Brazilian Journal of Microbiology 46, 1, 125-129 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246120120442

Research Paper

Synergistic effects of tacrolimus and azole antifungal compounds in fluconazole-susceptible and fluconazole-resistant *Candida glabrata* isolates

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Submitted: December 12, 2012; Approved: June 6, 2014.

Abstract

In vitro interaction between tacrolimus (FK506) and four azoles (fluconazole, ketoconazole, itraconazole and voriconazole) against thirty clinical isolates of both fluconazole susceptible and -resistant *Candida glabrata* were evaluated by the checkerboard microdilution method. Synergistic, indifferent or antagonism interactions were found for combinations of the antifungal agents and FK506. A larger synergistic effect was observed for the combinations of FK506 with itraconazole and voriconazole (43%), followed by that of the combination with ketoconazole (37%), against fluconazole-susceptible isolates. For fluconazole-resistant *C. glabrata*, a higher synergistic effect was obtained from FK506 combined with ketoconazole (77%), itraconazole (73%), voriconazole (63%) and fluconazole (60%). The synergisms that we observed *in vitro*, notably against fluconazole-resistant *C. glabrata* isolates, are promising and warrant further analysis of their applications in experimental *in vivo* studies.

Key words: *Candida glabrata*, azole resistance, FK506, combination therapy.

Introduction

In the last few decades, invasive fungal infections that are caused by *Candida* species have risen in parallel with the number of immunocompromised patients, such as those with AIDS, transplant recipients and cancer therapy patients (Bastert *et al.*, 2001). *Candida glabrata* is currently the second most common cause of candidemia in the United States (Pfaller *et al.*, 2007). In Brazil *C. glabrata* is an emerging pathogen especially in private hospitals, attributed mainly to the use of prophylactic therapy with fluconazole (Colombo *et al.*, 2013; Pasqualotto *et al.*, 2009). The increased number of *C. glabrata* systemic infections is very concerning due to the high mortality rate. *C. glabrata* fungemia is frequently difficult to treat due to its intrinsic or rapidly acquired resistance to azole antifungals (Pfaller *et al.*, 2007), which has emerged in clinical isolates from immunocompromised patients (Onyewu *et al.*, 2003).

The treatment for *Candida* infections that show resistance to fluconazole has been the use of other antifungal azoles such as voriconazole, posaconazole and ravuconazole and other classes of antifungal agents, such as amphotericin and echinocandins (Colombo *et al.*, 2013; Pappas *et al.*, 2009). However, the cross-resistance phenomenon among azoles is well-established (Pfaller *et al.*, 2007), as well as for amphotericin (Hull *et al.*, 2012) and echinocandins emergence of resistance has been detected when they are used for *C. glabrata* infection treatment (Alexander *et al.*, 2013; Niimi *et al.*, 2012; Pfaller *et al.*, 2012). Combination therapy is an alternative that can be used to improve the efficacy of antimicrobial therapy for difficult-to-treat infections, either by combining different antifungals or combining antifungal and non-antifungal agents, which can decrease antimicrobial resistance (Mukherjee *et al.*, 2005).

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FK506 is a calcineurin inhibitor that prevents T-cell proliferation and suppresses the immune responses that are involved in transplant rejection (Blankenship and Heitman, 2005). Recent studies have shown that calcineurin inhibitors, such as cyclosporin A and FK506 have synergistic interactions when combined with azole antifungal agents, resulting in fungicidal activity (Steinbach *et al.*, 2004). However, these antifungal activities against *C. glabrata* resistant isolates to azoles have not been evaluated. In this context, the aim of this study was to evaluated the *in vitro* activity of each antifungal (FLZ, KTZ, ITZ and VCR) alone and combined with FK506 against both fluconazole-susceptible and fluconazole-resistant *C. glabrata* isolates using the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008) standardised broth microdilution method.

Material and Methods

Microorganisms

We studied two groups of *Candida glabrata* isolates. The first included thirty fluconazole-susceptible (FS) clinical isolates that were recovered from AIDS patients. The second group included thirty fluconazole-resistant isolates obtained via induction of resistance, as previously described by Fekete-Forgacs *et al.* (2000). Strains were categorized as susceptible, susceptible dose dependent or resistant to fluconazole according to the interpretative breakpoints of CLSI M27-A3 criteria (≤ 8; 16-32; or ≥ 64 μg/mL respectively). All of the yeasts were identified using the commercial kit ID 32C (bioMérieux, Marcy l'Etoile, France). In addition, *Candida glabrata* (ATCC 2001) was used as quality control.

Chemicals

Ketoconazole (KTZ), itraconazole (ITZ) and FK506 (Janssen-Cilag Pharmaceutica, Belgium), voriconazole (VRC) (Pfizer, Inc., New York, NY) and fluconazole (FLZ) (Sigma Chemical Co., St. Louis, MO) were obtained as standard powders. A stock solution for FLZ was prepared by dissolving the powder in distilled water, and stock solutions for the other tested drugs were prepared by dissolving ITZ, KTZ and VRC in dimethyl sulphoxide and FK506 in methanol. The stock solutions were stored at -70 °C until use.

In vitro susceptibility and drug interaction tests

Susceptibility tests were performed according to the CLSI protocol M27-A3 microdilution technique (CLSI, 2008), and the highest concentrations used were 64.00 μg/mL for FK506, 512.00 μg/mL for fluconazole and 32.00 μg/mL for other the azoles. The interaction between FK506 and the azoles against thirty strains each of fluconazole-susceptible and -resistant *C. glabrata* was evaluated using the microdilution checkerboard method. Drug dilutions were prepared in order to obtain four times the final concentrations, and 50 μL of each concentration of azoles was added to columns 1 to 10 and 50 μL of each concentration of FK506 was added to rows A to G. The concentrations of FK506 ranged from 0.25 μg/mL to 32.00 μg/mL, and the concentrations of the azole antifungals ranged from 0.03 μg/mL to 256.00 μg/mL. The experiment was tested in triplicate. The fractional inhibitory concentration (FIC) was calculated for each agent by dividing the minimal inhibitory concentration (MIC) of each drug in combination by the MIC of the drug alone. The FIC values were then totalled to determine the fractional inhibitory concentration index (FICI) that resulted from the drug combinations, as the following equation: $FICI=FIC_A+FIC_B= C_A^{Comb}/MIC_A^{Alone} + C_B^{Comb}/MIC_B^{Alone}$, where MIC_A^{Alone} and MIC_B^{Alone} are the MICs of drugs A and B when acting alone and C_A^{Comb} and C_B^{Comb} are the concentrations of drugs A and B when combined. Synergism was defined as an $FICI \leq 0.5$, indifference was defined as $1.0 < FICI \leq 4$, and antagonism was defined as $FICI > 4$ (Johnson *et al.*, 2004). The statistical analysis of combinations was performed using one-way ANOVA followed by post-hoc Tukey's test. The statistical analysis used to evaluate the different groups (susceptible strains *vs.* resistant strains) when antifungal acting alone was T test. In all the statistical tests, it was considered a significance level of 5% ($p \leq 0.05$).

Results

Based on the parameters of susceptibility (MIC range, MIC_{50}, MIC_{90} and geometric mean), as shown in Table 1, fluconazole-susceptible (FS) strains showed lower MICs to ketoconazole (0.13-2.00 μg/mL), itraconazole (0.50-8.00 μg/mL), voriconazole (0.13-4.00 μg/mL) and fluconazole (1.00-32.00 μg/mL) compared to fluconazole-resistant (FR) strains, which showed MIC ranges of 0.50-16.00 μg/mL for ketoconazole, 1.00-16.00 μg/mL for itraconazole, 1.00-16.00 μg/mL for voriconazole and 64.00-256.00 μg/mL for fluconazole.

The statistical analysis showed significant differences between the susceptibility of FS *vs.* FR groups for all azole antifungal ($p < 0.0001$ (FLZ); $p < 0.0023$ (KTZ); $p < 0.01$ (ITZ and VCR)). Because *C. glabrata* fluconazole-resistant (FR) strains showed an increase in the MIC for all antifungal agents, cross-resistance appears to occur between fluconazole and the other azoles. FK506 did not show activity against FS and FR strains at the highest concentration tested. Table 2 shows the percentages of synergism, indifference and antagonism that resulted from the combinations of FK506 with ketoconazole, itraconazole, voriconazole and fluconazole against the FS and FR group.

FK506 combined with ketoconazole against the FR strains showed 77% of synergism, followed by the combinations with itraconazole (73%), voriconazole (63%) and fluconazole (60%). These results showed that the percent-

Table 1 - Susceptibility (μg/mL) of thirty fluconazole-susceptible (FS) and fluconazole-resistant (FR) *Candida glabrata* strains to fluconazole, ketoconazole, itraconazole and voriconazole.

Agents	Group of isolates	Geometric mean	MIC Range	MIC_{50}	MIC_{90}
Fluconazole	FS	5.039	1.00-32.00	4.00	32.00
	FR	147.033	64.00-256.00	128.00	256.00
Ketoconazole	FS	0.536	0.13-2.00	0.50	1.00
	FR	2.579	0.50-16.00	2.00	8.00
Itraconazole	FS	1.203	0.50-8.00	1.00	4.00
	FR	3.402	1.00-16.00	4.00	8.00
Voriconazole	FS	0.478	0.13-4.00	0.50	1.00
	FR	2.639	1.00-16.00	2.00	8.00

MIC_{50}: Mean the concentration at which 50% of tested strain cannot grow.
MIC_{90}: Mean the concentration at which 90% of tested strain cannot grow.

Table 2 - Percentages of synergism, indifference and antagonism that resulted from the combinations of tacrolimus (FK506) with fluconazole (FLZ), ketoconazole (KTZ), itraconazole (ITZ) and voriconazole (VRC) against fluconazole-susceptible (FS) and fluconazole-resistant (FR) *Candida glabrata* strains.

Agents	Group of isolates	Interactions (%)		
		Synergism	Indifference	Antagonism
FLZ + FK506	FS	3.33	50.00	46.67
	FR	60.00	40.00	0.00
KTZ + FK506	FS	37.00	63.00	0.00
	FR	77.00	23.00	0.00
ITZ + FK506	FS	43.00	57.00	0.00
	FR	73.00	27.00	0.00
VRC + FK506	FS	43.00	57.00	0.00
	FR	63.00	37.00	0.00

ages for the synergistic interactions against the FR strains were significantly similar for the four azoles ($p < 0.0001$). In contrast, the FS group showed lower synergism percentages for both voriconazole and itraconazole combined with FK506 (43%) as well as for the combination with ketoconazole (37%); these combinations didn't differ. ($p < 0.0001$). The combination with fluconazole showed a minimal synergic interaction (3.33%). The percentages of indifference were similar for the four combinations (approximately 56% with FS group, 38% (FLZ and VRC) and 25% (ITZ and KTZ) with FR group). Antagonism effects were not detected for combinations with ketoconazole, itraconazole and voriconazole, however the combination with fluconazole showed 46.67% to FS strains.

Discussion

Invasive fungal infections are a significant complication in transplant patients, and 62% to 91% of these infections are caused by *Candida* species. In liver transplants, the manipulation of the gastrointestinal tract can translocate this microorganism through the intestinal epithelium, resulting in the development of invasive candidiasis (Singh 2003).

FK506 is one of the most common and efficient immunosuppressants used to prevent transplant rejection (Onyewu *et al.*, 2003). This compound inhibits calcineurin, a phosphatase protein involved in lymphocyte activation that is responsible for the synthesis of cytokines, such as interleukin-2, and is primarily involved in NFAT (nuclear factor of activated T-cells) regulation. Calcineurin is activated by an increase in cytoplasmic Ca^{2+}, and its inhibition by immunosuppressants blocks the activation of T lymphocytes, which is critical for an immune response (Baksh and Burakoff, 2000).

Calcineurin governing fungal physiology, including the regulation of cell cycle progression, cation homeostasis, cell wall biosynthesis, antifungal drug resistance and virulence (Blankenship and Heitman, 2005). Immunosuppressants inhibit calcineurin function in several fungal species, including *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus,* affecting essential functions in cell fungal (Bader *et al.*, 2003).

Azole antifungals stimulate calcium influx and this activates the calcium signalling pathway, which is essential for yeast survival (Bader *et al.*, 2003). In *C. albicans,* calcineurin inhibition results in greater susceptibility to azole antifungal agents (Sun *et al.*, 2008), due to the high level of stress that is caused by influx of cations such as Ca^{2+} and Na^{2+} in the fungal cell (Blankenship and Heitman, 2005). Here, we showed that the combination of FK506 with azoles can show synergism against fluconazole-susceptible and -resistant *C. glabrata* strains.

Onyewu *et al.* (2003) demonstrated a synergistic effect with the combination of terbinafine and FK506 against *Candida glabrata*, and Sun *et al.* (2008) showed that the activity of azole antifungal agents against *C. albicans* can be enhanced by FK506. Cruz *et al.* (2002) proposed that the immunosuppressant FK506 and cyclosporin analogues can

be effective in combination with azoles for the treatment of invasive infections that are caused by *Candida spp*, which is in agreement with the present study showing that calcineurin inhibition can function as an adjuvant for treating invasive *Candida glabrata* infections.

Novel antifungal agents, such as posaconazole and echinocandins, have also been ineffective in the treatment of candidemias that is caused by *C. glabrata,* and susceptibility tests have confirmed drug resistance to posaconazole in these isolates (Auberger *et al.*, 2012). Among echinocandins, decreased *in vitro* susceptibility and resistance has been observed in *C. glabrata* strains with single *fks1* or *fks2* mutations, or both (Alexander *et al.*, 2013; Niimi *et al.*, 2012). Pfaller *et al.* (2012) reported a study were thereabout, 11% of *C. glabrata* fluconazole-resistant isolates were also resistant to one or more echinocandins, all of which contained an acquired mutation in *fks1* or *fks2*.

In conclusion, we demonstrated that the rates of synergism were higher among fluconazole-resistant isolates than those observed with fluconazole-sensitive *C. glabrata* isolates. Our results confirm that combinations of FK506 and azole antifungal agents may be promising and warrant further studies *in vivo* using an experimental *C. glabrata* infection.

Acknowledgments

We thank the Brazilian agencies CAPES for their support. Érico Silva Loreto is the recipient of a PNPD-CAPES fellowship. We have no conflicts of interest.

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Associate Editor: Carlos Pelleschi Taborda