Resistance reversal induced by a combination of fluconazole and tacrolimus (FK506) in *Candida glabrata*

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There is an increasing concern about *Candida glabrata* due to its high isolation frequency in candidiasis recently and notorious drug resistance to fluconazole. Drug combination is one effective approach to counteract drug resistance. This study aimed to test whether a combination of fluconazole and tacrolimus (FK506) had a synergistic effect on *C. glabrata*, and to seek the potential mechanisms underlying the synergistic effects. *In vitro* effects of fluconazole and FK506 against *C. glabrata* with different susceptibilities were investigated by a chequerboard method and a time–kill curve method. The mechanistic studies against the resistant *C. glabrata* were performed from two aspects: quantification of expression levels of fluconazole resistance genes (*ERG11*, *CDR1*, *PDH1* and *SNQ2*) by real-time quantitative PCR and functional assays of drug efflux pumps. The addition of FK506 resulted in a decrease in the MIC of fluconazole from 32 to 8 mg l\(^{-1}\) against the dose-dependent susceptible *C. glabrata*, and from 256 to 16 mg l\(^{-1}\) against the resistant *C. glabrata*, respectively. The synergy was further confirmed by the time-kill assay. The expression levels of the *ERG11* and *SNQ2* genes were significantly downregulated after exposure to the drug combination, whereas that of the *CDR1* gene was significantly upregulated, and no significant change in expression of *PDH1* gene was observed. Flow cytometric assays showed that FK506 reduced the efflux of fluconazole. Tacrolimus enhanced the susceptibility of fluconazole against resistant *C. glabrata* by reducing the expression levels of the *ERG11* and *SNQ2* genes and inhibiting fluconazole efflux.

INTRODUCTION

With the increase in the number of immunocompromised individuals and in the number of patients receiving interventional therapeutic procedures, there has been a significant increase in the incidence of opportunistic fungal infections in recent decades. *Candida* spp. account for the majority of fungal infections, and *Candida glabrata* has emerged as the second most prevalent human pathogen after *Candida albicans* in the USA over the past several years (Pfaller & Diekema, 2007). The isolation frequency of *C. glabrata* among fungal isolates has risen significantly, accounting for 15–20 % of all *Candida* infections (Kauffman et al., 2000; Ruan & Hsueh, 2009; Rodrigues et al., 2014). *C. glabrata* is also a major cause of life-threatening disease in immunocompromised patients, causing up to 30 % of all candidaemias (West et al., 2013). Although the ratio of isolated *C. glabrata* species in candidiasis is lower than that for *C. albicans*, *C. glabrata* intrinsically possesses a higher resistance to fluconazole (FLC). Moreover, coinciding with the widespread prophylactic and therapeutic use of azole...
antifungals in clinical settings, cross-resistance to azoles is constantly emerging in \textit{C. glabrata} (Panizo et al., 2009), and it was reported that resistance to echinocandin arose rapidly in \textit{C. glabrata}, which resulted in clinical treatment failure (Lewis et al., 2013). This makes the management of infections due to \textit{C. glabrata} more refractory than those caused by \textit{C. albicans}. Therefore, \textit{C. glabrata} is a focused pathogen and the notorious FLC resistance in \textit{C. glabrata} continues to be a major impediment in antimicrobial chemotherapy.

The number of efficient and less-toxic antifungal agents is limited, and the discovery of new antifungal lead compounds is far from easy, not to mention the financial and clinical requirements necessary for the development of any new antimicrobial drug. Accordingly, there is increasing interest in the study of the antifungal activity of clinically common non-antifungal drugs, and in the development of efficient antifungal combination therapy (Liu et al., 2014). Many researchers are dedicated to studying the combined antifungal effects of FLC and one non-antifungal drug (Shi et al., 2010; Gao et al., 2013), because FLC is the first-line drug for the treatment of \textit{Candida} infection by virtue of its oral availability, low price and good tolerance with few side effects. It was reported that FLC in combination with tacrolimus (FK506) had synergistic effects on \textit{C. albicans} biofilms in both \textit{in vitro} experiments and in an \textit{in vivo} rat catheter model (Uppuluri et al., 2008). Our previous research also demonstrated that FLC combined with FK506 exerted strong synergistic effects against resistant \textit{C. albicans} by different methods (Sun et al., 2008). This observation prompted us to consider whether FLC in combination with FK506 could have a synergistic effect on \textit{C. glabrata}. Therefore, one aim of the present study was to assess the \textit{in vitro} effects of FLC combined with FK506 against \textit{C. glabrata} with different susceptibilities. Another was to seek the potential mechanisms underlying the synergistic effects.

Investigation of drug resistance mechanisms in \textit{Candida} spp. is in constant progress and focuses mainly on \textit{C. albicans}. The FLC resistance mechanism in \textit{C. albicans} has been shown to involve three classes of membrane protein: ATP-binding cassette (ABC) pumps, major facilitator superfamily (MFS) pumps and the cytochrome P450 lanosterol 14-demethylase Erg11 (Albertson et al., 1996; Perea et al., 2001). Recently, it has been demonstrated that calcineurin and the molecular chaperone Hsp90 play important roles in the response to drug-induced stress in \textit{C. albicans} (Cruz et al., 2002; Cowen, 2009). Compared with \textit{C. albicans}, there are fewer studies on the mechanisms of drug resistance in \textit{C. glabrata}. It has been reported that the main mechanisms of FLC resistance in \textit{C. glabrata} include overexpression of the \textit{CDR1}, \textit{PDH1} and \textit{SNQ2} ABC efflux pump genes, as well as upregulation of \textit{ERG11} gene encoding the drug target enzyme (Sanglard et al., 2001; Jandric & Schüller, 2011). These processes lead to the increased production of drug efflux pumps and ergosterol. Efforts have been made to reverse the FLC resistance in \textit{C. glabrata} by blocking the processes as described above (Holmes et al., 2012). Further investigation is required to determine whether FK506 could enhance the susceptibility of resistant \textit{C. glabrata} to FLC by inhibiting these processes. In the present study, investigations of the potential mechanisms of the synergistic effects were conducted from the following two aspects: quantification of FLC resistance gene expression by quantitative real-time PCR (qRT-PCR) and functional assays of drug efflux pumps by flow cytometry.

**METHODS**

**Fungal isolates and suspension preparation.** Six \textit{C. glabrata} strains isolated from different specimens were tested in this work. Their susceptibilities were determined according to Clinical and Laboratory Standards Institute guidelines with \textit{C. parapsilosis} ATCC 22019 as a reference strain (CLSI, 2008), and the break points were described as follows: MIC \( \leq 32 \) \( \mu \)g ml\(^{-1}\) (susceptible dose dependent, SDD) and MIC \( \geq 64 \) \( \mu \)g ml\(^{-1}\) (resistant) at 24 h (Pfaffer & Diekema, 2012). The strains included four FLC-SDD \textit{C. glabrata} (CG1, CG4, CG8 and CG9) and two FLC-resistant \textit{C. glabrata} (CG2 and CG3). Frozen stocks of isolates were maintained at \(-70\)°C and subcultured at least twice before experiments to ensure purity and viability.

Prior to each experiment, all \textit{Candida} strains were propagated at 35°C for approximately 18 h on yeast extract–peptone–glucose (YPG) agar medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar. After 18 h, the fungal cells in the exponential phase of growth were harvested and suspended in sterilized PBS (pH 7.2). The concentrations of the \textit{Candida} suspension were measured using a haemocytometer.

**Preparation of drug stock solutions.** FLC was kindly provided by Cheng Chuang Pharmaceutical Co.; FK506 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China. A stock solution of FLC was prepared in sterile distilled water at a concentration of 2560 \( \mu \)g ml\(^{-1}\). A stock solution of FK506 was prepared in DMSO at a concentration of 6400 \( \mu \)g ml\(^{-1}\). All stock solutions were stored at \(-20\)°C until use.

**Determination of MICs against \textit{C. glabrata}.** The antifungal effects of FLC in combination with FK506 were evaluated against all \textit{Candida} isolates using a checkerboard broth microdilution method. Serial twofold dilutions were performed and 50 \( \mu \)l of each drug dilution was added to each well. The final concentration of FLC ranged from 0.25 to 256 \( \mu \)g ml\(^{-1}\) and of FK506 ranged from 0.008 to 0.5 \( \mu \)g ml\(^{-1}\) for SDD \textit{C. glabrata} or from 0.25 to 16 \( \mu \)g ml\(^{-1}\) for resistant \textit{C. glabrata}. The \textit{Candida} suspensions (4.5 \( \times \) 10\(^5\) c.f.u. ml\(^{-1}\)) were diluted, and 100 \( \mu \)l inoculum was added to 96-well polypropylene plates; the final concentration of the inoculum was 2.25 \( \times \) 10\(^5\) c.f.u. ml\(^{-1}\) for all strains. The plates were incubated at 35°C for 24/48 h. After 24/48 h, 50 \( \mu \)l reagent containing 1.25 mg 2,3-bis-(2-methoxy-4-nitro-5-sulphonyl)-2H-tetrazolium-5-carboxanilide (XTT) ml\(^{-1}\) and 5 \( \mu \)M menadione was added to each well, followed by 2 h incubation in the dark at 35°C. The colorimetric changes were measured at 492 nm with a microtitre plate reader (SpectraMax190; Molecular Devices). The MIC was defined as the lowest drug concentration that caused an 80% reduction in absorbance compared with that of the drug-free control well. All experiments were repeated three times.

**Drug interaction interpretation.** The \textit{in vitro} interaction of the drug combination against \textit{C. glabrata} was interpreted in terms of the fractional inhibitory concentration index (FICI). The non-parametric model FICI was expressed as follows: FICI=FICA\(_{A}\)+FICA\(_{B}\)=CA\(_{A}\)+CA\(_{B}\)+CB\(_{A}\)+CB\(_{B}\), where MIC\(_{A}\) and MIC\(_{B}\) are the MICs of drugs \( A \) and \( B \) used alone against \textit{C. glabrata} and \( C_{A} \) and \( C_{B} \) are the MICs of...
The interpretation of the FICI was as suggested by Odds (2003); the value of FICI \( \leq 0.5 \) was considered synergy, 0.5\(< FICI \leq 4 \) was considered no interaction, and FICI \( > 4 \) was considered antagonism.

**Plotting of the time–kill curve.** To investigate the effect of exposure time on the antifungal activities of FLC combined with or without FK506, a time–kill test was performed against CG4, at the starting inoculum of \( 10^7 \) c.f.u. ml\(^{-1} \). Time–kill studies were conducted in four groups: (i) drug-free control; (ii) FK506 alone; (iii) FLC alone; (iv) drug combination. FLC was used at a concentration of 10 \( \mu \)g ml\(^{-1} \) and FK506 was used at 0.5 \( \mu \)g ml\(^{-1} \). At predetermined time points (0, 6, 12, 24, and 48 h after incubation at 35°C), an aliquot (100 \( \mu \)l) was aspirated from each group and serially diluted in RPMI 1640. A 100 \( \mu \)l aliquot from each dilution was streaked onto a YPG plate. Colony counts were performed after incubation at 35°C for 48 h. The number of c.f.u. ml\(^{-1} \) for each incubation time point was plotted as the vertical ordinate. Synergy was defined as a decrease in c.f.u. ml\(^{-1} \) of \( \geq 2 \log_{10} \), and indifference as a decrease in c.f.u. ml\(^{-1} \) of \(<2 \log_{10} \) compared with the most active drug, and antagonism as an increase in c.f.u. ml\(^{-1} \) of \( \geq 2 \log_{10} \) compared with the most inactive drug (Sahuquillo Arce et al., 2006).

**FK506 reverses the resistance of C. glabrata to FLC**

The results of FLC alone and in combination with FK506 against C. glabrata are summarized in Table 2. The combination of FLC and FK506 exerted synergistic effects against C. glabrata at 24/48 h, especially against resistant C. glabrata. With regard to the SDD C. glabrata, the combined effects were different. When the MIC was 8 \( \mu \)g ml\(^{-1} \) (CG1 and CG9), addition of FK506 did not bring about a decrease in MIC, but when the MIC was 32 \( \mu \)g ml\(^{-1} \) (CG4 and CG8), addition of FK506 made the MIC of FLC decrease to a concentration of 8 \( \mu \)g ml\(^{-1} \) after incubation for 24 h. A strong synergistic effect was observed for all resistant C. glabrata strains (CG2 and CG3) at 24 h, with FICI values of \(<0.1 \). The addition of FK506 made the MIC of FLC decrease from 256 to 16 \( \mu \)g ml\(^{-1} \) against the resistant strain CG3, and this result is illustrated in Fig. 1. The MIC of the quality control strain, ATCC 22019, fell within the normal range.

**Expression of FLC resistance genes in C. glabrata is altered after drug intervention**

The results of the qRT-PCR are presented in Fig. 3. FK506 alone significantly reduced the expression levels of the

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**RESULTS**

**Expression of FLC resistance genes in C. glabrata**

The results of the qRT-PCR are presented in Fig. 3. FK506 alone significantly reduced the expression levels of the
ERG11 and SNQ2 genes, and FLC alone significantly induced ERG11 expression, whereas the combination of FLC and FK506 sharply downregulated the expression of ERG11 and SNQ2, which were reduced by 90 and 66%, respectively (Fig. 3a, d). Conversely, expression of the CDR1 gene was dramatically induced by the drug combination, despite the insignificant increase of CDR1 expression in the groups with FLC or FK506 alone by comparison with that in the control group (Fig. 3b). Although the increased expression of the PDH1 gene in the groups with FLC or FK506 alone appeared to be reduced by the combination of both drugs, there was no significant difference in the PDH1 expression level between any drug-treatment group and control group (Fig. 3c).

FK506 inhibits the efflux of rh6G

The efflux of rh6G in the CG3 cells was evaluated by flow cytometry (Fig. 4). The intracellular concentration of rh6G reached equilibrium at approximately 50 min after the first incubation, and the efflux of rh6G was then measured every 10 min over the course of a 50 min second incubation in glucose-supplemented medium. At the time point of 10 min, the fluorescence intensity in the two groups decreased dramatically compared with the balanced fluorescence intensity. The relative fluorescence intensity in the control group had a sharp drop at 20–30 min and decreased gradually thereafter (Fig. 4). However, the relative fluorescence intensity in the FK506-added group retained a relatively high level over 20–40 min, followed by

Table 1. Primers used to amplify the ERG11, CDR1, PDH1, SNQ2 and RDN5.8 genes in C. glabrata

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’–3’)</th>
<th>GenBank accession no.</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG11</td>
<td>F: CAAATCCAGCGTTGGGAC R: ATCTTAGCCAGGGACAGTTGG</td>
<td>L40389.1</td>
<td>285</td>
</tr>
<tr>
<td>CDR1</td>
<td>F: TAAACAGCTGTGCAGACAG R: CCACAAGTCAGGGGCCAGCCTTGGG</td>
<td>AF109723.1</td>
<td>240</td>
</tr>
<tr>
<td>PDH1</td>
<td>F: GCCGGCCCTTCATTCTGGG R: GATGCGGAACGTCAGCGACAT</td>
<td>AF251023.1</td>
<td>215</td>
</tr>
<tr>
<td>SNQ2</td>
<td>F: CTGGAGGAGGACACATCGG R: CAAGACAAAACCTTCCGGTGTC</td>
<td>AM849042.1</td>
<td>265</td>
</tr>
<tr>
<td>RDN5.8</td>
<td>F: TTGGGGGAACGGCTCTCTTTC R: GCCAGCAAGGGCTGTTAAA</td>
<td>KC597823.1</td>
<td>234</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.

Table 2. Combined drug effects evaluated by the FICI model against C. glabrata at 24/48 h

<table>
<thead>
<tr>
<th>Strain*</th>
<th>MIC† of drug (µg ml⁻¹)</th>
<th>FICI model‡</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FLCalone</td>
<td>FK506alone</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG1</td>
<td>8</td>
<td>256</td>
</tr>
<tr>
<td>CG9</td>
<td>8</td>
<td>256</td>
</tr>
<tr>
<td>CG4</td>
<td>32</td>
<td>512</td>
</tr>
<tr>
<td>CG8</td>
<td>32</td>
<td>512</td>
</tr>
<tr>
<td>CG3</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td>CG2</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG1</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>CG9</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>CG4</td>
<td>64</td>
<td>512</td>
</tr>
<tr>
<td>CG8</td>
<td>64</td>
<td>512</td>
</tr>
<tr>
<td>CG3</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>CG2</td>
<td>512</td>
<td>512</td>
</tr>
</tbody>
</table>

*CG, C. glabrata.
†MIC denotes the MIC₉₀₉₀ of each drug alone or in combination (comb) against C. glabrata at 24/48 h and is shown as the median of three independent experiments.
‡FICI values are shown as the median of three independent experiments. INT, interpretation; NI, no interaction; SYN, synergism.
a decrease at 50 min (Fig. 4). Furthermore, the relative fluorescence intensity was significantly higher than that in the control group at 30, 40 and 50 min (Fig. 4). At the time point of 40 min, the relative fluorescence intensity in the FK506-added group was 2.76-fold that in the control group. These results demonstrated a decreased efflux of rh6G in the FK506-treated C. glabrata cells.

**DISCUSSION**

The development of strategies to combat the innate FLC resistance and the fast acquisition of resistance in C. glabrata can be facilitated greatly by studying drug combinations of FLC and non-antifungal drugs. We examined whether FK506 could sensitize the effects of FLC against C. glabrata strains with different susceptibilities. Our results showed that a combination of FLC and FK506 had a synergistic effect against resistant and SDD (MIC $\leq 32$ μg ml$^{-1}$) C. glabrata, which expands on previously published results. Some earlier publications reported *in vitro* interactions between FK506 and FLC against two C. glabrata strains (Cruz et al., 2002; Kaur et al., 2004). Our study performed systematic and comprehensive investigation of FLC–FK506 interactions against C. glabrata after incubation for 24 and 48 h. The results indicated that the synergistic effects of the drug combination at 24 h were more obvious than those at 48 h. FLC in combination with FK506 exerted synergistic effects against the SDD C. glabrata strains at lower concentrations of FLC (8 μg ml$^{-1}$) and FK506 (0.5 μg ml$^{-1}$) at 24 h. Furthermore, the combined drug effects were synergistic against the resistant C. glabrata strains with FLC at 16 μg ml$^{-1}$ and FK506 8 μg ml$^{-1}$ at 24 h. It has...
been reported that most *C. glabrata* isolates are SDD (Pfaller *et al.*, 2006). Considering this point, the time–kill curve experiments were only applied to the SDD *C. glabrata* strain CG4. A time–kill curve can portray a more detailed picture of the kinetic effects of drug combinations on cell viability over time. The results of the dynamic experiments further confirmed the results obtained by the chequerboard method and the combined drug effect of FLC and FK506 against CG4 was the most potent at 24 h after drug treatment.

FLC acts by inhibition of cytochrome P450 lanosterol 14-demethylase Erg11, leading to reduced ergosterol biosynthesis. The decrease in *ERG11* expression levels by combination of FLC and FK506 may bring about the decrease in the amount of the target Erg11, which may potentiate the antifungal activity of FLC, by impairing the membrane. It has been postulated that membrane perturbation along with other stresses leads to the influx of Ca$^{2+}$ in *Saccharomyces cerevisiae* (Edlind *et al.*, 2002). It may be extrapolated that the increased membrane stress by combination of FLC and FK506 leads to the increased uptake of Ca$^{2+}$. As another point of view, FK506 dramatically induces Ca$^{2+}$ uptake in *C. glabrata* cells in the presence of FLC by blocking the calcineurin interacting partner Cpr1 (Kaur *et al.*, 2004), and FK506 enhances the activity against *C. glabrata* of terbinafine and fenpropimorph that target other enzymes in the ergosterol biosynthetic pathway (Onyewu *et al.*, 2003). Therefore, the ergosterol biosynthesis pathway may be correlated with...
the calcineurin pathway in *C. glabrata*, and inhibition of the calcineurin pathway could enhance the antifungal activities of drugs that target the ergosterol biosynthesis pathway. In addition, it has been reported that FK506 addition or calcineurin gene *CMP2* disruption specifically reverses the β-1,3-glucan synthase Fks2-mediated resistance of *C. glabrata* to echinocandin (Katiyar et al., 2012). Therefore, the biological processes switched on by addition of FK506 are complex, and inhibition of the calcineurin pathway may be an important approach for overcoming the resistance of *C. glabrata* to antifungal agents.

The precise molecular mechanism underlying FLC resistance in *C. glabrata* is not well understood. Up-to-date research has highlighted the important role that complex resistance gene overexpression interactions play in FLC resistance in *C. glabrata*, and has demonstrated that overexpression of ABC membrane transporter genes is a pivotal FLC resistance mechanism in *C. glabrata* clinical isolates (Abbes et al., 2013). Although the MFS transporter genes *FLR1* and *QDR2* in *C. glabrata* are known to be involved inazole efflux, the expression of *FLR1* and *QDR2* in *C. glabrata* was not found to confer resistance to FLC (Chen et al., 2007; Costa et al., 2013). Therefore, the investigation of FLC resistance gene expression did not involve the MFS transporter genes in this work. After exposure to the drug combination, expression of the *CDR1* gene was upregulated, whilst a significant decrease in the expression level of the *SNQ2* gene was observed by comparison with the control group. It has been revealed that, during azole resistance, the *SNQ2* gene exerts a major independent effect on FLC resistance in *C. glabrata* according to a multivariate analysis (Abbes et al., 2013), and our results indicated that the *SNQ2* gene expression was reduced threefold by the drug combination. Moreover, further functional assays confirmed that FK506 reduced the efflux of FLC. Additionally, another study indicated that FK506 can enhance the antifungal effect of FLC against a strain overexpressing the *CDR1* gene in *C. glabrata* (Lamping et al., 2007). These results demonstrate that a combination of FLC and FK506 may act synergistically against *C. glabrata* at both the gene and protein levels.

Recently, FK506 has been widely used in organ and haematopoietic stem-cell transplant patients, although these patients are vulnerable to *C. glabrata* infections (Montejo, 2011). Therefore, the FLC–FK506 combination may be applicable as an optional therapy for organ and haematopoietic stem-cell transplant patients with *C. glabrata* infections.

Taken as a whole, the combination of FLC and FK506 exerts synergistic effects against *C. glabrata*. The antifungal mechanism of the FLC–FK506 combination against resistant *C. glabrata* is related to the decrease in *ERG11* and *SNQ2* expression levels and the inhibition of FLC efflux. Nevertheless, the precise synergistic mechanisms require further investigation owing to the unclear resistance mechanism in the tested strain CG3 and the complexity of mechanisms of the drug combination against *C. glabrata*. This study provides some new information about the antifungal mechanism of this drug combination, as well as insights into antifungal agent discovery. Furthermore, the FLC–FK506 combination will hopefully be a new approach against antifungal drug resistance.

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