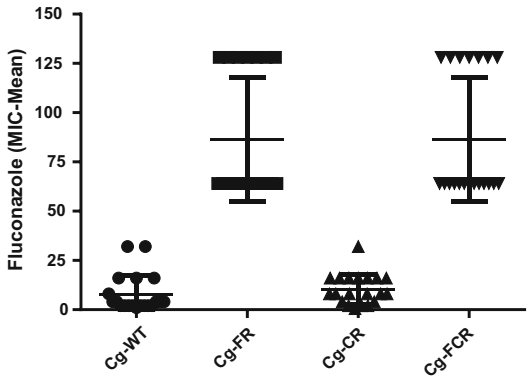
[a]Number of isolates for which the echinocandin/azole MICs were intermediate (I) (anidulafungin and caspofungin MICs of 0.25 μg mL^{-1}; micafungin MIC of 0.12 μg mL^{-1}; voriconazole and posaconazole MICs of 2.0 μg mL^{-1}; fluconazole MIC of 32.0 μg mL^{-1}) or resistant (R) (anidulafungin and caspofungin MICs ≥ 0.5 μg mL^{-1}; micafungin MIC ≥ 0.25 μg mL^{-1}; voriconazole and posaconazole MICs ≥ 4.0 μg mL^{-1}; fluconazole ≥ 64.0 μg mL^{-1})

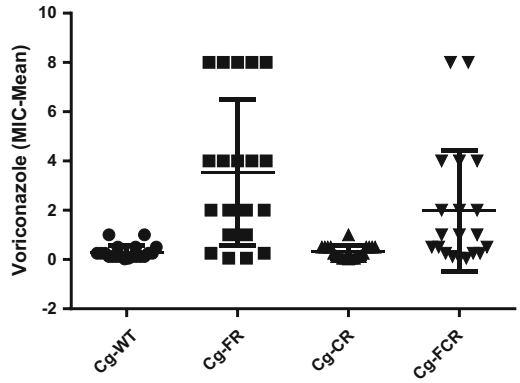
Fig. 1 Minimal Inhibitory Concentrations (MIC) means of azole antifungals, echinocandins, and amphotericin B against *Candida glabrata* groups (*Cg*-FR, *Candida glabrata* fluconazole-resistant; *Cg*-CR, *Candida glabrata* caspofungin-resistant; *Cg*-FCR, *Candida glabrata* fluconazole/caspofungin-resistant

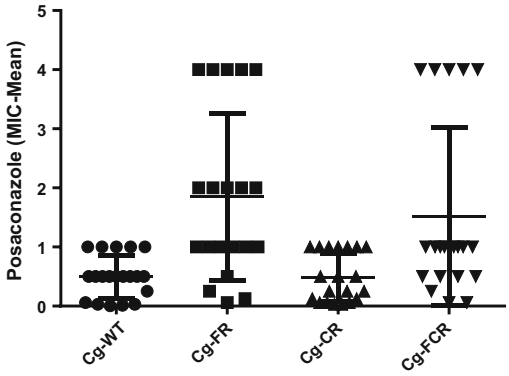
The three echinocandins had good active against the *Cg*-Wt and *Cg*-FR groups. The MIC at which 90% (MIC_{90}) of the both groups were inhibited was 0.125 μg mL^{-1} for caspofungin, 0.030 μg mL^{-1} for anidulafungin and 0.008 μg mL^{-1} for micafungin (Table 1).

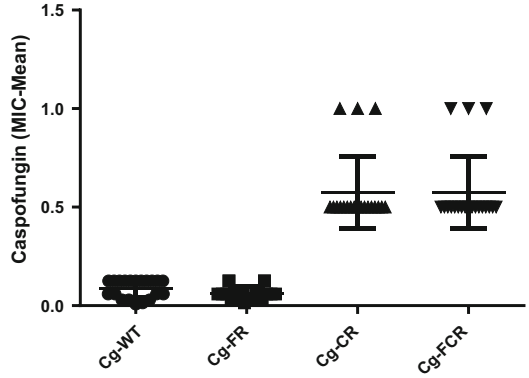
Despite the good activity of echinocandins against the wild-group and fluconazole-resistant group, when they were tested against the *Cg*-CR there was a significant increase in anidulafungin and micafungin averages ($P = 0.0389$) (Fig. 1). Five strains showed intermediate MIC values and three strains were resistant to anidulafungin. Three strains were resistant to micafungin. In *Cg*-CFR strains, the anidulafungin MICs remained high while the micafungin MICs did not differ significantly between the wild and fluconazole-resistant groups; however, a resistance profile for any of the echinocandins (anidulafungin and micafungin) (Table 2; Fig. 1) was not observed.

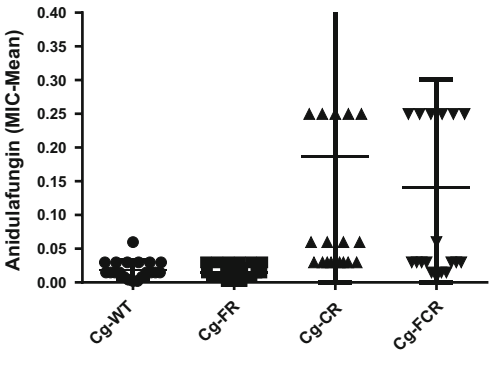
The results of the in vitro checkerboard analysis of the combined antifungal effects against the *Cg*-CFR isolates are summarized in Table 3 and Fig. 2. The association of caspofungin and posaconazole led to synergism in 85% (FIC indexes mean = 0.271) of the isolates, indifference in 15%, and lack of antagonism. The association caspofungin *plus* voriconazole was synergistic to 35% (FIC indexes mean = 0.811) and indifferent to 65% of the isolates and caspofungin *plus* fluconazole showed 85% (FIC indexes mean = 1.905) of indifference and no synergism. The association micafungin *plus* posaconazole was synergistic to 30% and indifferent to 70% of isolates (FIC indexes mean = 1.165). When micafungin was associated with voriconazole, the observation was synergism in 15%, indifference in 60%, and antagonism in 25% of

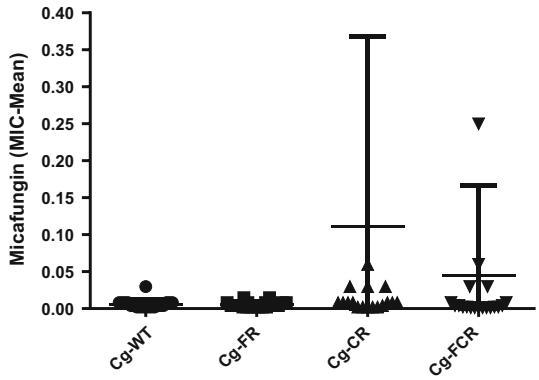

Fluconazole (MIC-Mean)
150
125
100
75
50
25
0
Cg-WT
Cg-FR
Cg-CR
Cg-FCR


Voriconazole (MIC-Mean)
10
8
6
4
2
0
-2
Cg-WT
Cg-FR
Cg-CR
Cg-FCR


Posaconazole (MIC-Mean)
5
4
3
2
1
0
Cg-WT
Cg-FR
Cg-CR
Cg-FCR


Caspofungin (MIC-Mean)
1.5
1.0
0.5
0.0
Cg-WT
Cg-FR
Cg-CR
Cg-FCR


Anidulafungin (MIC-Mean)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Cg-WT
Cg-FR
Cg-CR
Cg-FCR


Micafungin (MIC-Mean)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Cg-WT
Cg-FR
Cg-CR
Cg-FCR

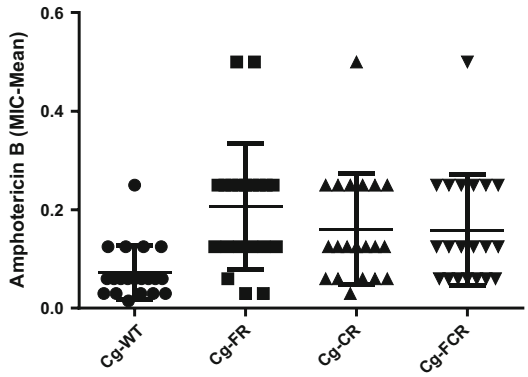

Amphotericin B (MIC-Mean)
0.6
0.4
0.2
0.0
Cg-WT
Cg-FR
Cg-CR
Cg-FCR

Table 3 Interactions (%) of echinocandins with fluconazole, posaconazole, voriconazole and amphotericin B against *Candida glabrata* fluconazole/caspofungin resistant (*Cg*-FCR) ($n = 20$)

Combinations	*Candida glabrata* fluconazole/caspofungin resistant		
	Synergy (%)	Indifference (%)	Antagonism (%)
CAS *plus* FLZ	00	85	15
MFG *plus* FLZ	30	55	15
ANF *plus* FLZ	45	45	10
CAS *plus* PCZ	85	15	00
MFG *plus* PCZ	30	70	00
ANF *plus* PCZ	70	30	00
CAS *plus* VRC	35	65	00
MFG *plus* VRC	15	60	25
ANF *plus* VRC	70	30	00
CAS *plus* AMB	20	70	10
MFG *plus* AMB	15	35	50
ANF *plus* AMB	35	55	10

AMB: amphotericin B, ANF: anidulafungin, CAS: caspofungin, MFG: micafungin, FLZ: fluconazole, PCZ: posaconazole, VRC: voriconazole

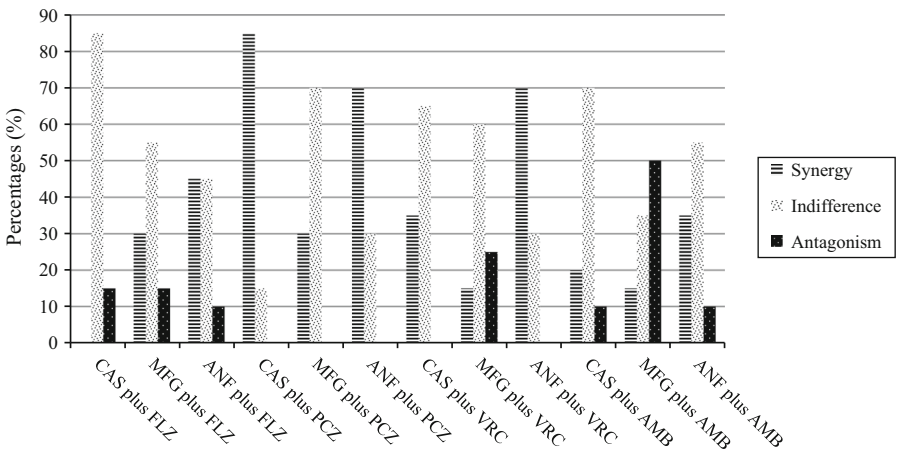


Fig. 2 Results obtained with antifungal combinations against *Candida glabrata* fluconazole/caspofungin resistant (*Cg*-FCR). AMB: amphotericin B, ANF: anidulafungin, CAS: caspofungin, MFG: micafungin, FLZ: fluconazole, PCZ: posaconazole, VRC: voriconazole

the isolates (FIC indexes mean = 3.463). Micafungin *plus* fluconazole was synergistic to 30% and indifferent to 55% of the isolates (FIC indexes mean = 2.288). In the associations of anidulafungin *plus* posaconazole and anidulafungin *plus* voriconazole, synergism was observed in 70% of the isolates with FIC indexes mean of 0.463, and 0.627, respectively; in these associations, no antagonism was observed. When anidulafungin was associated with fluconazole the same percentage (45%) of synergism and indifference was observed (FIC indexes mean = 1.633). In the associations of echinocandins with amphotericin B, synergism was observed in 20% (FIC indexes mean = 1.276), 15% (FIC indexes mean = 4.263), and 35% (FIC indexes mean = 1.200) of the isolates for caspofungin, micafungin and anidulafungin associations, respectively. Micafungin *plus* amphotericin B was antagonistic to 50% of the isolates.

Discussion

According to the current international guidelines, prophylactic therapy with azole antifungals is a risk factor for the development of reduced susceptibility or even resistance in *Candida* spp. isolates. On the other hand, pre-exposure to echinocandins has not been

considered a risk so far [4]. However, this situation might be changing. A survey carried out in France reports that recent exposure to caspofungin influences the epidemiology of candidemia. In this study caspofungin MICs increased significantly for five major *Candida* species including *C. glabrata* [18].

In newer studies, it has been found that echinocandin resistance is most common in *C. glabrata* compared to that in other species, with rates exceeding 10% at selected institutions [19, 20]. This rate can be attributed to the high potential of *C. glabrata* for developing resistance mutations [21]. It has been reported that the reduced susceptibility to echinocandins is due to mutations in regions of the *FKS1* and *FKS2* genes; these genes encode subunits of the glucan synthase enzyme complex [22–25]. Echinocandin resistance in *C. glabrata* is related to pre-exposure to echinocandins and/or *FKS* mutant isolates [26].

Alexander et al. [11] analyzed 313 *C. glabrata* strains from bloodstream infections isolated at Duke Hospital, Iowa, USA. They found that between 2001 and 2010, echinocandin resistance increased from 4.9 to 12.3%, while fluconazole resistance increased from 18 to 30%. Among the isolates resistant to fluconazole, 14.1% were also resistant to one or more echinocandins. Further, 8% of patients infected with FKS mutant strains showed intermediate or resistant MICs and failure to treatment with any of the echinocandins.

Here we demonstrated cross-resistance between azole antifungals. On the other hand, all of them showed good activity against the caspofungin-resistant strains, in the same way as the echinocandins exhibited good activity against the *Cg*-FR strains. Multidrug-resistance between azoles and echinocandins is uncommon because they have different mechanisms of action; nevertheless Pfaller et al. [10] showed that 9.3, 9.3, and 8% of 162 fluconazole-resistant *C. glabrata* isolates were resistant to anidulafungin, caspofungin, and micafungin, respectively. This relationship highlights a possible co-resistance between these antifungal classes, which was not observed here.

We found an increase in anidulafungin and micafungin MICs against *Cg*-CR isolates. Thus constant exposure of wild-type *C. glabrata* strains to caspofungin, besides developing resistance to caspofungin, can also develop cross-resistance between other echinocandins. In *Cg*-CFR isolates, anidulafungin also showed higher MICs, while the average for micafungin significantly remained the same for the *Cg*-FR and *Cg*-Wt strains. Resistance profiles were not observed for any of the echinocandins. This difference indicates that micafungin can have a better effect than anidulafungin in fluconazole/caspofungin-resistant isolates. In contrast to our findings, a study performed by Farmakiotis et al. [27] showed that *C. glabrata* isolates from cancer patients that were resistant to fluconazole and caspofungin were also resistant to anidulafungin and micafungin.

Concerning the combinations of echinocandins with other antifungal agents against *Cg*-CFR, it has been shown that the highest percentages of synergistic outcomes were in the associations of posaconazole with caspofungin and anidulafungin as well as the association of voriconazole with anidulafungin.

The combination therapy of posaconazole with echinocandins is probably advantageous against fluconazole/caspofungin-resistant *C. glabrata,* since synergism was seen in up to 50% of the isolates. The synergism was less (30%) in the combination with micafungin, but antagonism was absent. Moreover, posaconazole appears to reduce the resistance of *C. glabrata* to caspofungin. Oliveira et al. [28] has already reported synergy of posaconazole and caspofungin against 18% of *C. glabrata* sensitive isolates ($n = 199$) and 4% of fluconazole resistant isolates, without evidence of antagonism.

Furthermore, the combination posaconazole *plus* caspofungin also shows in vivo synergy against *C. albicans* echinocandin-resistant isolates suggesting potential therapeutic usage for these combinations [29]. In addition, co-administration of posaconazole with echinocandins was seen to be well tolerated without any effect on either agent's pharmacokinetics [30].

The associations of caspofungin with voriconazole, fluconazole and amphotericin B showed indifferent results. Our results were according to those of Barchiesi et al. [31], who reported indifferent results about the effects of caspofungin combined with amphotericin B against *C. glabrata*. The combination of caspofungin with fluconazole has no synergy; this outcome shows that the combined use of these antifungal agents is not recommended when resistance to them is established. Although the combination of caspofungin and voriconazole has not shown high percentages of synergism against *Cg*-CFR, this combination has been considered the preferred therapy for

subsets of organ transplant recipients with invasive aspergillosis [32]. This combination deserves further studies to clarify its activity against *C. glabrata.*

Anidulafungin was seen to be a good option to combine with azole antifungals. In a study conducted by Karlowsky et al. [33], an additive effect was observed when anidulafungin was combined with fluconazole against *C. glabrata*. Combinations studies of anidulafungin with voriconazole have been most exploited against *Aspergillus;* they showed partial synergy and a lack of antagonism [34, 35]. According to our knowledge, this is the first study to evaluate the combination of anidulafungin and posaconazole against multi-resistant *C. glabrata* isolates. The high synergism found leads us to believe that this combination is a promising option for the treatment of infections caused by resistant *C. glabrata*. We also understand that further studies are needed to confirm our findings.

The association of micafungin with azoles and amphotericin B did not show significant synergism percentages; however, other authors have found positive results when micafungin was associated with voriconazole or amphotericin B. Baltch et al. [36] described that in human macrophages infected by *C. glabrata*, the combination of micafungin and voriconazole was more effective than single drugs. Olson et al. [37] showed that the combination of amphotericin B with micafungin markedly improved the therapeutic outcome in murine *C. glabrata* systemic infection. In addition, co-administration of micafungin with amphotericin B does not affect the pharmacokinetic action of micafungin; therefore, this combination is a safe option for treatment [38].

Considering the substantial benefit of combination therapy, combinations of echinocandins and azole antifungals are promising in the treatment of infections caused by multi-resistant *C. glabrata*. However, the interactions between amphotericin B and echinocandins did not show any advantage as compared to the use of amphotericin B alone. Since the results presented here are obtained in vitro, and checkerboard assay is a preliminary study of antimicrobial interactions, in vivo studies are needed to validate the safe use of these combinations.

As well as, more studies are needed to demonstrate the cross-resistance of *C. glabrata* to echinocandins. This is just an in vitro demonstration that pre-exposure to caspofungin may result in resistance to caspofungin, and cross-resistance may occur between other echinocandins; such a cross-resistance has already happened with the azole antifungals.

Acknowledgements This study was supported by the Higher Education Personnel Training Coordination (CAPES), Brazil. Alves SH thanks the financial support provided by the Brazilian Agencies FAPERGS (Grant Proc. 2261-12) and CNPq (Grant Proc. 470229/2012-8).

Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest.

References

1. Pfaller MA, Diekema DJ. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. Clin Microbiol Infect. 2004;10(Supp 1):11–23.
2. Wisplinghoff H, Seifert H, Wenzel RP, Edmond MB. Inflammatory response and clinical course of adult patients with nosocomial bloodstream infections caused by *Candida* spp. Clin Microbiol Infect. 2006;12:170–7.
3. Castanheira M, Messer SA, Jones RN, Farrel DJ, Pfaller MA. Activity of echinocandins and triazoles against a contemporary (2012) worldwide collection of yeast and moulds collected from invasive infections. Int J Antimicrob Agents. 2014;44:320–6.
4. Pappas PG, Kauffman CA, Andes D, Benjamin DK Jr, Calandra TF, Edwards JE, et al. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis. 2009;48:503–35.
5. Perlin DS. Current perspectives on echinocandin class drugs. Future Microbiol. 2011;4:441–57.
6. Thompson GR 3rd, Wiederhold NP, Vallor AC, Villareal NC, Lewis JS 2nd, Patterson TF. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection. Antimicrob Agents Chemother. 2008;52:3783–5.
7. Pham CD, Iqbal N, Bolden CB, Harrison LH, Farley MM, Schaffner W, et al. Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. Antimicrob Agents Chemother. 2014;58:4690–6.
8. Lewis JS II, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH. Rapid emergence of echinocandin resistance in *Candida glabrata* resulting in clinical and microbiologic failure. Antimicrob Agents Chemother. 2013;57:4559–61.
9. Niimi K, Woods MA, Maki K, Nakayama H, Hatakenaka K, Chibana H, et al. Reconstitution of high-level micafungin resistance detected in a clinical isolate of *Candida glabrata* identifies functional homozygosity in glucan synthase gene expression. J Antimicrob Chemother. 2012;67:1666–76.
10. Pfaller MA, Castanheira M, Lockhart SR, Ahlquist AM, Messer SA, Jones RN. Frequency of decreased

susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of *Candida glabrata*. J Clin Microbiol. 2012;50:1199–203.
11. Alexander BD, Johnson MD, Pfeiffer CD, Jiménez-Ortigosa C, Catania J, Booker R, et al. Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis. 2013;56: 1724–32.
12. Fekete-Forgács K, Gyurc L, Lenkey B. Chances of virulence factors accompanying the phenomenon of induced fluconazole e resistance in *Candida albicans*. Mycoses. 2000;43:273–9.
13. Clinical Laboratory and Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved standard, 3rd ed, document M27-A3. Wayne: Clinical Laboratory and Standards Institute; 2008.
14. Clinical Laboratory and Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Informational Supplement. CLSI document M27-S4. Wayne: Clinical Laboratory and Standards Institute; 2012.
15. Pfaller MA, Diekema DJ, Rex JH, Espinel-Ingroff A, Johnson EM, Andes D, et al. Correlation of MIC with outcome for *Candida* species tested against voriconazole: analysis and proposal for interpretive breakpoints. J Clin Microbiol. 2006;44:819–26.
16. Pfaller MA, Messer SA, Boyken L, Tendolkar S, Hollis RJ, Diekema DJ. Selection of a surrogate agent (fluconazole or voriconazole) for initial susceptibility testing of posaconazole against *Candida* spp.: results from a Global Antifungal Surveillance Program. J Clin Microbiol. 2008;46:551–9.
17. Johnson MD, MacDougall C, Ostrosky-Zeichner L, Perfect JR, Rex JH. Combination antifungal therapy. Antimicrob Agents Chemother. 2004;48:693–715.
18. Lortholary O, Desnos-Ollivier M, Sitbon K, Fontanet A, Bretagne S, Dromer F, et al. Recent exposure to caspofungin or fluconazole influences the epidemiology of candidemia: a prospective multicenter study involving 2,441 Patients. Antimicrob Agents Chemother. 2011;55:532–8.
19. Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Cassaing S, Baixench MT, et al. *Candida* spp. with acquired echinocandin resistance, France, 2004–2010. Emerg Infect Dis. 2012;18:86–90.
20. Matsumoto E, Boyken L, Tendolkar S, McDanel J, Castanheira M, Pfaller M, Diekema D. Candidemia surveillance in Iowa: emergence of echinocandin resistance. Diagn Microbiol Infect Dis. 2014;79:205–8.
21. Arendrup MC, Perlin DS. Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis. 2014; 27:484–92.
22. Katiyar S, Pfaller M, Edlind T. *Candida albicans* and *Candida glabrata* clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob Agents Chemother. 2006;50:2892–4.
23. Kahn JN, Garcia-Effron G, Hsu MJ, Park S, Marr KA, Perlin DS. Acquired echinocandin resistance in a *Candida krusei* isolate due to modification of glucan synthase. Antimicrob Agents Chemother. 2007;51:1876–8.
24. Perlin DS. Resistance to echinocandins-class antifungal drugs. Drug Resist Updat. 2007;10:121–30.
25. Costa-de-Oliveira S, Marcos Miranda I, Silva RM, Pinto E, Silva A, Rocha R, Amorim A, et al. FKS2 mutations associated with decreased echinocandin susceptibility of *Candida glabrata* following anidulafungin therapy. Antimicrob Agents Chemother. 2011;55:1312–4.
26. Shields RK, Nguyen MH, Press EG, Updike CL, Clancy CJ. Caspofungin MICs correlate with treatment outcomes among patients with *Candida glabrata* invasive candidiasis and prior echinocandin exposure. Antimicrob Agents Chemother. 2013;57:3528–35.
27. Farmakiotis D, Tarrand JJ, Kontoyiannis DP. Drug-resistant *Candida glabrata* infection in cancer patients. Emerg Infect Dis. 2014;20:1833–40.
28. Oliveira ER, Fothergill AW, Kirkpatrick WR, Coco BJ, Patterson TF, Redding SW. In vitro interaction of posaconazole and caspofungin against clinical isolates of *Candida glabrata*. Antimicrob Agents Chemother. 2005;49:3544–5.
29. Chen YL, Lehman VN, Averette AF, Perfect JR, Heitman J. Posaconazole exhibits in vitro and in vivo synergistic antifungal activity with caspofungin or FK506 against *Candida albicans*. PLoS ONE. 2013;8:e57672.
30. Krishna G, Vickery D, Ma L, Yu X, Noren C, Power E, et al. Lack of pharmacokinetic drug interaction between oral posaconazole and caspofungin or micafungin. J Clin Pharmacol. 2011;51:84–92.
31. Barchiesi F, Spreghini E, Fothergill AW, Kiremitci A, Kasifoglu N, Akgun Y. Caspofungin in combination with amphotericin B against Candida glabrata. Antimicrob Agents Chemother. 2005;49:2546–9.
32. Singh N, Limaye AP, Forrest G, Safdar N, Muñoz P, Pursell K, et al. Combination of voriconazole and caspofungin as primary therapy for invasive aspergillosis in solid organ transplant recipients: a prospective, multicenter, observational study. Transplantation. 2006;81:320–6.
33. Karlowsky JA, Hoban DJ, Zhanel GG, Goldstein BP. In vitro interactions of anidulafungin with azole antifungals, amphotericin B and 5-fluorocytosine against *Candida* species. Int J Antimicrob Agents. 2006;27:174–7.
34. van de Sande WW, Mathot RA, ten Kate MT, van Vianen W, Tavakol M, Rijnders BJ, et al. Combination therapy of advanced invasive pulmonary aspergillosis in transiently neutropenic rats using human pharmacokinetic equivalent doses of voriconazole and anidulafungin. Antimicrob Agents Chemother. 2009;53:2005–13.
35. Krishnan-Natesan S, Wu W, Chandrasekar PH. In vitro efficacy of the combination of voriconazole and anidulafungin against voriconazole-resistant *cyp*51A mutants of *Aspergillus fumigatus*. Diagn Microbiol Infect Dis. 2012; 73:135–7.
36. Baltch AL, Ritz W, Bopp LH, Michelsen PB, Smith RP. Time kill studies with micafungin and voriconazole against *Candida glabrata* intracellularly in humanmonocyte-derived macrophages and extracellularly in broth. Diagn Microbiol Infect Dis. 2011;70:468–74.

37. Olson JA, Adler-Moore JP, Smith PJ, Proffitt RT. Treatment of *Candida glabrata* infection in immunosuppressed mice by using a combination of liposomal amphotericin B with caspofungin or micafungin. Antimicrob Agents Chemother. 2005;49:4895–902.

38. Undre NA, Stevenson P, Wilbraham D. Pharmacokinetic profile of micafungin when co-administered with amphotericin B in healthy male subjects. Int J Clin Pharmacol Ther. 2014;52:237–44.