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In Vitro Activities of Retigeric Acid B Alone and in Combination with Azole Antifungal Agents against *Candida albicans*▽

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The vitro antifungal activity of retigeric acid B (RAB), a pentacyclic triterpenoid from the lichen species *Lobaria kurokawae*, was evaluated alone and in combination with fluconazole, ketoconazole, and itraconazole against *Candida albicans* using checkerboard microdilution and time-killing tests. The MICs for RAB against 10 different *C. albicans* isolates ranged from 8 to 16 μg/ml. A synergistic action of RAB and azole was observed in azole-resistant strains, whereas synergistic or indifferent effects were observed in azole-sensitive strains when interpreted by a separate approach of the fractional inhibitory concentration index and Δ*E* model (the difference between the predicted and measured fungal growth percentages). In time-killing tests, we used both colony counts and a colorimetric assay to evaluate the combinational antifungal effects of RAB and azoles, which further confirmed their synergistic interactions. These findings suggest that the natural product RAB may play a certain role in increasing the susceptibilities of azole-resistant *C. albicans* strains.

The incidence of candidiasis has increased during the last several decades due to the widespread use of antibacterials, corticosteroids, immunosuppressive agents, radiotherapy, and antitumoral chemotherapy (2, 4, 21, 23). The azole antifungal agents have excellent efficacy-toxicity profiles and play an important role in the treatment of *Candida* infections (12). However, concomitant with their widespread use, reports of clinical failure and correlations with elevated MICs to azole have begun to appear (3, 21). Moreover, some of these clinical isolates exhibit cross-resistance to a variety of different azole drugs (15). At present, although three echinocandins (caspofungin, micafungin, and anidulafungin) and voriconazole are available for the treatment of infection caused by azole-resistant isolates, the cost is too high for the patients. New antifungal agent research and development is still needed. Moreover, identification of small molecules that synergize with current antifungals against azole-resistant *Candida* strains may be a better way to overcome antifungal drug resistance.

Natural products with diverse bioactivities and structures are an important source of novel chemicals with pharmaceutical potentials (1, 26). Lichens, the symbiotic organisms of fungi and algae, are found commonly worldwide and can survive a variety of harsh environmental conditions. Lichens are inherently resistant to microbial infection due to the production of large numbers of unique secondary metabolites (6, 9). Therefore, we have focused our attention on lichens and their metabolites in an effort to find novel, naturally occurring antifungal potentiators. A thin-layer chromatography-bioautography screening guided phytochemical investigation for antifungal constituents from a lichen, *Lobaria kurokawae* Yoshim., led to the isolation of a pentacyclic triterpenoid, retigeric acid B (RAB) (Fig. 1), as the main active constituent (27). Interestingly, *Lobaria kurokawae* Yoshim. has been applied as a folk medicine for the treatment of hypopepsia, malnutritional stagnation, and abdominal distention in South China.

Preliminary studies using the agar disk diffusion method have shown that RAB has excellent anti-*Candida* activity when combined with azoles (data not shown). In the present study, we investigated the antifungal activity of RAB alone and in combination with azoles, mainly fluconazole (FLC), ketoconazole (KCZ), and itraconazole (ITR), against clinical isolates of *Candida albicans* by the checkerboard broth microdilution method and time-killing tests.

MATERIALS AND METHODS

Strains. The 10 clinical isolates of *C. albicans* used in this study were kindly provided by the Shandong Provincial Qianfoshan Hospital, Jinan, China. Their sensitivities to the tested azoles were evaluated according to the CLSI standard M27-A2 guidelines (16). Quality control was performed on each day of testing using CLSI-recommended reference strain *C. albicans* ATCC 10231. Frozen stocks of isolates were stored at −70°C in culture medium supplemented with 10% (vol/vol) glycerol and were subcultured twice at 35°C before each experiment.

Chemicals. FLC was obtained from the Institute of Biopharmaceuticals of Shandong, KCZ was purchased from the National Institute for the Control of Pharmaceutical Biological Products, and ITR was obtained from Xian-Janssen Pharmaceutical Co., Ltd., China. RAB was isolated from the lichen *L. kurokawae* in our laboratory, and its purity is over 96% as analyzed by high-performance liquid chromatography. Stock solutions were prepared in dimethyl sulfoxide at 5,120 μg/ml for KCZ and ITR and 20,480 μg/ml for RAB. Stock solutions of FLC at 5,120 μg/ml were made in sterile distilled water.

Antifungal activities of RAB and azoles. The antifungal activities of all tested drugs were tested by the broth microdilution method according to CLSI standard M27-A2 (16) with a final inoculum of 0.5×10^3 to 2.5×10^3 cells/ml. The test was carried out in RPMI 1640 medium (adjusted to pH 7.0 with 0.165 M morpholinepropanesulfonic acid [MOPS] buffer) in 96-well flat-bottomed microtitration plates (Costar). After incubation at 35°C for 48 h, MICs were determined by measuring the optical density at 490 nm with a spectrophotometer, and background optical densities were subtracted from that of each well. The MICs were defined as the concentrations of drug that reduced growth by 80% compared to that of organisms grown in the absence of drug. All experiments were performed in triplicate.

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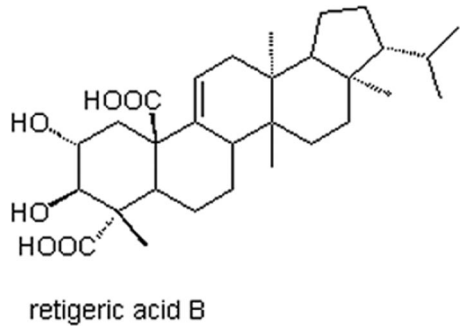

FIG. 1. Structure of RAB.

Interactions between RAB and azoles. Drug interactions were assessed by broth microdilution checkerboard assays (24). Drugs dilutions were initially prepared at four times the desired final concentration. Aliquots of 50 μl of each concentration of azoles were added to columns 2 to 12, and then 50 μl of RAB was added to rows A to G. Row H and column 1 contained only the azole and RAB, 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. An exploratory study was carried out to choose the appropriate range of concentrations for different drugs against strains with different susceptibilities. The final drug concentrations after the addition of 100 μl of inoculum ranged from 0.008 to 8 μg/ml for FLC, 0.001 to 1 μg/ml for KCZ and ITR, and 0.25 to 16 μg/ml for RAB, and the final inoculum size was 0.5×10^3 to 2.5×10^3 CFU/ml. The microtiter plates were incubated at 35°C for 48 h. The growth in each well was quantified by a spectrophotometer in a manner similar to that for the sensitivity assay. The growth inhibitory effects of the drugs alone and in combination were then calculated based on the results. All the experiments were performed in triplicate.

Drug interaction models. To assess the in vitro interactions between the three azoles and RAB, the data obtained from the checkerboard tests were analyzed by nonparametric models based on the following two no-interaction theories: the Loewe additivity model (LA) and the Bliss independence (BI) theory. 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 independently with the probabilistic sense of independence (13, 25).

LA-based model. The nonparametric approach of fractional inhibitory concentration index (FICI) was used and expressed as follows: $\sum \text{FIC} = \text{FIC}_A + \text{FIC}_B = \text{MIC}_{AB}/\text{MIC}_A + \text{MIC}_{BA}/\text{MIC}_B$, where MIC_A and MIC_B are the MICs of drugs A and B when acting alone and MIC_{AB} and MIC_{BA} are the MICs of drugs A and B when acting in combination, respectively. Among all of the $\sum$ FIC values calculated for each data set, the FICI was determined as $\sum \text{FIC}_{min}$ (the lowest $\sum$ FIC) when $\sum \text{FIC}_{max}$ (the highest $\sum$ FIC) was less than 4; otherwise, the FICI was determined as $\sum \text{FIC}_{max}$. Synergy was defined as an FICI of ≤0.5, while antagonism was defined as an FICI value of >4. An FICI result between 0.5 and 4 ($0.5 < \text{FICI} \leq 4$) was considered indifferent (17). In addition, isobolograms were plotted. The characteristic shape of the isobologram was used to visualize synergistic and antagonistic drug interactions (8).

BI-based model. The nonparametric approach of BI is based on the Prichard model, defined as $E_i = E_A \times E_B$, where E_i is the predicted percentage of growth of the theoretical noninteractive combination of the drugs A and B and E_A and E_B are the experimental percentages of growth of each drug acting alone. Interaction is defined by the difference (ΔE) between the predicted and measured percentages of growth with drugs at various concentrations ($\Delta E = E_{predicted} - E_{measured}$). In each of the three independent experiments, the observed percentage of growth obtained from the experimental data was subtracted from the predicted percentage, and then the average difference of three experiments was calculated. When the average difference as well as its 95% confidence interval

TABLE 1. Susceptibilities of drugs alone and in combination against 10 clinical isolates of *C. albicans* by checkerboard microdilution assay

Drug	Strain	Median MIC (range) of drug (μg/ml) used:			
		Alone		In combination	
		Azole	RAB	Azole	RAB
FLC	CA1	0.25 (0.25–0.5)	8	0.062 (0.062–0.125)	2
	CA2	0.5 (0.25–0.5)	16	0.062 (0.062–0.125)	2
	CA3	0.25 (0.25–0.5)	16	0.062 (0.062–0.125)	4
	CA4	1 (0.5–1)	8	0.125 (0.125–0.5)	1
	CA127	1 (0.5–1)	16	0.125 (0.125–0.25)	4
	CA132	0.5 (0.5–1)	8	0.25	2
	CA10	256 (256–512)	16	0.25	2
	CA135	128 (64–128)	16	0.25 (0.125–0.25)	2
	CA137	64 (64–128)	16	0.125 (0.125–0.25)	4
	CA138	128 (128–256)	8	0.25	2
KCZ	CA1	0.031 (0.031–0.062)	8	0.004 (0.004–0.008)	2
	CA2	0.016 (0.016–0.031)	16	0.002 (0.002–0.004)	2
	CA3	0.062 (0.062–0.125)	16	0.004 (0.004–0.008)	2
	CA4	0.062 (0.062–0.125)	8	0.031 (0.031–0.062)	2
	CA127	0.031 (0.031–0.062)	16	0.004 (0.004–0.008)	8
	CA132	0.031 (0.031–0.062)	8	0.004 (0.004–0.008)	4
	CA10	16	16	0.031 (0.031–0.062)	4
	CA135	16 (8–16)	16	0.002	2
	CA137	16 (8–16)	16	0.002	4
	CA138	16	8	0.062 (0.031–0.062)	2
ITR	CA1	0.125 (0.125–0.25)	8	0.031 (0.031–0.062)	4
	CA2	0.125 (0.125–0.25)	16	0.016 (0.016–0.031)	4
	CA3	0.031 (0.031–0.062)	16	0.004	4
	CA4	0.031 (0.031–0.062)	8	0.004	4
	CA127	0.016 (0.016–0.031)	16	0.004	2
	CA132	0.031 (0.031–0.062)	8	0.016 (0.016–0.031)	2
	CA10	128 (64–128)	16	0.125 (0.125–0.25)	4
	CA135	32 (16–32)	16	0.125 (0.125–0.25)	4
	CA137	16 (16–32)	16	0.125 (0.125–0.25)	4
	CA138	64 (64–128)	8	0.25	2

A

RAB (μg/ml)	% Growth at FLC (μg/ml)											
	0	0.008	0.016	0.031	0.062	0.125	0.25	0.5	1	2	4	8
16	0.9	0.5	0.5	0.4	0.3	0.2	0.3	0.2	0.3	0.3	0.2	0.3
8	66.4	**9.8**	**0.5**	**0.4**	0.4	0.3	0.4	0.4	0.3	0.3	0.3	0.3
4	96.1	95.4	64.5	22.7	**4**	**2.4**	1.3	1.3	1.1	0.7	0.7	0.5
2	97.1	97	79.4	60.5	50.7	39.7	**19.5**	**19.5**	**19.1**	**14.9**	**14.8**	**14.6**
1	98.3	98.3	86.5	57.7	44.2	43	33.4	28.8	24.1	22.6	23.1	20.3
0.5	100	97.8	94.5	92.8	74.2	59.3	45.6	43.1	40.2	45.6	40.9	29.8
0.25	99.7	98.5	93.6	92.1	87.5	63.6	53.4	45.7	42.6	40.1	42.2	40.9
0	100	100.6	99.7	97.9	91.2	75.3	62.7	61.7	59	59.6	56.9	51

B

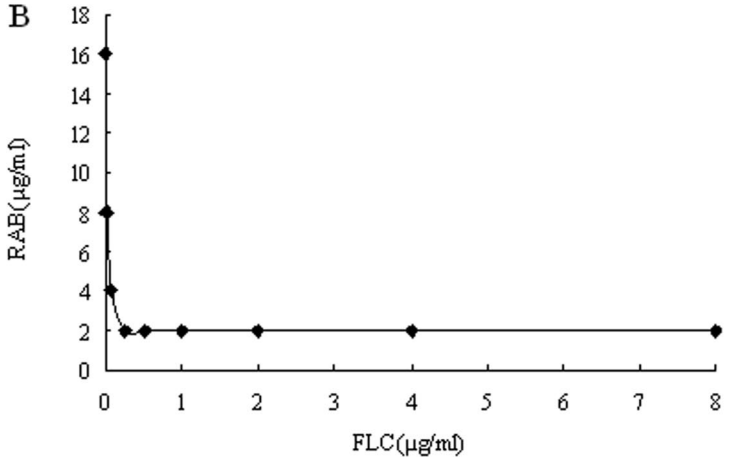

FIG. 2. In vitro assessment of the interaction between FLC and RAB against the clinical azole-resistant strain CA10 based on the FICI. (A) Checkerboard showing the percentage of growth for each combination and the combination with more than 20% growth (light gray area). The isoeffective combinations, on the basis of which the FICIs were calculated, are shown in bold. Among all the FICIs calculated based on the isoeffective combinations, $FICI_{max}$ was <4, and so $FICI_{min}$ (0.126) was reported as the FICI. The underlined combination is that with the lowest FICI. (B) Corresponding isobologram of the MICs obtained with combinations of FLC and RAB. The isobole is concave, which indicates synergism.

among the three replicates was positive, statistically significant synergy was defined; when the difference as well as its 95% confidence interval was negative, significant antagonism was defined. In any other case, BI was concluded. The ΔE value obtained for each combination can be depicted on the *z* axis to construct a 3-D surface plot. Peaks above and below the zero plane indicate synergistic and antagonistic combinations, respectively, while the zero plane indicates the absence of statistically significant interaction.

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, while interactions with >200% were considered strong, as described previously (24). In addition, the numbers of statistically significant synergistic and antagonistic combinations among the 77 combinations tested were calculated for each strain.

Time-killing test. In order to further evaluate the effect of RAB alone and in combination with azoles on the resistant strain, CA10 was used for the time-kill experiments. CA10 was grown in RPMI 1640 medium at the starting inoculum of 10^5 CFU/ml. The concentrations for RAB, FLC, and ITR were all 8 μg/ml and that for KCZ was 2 μg/ml, respectively. A drug-free sample served as a growth control. Dimethyl sulfoxide comprised <1% of the total testing volume. Samples (100 μl) were removed from the cultures just before treatment and at 6 h, 12 h, 24 h, and 48 h. The samples were then diluted, plated, and incubated at 35°C for 48 h for colony counts.

Meanwhile, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) quantification of metabolic activity was performed with a different 100-μl sample of culture in order to estimate the cell viability after drug treatment according to methods described previously (10). Briefly, at each time point, a 100-μl aliquot was removed from every treatment mixture and transferred to a well of a 96-well microplate, and then a 100-μl aliquot of XTT-menadione solution was added. (XTT and menadione were purchased from Sigma Chemical Co. Prior to each assay, XTT was dissolved in a saturated solution at 0.5 mg/ml in Ringer's lactate. The solution was filtered through a 0.22-μm-pore-size filter. Menadione was prepared as a 10-mM stock solution in acetone and stored at −20°C.) The final concentrations of XTT and menadione were 0.25 mg/ml and 5 μM, respectively. The plate was then incubated in the dark for up to 2 h at 35°C. After incubation, the absorbance of the XTT reduction product, formazan, was read at 490 nm with a microtiter plate reader. All experiments were done in triplicate, and the results were presented as mean values. Thus, growth and metabolic inhibitory effects of each drug alone and in combination were measured based on colony counts and absorbance.

Synergism and antagonism were defined as a ≥ 2 $\log_{10}$ CFU/ml increase or a ≥ 2 $\log_{10}$ CFU/ml decrease of antifungal activity produced by the combination compared with that by the most active agent alone, respectively. If a <2 $\log_{10}$ CFU/ml change was observed, the interaction was considered indifferent (22). Each experiment was performed in triplicate, and the results were presented as mean values.

The correlation between the viable cell counts determined by colony counting and the optical density values by XTT-menadione colorimetric readings was evaluated before interpretation of results.

RESULTS

Susceptibility and interaction of drugs. The MICs of RAB and azoles, alone and in combination against *C. albicans*, are shown in Table 1. The MIC of FLC against the quality control

TABLE 2. In vitro interactions between RAB and azoles as determined by nonparametric methods FICI and the ΔE model[a]

Drug used in combination with RAB	Strain	FICI		ΔE model[b]		
		Median (range)	INT	∑SYN (*n*)	∑ANT (*n*)	INT
FLC	CA1	0.5	SYN	751.2 (51)	−77.2 (13)	SYN
	CA2	0.25 (0.25–0.375)	SYN	712.6 (42)	−71.1 (20)	SYN
	CA3	0.5	SYN	150.7 (13)	−62.8 (12)	SYN
	CA4	0.625 (0.375–0.625)	IND	41.0 (21)	−20.3 (15)	IND
	CA127	0.5 (0.375–0.5)	SYN	211.7 (39)	−37.7 (14)	SYN
	CA132	0.75 (0.5–0.75)	IND	88.0 (21)	−85.8 (24)	IND
	CA10	0.126 (0.125–0.126)	SYN	1,365.6 (38)	0	SYN
	CA135	0.127 (0.126–0.127)	SYN	877.7 (51)	−91.8 (13)	SYN
	CA137	0.252	SYN	1,464.7 (64)	0	SYN
	CA138	0.252 (0.251–0.252)	SYN	981.3 (54)	−12.5 (14)	SYN
KCZ	CA1	0.375	SYN	42.8 (35)	−16.0 (30)	IND
	CA2	0.253 (0.137–0.253)	SYN	25.0 (20)	−64.8 (53)	IND
	CA3	0.25 (0.187–0.25)	SYN	106.8 (45)	−40.0 (30)	SYN
	CA4	0.75	IND	39.5 (16)	−21.3 (6)	IND
	CA127	0.625 (0.562–0.75)	IND	116.4 (47)	−23.8 (27)	SYN
	CA132	0.625 (0.562–0.75)	IND	21.2 (30)	−10.7 (25)	IND
	CA10	0.254 (0.252–0.254)	SYN	910.7 (39)	−68.1 (31)	SYN
	CA135	0.125	SYN	728.3 (39)	−26.8 (18)	SYN
	CA137	0.25 (0.25–0.251)	SYN	814.2 (51)	−28.8 (18)	SYN
	CA138	0.254 (0.252–0.254)	SYN	413.5 (52)	−23 (16)	SYN
ITR	CA1	0.75	IND	116.3 (36)	−38.8 (32)	SYN
	CA2	0.375 (0.313–0.5)	SYN	197.7 (36)	−84.4 (33)	SYN
	CA3	0.375 (0.312–0.375)	SYN	105.7 (37)	−15.1 (9)	SYN
	CA4	0.625 (0.562–0.625)	IND	69.6 (22)	−58.4 (14)	IND
	CA127	0.375 (0.25–0.375)	SYN	321.1 (42)	−20.8 (35)	SYN
	CA132	0.75	IND	60.5 (22)	−68.7 (25)	IND
	CA10	0.252 (0.251–0.252)	SYN	520.9 (48)	−15.9 (20)	SYN
	CA135	0.254 (0.254–0.258)	SYN	905.6 (44)	−25.3 (8)	SYN
	CA137	0.258	SYN	541.3 (60)	−14.2 (17)	SYN
	CA138	0.254 (0.252–0.254)	SYN	553.0 (41)	−20.7 (30)	SYN

[a] INT, interpretation; IND, indifference. Synergy was defined as an FICI of ≤0.5, antagonism was defined as an FICI of >4.0, and indifference was defined as an FICI of >0.5 to 4 (i.e., no interaction).

[b] *n*, number of drug combinations (among the 77 drug combinations for each strain) with statistically significant synergy or antagonism.

strain *C. albicans* ATCC 10231 was 0.5 to 2 μg/ml, within the reference range (11). According to the interpretive breakpoints for FLC (≤8 and ≥64 μg/ml, respectively), ITR (≤0.1 and ≥1 μg/ml, respectively) (24), and KCZ (≤0.125 and ≥1 μg/ml, respectively) (14), *C. albicans* isolates CA10, CA135, CA137, and CA138 are resistant to FLC, KCZ, and ITR; the others are sensitive. RAB has antifungal activity against sensitive and resistant strains alike, especially considering it is a natural product. The MIC range of RAB for all tested strains, based on an 80% reduction in growth, was 8 to 16 μg/ml as reported in Table 1. When a MIC-like assay was performed for the azoles in the presence of a fixed subinhibitory concentration of RAB, the median MICs of FLC, KCZ, and ITR decreased from two- to 16-fold for azole-sensitive strains while even greater reductions were observed against the azole-resistant strains. In fact, the MICs of azoles against the resistant strains in the presence of RAB were comparable to those of the sensitive strains. For example, in combination with RAB, the MICs of FLC, KCZ, and ITR against the azole-resistant *C. albicans* strain CA10 decreased more than 1,000-fold, 500-fold, and 1,000-fold, respectively, with RAB. The interaction between FLC and RAB against the azole-resistant strain CA10 is shown in Fig. 2A, and the corresponding isobologram is presented in Fig. 2B.

The results of the checkerboard analysis interpreted by the FICI and ΔE method are summarized in Table 2. In the checkerboard microtiter plate format, synergism was consistently concluded in all four resistant isolates analyzed by the FICI and ΔE models for FLC, KCZ, and ITR. However, different interpretations were obtained for the azole-sensitive isolates when the conclusions from the FICI and ΔE models were compared for the different azole drug treatments. For the RAB-FLC combination, indifference was observed against two strains and synergism was observed against four strains analyzed by both models. For the RAB-KCZ combination, synergism was observed in three strains by FICI and in two strains by ΔE; others showed indifference. For the RAB-ITR combination, synergism was observed in three strains by FICI and in four strains by ΔE; others revealed indifference.

Additionally, the ΔE values obtained for each combination were illustrated in a 3-D plot made by MATLAB7 (Fig. 3). Peaks above and below the zero plane indicate synergistic and antagonistic combination, respectively, while the zero plane indicates the absence of statistically significant interaction.

Time-killing test. Time-kill curves showed the effect of drug combination on cell viability and supported the results of the broth checkerboard microdilution assays. First, we demonstrated that the XTT viability assay correlated to viable cell

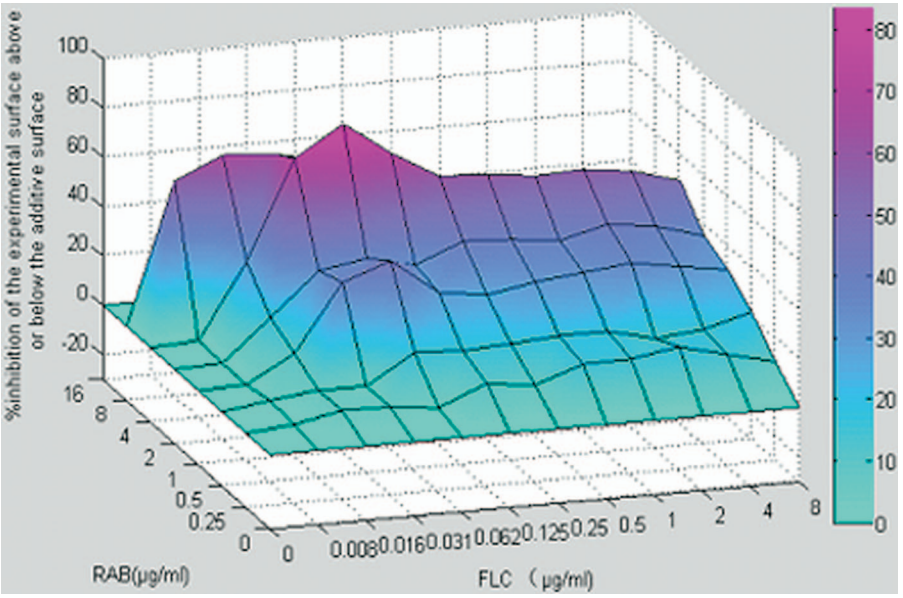

FIG. 3. The 3-D plot of the percent synergy calculated with the Δ*E* model. The mean Δ*E* values obtained for three separate experiments are shown on the *z* axis of the graph. Higher Δ*E* values suggested stronger synergistic interaction between FLC and RAB.

count with a correlation coefficient (R^2) of 0.9664. The results of RAB combined with azoles from the time-kill study are shown in Table 3. Given the initial inoculums of 10^5 CFU/ml, RAB alone had a very weak antifungal effect at 8 μg/ml at 48 h, but the combination yielded a 2.03-$\log_{10}$ CFU/ml decrease compared with 8 μg/ml FLC alone, a 2.10-$\log_{10}$ CFU/ml decrease compared with 2 μg/ml KCZ alone, and a 2.11-$\log_{10}$ CFU/ml decrease compared with 8 μg/ml ITR alone at 48 h. There was no obvious difference in the features of drug action among the three combinations.

DISCUSSION

Methods for studying antifungal combinations in vitro have differed considerably over time and among investigators (17, 19, 20). Different results might be obtained by choosing different approaches and models for the assessment of in vitro drug interaction (17, 20). To accurately evaluate the combinational effect of drugs, we used a spectrophotometric method in the broth checkerboard microdilution assay, which is a more objective measurement of yeast growth in the presence of inhibitory agents than subjective visual assessment. The data analysis was done with Microsoft Excel, which makes endpoint readings quantitative and more objective than visual endpoint determination. In this study, we chose the more stringent endpoint of 80% inhibition in growth rather than the standard 50%. For the antifungal agents that did not have sharp endpoints, an 80% endpoint was found to be useful for resolving problems of reproducibility (7). In addition, we found good reproducibility when susceptibility tests were repeated. Two nonparametric approaches, FICI and Δ*E* models, were used to assess the nature and intensity of the in vitro interactions between the three azoles and RAB. Between them, the FICI approach is popular among bacteriologists and mycologists to quantify drug interactions, although the interpretation of the FICI model in concluding synergy or antagonism can be problematic (8). For example, the results obtained with the FICI model are dependent on the MIC endpoints and the cutoff values by which synergism and antagonism are defined. To overcome these problems, a response surface approach based on the BI theory (Δ*E* model) was then used to conclude the interactions between RAB and the three azoles. The Δ*E* model has been used extensively to describe drug-drug interactions, especially in the area of antiviral drugs (5, 18) and has considerable advantages over conventional methods. The fitting of a model to the whole data surface not only allows the optimal use of information in the data but also allows the determination of error estimates of the interaction coefficient, thereby indicating whether the interaction is significant or not (25). In our study, the Δ*E* model and the FICI model showed good agreement in the interpretation of the results.

Besides the checkerboard method, we used the time-killing test to assess antimicrobial combination in vitro. We performed the time-killing test by both the XTT assay and colony counts. Repetitive counting of CFU is labor intensive and tedious, which seriously limits the number of antifungal concentrations and combinations that can be tested in any one experiment. The XTT assay largely avoided these disadvan-

TABLE 3. Decrease in $\log_{10}$ CFU/ml of strain CA10 using RAB combined with azoles at 48 h[a]

Drug in combination with RAB	Mean (±SD) decrease in $\log_{10}$ CFU/ml as measured by:	
	Colony count	XTT reduction assay
FLC	2.15 (0.09)	2.03 (0.02)
KCZ	2.12 (0.12)	2.10 (0.10)
ITR	2.15 (0.09)	2.11 (0.05)

[a] All drug combinations were interpreted as synergistic.

tages, showing an excellent correlation between colorimetric readings and cell numbers, and the data from the XTT assay correlate well with the colony-counting results.

In conclusion, the findings of the present study are very encouraging. RAB exhibited antifungal activity alone against both azole-sensitive and -resistant *C. albicans* isolates. Furthermore, when RAB was combined with azoles, strong synergy was observed against azole-resistant strains, with synergistic or indifferent effects observed against azole-sensitive strains, analyzed by both the FICI and ΔE models. RAB is an acid with antifungal activity that possibly has activity either in facilitating the uptake of azoles or in enhancing the membrane damage associated with the action of the azoles. Further studies will be performed to discern the mechanism of growth inhibition by RAB alone and the mechanism of synergy between RAB and azoles.

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