

## In Vitro Interactions between Tacrolimus and Azoles against *Candida albicans* Determined by Different Methods<sup>∇</sup>

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Combination therapy could be of use for the treatment of fungal infections, especially those caused by drug-resistant fungi. However, the methods and approaches used for data generation and result interpretation need further optimizing. The fractional inhibitory concentration index (FICI) is the most commonly used method, but it has several drawbacks in characterizing antifungal drug interaction. Alternatively, some new methods can be used such as the  $\Delta E$  model (difference between the predicted and measured fungal growth percentages) and the response surface approach, which uses the concentration-effect relationship over the whole concentration range instead of just the MIC. In the present study, in vitro interactions between tacrolimus (FK506) and three azoles—fluconazole (FLC), itraconazole (ITR), and voriconazole (VRC)—against *Candida albicans* were evaluated by the checkerboard microdilution method and time-killing test. The intensity of the interactions was determined by visual reading and the spectrophotometric method in a checkerboard assay, and the nature of the interactions was assessed by nonparametric models of FICI and  $\Delta E$ . Colony counting and colorimetric viable detection methods (2,3-bis {2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium hydroxide} [XTT] reduction test) were used for evaluating the combination antifungal effects over time. Synergistic and indifferent effects were found for the combination of FK506 and azoles against azole-sensitive strains, while strong synergy was found against azole-resistant strains analyzed by FICI. The  $\Delta E$  model gave more consistent results with FICI. The positive interactions were also confirmed by the time-killing test. Our findings suggest a potential role for combination therapy with calcineurin pathway inhibitors and azoles to augment activity against resistant *C. albicans*.

The incidence of invasive fungal infections has increased significantly in the last several decades, paralleling the increase in the number of immunocompromised patients (15, 34). *Candida* infections represent an increasing challenge for clinicians. The epidemiology of the last decade shows that these infections are continuously increasing (21), especially in patients with compromised immune systems. *Candida albicans* is the causative agent of most candidiasis (33).

Azoles are a widely applied class of antifungal agents, and fluconazole (FLC) has been shown to be as effective as amphotericin B in the treatment of candidemia in nonneutropenic patients (42). Since amphotericin B is toxic in its conventional form and very expensive in its new lipidic forms, azole antifungal agents are currently used as first-line drugs (13) because of their excellent oral bioavailability, stable parenteral formulation, and especially their low toxicity. However, with the increasing clinical use of azole, resistance is emerging in clinical isolates from immunocompromised patients. In addition, azole is only fungistatic; this characteristic probably contributes to the development of resistance. The emergence of *C. albicans* strains with decreased susceptibility complicates the

management of these infections (9, 29). Therefore, new approaches for treating these infections are warranted.

Combination therapy is one approach that can be used to improve the efficacy of antimicrobial therapy for difficult-to-treat infections (1). Attempts have been made to cope with treatment failures either by combining different antifungals or by combining antifungals with nonantifungals (1, 2, 20, 21, 24, 26, 33). However, assessing the nature and intensity of drug interactions is still a debated issue. The observed in vitro interaction of two agents depends on different methodology for data generation and different approaches for data analysis, resulting in variable as well as controversial conclusions (5, 14, 37). In the present study, we investigated the combined effects of three azoles and FK506 against *C. albicans* by the checkerboard microdilution method and the time-killing test. New methods and interpretation models such as the spectrophotometric method and the  $\Delta E$  model were employed in comparison with the traditional methods of MIC visual reading and fractional inhibitory concentration index (FICI) combination interpretation. The colorimetric method was compared with colony counting in a time-killing study.

### MATERIALS AND METHODS

**Strains.** Ten clinical isolates of *C. albicans* were tested in this study, including five azole-susceptible isolates (CA5, CA8, CA12, CA14, and CA129) and five azole-resistant isolates (CA10, CA15, CA16, CA135, and CA137). All the strains were isolates from patients with invasive candidiasis from our hospital and were confirmed according to standard mycological methods (3, 12, 35) by the Microbiological Research Laboratory, the Center of Health Research and Epidemic

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Prevention, Shandong Province. Their susceptibilities to azoles were tested according to CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS) method M27-A2 (27). In addition, *Candida parapsilosis* (ATCC 22019) and *Candida albicans* (ATCC 10231) were used as quality controls. All the isolates were stored at  $-70^{\circ}\text{C}$ .

**Medium.** The medium used for the broth microdilution method was RPMI 1640 (pH 7.0; with L-glutamine but without sodium bicarbonate; GIBCO BRL, Life Technologies, Woerden, The Netherlands) supplemented with dextrose to a final concentration of 2% and 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich Chemie GmbH, Steinheim, Germany); the pH of the medium was adjusted with 0.1 M NaOH to  $7.0 \pm 0.1$  at  $22^{\circ}\text{C}$ . The medium used for the colony counting was Sabouraud dextrose agar (Tian He Microbiological Agent Co. Ltd., Hang Zhou, China).

**Inoculum preparation.** Each isolate from deep-frozen stock cultures had been grown for 7 days on Sabouraud dextrose agar at room temperature and was then subcultured on the same medium for at least three generations at  $35^{\circ}\text{C}$ . Cells were collected with a stick and suspended in 0.9% NaCl water. After thorough mixing, the turbidity of the suspension was compared with the isolate density standard (National Institute for Drug and Biological Products Identification, China). Then, each suspension was diluted in RPMI 1640 to obtain two times the final inoculum size of  $2.5 \times 10^5$  CFU/ml. The inoculum size was verified by determination of the number of viable CFU after serial dilutions of the inoculum were plated onto Sabouraud dextrose agar.

**Drugs and stock solution preparation.** The three azoles used in the present study were FLC, itraconazole (ITR), and voriconazole (VRC). FLC was kindly provided by Cheng Chuang Pharmaceutical Co., Ltd., China; ITR was bought from Xian-Jansen Pharmaceutical Co., Ltd., China; and VRC was obtained from Chengdu Qihe Pharmaceutical Co., Ltd., China. FK506 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China. A 2,560- $\mu\text{g}/\text{ml}$  stock solution was prepared for FLC by dissolving the powder in distilled water, while a 25,600- $\mu\text{g}/\text{ml}$  stock solution for the other tested drugs was prepared by dissolving ITR and VRC in dimethyl sulfoxide and FK506 in methanol. The stock solutions were stored at  $-70^{\circ}\text{C}$  until use.

**Drug susceptibility testing.** The susceptibilities to all tested drugs were studied by the broth microdilution method according to the CLSI standard M27-A2 (27). The test was carried out in 96-well flat-bottomed microtiter plates. In order to choose the appropriate range of concentrations for different drugs against strains with different susceptibilities, the MICs of individual drugs were determined in an exploratory study for each strain. After agitation for 15 s, the plates were incubated at  $35^{\circ}\text{C}$  without shaking, and readings were performed following 48 h of incubation by both visual reading and optical density (OD) determination with a spectrophotometer. For the visual reading, growth was graded on a scale from 0 to 5. For the OD determination, each plate was shaken for 5 min and the OD values at 492 nm of each well were read with a microtiter plate reader (Thermolabsystems Multiskan MK3). The MIC<sub>80</sub> was defined as the lowest drug concentration that resulted in an 80% decrease in absorbance compared with that of the control (no drug). All experiments were performed in triplicate.

**Interactions between FK506 and azoles.** The interaction between FK506 and three azoles against five susceptible *C. albicans* strains and five resistant *C. albicans* strains was tested by a microdilution checkerboard technique. Drug dilutions were prepared to obtain four times the final concentration. A total of 50  $\mu\text{l}$  of each concentration of azoles was added to columns 2 to 12, and then 50  $\mu\text{l}$  of FK506 was added to rows A to G. In the wells of column 1, 50  $\mu\text{l}$  of the medium containing 1% of the corresponding azole solvent was added, and in wells of row H, 50  $\mu\text{l}$  of the medium containing 1% of the FK506 solvent was added. Thus, row H and column 1 contained only the azole and FK506, respectively, and the well at the intersection of row H and column 1 (well H1) was the drug-free well that served as the growth control. The final drug concentrations after the addition of 100  $\mu\text{l}$  of inoculum ranged from 0.125 to 128  $\mu\text{g}/\text{ml}$  for FLC, 0.008 to 8  $\mu\text{g}/\text{ml}$  for ITR and VRC, and 0.002 to 0.125  $\mu\text{g}/\text{ml}$  for FK506, and the final inoculum size was  $2.5 \times 10^5$  CFU/ml. Plates were incubated at  $35^{\circ}\text{C}$  for 48 h. Then visual reading of MICs was performed, and OD values were measured at 492 nm. The value of background OD was subtracted from that of each well. Each isolate was tested in triplicate on different days. 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 microtiter plates processed in the same manner as the inoculated plates.

**Drug interaction interpretation.** In order to assess the nature of the in vitro interactions between the three azoles and FK506 against each isolate, the data obtained by the spectrophotometric method were analyzed using two models, FICI and  $\Delta E$ , that have been used to characterize antifungal drug interactions (24, 48).

Many models and approaches have been described for the assessment of in

vitro drug interactions and extensively reviewed (14, 38). The assumption of no interaction has a central position in these debates, since synergy and antagonism are defined as departures from this. Thus, when the observed effects are more or less than the effect predicted from the no-interaction theory, synergy or antagonism, respectively, is claimed. Among the various no-interaction theories, the Loewe additivity (LA) and Bliss independence (BI) theories are the two major competitor candidates for referenced models (14, 37–39). FICI and  $\Delta E$  are nonparametric models based on LA theory and BI theory, respectively, while the response surface approach is a parametric model based on LA theory which has been proven to be inadequate for this study because one of the combined agents has little or no antifungal activity (1). In the FICI model, based on LA theory, concentrations of the drugs, alone or in combination, which produce the same effect are prepared, while in the BI-based  $\Delta E$  model, the estimates of the combined effects based on the effects of the individual drugs were compared with those obtained from the experiment.

**FICI.** Based on LA theory, the FICI method, a nonparametric model, was defined as the following equation:  $\text{FICI} = \text{FICI}_A + \text{FICI}_B = C_A^{\text{comb}}/\text{MIC}_A^{\text{alone}} + C_B^{\text{comb}}/\text{MIC}_B^{\text{alone}}$ , where  $\text{MIC}_A^{\text{alone}}$  and  $\text{MIC}_B^{\text{alone}}$  are the MICs of drugs A and B when acting alone and  $C_A^{\text{comb}}$  and  $C_B^{\text{comb}}$  are concentrations of drugs A and B at the isoeffective combinations, respectively (24, 48). Off-scale MICs were converted to the next highest or the next lowest doubling concentration. Among all FICIs calculated for each data set, the  $\text{FICI}_{\text{min}}$  was reported as the FICI in all cases unless the  $\text{FICI}_{\text{max}}$  was higher than 4; in these cases, the  $\text{FICI}_{\text{max}}$  was reported as the FICI.

The interpretation of the FICI was as follows: a FICI value of  $\leq 0.5$  revealed synergy, a value of 1 to 4 revealed indifference, and a value of  $>4$  represented antagonism (30).

**$\Delta E$  model.** The model of difference in fungal growth percentage was based on BI theory, which is described by the equation  $I_i = (I_A + I_B) - (I_A \times I_B)$ , where  $I_i$  is the predicted percentage of inhibition of the theoretical combination of drug A and B, and  $I_A$  and  $I_B$  are the experimental percentages of inhibition of each drug acting alone, respectively. Since  $I$  is equal to  $1 - E$ , where  $E$  is the percentage of growth, and by substitution into the former equation, the following equation is derived:  $E_i = E_A \times E_B$ , where  $E_i$  is the predicted percentage of growth of the theoretical combination of drug A and drug B, respectively, and  $E_A$  and  $E_B$  are the experimental percentages of growth of each drug action alone, respectively. Interaction is described by the difference  $\Delta E$  between the predicted and the measured percentages of growth at various concentrations ( $\Delta E = E_{\text{predicted}} - E_{\text{measured}}$ ). By the nonparametric approach described by Prichard et al. (39, 40),  $E_A$  and  $E_B$  are obtained directly from the experimental data. Because of the nature of interaction, testing with microtiter plates and a twofold dilution of either drug results in a  $\Delta E$  for each drug combination. In each of the three independent experiments, the observed percentages of growth obtained from the experimental data were subtracted from the predicted percentages, and then the average difference of three experiments was calculated. When the average difference as well as its 95% confidence interval among the three replicates was positive, statistically significant synergy was claimed; when the difference as well as its 95% confidence interval was negative, significant antagonism was claimed. In any other case, BI was concluded.

To summarize the interaction surface, the sums of the percentages of all statistically significant synergistic ([sum]SYN) and antagonistic ([sum]ANT) interactions were calculated. Interactions with  $<100\%$  statistically significant interactions were considered weak, interactions with 100% to 200% statistically significant interactions were considered moderate, and interactions with  $>200\%$  statistically significant interactions were considered strong, as described previously (24). In addition, the numbers of statistically significant synergistic and antagonistic combinations among all tested drug combinations were calculated for each strain.

**Time-killing test.** We performed an in vitro time-killing test to investigate the activity of the calcineurin inhibitor FK506 with and without azoles against one susceptible strain, CA14, and one resistant strain, CA10, at the starting inoculum of  $10^5$  CFU/ml. The concentrations were 10  $\mu\text{g}/\text{ml}$  for FLC, 1  $\mu\text{g}/\text{ml}$  for ITR and VRC, and 0.016  $\mu\text{g}/\text{ml}$  for FK506 (the concentrations of all drugs were within the range achievable in human plasma). A drug-free sample served as a growth control. Dimethyl sulfoxide comprised  $<1\%$  of the total test volume. At predetermined time points (0, 6, 12, 24, and 48 h after incubation at  $35^{\circ}\text{C}$ ), (i) a 100- $\mu\text{l}$  aliquot was removed from every solution and serially diluted in RPMI 1640. A 100- $\mu\text{l}$  aliquot from each dilution was streaked on the Sabouraud dextrose agar plate. Colony counts were performed after incubation at  $35^{\circ}\text{C}$  for 48 h. (ii) At the same time, the XTT test was performed to detect the cell viability after different treatments according to the method described previously (16, 41). Briefly, at each time point, a 100- $\mu\text{l}$  aliquot was removed from every treatment mixture and transferred to a well of a 96-well microplate, and then a 100- $\mu\text{l}$  aliquot of

TABLE 1. Susceptibilities of *C. albican* strains against azoles alone and in combination with FK506 obtained by the spectrophotometric method<sup>a</sup>

Drug in combination and strain	Median MIC (range) of drug ( $\mu\text{g/ml}$ )			
	Alone		In combination	
	Azole	FK506	Azole	FK506
<b>FLC</b>				
Susceptible				
CA5	0.25 (0.25–0.5)	512	0.25 (0.125–0.5)	0.002 (0.002)
CA8	0.25 (0.125–0.5)	512	0.125 (0.125–0.25)	0.002 (0.002)
CA12	4 (2–4)	512	2 (1–2)	0.008 (0.004–0.008)
CA14	0.5 (0.25–0.5)	512	0.25 (0.25–1)	0.002 (0.002–0.004)
CA129	0.5 (0.5–1)	512	0.25 (0.25–0.5)	0.008 (0.004–0.016)
Resistant				
CA10	256 (256–512)	512	0.25 (0.125–0.5)	0.063 (0.016–0.125)
CA15	128 (64–128)	512	0.5 (0.25–0.5)	0.008 (0.004–0.016)
CA16	64 (64–128)	512	0.5 (0.25–2)	0.008 (0.008–0.016)
CA135	64 (64–128)	512	0.25 (0.125–0.5)	0.004 (0.004–0.008)
CA137	64 (64–128)	512	0.5 (0.25–0.5)	0.002 (0.002–0.008)
<b>ITR</b>				
Susceptible				
CA5	0.031 (0.031–0.063)	512	0.031 (0.031–0.063)	0.063 (0.016–0.063)
CA8	0.016 (0.008–0.031)	512	0.008 (0.008–0.016)	0.002 (0.002)
CA12	0.063 (0.063–0.125)	512	0.031 (0.031–0.063)	0.125 (0.063–0.125)
CA14	0.063 (0.031–0.125)	512	0.031 (0.031–0.125)	0.002 (0.002)
CA129	0.063 (0.063–0.125)	512	0.031 (0.031–0.063)	0.063 (0.063–0.125)
Resistant				
CA10	128 (64–128)	512	0.031 (0.031–0.063)	0.125 (0.063–0.125)
CA15	32 (16–64)	512	0.063 (0.031–0.125)	0.063 (0.031–0.125)
CA16	32 (16–64)	512	0.016 (0.008–0.031)	0.125 (0.125)
CA135	16 (16–32)	512	0.016 (0.016–0.031)	0.063 (0.031–0.125)
CA137	16 (16–32)	512	0.063 (0.031–0.063)	0.008 (0.008–0.031)
<b>VRC</b>				
Susceptible				
CA5	0.016 (0.016–0.031)	512	0.016 (0.016–0.031)	0.002 (0.002)
CA8	0.008 (0.008–0.016)	512	0.016 (0.008–0.031)	0.002 (0.002)
CA12	0.031 (0.008–0.031)	512	0.016 (0.008–0.031)	0.008 (0.004–0.016)
CA14	0.008 (0.004–0.016)	512	0.008 (0.008)	0.002 (0.002)
CA129	0.016 (0.016–0.031)	512	0.008 (0.008–0.016)	0.008 (0.008–0.125)
Resistant				
CA10	64 (32–64)	512	0.008 (0.008–0.016)	0.063 (0.031–0.063)
CA15	8 (4–16)	512	0.008 (0.008–0.016)	0.125 (0.063–0.125)
CA16	8 (4–8)	512	0.008 (0.008–0.016)	0.031 (0.031–0.063)
CA135	8 (4–8)	512	0.016 (0.008–0.031)	0.031 (0.016–0.063)
CA137	8 (4–16)	512	0.008 (0.008–0.016)	0.016 (0.008–0.016)

<sup>a</sup> Strains were categorized as susceptible or resistant according to the interpretive breakpoints of CLSI criteria for FLC ( $\leq 8$  and  $\geq 64$   $\mu\text{g/ml}$ , respectively), ITR ( $\leq 0.1$  and  $\geq 1$   $\mu\text{g/ml}$ , respectively), and VRC ( $\leq 1$  and  $\geq 4$   $\mu\text{g/ml}$ , respectively) (11). For azole-susceptible isolates, when drugs were used in combination, MICs of azoles showed no apparent changes, while for azole-resistant strains, MICs of azoles decreased markedly when drugs were used in combination.

XTT-menadione solution was added. (XTT was purchased from Sigma. Prior to each assay, XTT was dissolved in a saturated solution at 0.5 g/liter in Ringer's lactate. The solution was filter sterilized through a 0.22- $\mu\text{m}$  filter, and then 100 mM menadione in acetone was added to a final concentration of 10  $\mu\text{M}$ .) The plate was then incubated in the dark for up to 2 h at 35°C. After that, the absorbance of the XTT reduction product, formazan, was read at 492 nm for each well with a microtiter plate reader (Thermolabsystems Multiskan MK3). All experiments were conducted in triplicate, and the results were reported as mean values. Thus, growth- and metabolism-inhibitory effects of the drugs alone and in combination were observed based on the results of colony counting and spectrophotometric methods.

For antifungal interactions tested by the time-killing method, the following criteria were used: synergism and indifference were defined as a decrease of  $\geq 2$   $\log_{10}$  and  $< 2$   $\log_{10}$  CFU/ml with respect to the most active drug, and antagonism

was defined as an increase of  $\geq 2$   $\log_{10}$  CFU/ml with respect to the least active drug (43).

The correlation between viable cell counts determined by colony counting and OD value by XTT-menadione colorimetric readings was studied for the OD data transformed before interpretation of results.

## RESULTS

**Drug susceptibility alone and in combination.** According to the criteria of CLSI M27-A2, five susceptible and five resistant isolates were selected for this study. The MICs of two control strains, ATCC 22019 and ATCC 10231, were within the range

TABLE 2. Checkerboard showing the effect of each combination of FLC and FK506 on the spectrophotometrically measured growth of drug-resistant *C. albicans* CA10<sup>a</sup>

FK506 concn ( $\mu\text{g/ml}$ )	% Growth at FLC concn ( $\mu\text{g/ml}$ ):											
	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0.125	95	25	<b>7</b>	6	3	12	5	2	3	0	0	0
0.063	93	27	<u>11</u>	13	18	13	13	12	7	1	0	5
0.031	93	36	<b>23</b>	<b>19</b>	<b>17</b>	12	16	18	15	16	13	14
0.016	96	38	23	21	24	<b>20</b>	<b>18</b>	<b>19</b>	<b>19</b>	<b>18</b>	<b>16</b>	<b>18</b>
0.008	96	60	39	34	25	22	30	28	24	25	21	39
0.004	99	67	42	38	29	32	46	34	35	30	27	45
0.002	97	76	61	55	50	48	44	38	35	36	28	44
0	100	75	63	53	56	54	53	55	50	48	48	47

<sup>a</sup> The isoeffective combinations on the basis of which the FICIs were calculated are shown in bold. Among all the FICIs calculated based on the isoeffective combinations,  $\text{FICI}_{\text{max}}$  was  $<4$ , and so  $\text{FICI}_{\text{min}}$  (0.0006) was reported as the FICI. The combination with the lowest FICI is shown by underlining.

provided by CLSI. The results for the tested drug alone and in combination against the tested 10 isolates are summarized in Table 1. By itself, FK506 has little or no effect on the tested strains. However, when it was combined with azoles, a potent effect was revealed, especially on the resistant strains. In terms of  $\text{MIC}_{80}$ , the MICs of either individual agent were reduced by one to two dilutions against the susceptible strains, while remarked reductions were observed for three azoles against resistant strains when combined with FK506. The MICs of azoles against the resistant strains became almost the same as those against the susceptible strains. The result from the visual reading has no obvious difference from those from the OD reading (data not shown).

The percentages of fungal growth for each combination were calculated by comparing the OD of the drug-containing well with that of the drug-free well after subtracting the background OD obtained from the microorganism-free well. The interaction between FLC and FK506 against azole-resistant strain CA10 is presented in Table 2.

**Interpretation of drug interactions.** The results of the checkerboard analysis interpreted by the nonparametric methods based on both the LA and BI theories are summarized in Table 3. In the checkerboard microtiter plate format, strong synergism was concluded in all five resistant isolates analyzed by both models, FICI and  $\Delta E$ , and the two models correlated very well. However, different interpretations were obtained for the five susceptible isolates. Synergisms were observed in two strains by FICI and in one strain by  $\Delta E$  for FK506-FLC and FK506-ITR combinations, and indifference was observed in other strains, while for the FK506-VRC combination, synergism was observed in one strain by FICI and in two strains by  $\Delta E$ ; others revealed indifference. The  $\Delta E$  value of each well was the average of triplicate results. When the  $\Delta E$  value was positive, synergy was concluded, and higher  $\Delta E$  values suggested stronger synergistic interactions. [summ]SYN and [summ]ANT were the sums of the percentages for all statistically significant synergistic and antagonistic interactions among a whole checkerboard combination with different concentrations. The interaction was considered weak, moderate, and strong synergism when the [summ]SYN was  $<100\%$ ,  $100$  to  $200\%$ , and  $>200\%$ , respectively.

In addition, the values obtained for each combination based on BI theory were used to construct a three-dimensional plot. Thus, a surface plot was obtained by use of a three-dimen-

sional plot, with  $\Delta E$  depicted on the z axis (Fig. 1). The contour plot was also constructed in order to visualize the drug concentrations that produce an interaction.

**Time-killing curves.** Positive interactions were confirmed by time-kill curves. The OD value obtained from the XTT reduction assay was transformed to a colony count according to the standard curve (Fig. 2). The intensity and nature of interactions between FK506 and azoles by time-kill study are shown in Table 4, and the representative plots of the  $\log_{10}$  CFU/ml versus time for FK506 alone or in combination with three azoles are depicted in Fig. 3. In general, indifference was displayed for all three combinations against the susceptible strains, while synergism was revealed for the resistant strain. The result by metabolism inhibition (XTT reduction assay) displayed no difference from the interpretation based on colony counting except for the combination of ITR plus FK506 as shown in Table 4. Figure 3 revealed that FK506 itself can hardly affect the growth at  $0.016 \mu\text{g/ml}$  after 48 h, but the antifungal activity of azoles against the resistant strain was dramatically enhanced by addition of it. Given the initial inoculum of  $10^5$  CFU/ml, the combination yielded a  $2.37\text{-log}_{10}\text{-CFU/ml}$  decrease compared with  $10 \mu\text{g/ml}$  FLC alone (Fig. 3, R-A), a  $2.46\text{-log}_{10}\text{-CFU/ml}$  decrease compared with  $1 \mu\text{g/ml}$  ITR alone (Fig. 3, R-B), and a  $2.26\text{-log}_{10}\text{-CFU/ml}$  decrease compared with  $1 \mu\text{g/ml}$  VRC alone at 48 h (Fig. 3, R-C).

## DISCUSSION

Transplant recipients are one of the highest-risk patient groups to develop fungal infection, and it is this group of patients who are generally already receiving calcineurin inhibitors as part of their immunosuppressive regimen. FK506 is a new immunosuppressant used clinically in the prevention of allograft rejection. It can suppress T-cell-proliferative responses and inhibit calcineurin, a  $\text{Ca}^{2+}$ -calmodulin-dependent protein phosphatase that is important in cell signaling (6, 19). The calcineurin pathway has been shown to be critical in fungal survival and stress responses in several fungi (32, 44), including in vitro antifungal activity against *Saccharomyces cerevisiae*, *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (7, 8, 31).

Previous study has shown that FK506 and caspofungin exhibited a clear in vitro positive interaction against *A. fumigatus* (45, 46) and a potent fungicidal synergism with the ergosterol

TABLE 3. In vitro interaction between FK506 and azoles

Drug in combination, strain, and median	Nonparametric method <sup>a</sup>				
	FICI		$\Delta E$ model		
	Mean (range)	Interpretation	$\Sigma$ SYN ( <i>n</i> )	$\Sigma$ ANT ( <i>n</i> )	Interpretation
<b>FLC</b>					
Susceptible					
CA5	2.00 (1.00–2.00)	IND	16 (7)	–51 (11)	IND
CA8	1.00 (0.5–1.00)	IND	31 (16)	–43.2 (33)	IND
CA12	0.5 (0.25–0.50)	SYN	103.5 (23)	–45.3 (9)	SYN
CA14	1.00 (0.50–2.00)	IND	16.8 (7)	–33.9 (21)	IND
CA129	0.50 (0.25–0.50)	SYN	65.6 (8)	–36 (16)	IND
Median	1.00		31	–43.2	
Resistant					
CA10	0.00 (0.00)	SYN	1,032 (38)	–55.7 (11)	SYN
CA15	0.00 (0.00)	SYN	962.7 (34)	–28.4 (8)	SYN
CA16	0.00 (0.00–0.03)	SYN	1,322.4 (49)	–13.5 (14)	SYN
CA135	0.00 (0.00)	SYN	956 (42)	–33.4 (10)	SYN
CA137	0.00 (0.00)	SYN	1,154.3 (52)	–15.6 (6)	SYN
Median	0.00		1,032	–28.4	
<b>ITR</b>					
Susceptible					
CA5	1.00 (0.50–1.00)	IND	11.8 (4)	–38.4 (15)	IND
CA8	2.00 (0.50–2.00)	IND	13.7 (4)	–78.4 (21)	IND
CA12	0.50 (0.25–0.50)	SYN	137.6 (29)	–49.7 (16)	SYN
CA14	1.00 (0.50–1.00)	IND	33.7 (13)	–54.8 (23)	IND
CA129	0.25 (0.25–0.50)	SYN	98.1 (54)	–53 (9)	IND
Median	1.00		33.7	–53	
Resistant					
CA10	0.00 (0.00)	SYN	1,384.2 (56)	–33.4 (10)	SYN
CA15	0.00 (0.00)	SYN	1,618 (49)	–56.1 (13)	SYN
CA16	0.00 (0.00)	SYN	1,142 (57)	–43.8 (7)	SYN
CA135	0.00 (0.00)	SYN	1,512.3 (32)	–4.7 (2)	SYN
CA137	0.00 (0.00)	SYN	1,035.2 (38)	–38.1 (13)	SYN
Median	0.00		1,384.2	–38.1	
<b>VRC</b>					
Susceptible					
CA5	1.00 (1.00–2.00)	IND	42.9 (6)	–64.3 (11)	IND
CA8	1.00 (0.50–2.00)	IND	91.3 (22)	–6.5 (3)	SYN
CA12	1.00 (0.50–1.00)	IND	100.6 (26)	–5.7 (5)	SYN
CA14	1.00 (0.50–2.00)	IND	35.4 (21)	–83.2 (18)	IND
CA129	0.25 (0.25–0.50)	SYN	89.1 (27)	–32.5 (12)	IND
Median	1.00		89.1	–32.5	
Resistant					
CA10	0.00 (0.00)	SYN	1,024 (62)	–3.4 (1)	SYN
CA15	0.00 (0.00)	SYN	857.8 (42)	–33.5 (13)	SYN
CA16	0.00 (0.00)	SYN	912.1 (44)	–15.6 (6)	SYN
CA135	0.00 (0.00–0.01)	SYN	1,328 (52)	–97.4 (23)	SYN
CA137	0.00 (0.00)	SYN	764.2 (34)	–28.4 (15)	SYN
Median	0.00		912.1	28.4	

<sup>a</sup> SYN, synergism; ANT, antagonism; IND, indifference. For the FICI model, synergy was defined as a FICI of  $\leq 0.5$ , antagonism was defined as a FICI of  $> 4.0$ , and indifference was defined as a FICI of  $> 0.5$  to 4 (i.e., no interaction). For the  $\Delta E$  model,  $\Sigma$ SYN and  $\Sigma$ ANT were the sums of the percentages of all statistically significant synergistic and antagonistic interactions. Interactions with  $< 100\%$  statistically significant interactions were considered weak synergism, those with 100 to 200% statistically significant interactions were considered moderate, and those with  $> 200\%$  statistically significant interactions were considered strong.

biosynthesis inhibitors fenpropimorph and terbinafine against *C. albicans* determined by microdilution checkerboard assay and analyzed by FICI (33). The authors of another report showed that FK506 has a synergism effect with azole (fluconazole, ketoconazole, and itraconazole) against azole-resistant *C. albicans* strains by performing the time-killing test and eval-

uating the impact on azole accumulation (20). However, no formal MIC study was performed, and no study was reported on the interaction of FK506 and VRC.

In this study we evaluated comprehensive interactions between FK506 and three azoles (FLC, ITR, and VRC) against newly clinically isolated *C. albicans* strains by different methods

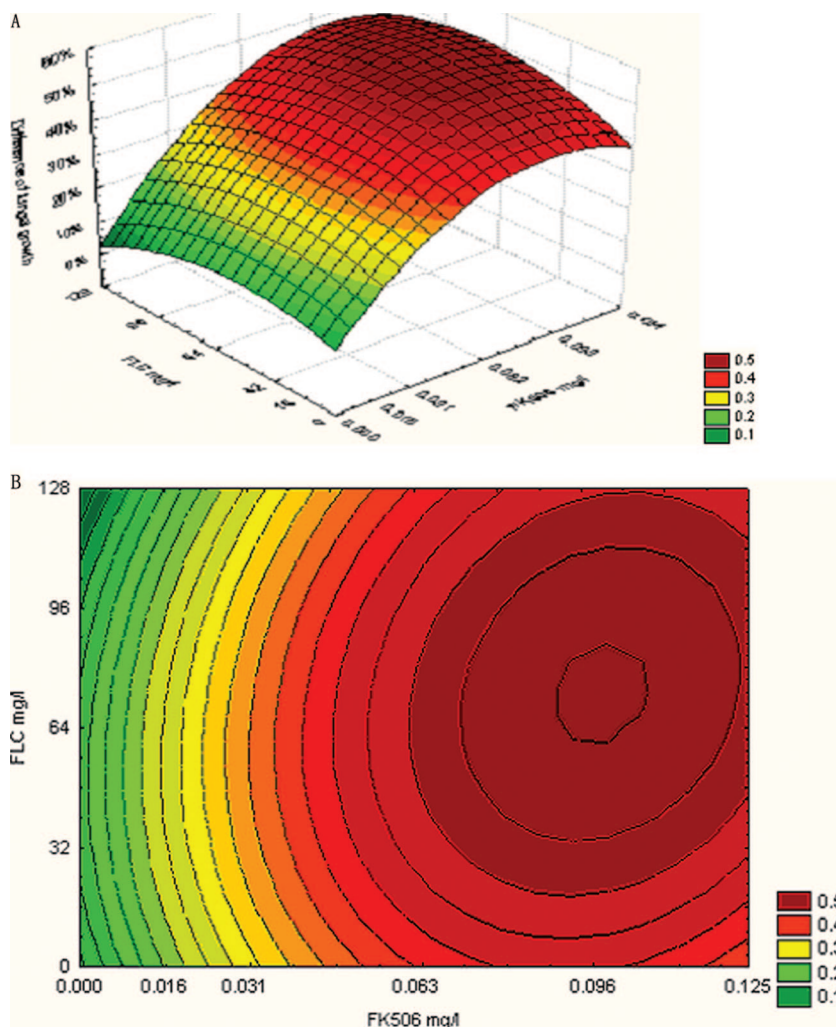


FIG. 1. The three-dimensional (A) and contour (B) plots of interaction between FLC and FK506 against resistant *C. albicans* CA10 based on the  $\Delta E$  model. The difference between the predicted and the measured percentage of fungal growth ( $\Delta E = E_{\text{predicted}} - E_{\text{measured}}$ ) is shown on the z axis.  $E_{\text{predicted}} = E_A \times E_B$ , where  $E_A$  and  $E_B$  were the experimental percentages of growth of each drug action alone, respectively.  $E_{\text{measured}}$  is the percentage of fungal growth when drugs were used in combination. The  $\Delta E$  value of each well was the average of triplicate results. Higher  $\Delta E$  values suggested stronger synergistic interactions between FLC and FK506.

and models. The interactions were evaluated with the checkerboard microdilution assay based on antifungal growth, which is assessed either visually by grading turbidity or spectrophotometrically by measuring OD.  $\text{MIC}_{80}$  was used as an endpoint,

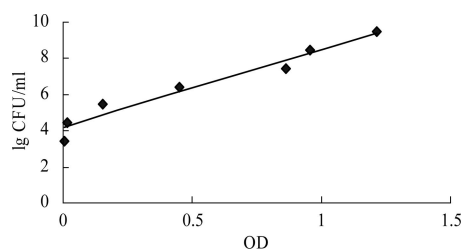


FIG. 2. Correlation between viable cell counts determined by colony counting and OD values determined by XTT-menadione colorimetric readings. Results are expressed as  $Y = 4.2610x + 4.2087$ , with a correlation coefficient  $r$  of 0.9622.

and in order to make the results from the two methods comparable, the fungal growth was graded 0 to 5 for the visual reading. The results were interpreted by two nonparametric models, FICI, based on LA, and  $\Delta E$ , based on BI no-interaction theories. The time-killing test was performed by colony counting and XTT reduction assay.

As we know, different results may be obtained by different methods. In recent years, considerable progress has been made in the methods of evaluating drug interactions. Spectrophotometric and colorimetric methods have been developed in recent years. They can quantify the fungal growth more precisely and are able to detect small changes in metabolic activity of fungi (22, 23). Based on these methods, the effects of antifungal drugs either on the fungal biomass or on the metabolic status of fungi can be measured. Our results revealed that the data obtained by the spectrophotometric method correlate well with the data from visual reading. Furthermore, the method is easier to perform and the results can be interpreted by more new models.

TABLE 4. Intensity and nature of interactions between FK506 and azoles determined by time-kill study

Drug combined with FK506	Colony counting				XTT method <sup>c</sup>			
	CA14 colony count <sup>a</sup>	Interpretation <sup>b</sup>	CA10 colony count <sup>a</sup>	Interpretation <sup>b</sup>	CA14 colony count <sup>a</sup>	Interpretation <sup>b</sup>	CA10 colony count <sup>a</sup>	Interpretation <sup>b</sup>
FLC	1.98	IND	2.03	SYN	1.51	IND	2.37	SYN
ITR	2.13	SYN	2.12	SYN	1.40	IND	2.46	SYN
VRC	1.49	IND	2.15	SYN	1.37	IND	2.26	SYN

<sup>a</sup> Colony counts are decreases in colony numbers [ $\log(48 \text{ h})$ ] compared with the value for the most active drug.

<sup>b</sup> SYN, synergism; ANT, antagonism; IND, indifference.

<sup>c</sup> The data from the XTT reduction assay were calculated according to the equation  $Y = 4.2610x + 4.2087$ .

FICI is the most frequently used model to interpret the interaction between antifungal drugs. However, the interpretation of FICI in defining synergy or antagonism is a problem in itself. Several different definitions have been described in the literature (4, 25, 28), but there is no consensus on which definition to use in interaction studies. According to the work of Odds (30), for the interpretation of a single FICI, a value of  $>4$  is usually considered antagonism, and a value of  $\leq 0.5$  is usually considered synergy. In this study we interpreted the results according to the definition of Odds. However, because we performed triplicate experiments for each strain studied, we interpreted the results of all replicates as one outcome, thereby constraining the interexperimental error. Thus, when the results of all three replicates were concordant, synergy or antagonism was defined if FICIs were below 1 or above 1, respec-

tively. In all other cases interactions were defined as indifference (47). Ease of use, simplicity, and feasibility of performance make FICI still the method of choice for analyses of drug-drug interactions, although it has some disadvantages.

The  $\Delta E$  model is the nonparametric method based on BI theory. When it was used in assessing the interaction between FK506 and three azoles, the percentages of fungal growth were derived from the experimental data directly, and good agreement was found with the FICI model, particularly against azole-resistant strains. Therefore, the  $\Delta E$  model, developed in recent years, is a useful approach in analyzing the nature of interactions between different drugs based on a checkerboard method.

Time-kill curves can provide growth kinetic information over time and give a more detailed picture of the effect of drug combinations on cell viability (26). 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 (10, 18, 36). For fungal growth evaluation, besides visual reading of turbidity and OD measurement, growth can be determined colorimetrically using indicator substances that are reduced to colored products by viable microorganisms (23). XTT is a new yellow tetrazolium salt which can be converted by mitochondrial dehydrogenases of viable fungi to a water-soluble orange formazan product, which can be measured spectrophotometrically at 492 nm (17). Therefore, we performed the time-kill experiment by the XTT assay and compared it with colony counting. The data from time-kill curves based on cell colony counting and metabolism-inhibitory effects confirmed the positive interactions observed in the checkerboard test. The potency of all the tested azoles can be enhanced by combining them with FK506 against *C. albicans*, especially against the resistant isolates. The data from the XTT test correlate well with the colony counting results.

In conclusion, our study revealed that the activity of azole antifungal agents that target ergosterol biosynthesis can be enhanced by FK506, an inhibitor of the calcineurin signaling pathway, especially against azole-resistant *C. albicans* in vitro. The results confirmed those earlier findings by using a normal MIC test with different interpretation models and a time-kill study to demonstrate combined effects against *C. albicans* and suggested differences in the combination effects against sensitive and resistant isolates. In addition, the isolates that we tested were newly isolated from clinical sources and were resistant to all three tested azoles, so our results also expanded on the potent effect of FK506 with VRC against resistant *C. albicans*. This result provides an alternative approach by which

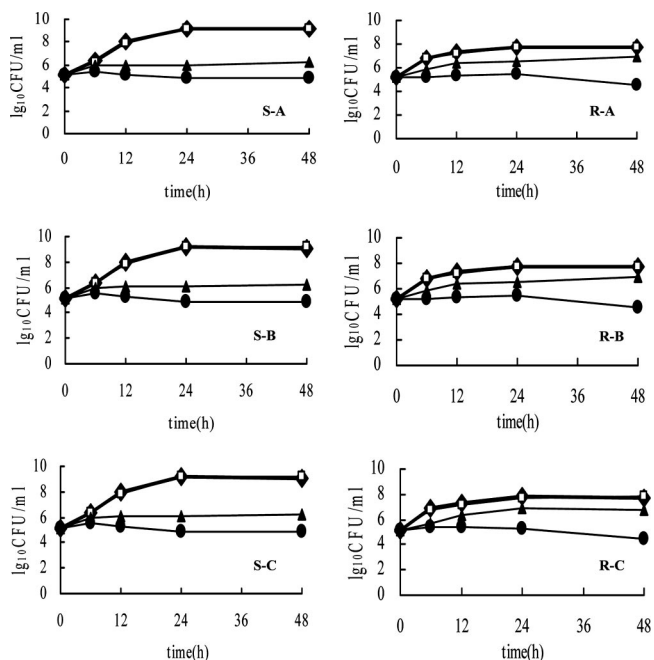


FIG. 3. Time-kill curves of azoles and FK506 alone and in combination against azole-susceptible strain (S) CA14 and azole-resistant strain (R) CA10. The strains at starting inocula of  $10^5$  CFU/ml were exposed to in vivo-achievable concentrations of 10  $\mu\text{g/ml}$  FLC (A), 1  $\mu\text{g/ml}$  ITR (B), and 1  $\mu\text{g/ml}$  VRC (C) alone and in combination with 0.016  $\mu\text{g/ml}$  FK506. At 0, 6, 12, 24, and 48 h, aliquots were removed from each test tube to examine the cell metabolism ability by XTT reduction assay. Symbols:  $\blacklozenge$ , growth control;  $\square$ , FK506;  $\blacktriangle$ , azole;  $\bullet$ , azole plus FK506. The OD value obtained by the XTT test was transformed to viable cells according to the equation  $Y = 4.2610x + 4.2087$ .

to overcome antifungal drug resistance. However, the potential of using this combination therapy in vivo warrants investigation, and further study is needed to determine the underlying mechanism of the synergistic action.

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