In vitro interaction between azoles and cyclosporin A against clinical isolates of Candida albicans determined by the chequerboard method and time–kill curves

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Objectives: To investigate the in vitro interaction between three azoles (fluconazole, itraconazole and voriconazole) and cyclosporin A against five azole-susceptible (azole-S) and five azole-resistant (azole-R) clinical Candida albicans isolates.

Methods: By using a chequerboard technique and time–kill curves, synergistic, indifferent or antagonistic effects when drugs were used in combination were assessed. In the chequerboard assay, the antifungal activity of drug combinations was determined by the microdilution method based on the CLSI M27-A2 guidelines. The effects of the interactions were assessed by two non-parametric approaches (fractional inhibitory concentration index model and ΔE model). In the time–kill assay, a colony counting method was employed against one azole-S strain and one azole-R strain at 0, 6, 12, 24 and 48 h of incubation at 35°C.

Results: Good concordance was found between the chequerboard method and time–kill curves. Indifference or synergism was observed for azole-S isolates in interactions of azoles and cyclosporin A, while strong synergism was observed for azole-R isolates in all drug combinations.

Conclusions: Cyclosporin A showed potent synergism when combined with the three azoles, especially against azole-R C. albicans strains, and there was good agreement between various methods used in this study.

Keywords: microdilution, spectrophotometric method, non-parametric approaches, colony counting

Introduction

Opportunistic yeast infections, particularly caused by Candida species, are the most prevalent fungal infections of humans and are a serious concern for patients with compromised immune systems, such as cancer patients, transplant recipients or human immunodeficiency virus (HIV)-infected patients.¹,² Of all the Candida infections, Candida albicans represents an increasing challenge for clinicians. Epidemiology shows that infections caused by C. albicans are continuously increasing,³ owing to more aggressive management of medical and surgical cases.

Compared with bacterial infections, few drugs are available to treat fungal infections. This is largely attributable to the eukaryotic nature of fungal cells and the difficulty in identifying unique targets not shared with human hosts. Azole antifungal agents have been shown to be effective in the treatment of candidemia and are currently used as first-line drugs. But the azole derivatives have a drawback in antifungal activity, that is, they are only fungistatic. Their efficacy relies on the function of the host defences and is limited in cases of profound neutropenia or immunocompromised patients. This characteristic probably contributes to the development of resistance seen in clinical isolates from immunocompromised patients, which continues to represent major challenges; therefore, new treatments for systemic infections need to be developed.⁴,⁵ Thus, data on the antifungal effect of azoles in combination with partner drugs would be very useful.

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Cyclosporin A is a natural product from a soil fungus that exhibits antifungal activity, it has subsequently been found to have potent immunosuppressive activity and has revolutionized modern transplantation. The immunosuppressant cyclosporin A can suppress T cell proliferative responses by inhibiting calcineurin, a Ca"^+"-calmodulin-dependent protein phosphatase important in cell signalling. The calcineurin pathway has been shown to be critical in fungal survival and stress responses in several fungi, including C. albicans, Saccharomyces cerevisiae and Cryptococcus neoformans. In C. albicans, it has previously been shown that calcineurin activity is required for survival during membrane perturbation with azoles. Previous studies showed that calcineurin inhibitor agents exhibited a potent fungicidal synergism with fluconazole in both in vitro and in vivo experiments against Aspergillus fumigatus and Candida spp. These studies stimulated our interest to research further.

In vitro activities between drugs can be evaluated by several approaches, such as the chequerboard method, time−kill curves and the Etest method. Various methods may yield different results. In this study, we used two kinds of method (chequerboard and time−kill curves) and two interaction models to assess the in vitro interactions between tested drugs. The two models were the fractional inhibitory concentration index (FICI) model and the change in percentage growth (ΔE) model, which were based on Loewe additivity (LA) and Bliss independence (BI) theories, respectively. The LA theory is based on the idea that a drug cannot interact with itself, while the BI theory is based on the idea that two drugs act in ways that are probably independent. Our aim was dual: (i) to investigate the in vitro interactions between cyclosporin A and three azoles (fluconazole, itraconazole and voriconazole) against clinical isolates of C. albicans susceptible or resistant to azoles; and (ii) to compare the agreement of different methods.

**Materials and methods**

**Strains and media**

Ten C. albicans strains were tested in this study. These included five azole-susceptible (azole-S) isolates (CA5, CA8, CA12, CA14 and CA129) and five azole-resistant (azole-R) isolates (CA10, CA15, CA16, CA135 and CA137). Isolate screening was performed according to CLSI M27-A2 guidelines, in which MICs of the three azoles against tested strains were defined as: fluconazole MIC ≤ 8 µg/mL, itraconazole MIC ≤ 0.125 µg/mL and voriconazole MIC ≤ 1 µg/mL for azole-S strains; and fluconazole MIC ≥ 64 µg/mL, itraconazole MIC ≥ 1 µg/mL and voriconazole MIC ≥ 4 µg/mL for azole-R strains. All of the tested strains were clinical isolates from patients with invasive fungal infections in our hospital. Candida parapsilosis (ATCC 22019) and C. albicans (ATCC 10231) were used as quality control strains. The isolates were stored at 4°C on Sabouraud dextrose agar plates and subcultured at least twice at 35°C before each experiment to ensure viability and purity. The liquid medium used was RPMI 1640 (with L-glutamine, without sodium bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands) supplemented with 0.165 M MOPS (morpholinoenepanesulfonic acid) buffer (Pengyuan biological technology limited company, Jinan, China) and dextrose to a final concentration of 2% with the pH adjusted to 7.0 ± 0.1 at 22°C. Sabouraud dextrose with 2% agar (Tian He microbiological agent limit company, Hang Zhou, China) was used to subculture the tested strains and for colony counting.

**Preparation of drug stock solutions**

All solutions were prepared with powders from the same lot. Fluconazole was provided by Cheng Chuang Pharmaceutical Co., Ltd, China, itraconazole was provided by Xian-Jansen Pharmaceutical Co., Ltd, China, voriconazole was provided by Chengdu Qihe Pharmaceutical Co., Ltd, China and cyclosporin A was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. Fluconazole was dissolved in distilled water at a final concentration of 2560 mg/L. Itraconazole, voriconazole and cyclosporin A were dissolved in dimethyl sulphoxide at final concentrations of 25 600 mg/L, respectively. The stock solutions were stored at −70°C until use. Serial 2-fold dilutions were prepared according to CLSI (formerly NCCLS) M27-A2. Dilutions were made in RPMI 1640 medium.

**Inoculum preparation**

A single colony of each isolate to be tested was grown overnight on Sabouraud dextrose agar at 35°C and was then subcultured on the same medium for a further 24 h at 35°C. The inoculum was prepared by diluting the overnight culture with 0.9% NaCl to 4.5 × 10^8 cfu/mL by comparing to the isolate density standard (National Institute for Drug and Biological Products Identification, China). Then the yeast suspension was further diluted in RPMI 1640 to a final inoculum ranging between 0.5 × 10^3 and 2.5 × 10^3 cfu/mL. Inoculum size was verified by plating 100 µL of serial dilutions of each inoculum onto a Sabouraud dextrose agar plate and incubation until the colony growth became visible.

For the quality controls, the same procedure as above was followed to obtain a final inoculum ranging from 0.5 × 10^3 to 2.5 × 10^3 cfu/mL.

**Susceptibility testing**

In order to choose the appropriate range of drug concentrations for the combination studies, the MICs of the individual drugs were determined for each strain by a broth microdilution method described in the CLSI M27-A2 for yeasts. The test was performed in 96-well flat-bottomed microtitration plates, which were incubated at 35°C without shaking for 48 h. The fungal growth was quantified by using a visual observation method and a spectrophotometric method after 48 h of incubation at 35°C, with MIC-1 and MIC-2 obtained from the two methods, respectively. Growth was graded on a scale from 0 to 4, as follows: 4 indicated no reduction in growth, 3 indicated a 25% reduction of growth, 2 indicated a 50% reduction of growth, 1 indicated a 75% reduction of growth and 0 indicated an optically clear well. Optical density (OD) was read with a Microplate Reader (Thermolabsystems Multiskan MK3) at 492 nm following incubation for 48 h. The percentage of growth in each well was calculated as the OD of each well/OD of the drug-free well after subtracting the background OD obtained from microorganism-free microtitre plates processed in the same manner as the inoculated plates. The MIC endpoint was defined as the lowest concentration of the drugs (alone or in combination) showing 80% inhibition of growth compared with that of the drug-free control. Each experiment was performed in triplicate, and the results were reported as geometric means and ranges.

**Chequerboard method**

The combination studies were performed using a broth microdilution method according to CLSI M27-A2 guidelines for yeasts. Briefly,
each drug was serially diluted 2-fold in RPMI 1640 medium. The final drug concentrations after the addition of 100 μL of inoculum ranged from 128 to 0.125 mg/L for fluconazole, 8 to 0.008 mg/L for itraconazole and voriconazole, and 2 to 0.031 mg/L for cyclosporin A. Then the microtitration plates were incubated for 48 h at 35°C. The growth in each well was quantified by using a visual observation method or a spectrophotometric method in the same manner as the susceptibility testing. Therefore, the growth inhibitory effects of the drugs alone or in combinations were estimated based on the results of two methods. Each experiment was performed in triplicate, and the results were reported as medians and ranges.

Interaction models
In order to assess the nature of the in vitro interactions between the three azoles and cyclosporin A against each C. albicans strain, the data obtained were analysed using two non-parametric models, the FICI model and the ΔE model, based on two no-interaction theories (LA and BI theories, respectively). In the LA-based model, concentrations of the drugs, alone or in combination, which produce the same effect, are compared, while in the BI-based models, the estimates of the combined effect based on the effect of the individual drugs were compared with those obtained by the experiment.

FICI model
Based on LA theory, the FICI was described by the following equation:

$$\Sigma \text{FIC} = \text{FIC}_A + \text{FIC}_B = \frac{C_{A,\text{comb}}}{C_{A,\text{alone}}} + \frac{C_{B,\text{comb}}}{C_{B,\text{alone}}}$$

In the formula above, $C_{A,\text{alone}}$ and $C_{B,\text{alone}}$ are the MICs of the drugs A and B when acting alone and $C_{A,\text{comb}}$ and $C_{B,\text{comb}}$ are concentrations of the drugs A and B at the iso-effective combinations, respectively. Since each triplicate experiment yielded several $\Sigma$FICs, among all $\Sigma$FICs calculated for each replicate, the FICI was determined as the $\text{FIC}_{\text{min}}$ (the lowest FIC) when the $\text{FIC}_{\text{max}}$ (the highest FIC) was $<4$; otherwise, the FICI was determined as the $\text{FIC}_{\text{max}}$. The percentages of fungal growth for each combination were calculated by comparing the OD of the drug-containing well obtained directly from the experimental data. The FICI of growth of the theoretical non-interactive combination of the drugs A and B, respectively, and $E_A$ and $E_B$ are the experimental percentages of growth of each drug acting alone, respectively. Interaction between drugs is described by the difference ($\Delta E$) between the predicted and measured percentages of growth with drugs at various concentrations. Because of the nature of interaction testing using microtitre plates with 2-fold dilutions of either drug, this results in a $\Delta E$ for each drug combination. In the non-parametric response surface approach described by Prichard et al., $E_A$ and $E_B$ are obtained directly from the experimental data.

For each combination of the two drugs in each of the three independent experiments, the observed percentage growth obtained from the experimental data was subtracted from the predicted percentage. When the average difference was positive and the 95% CI among the three replicates did not include 0, SS (statistically significant) synergy was claimed; when the difference was negative and the 95% CI did not include 0, SS antagonism was claimed. In any other case, BI was concluded.

To summarize the interaction surface of the three replicate experiments, the sums of the percentages of all SS synergistic ($\Sigma SYN$) and antagonistic ($\Sigma ANT$) interactions were calculated. Interactions with $<100\%$ SS interactions were considered weak, those with 100% to 200% of SS interactions were considered moderate and those with $>200\%$ of SS interactions were considered strong. In addition, the number of SS synergistic and antagonistic combinations among the 77 combinations tested was calculated for each strain.

Time–kill curves
According to the method described previously, the time–kill curves were performed for the combination of three azoles plus cyclosporin A against two isolates: one was susceptible to azoles (CA14, fluconazole MIC 0.25–0.5 mg/L) and another was resistant (CA10, fluconazole MIC ≥256 mg/L). Tubes (5 mL volume) containing cyclosporin A (0.5 mg/L), fluconazole (10 mg/L), itraconazole (1 mg/L), voriconazole (1 mg/L), fluconazole/cyclosporin A (10 and 0.5 mg/L, respectively), itraconazole/cyclosporin A (1 and 0.5 mg/L, respectively) and voriconazole/cyclosporin A (1 and 0.5 mg/L, respectively) at the concentrations achievable in vivo and 10^5 cfu/mL of the tested isolates were incubated at 35°C. A drug-free tube served as a growth control. To study the effects of these different treatments on fungal growth after 0, 6, 12, 24 and 48 h of incubation, an aliquot (100 μL) from each test tube was subcultured after serial dilution on Sabouraud dextrose agar plates and incubated for 48 h at 35°C. The cfu for each incubation time point per mL is plotted as a function of time, resulting in ‘time–kill’ curves for each drug combination tested. The results were reported as the mean colony counts from triplicate experiments.

Synergism was defined as a decrease in cfu/mL of $\geq 2 \log_{10}$ and indifference as a decrease in cfu/mL of $<2 \log_{10}$ compared with the most active drug, and antagonism as an increase in cfu/mL $\geq 2 \log_{10}$ compared with the most inactive drug.

Results
Chequerboard method
The in vitro antifungal effects of three azoles and cyclosporin A against 10 C. albicans isolates were tested alone and in combination at concentrations of 128–0.125 mg/L for fluconazole, 8–0.008 mg/L for itraconazole and voriconazole and 2–0.031 mg/L for cyclosporin A, respectively. The MICs of fluconazole against
ATCC 22019 and ATCC 10231 were 2–4 and 0.5–2 mg/L, respectively, both within the reference ranges.24,25 MIC-1 (data not shown) and MIC-2 were obtained using the two methods described above (Table 1). Figure 1 shows the fungal inhibition of fluconazole when used in combination with cyclosporin A against azole-R isolate CA10.

Fluconazole/cyclosporin A combination. There was good agreement between the FICI and ΔE methods. For the azole-S isolates tested, the fluconazole/cyclosporin A combination displayed synergism against three strains (FICI-1) or two strains (FICI-2), respectively (range, 0.25–2.00) by the FICI method. By the ΔE method, three strains showed very low percentages of synergistic and antagonistic interactions, while two strains showed relatively high percentages of synergistic interactions, ranging from 13% to 176% and −86% to −17.4%, respectively. For the azole-R isolates, the interaction between fluconazole and cyclosporin A was synergistic by the FICI method, with FICIs ranging from 0 to 0.20. By the ΔE method, all five resistant strains showed very high percentages of synergistic interactions, ranging from 952.7% to 1503% and −72.5% to −5.7%, respectively (Table 2).

Itraconazole/cyclosporin A combination. For the azole-S isolates tested, the itraconazole/cyclosporin A combination displayed indifference against four strains and synergism against one strain based on FICI-2 (range, 0.50–1.00) by the FICI method. By the ΔE method, two strains showed very low percentages of synergistic and antagonistic interactions, and three strains showed relatively high percentages of synergistic interactions, ranging from 26.3% to 180.4% and 13% to 176%, respectively. For the azole-R isolates, the interaction between itraconazole and cyclosporin A was synergistic by the FICI method, with FICIs ranging from 0–0.25. By the ΔE method, all five strains showed very high percentages of synergistic interactions, ranging from 719% to 1760.6% and −57.5% to 0%, respectively (Table 3).

Voriconazole/cyclosporin A combination. For the azole-S isolates tested, the FICI-1 and FICI-2 showed good consistency...
with respect to the voriconazole/cyclosporin A combination displaying indifference against four strains and synergism against one strain (range, 0.25–2.02) based on the FICI method. By the DE method, two strains showed very low percentages of synergistic and antagonistic interactions, while three strains showed relatively high percentages of synergistic interactions, ranging from 59.4% to 223.5% and −94.5% to −1%, respectively. For the azole-R isolates, the interaction between voriconazole and cyclosporin A was synergistic by both the FICI method and the DE method against all five strains (Table 4).

In general, by using the FICI model, the combinations of the three azoles and cyclosporin A displayed indifference or synergism against azole-S strains, while displaying strong synergism against azole-R strains. By the DE method, the greatest synergistic interaction was observed for itraconazole and cyclosporin A, followed by fluconazole and cyclosporin A against azole-R strains.

**Time–kill curves**

The time–kill plots of the three azoles and cyclosporin A alone and in combination are shown in Figure 2. The viable counts for the growth controls of both strains were comparable. The antifungal effect of azoles was more marked against the azole-S strain than the azole-R strain. Cyclosporin A, however, showed no antifungal activity against either the azole-S or the azole-R strain when used alone.

**Fluconazole/cyclosporin A combination.** Against the azole-S strain, the combination of fluconazole and cyclosporin A showed an indifferent effect, with mean viable counts after 48 h of incubation of $1.0 \times 10^3$ cfu/mL. Compared with the most active drug (fluconazole) alone, there was a $1.98 \log_{10}$ cfu/mL decrease when drug was used in combination (Figure 2a). However, a synergistic effect was observed when fluconazole was combined with cyclosporin A against the azole-R strain, with mean viable counts after 48 h of incubation of $1.0 \times 10^4$ cfu/mL. Therefore, there was a $2.51 \log_{10}$ cfu/mL decrease when cyclosporin A was combined with fluconazole compared with fluconazole alone (Figure 2b).

**Itraconazole/cyclosporin A combination.** For the itraconazole/cyclosporin A combination, a synergistic effect was found against both azole-S and azole-R isolates. For the azole-S isolate, the mean viable counts after 48 h of incubation were $1.0 \times 10^3$ cfu/mL when challenged by the itraconazole/cyclosporin A combination. So compared with itraconazole alone, there was a $2.43 \log_{10}$ cfu/mL decrease when drug was used in combination. For the azole-R strain, the mean viable counts after 48 h of incubation were $2.0 \times 10^4$ cfu/mL when challenged by the drug combination. There was a $2.15 \log_{10}$ cfu/mL decrease when cyclosporin A was combined with itraconazole compared with itraconazole alone.

**Voriconazole/cyclosporin A combination.** The combination of voriconazole and cyclosporin A showed an indifferent effect on the azole-S strain (mean viable counts after 48 h of incubation of $1.2 \times 10^4$ cfu/mL). Compared with voriconazole alone, there was a $1.38 \log_{10}$ cfu/mL decrease when drug was used in combination. However, a synergistic effect was observed when voriconazole was combined with cyclosporin A against the azole-R strain (mean viable counts after 48 h of incubation of $1.0 \times 10^4$ cfu/mL). Compared with voriconazole alone, there was a $2.60 \log_{10}$ cfu/mL decrease when cyclosporin A was used in combination with voriconazole.

**Discussion**

From the data above, we can conclude that cyclosporin A shows a potent antifungal effect against clinical *C. albicans* isolates, particularly against azole-R strains, when combined with three azoles—namely, fluconazole, itraconazole and voriconazole, although it shows very weak antifungal activity when used alone. These results were verified by a chequerboard method and time–kill curves.

In the chequerboard assay, to analyse the interaction between the three azoles and cyclosporin A, we used two non-parametric approaches, FICI and DE models, based on LA and BI no-interaction theories, respectively.

The FICI model is the most frequently used method to determine the interaction between antifungal drugs. However, the interpretation of the FICI in concluding synergy or antagonism
is a problem in itself. Since the determination of the MIC is sensitive to dilution errors and 2-fold dilutions are used in determining MICs, the MIC is usually taken to be accurate to within one dilution. Since the FICIs are determined from two drugs, the value of the FICI may also differ between experiments. In the interpretation of a single FICI, therefore, a value of \( \frac{1}{2} \) is usually considered to indicate antagonism and a value of \( \geq 2 \) is usually considered to indicate synergy. This is one of the approaches we also took in interpreting the results. However, because we performed triplicate experiments for each strain combination studied, we were also able to interpret the results of the FICI model for all replicates as one outcome (synergy, antagonism or indifference), thereby constraining the inter-experimental error. Thus, when the results of all three replicates were concordant, synergy or antagonism was claimed if FICIs were below 1 or above 1, respectively. In all other cases indifference was concluded. Another alternative approach would have been a more statistical approach, using the mean of the three replicates and the 95% CI; if the 95% CI does not include 1, synergism or antagonism could be concluded. However, because the distribution of the FICIs is not normal, we preferred the approach described above.

Table 2. *In vitro* interaction between fluconazole and cyclosporin A against *C. albicans*

<table>
<thead>
<tr>
<th>Strain</th>
<th>FICI model [median (range)]</th>
<th>ΔE model</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FICI-1</td>
<td>INT</td>
<td>FICI-2</td>
</tr>
<tr>
<td>CA5</td>
<td>0.5 (0.5–2)</td>
<td>IND</td>
<td>1 (1–2)</td>
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<tr>
<td>CA8</td>
<td>0.5 (0.5–1)</td>
<td>SYN</td>
<td>1 (0.5–1)</td>
</tr>
<tr>
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<td>0.5 (0.25–0.5)</td>
<td>SYN</td>
<td>0.25 (0.25–0.5)</td>
</tr>
<tr>
<td>CA14</td>
<td>2 (1–4)</td>
<td>IND</td>
<td>2 (1–4)</td>
</tr>
<tr>
<td>CA129</td>
<td>0.5 (0.5)</td>
<td>SYN</td>
<td>0.5 (0.25–0.5)</td>
</tr>
<tr>
<td>Median</td>
<td>0.5</td>
<td>1</td>
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</tr>
<tr>
<td>CA10</td>
<td>0 (0)</td>
<td>SYN</td>
<td>0 (0)</td>
</tr>
<tr>
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<td>0 (0–0.01)</td>
<td>SYN</td>
<td>0 (0)</td>
</tr>
<tr>
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<td>0.01 (0–0.02)</td>
<td>SYN</td>
<td>0.01 (0–0.01)</td>
</tr>
<tr>
<td>CA135</td>
<td>0.02 (0–0.02)</td>
<td>SYN</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CA137</td>
<td>0 (0)</td>
<td>SYN</td>
<td>0 (0)</td>
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<tr>
<td>Median</td>
<td>0</td>
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INT, interpretation; IND, indifference; SYN, synergism; ANT, antagonism.

Table 3. *In vitro* interaction between itraconazole and cyclosporin A against *C. albicans*

<table>
<thead>
<tr>
<th>Strain</th>
<th>FICI model [median (range)]</th>
<th>ΔE model</th>
<th></th>
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<tr>
<td></td>
<td>FICI-1</td>
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<td>FICI-2</td>
</tr>
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<td>CA5</td>
<td>1 (1)</td>
<td>IND</td>
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<td>Median</td>
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<td>0 (0)</td>
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<td>SYN</td>
<td>0 (0)</td>
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<td>SYN</td>
<td>0 (0)</td>
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<td>0 (0–0.02)</td>
<td>SYN</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CA137</td>
<td>0 (0)</td>
<td>SYN</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
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INT, interpretation; IND, indifference; SYN, synergism; ANT, antagonism.
**Table 4. In vitro interaction between voriconazole and cyclosporin A against C. albicans**

| Strain | FICI model [median (range)] | ΔE model | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FICI-1 | INT | FICI-2 | INT | ΣSYN (n) | ΣANT (n) | INT |
| CA5 | 1 (1) | IND | 1 (1–2) | IND | 59.4 (2) | −94.5 (8) | IND |
| CA8 | 1 (0.5–2) | IND | 1 (0.25–1) | IND | 223.5 (34) | −1 (1) | SYN |
| CA12 | 0.5 (0.5–1) | IND | 0.5 (0.25–1) | IND | 107.3 (19) | −3 (6) | SYN |
| CA14 | 2 (1–2) | IND | 0.5 (0.25–1) | IND | 77.9 (24) | −37.8 (7) | IND |
| CA129 | 0.25 (0.25–0.5) | SYN | 0.25 (0.25–0.5) | SYN | 195.2 (14) | −31.5 (3) | SYN |
| Median | 1 | 1 | | | 77.9 | −31.5 | |
| CA10 | 0 (0) | SYN | 0 (0) | SYN | 858.8 (39) | 0 (0) | SYN |
| CA15 | 0 (0) | SYN | 0 (0) | SYN | 695.8 (35) | −40.5 (5) | SYN |
| CA16 | 0 (0) | SYN | 0.02 (0–0.02) | SYN | 787.6 (31) | −6.9 (2) | SYN |
| CA135 | 0 (0) | SYN | 0.01 (0–0.02) | SYN | 712.1 (34) | −53.4 (6) | SYN |
| CA137 | 0.01 (0–0.01) | SYN | 0 (0) | SYN | 516 (30) | −40 (6) | SYN |
| Median | 0 | 0 | | | 712.1 | −40 | |

INT, interpretation; IND, indifference; SYN, synergism; ANT, antagonism.

Figure 2. Time–kill curve plots of azoles (fluconazole, itraconazole, voriconazole) and cyclosporin A alone and in combination against azole-S isolate CA14 (a, c and e) and azole-R isolate CA10 (b, d and f) for 48 h with drug concentrations of 10 mg/L fluconazole (a, b), 1 mg/L itraconazole (c, d), 1 mg/L voriconazole (e, f) and 0.5 mg/L (cyclosporin A). Filled squares, control; diamonds, cyclosporin A; triangles, azole; open squares, azole+cyclosporin A. The starting inoculum was ~10⁷ cfu/mL. At 0, 6, 12, 24 and 48 h, aliquots were removed from each test tube to determine the number of cfu/mL.
experiment was also performed in triplicate. For the non-parametric method of this model, the percentages of fungal growth were derived from the experiment data directly. Good agreement was found between the FICI and $\Delta E$ models. Therefore, the $\Delta E$ model, developed in recent years, is a useful instrument for analysing the nature of interactions between different drugs based on a checkerboard method.

In addition, by using visual observation and OD determination methods, MIC-1 and MIC-2 were obtained, respectively. There was also good concordance between the two approaches for endpoint reading, showing that OD determination was suitable for interaction studies as well.

Interaction effect between drugs was confirmed by time–kill curves. This method is capable of detecting differences in the rate and extent of antifungal activity over time and has been widely used in recent years.\textsuperscript{26–28} In this method, a starting inoculum of $10^5$ cfu/mL was exposed to drugs for testing at special concentrations achievable \textit{in vivo}. The time–kill tubes were vortexed before the removal of each sample for colony count determination, and colony count samples were obtained at 0, 6, 12, 24 and 48 h. According to the results, either synergism (itraconazole/cyclosporin A) or indifference (fluconazole/cyclosporin A and voriconazole/cyclosporin A) was observed for drug combinations against theazole-S strain CA14, while the combination of the three azoles and cyclosporin A produced potently synergistic action against theazole-R strain CA10 \textit{in vitro}. Therefore, there was some disagreement between the conclusions drawn by the FICI method and the time–kill curves for the azole-S strain, while the two methods produced the same results for the azole-R strain.

There are previous studies showing a synergistic effect of cyclosporin A in combination with fluconazole against \textit{C. albicans}.\textsuperscript{3,12} These studies confirmed the synergism of cyclosporin A with fluconazole and investigated the mechanisms, but they did not involve other azole agents and they did not assess the interaction between drugs by LA or BI theories. Our \textit{in vitro} studies showed that when used in combination with the three azoles (fluconazole, itraconazole and voriconazole), cyclosporin A exhibited a strong synergism againstazole-R isolates of \textit{C. albicans}. Besides, this study also shows that an OD determination method and consequent analysis by using two non-parametric models are useful approaches to determine the interaction between drugs against \textit{C. albicans}, and there was good agreement between various methods. Because the drug concentrations used in this study are achievable \textit{in vitro}, this new finding of combining three azoles with cyclosporin A might provide a clue for the treatment of fungal infections. However, the correlation between \textit{in vitro} and \textit{in vivo} studies still needs to be studied further.

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Transparency declarations

None to declare.

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