Possible mechanisms of the antifungal activity of fluconazole in combination with terbinafine against Candida albicans

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Abstract

Context: Candidiasis is a term describing infections by yeasts from the genus Candida, the majority Candida albicans. Treatment of such infections often requires antifungals such as the azoles, but increased use of these drugs has led to selection of yeasts with increased resistance to these drugs.

Objective: Combination therapy would be one of the best strategies for the treatment of candidiasis due to increased resistance to azoles.

Materials and methods: The antifungal activities of fluconazole and terbinafine were evaluated in vitro alone and in combination using broth microdilution test and time killing study. Eventually, the expression level of selected genes involved in ergosterol biosynthesis of Candida was evaluated using semi-quantitative RT-PCR.

Results: The obtained results showed the significant MICs ranging from 0.25 to 8 μg/mL followed by FICs ranged from 0.37 to 1 in combination with fluconazole/terbinafine. Our findings have demonstrated that the combination of fluconazole and terbinafine could also significantly reduce the expression of ERG1, 3, and 11 in the cell membrane of Candida in all concentrations tested ranging from 1.73- to 6.99-fold.

Discussion and conclusion: This study was undertaken with the ultimate goal of finding the probable targets of fluconazole/terbinafine in C. albicans by looking at its effects on cell membrane synthesis.

Introduction

Candidiasis is a common term that usually results from overgrowth of Candida albicans in the human body treated by antifungal agents such as azoles (like fluconazole) and allylamines (like terbinafine) as the common antifungal drugs (Ferahbas et al., 2006; Rossie & Guggenheimer, 1997). The primary target of azoles may be the heme protein, which cocaatalyzes cytochrome-P450-dependent 14α-demethylation of lanosterol in the last stage of ergosterol biosynthesis, while allylamines act by inhibiting the early stages. Indeed, the inhibition of squalene epoxidase by terbinafine (early step) or 14α-demethylase by fluconazole (last step) of ergosterol’s biosynthesis has principal role in a play of depletion of ergosterol and agglomeration of sterol precursors, resulting some alteration in the structure and function of cell membrane in Candida cells (Borecká-Melkusová et al., 2009; Espinel-Ingroff, 2008; Ghannoun & Rice, 1999; Spampinato & Leonardi, 2013).

Sometimes, Candida seen in immunocompromised or hospitalized individuals is resistant to main types of antifungal agents (Park & Perlin, 2005). Ergosterol biosynthesis genes including ERG1, ERG3, and ERG11 are the most significant genes involved in the resistance to azoles and the other antifungals such as allylamines. In contrast, the up-regulation of these genes resulting in alteration of enzyme targeted by fluconazole (encoded by ERG11) or terbinafine (encoded by ERG1) which may result in the resistance to drugs. Moreover, mutation of these genes could lead to resistance to these antifungals (Borecká-Melkusová et al., 2009; Espinel-Ingroff, 2008).

Nowadays increasing incidence of resistance to antifungal therapy has required that novel therapies be used. Some reports have shown that combination of fluconazole/terbinafine was effective to inhibiting Candida growth in vitro (Ghannoun & Elewski, 1999; Perea et al., 2002). In the present study, in vitro antifungal activities of fluconazole and terbinafine alone and in combination against C. albicans were examined. Subsequently, the expression of significant genes involved in ergosterol biosynthesis of Candida such as ERG1, ERG3, and ERG11 were analyzed with fluconazole/terbinafine combination therapy.
Materials and methods

Inoculum

Five clinical isolates of *C. albicans* which were obtained from systemic candidiasis patients and *C. albicans* ATCC 10231 as a reference quality-control strain were used. All isolates grown on Sabouraud dextrose agar and the cell density was adjusted at 530 nm wavelength to acquire the yeast stock suspension containing 1–5 × 10^6 cells/mL.

Antifungals

Fluconazole and terbinafine were obtained from Sigma Chemicals Co. (St. Louis, MO) and were dissolved in dimethylsulfoxide at 5 mg/mL according to the manufacturer’s recommendations.

Determination of MIC using broth microdilution assay

According to CLSI document M27-A3 for yeast, 50 or 100 μL of the two-fold dilution of the drugs alone or in combination, respectively, dissolved in a standard RPMI 1640 medium with 0.2% glucose [buffered to pH 7.0 with 0.165 M morpholinoephosph- phonyl sulfate (MOPS)] using 96-well microplates. Subsequently, 100 μL of a suspension containing 5 × 10^2–2.5 × 10^3 yeast cells/mL was added to the former mixture and incubated at 35 °C for 24 h. The endpoint was calculated as the lowest concentration of each drug that caused at least 50 and 90% growth inhibition compared with the control-growth.

Time kill study

Four mL of *C. albicans* ATCC 10231 (1 × 10^6 cells/mL) were dissolved in RPMI 1640 (as described above) and mixed with 2, 4, and 0.25 μg/mL of fluconazole, terbinafine, and fluconazole/terbinafine, respectively, and grown at 35 °C. After different time intervals, 100 μL of each mixture was loaded and plated on Sabouraud dextrose agar and was incubated at 35 °C for 48 h. Eventually, colonies were counted and the CFU were calculated.

RNA extraction and cDNA synthesis

Total RNA of each sample contained *C. albicans* ATCC 10231, fluconazole and terbinafine alone and in combination was extracted using RNeasy mini kit (Qiagen, Hilden, Germany) with slight modifications for yeast cells. Subsequently, 2 mL of sorbitol lysis buffer (1 M sorbitol and 0.1 M EDTA pH 7.4) was added to each sample and then 50U lyticase/zymolyase (ICN Chemicals, Newburyport, MA) and 10 μL of β-mercaptoethanol were added, respectively, to the former mixture. The extracted RNA was also treated using 1U DNase I (Promega, Southampton, UK) according to the manufacturer’s operating instructions. RNA quality, concentrations, and absorbance ratio were checked using 1.2% (w/v) formaldehyde-denaturing agarose gel electrophoresis and Nanodrop ND-1000 spectrophotometer, respectively.

In the next step, 1 μg extracted RNA was used to achieve the single-stranded cDNA according to the manufacturer’s protocol as described in our previous work (Khodavandi et al., 2011).

Semi-quantitative RT-PCR for comparing *ERG1, ERG3, and ERG11* gene expression levels

*ERG1, ERG3,* and *ERG11* genes of *C. albicans* ATCC 10231 were amplified using the synthesized cDNA as prepared in former stages. β-Actin gene was also amplified as a housekeeping gene and an internal control as explained entirely in our previous work (Khodavandi et al., 2011). The primer sequences which were used in this work are listed in Table 1.

Finally, the PCR products were analyzed by gel electrophoresis and visualized using an Alpha Imager HP imaging system. The concentration of PCR products was measured and the relative quantification was calculated using the following formula (Khodavandi et al., 2011):

$$\text{Fold change in target gene expression} = \frac{\text{Ratio of target gene expression (experiment/untreated control)}}{\text{Ratio of reference gene expression (experiment/untreated control)}}$$

All statistical analyses were done using the SPSS software (version 21; SPSS Inc., Chicago, IL). We used normality and ANOVA test to analyze the gene expression. In all statistical analyses, *p* < 0.05 was considered significant.

Results

The MICs range of fluconazole against *C. albicans* was 0.5–128 μg/mL. Indeed, except *C. albicans* clinical isolate number 5 (CI-5), 80% of isolates were susceptible to fluconazole (MIC < 64 μg/mL). However, no specific breakpoints were proposed based on the terbinafine, all isolates have shown MICs less 16 μg/mL and ranged from 0.5 to 32 μg/mL (Table 2).

Table 2 also indicates that the combination of fluconazole/terbinafine was highly active. While 50% of isolates show...
strong synergistic activity after treatment by fluconazole in combination with terbinafine, only 33 and 16.7% of the combination of fluconazole/terbinafine were partial synergism and additive activities, respectively (Khodavandi et al., 2011).

The inhibitory effect of antifungals tested is shown in Figure 2. Both fluconazole and terbinafine decreased the number of cells after time intervals, significantly. The combination of fluconazole/terbinafine was significantly more efficacious than the antifungal tested alone ($p < 0.05$).

Gene expression analysis data indicated significant down-regulation ($p < 0.05$) of selected genes in all concentration tested for both fluconazole and terbinafine alone. Indeed the expression level of ERG3 and ERG11 was down-regulated 2.02–3.96- and 2.39–4.46-fold, respectively, for different concentrations of fluconazole alone based on MIC (data not shown). Terbinafine was also effective to decrease the expression level of ERG1 ranged from 1.63 to 3.05 (data not shown). Moreover, antifungals with combination could able to down-regulate all selected genes as well as fluconazole and terbinafine alone. ERG1, ERG3, and ERG11 were down-regulated 1.73–4.11-, 2.29–4.85-, and 3.95–6.99-fold, respectively, for different concentrations of fluconazole/terbinafine based on MIC (Figures 3 and 4).

**Discussion**

Antifungal drugs including azoles such as fluconazole, polyenes, allylamines such as terbinafine and flucytosine are mostly commonly used to treat *Candida* infections. Most of these antifungal agents are phenolic structured. The phenolic compounds show the highest anti-*Candida* bioactivity (Figure 1) (Yang, 2003). In the recent years, treatment failure of fungal infections such as candidiasis is apparently increasing due to low efficacy of anticandidal drugs and occurrence of drug-resistance in the number of immunocompromised patients (Al-Mohsen & Hughes, 1998; Bruzual et al., 2007; Odds, 1996). Therefore, there is a need to find the new ways to increase the efficacy of antifungals with minimal drug resistance to treat *Candida* infections. Towards this goal, combination of drugs with synergistic effect and understanding the molecular mechanisms are fundamental to discover and develop of new anti-infective strategies.

Indeed, the combination of fluconazole with terbinafine was shown to be significant in terms of growth inhibition in *C. albicans* by some reports (Ghannoum & Elewski, 1999; Ghannoum & Rice, 1999; Perea et al., 2002; Scheid et al., 2012).

Determination of MICs is a simple and reproducible approach that can be applied to show the ability of growth

<table>
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<tr>
<th>Isolates</th>
<th>Fluconazole&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Terbinafine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fluconazole/Terbinafine&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>MIC&lt;sub&gt;50&lt;/sub&gt;/MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC range</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
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<td><em>C. albicans</em> ATCC 10231</td>
<td>1/2</td>
<td>0.5/4</td>
<td>0.5/4</td>
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<td><em>C. albicans</em> CL&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8/32</td>
<td>4/32</td>
<td>4/16</td>
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<tr>
<td><em>C. albicans</em> CL&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16/32</td>
<td>8/32</td>
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<tr>
<td><em>C. albicans</em> CL&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8/16</td>
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<td>1/4</td>
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<td><em>C. albicans</em> CL&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16/32</td>
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<td><em>C. albicans</em> CL&lt;sub&gt;5&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32/64</td>
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<sup>a</sup>µg/mL.
<sup>b</sup>CI: Clinical isolates.
inhibition of *Candida* cells by antifungals. The MICs values for fluconazole alone ranged from 0.5 to 128 μg/mL (Table 2). Interestingly, most of clinical isolates were susceptible to fluconazole except one clinical isolate, while MICs of drugs in combination was significantly decreased and resistant was not observed. One of the best examples of this is fluconazole-resistant *C. albicans* clinical isolate (CI-5), where the MIC of this isolate is markedly decreased (512-fold) with the combination of fluconazole/terbinafine (Table 2). Meanwhile, FICs values also were demonstrated that terbinafine could be able to increase the antifungal activities of fluconazole when they were used in combination. This significant synergistic effect was observed in most of isolates tested and ranged from 0.13 to 0.25 (Table 2).

The time kill curve showed a significant reduction (*p*<0.05) the number of *Candida* comparing untreated controls for those fluconazole and terbinafine alone and in combination after 6h incubation at 35°C. Interestingly, the terbinafine was almost as efficiently as fluconazole in terms of the growth of the yeast cells (*p*<0.05) until 36h incubation at 35°C, while fluconazole was significantly stronger than terbinafine (*p*<0.05) after 42h incubation at 35°C (Figure 2). This may be due to potential of azoles to destroy the membrane of *Candida* through the inhibition activities of both critical enzymes in ergosterol biosynthesis such as 14α-demethylase and desaturase, while allylamine could inhibit the activity of squalene epoxidase only during ergosterol biosynthesis (Ghannoum & Rice, 1999; Lupetti et al., 2002).

The combination therapy may be one of the viable therapeutic options due to increase of azole-resistance phenomena. The *in vitro* activities of fluconazole with terbinafine were reported by several investigations (Cantón et al., 2005; Cavalheiro et al., 2009; Ghannoum & Elewski, 1999; Perea et al., 2002). All of them were persisted that the antifungal activity of fluconazole in combination with terbinafine could increase significantly and no antagonistic effects were observed. Nonetheless, the possible molecular mechanisms of those activities are not understood. In the present study, we have attempted to clear one of the probable mechanisms with a standard and accessible method. In fact, the relative expression level of transcript mRNA of genes involved in
biosynthesis of ergosterol in the cell membrane of *C. albicans* was evaluated with fluconazole and terbinafine monotherapy or in combination using semi-quantitative RT-PCR.

As explained above the main target of both fluconazole and terbinafine is the cell membrane of *Candida*. Indeed, squalene, a precursor of ergosterol, is converted to lanosterol with squalene epoxidase encoded by *ERG1* gene. Squalene epoxidase is inhibited in the presence of allylamines resulting in cell membrane destruction (Lupetti et al., 2002; Pasrija et al., 2005). Our results showed that the expression level of *ERG1* was significantly down-regulated (*p* < 0.05) after treatment of *C. albicans* ATCC 10231 (1.63–3.05 folds) with terbinafine alone, while the expression of those *ERG3, 11* was not changed as expected (data not shown). In contrast, the expression level of *ERG3, 11* was down-regulated significantly (*p* < 0.05) after treatment of *C. albicans* ATCC 10231 using fluconazole alone (2.02–3.96 and 2.39–4.46-fold, respectively) and *ERG1* was not significantly changed (data not shown). This is also justified by the role of fluconazole to inhibit the possible target genes including *ERG3 and ERG11* (Geber et al., 1995; Ghannoum & Rice, 1999; Lupetti et al., 2002).

Combination of fluconazole/terbinafine was decreased the expression level of all genes tested significantly at level *p* ≤ 0.05 in compared with the untreated control (Figures 3 and 4). Clearly, significant genes involved in ergosterol biosynthesis of *C. albicans* ATCC 10231 including *ERG1, 3, and 11* were down-regulated ranged from 1.73- to 6.99-fold with combination therapy of fluconazole/terbinafine. Hence combination of fluconazole/terbinafine could destroy the cell membrane through the inhibition of all three key enzymes in ergosterol biosynthesis of *C. albicans*. Therefore, the ergosterol suppression by combination of fluconazole/terbinafine seems to be related to *ERG* genes.

Further experiments need to be performed in order to investigate the effect of these combinations on other significant genes contributing to the cell membrane of *C. albicans* with different techniques such as relative real time RT-PCR and microarray tests.

**Declaration of interest**

The authors declare that they have no competing interests.

**References**


