Original article

In vitro synergy of pseudolaric acid B and fluconazole against clinical isolates of Candida albicans

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Summary

Candida albicans is the most common fungal pathogen in humans. The emergence of resistance to azole antifungals has raised the issue of using such antifungals in combination to optimise therapeutic outcome. The objective of this study was to evaluate *in vitro* synergy of pseudolaric acid B (PAB) and fluconazole (FLC) against clinical isolates of *C. albicans*. The *in vitro* antifungal activity of PAB, a diterpene acid from *Pseudolarix kaempferi* Gordon, was evaluated alone and in combination with FLC against 22 FLC-resistant (FLC-R) and 12 FLC-susceptible (FLC-S) *C. albicans* using the chequerboard microdilution method and time-killing test assays. Synergism was observed in all 22 (100%) FLC-R strains tested as determined by both fractional inhibitory concentration index (FICI) with values ranging from 0.02 to 0.13 and bliss independence (BI) models. Synergism was observed in two of 12 (17%) FLC-S strains as determined by FICI model with values ranging from 0.25 to 0.5 and in three of 12 (18%) FLC-S strains as determined by BI model. For FLC-R strains, the drug concentrations of FLC and PAB, where synergistic interactions were found, ranged from 0.06 to 4 μg ml^{-1} and 0.5 to 4 μg ml^{-1} respectively. For FLC-S strains, the drug concentrations of FLC and PAB were 1–8 μg ml^{-1} and 0.5–4 μg ml^{-1} respectively. The BI model gave results consistent with FICI, but no antagonistic activity was observed in any of the strains tested. These interactions between PAB and FLC were confirmed using the time-killing test for the selected strains. Fluconazole and PAB exhibited a good synergism against azole-R isolates of *C. albicans*.

Key words: Pseudolaric acid B, *Candida albicans*, antifungal susceptibility, synergy, chequerboard method, time–kill curves.

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Introduction

Candida albicans is a major human fungal pathogen causing various forms of candidiasis ranging from chronic superficial mycoses such as vaginitis to severe and life-threatening systemic infections, predominantly in patients with a compromised immune system.[1] *Candida albicans* has been reported to be the fourth leading cause of nosocomial infections.[2]

Over the years, the polyene fungicidal agent Amphotericin B has become the standard treatment for candidal infections, but the severe nephrotoxicity of its conventional form and the costs of its lipid forms limit its widespread use. Currently, the azole antifungal

compounds such as fluconazole (FLC) have emerged as the principal and first-line drugs used in the treatment of candidal infections in non-neutropenic patients. Fluconazole has the advantage of being stable parenteral formulation, and having excellent oral bioavailability and efficacy–toxicity profiles.[3,4] However, resistance to FLC is increasing in *C. albicans* and other species.[5] The emergence of drug resistance can probably be ascribed to the fungistatic rather than fungicidal characteristics of FLC action.[6] Combination therapy is one approach that can be used to improve the efficacy of antifungal therapy for difficult-to-treat infections.[7]

The root and trunk bark of *Pseudolarix kaempferi* Gordon (Pinaceae), known as 'Tu-Jin-Pi' in China, have been traditionally used as a remedy for fungal infections of the skin. Pseudolaric acid B (PAB), which is a diterpene acid, was isolated and identified as the main antifungal constituent of *P. kaempferi* Gordon.[8] It has been reported that PAB was active against *Trichophyton mentagrophytes*, *Torulopsis petrophilum*, *Microsporum gypseum* and *Candida* spp.[9] A previous report also showed that PAB reduced the number of recovered colony-forming units significantly at different dosages in a murine model of disseminated candidiasis, while infected mice treated intravenously with PAB had a longer survival time than those treated with the vehicle alone.[9]

In an attempt to improve the antifungal effect of FLC, we investigated the combined effects of FLC and PAB against clinical isolates of *C. albicans* using alternative methods.

Materials and methods

Fungal strains and materials

Twenty-two clinical isolates of the FLC-R *C. albicans* strains[10] were kindly provided by Jiang Y.Y. and 12 clinical isolates of the FLC-S *C. albicans* were obtained from the First Hospital of Jilin University and used in this study. In addition, three ATCC type *Candida* strains (*C. albicans* ATCC 10231, *Candida parapsilosis* ATCC 90018 and *Candida krusei* ATCC 6258) were acquired from the American Type Culture Collection (ATCC, Gaithersburg, MD, USA). The components of YPD broth (1% yeast extract, 2% peptone, and 2% glucose) were purchased from BD Biosciences, Inc. (Sparks, MD, USA). Pseudolaric acid B was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), FLC was obtained from Pfizer Inc. (New York, NY, USA) and stock solutions of varying concentrations were prepared in dimethyl

sulphoxide (DMSO). Pseudolaric acid B and FLC were prepared using RPMI 1640 with glutamine broth medium, buffered to pH 7.0 with 0.165 mmol l^{-1} morpholinepropanesulphonic acid.[11]

Antifungal susceptibility testing

The minimum inhibitory concentrations (MICs) of PAB and FLC against the *Candida* strains mentioned above were determined by broth microdilution using twofold serial dilutions in RPMI 1640 medium as described by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) method M27-A.[12] The quality control (QC) strain, *C. krusei* ATCC 6258, and the reference strain, *C. parapsilosis* ATCC 90018, were included in each batch of susceptibility tests to ensure QC. The test was carried out in 96-well flat-bottomed microtitration plates. After agitation for 15 s, the plates were incubated at 35 °C without shaking, and readings were performed after 48 h of incubation by both visual reading and optical density (OD) determination.

For the visual reading, the MICs of FLC were determined according to CLSI procedure. For PAB, the MIC was defined as the lowest concentration showing 100% growth inhibition.[9]

For the OD determination, the MICs was defined as the lowest concentration of antifungal which resulted in 80% inhibition of growth compared with that of the drug-free control for FLC, and the MICs was defined as the lowest concentration showing 100% growth inhibition for PAB.[9]

Chequerboard method

The interaction between PAB and FLC against the 34 clinical isolates and the *C. albicans* ATCC 10231 strains mentioned above was assayed using a microdilution chequerboard technique.[13,14] Drug dilutions were prepared to obtain four times the final concentration. A total of 50 µl of each FLC concentration was added to columns 2 to 12, and then 50 µl of PAB was added to rows B to H. To column 1, 50 µl of the medium containing the PAB solvent was added, and to row A, 50 µl of the medium containing the FLC solvent was added. The solvent DMSO in the medium comprised <1% of the total test volume. Thus, row A and column 1 contained only the azole and PAB, respectively, and the well at the intersection of row A and column 1 (well A1) was the drug-free well that served as the growth control. The final concentrations ranged from 0.0078 to 8 µg ml^{-1} for FLC, 0.125 to 8 µg ml^{-1} or 0.5 to 32 µg ml^{-1} for PAB, and the final inoculum size was

2.5×10^3 cfu ml^{-1}. After incubation, visual analysis of the MICs was performed and the OD values were measured at 595 nm. The percentage of growth in each well was calculated as the OD of each well. The background OD was subtracted from the OD of each well. Each isolate was tested in triplicate on different days. The background OD to be subtracted from the growth after incubation includes that of all the inoculated wells taken at time zero before incubation. This has to be distinct from the difference between the growth inhibition and the growth control after incubation.

Interpretation of drug interaction

To assay the *in vitro* interactions between FLC and PAB against each strain, the data obtained by the spectrophotometric method were analysed using two models, FICI and BI, both of which have been used previously to characterise antifungal drug interactions.[15] Fractional inhibitory concentration index and BI are non-parametric models based on the Loewe additivity (LA) and BI theories respectively.[13]

Fractional inhibitory concentration index

The FICI method was defined by the following equation: $\text{FICI} = \text{FICA} + \text{FICB} = C_A{}^{\text{comb}}/\text{MIC}_A{}^{\text{alone}} + C_B{}^{\text{comb}}/\text{MIC}_B{}^{\text{alone}}$, where $\text{MIC}_A{}^{\text{alone}}$ and $\text{MIC}_B{}^{\text{alone}}$ are the MIC values 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 isoeffective combinations respectively.[13] Low off-scale MIC values were converted to the lowest tested doubling concentration. Among all the FICIs calculated for each data set, the FICI$_{\text{min}}$ was reported as the FICI in all cases unless the FICI$_{\text{max}}$ was >4, in which case the FICI$_{\text{max}}$ was reported as the FICI. The interpretation of the FICI was as follows: an FICI value of ≤0.5 represented synergy, an FICI value between 1 and 4 represented indifference and an FICI value >4 represented antagonism.[16]

Bliss independence analysis

Bliss independence model 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 drugs A and B, and I_A and I_B are the experimental percentages of inhibition for each drug acting alone. As $I = 1 - E$, where E is the percentage of growth, 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 com-

bination of drugs A and B, respectively, and E_A and E_B are the experimental percentages of growth of each drug alone. An interaction is described by the difference (ΔE) between the predicted and measured percentages of growth at various concentrations ($\Delta E = E_{\text{predicted}} - E_{\text{measured}}$). Using the non-parametric approach described by Prichard *et al.* [17], E_A and E_B are obtained directly from the experimental data. Because of the nature of the interaction, testing with microtitre plates and a twofold dilution of either drug results in a ΔE 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 after which the average difference of three experiments was calculated. When the average difference and the 95% confidence interval for the three replicates were positive, statistically significant synergy was claimed. When the difference and the 95% confidence interval were negative, significant antagonism was claimed. In any other case, BI was concluded. The BI model was derived by calculating the sum of the percentages of all statistically significant synergistic ($\sum$SYN) and antagonistic ($\sum$ANT) interactions. Interactions with <100% statistically significant interactions were considered weak, interactions with 100–200% statistically significant interactions were considered moderate and interactions with >200% statistically significant interactions were considered strong, as described previously.[15] In addition, the numbers of statistically significant SYN and ANT combinations among the 77 combinations of drug concentrations tested were calculated for each strain.

Time–kill curves

Candida albicans in RPMI 1640 medium was prepared at the starting inoculum density of 10^5 cfu ml^{-1}.[18] For one chosen clinical isolate FLC-R *C. albicans* YL313, the concentrations used were 16 µg ml^{-1} ($1/2 \times$ MIC) for PAB and 256 µg ml^{-1} ($1/2 \times$ MIC) for FLC.[19] For clinical isolate FLC-S *C. albicans* YL381, the concentrations used were 256 µg ml^{-1} ($1/2 \times$ MIC) for PAB and 4 µg ml^{-1} ($1/2 \times$ MIC) for FLC. Dimethyl sulphoxide comprised <1% of the total test volume. At various predetermined time points (0, 12, 24, 36 and 48 h after incubation with agitation at 35 °C), 100 µl of aliquot was removed from each solution and serially diluted 10-fold in sterile water. Subsequently, 100 µl of each dilution was streaked on a Sabouraud dextrose agar plate. Colony counts were determined after incubation at 35 °C for 48 h. The experiment was performed in triplicate. Synergism and antagonism were defined as

Table 1 *In vitro* interaction between pseudolaric acid B and fluconazole against clinical isolates of *Candida albicans*.

| | Median MIC (range) of drug alone (μg ml⁻¹) | | Results according to theory | | | | | | |
| | | | LA | | Result | | BI | | |
Strains	FLC	PAB	Median MIC (range) in combination (μg ml⁻¹) FLC	PAB	FICI	INT	∑SYN(n)	∑ANT	INT
Clinical isolates of FLC-R *Candida albicans*									
YL 313	512 (512)	32 (32)	0.125 (0.06–0.25)	1 (0.5–1)	0.03 (0.02–0.03)	SYN	1336 (50)	−35.5 (10)	SYN
YL 317	512 (256–512)	32 (16–32)	1 (0.5–1)	1 (0.5–1)	0.03 (0.03–0.06)	SYN	920.2 (30)	−35.5 (10)	SYN
YL 318	512 (512)	32 (16–32)	1 (1)	1 (1)	0.03 (0.03–0.06)	SYN	1011.8 (39)	−15.7 (14)	SYN
YL 319	512 (256–512)	32 (32)	0.25 (0.25–0.5)	1 (0.5–1)	0.03 (0.02–0.03)	SYN	720.9 (17)	−8.2 (4)	SYN
YL 325	512 (512)	32 (16–32)	2 (0.5–4)	1 (0.5–1)	0.03 (0.03–0.06)	SYN	980.7 (30)	−29.2 (5)	SYN
YL 328	512 (512)	32 (16–32)	1 (0.5–1)	1 (1)	0.03 (0.03–0.06)	SYN	856.8 (38)	−5.8 (2)	SYN
YL 331	512 (128–512)	32 (16–32)	0.125 (0.06–0.5)	1 (1)	0.03 (0.03–0.06)	SYN	1146.4 (52)	−40.4 (7)	SYN
YL 336	512 (512)	32 (32)	2 (1–4)	2 (1–4)	0.06 (0.03–0.04)	SYN	1180.6 (38)	−4.6 (2)	SYN
YL 338	>512	32 (32)	1 (1–2)	2 (1–4)	0.06 (0.03–0.13)	SYN	750.2 (32)	−25.4 (13)	SYN
YL 339	>512	32 (32)	0.5 (0.25–1)	1 (1–2)	0.03 (0.03–0.06)	SYN	980.6 (31)	−55.8 (4)	SYN
YL 342	512 (256–512)	16 (32)	2 (1–4)	2 (1–4)	0.06 (0.03–0.04)	SYN	518.8 (28)	−35.8 (6)	SYN
YL 345	>512	32 (16–32)	2 (1–4)	2 (1–4)	0.07 (0.03–0.13)	SYN	582.1 (29)	−43.1 (7)	SYN
YL 346	>512	32 (32)	1 (1)	2 (1–4)	0.06 (0.03–0.13)	SYN	630.8 (35)	−40.8 (5)	SYN
YL 349	256 (256)	32 (32)	0.25 (0.25–0.5)	2 (2–4)	0.06 (0.06–0.13)	SYN	250.5 (14)	−15.3 (4)	SYN
YL 351	512 (256–512)	32 (32)	1 (0.5–2)	1 (1–2)	0.03 (0.03–0.07)	SYN	960.7 (32)	−30.4 (7)	SYN
YL 353	>512	32 (16–32)	2 (1–4)	2 (1–4)	0.06 (0.03–0.13)	SYN	660.8 (36)	−42.1 (8)	SYN
YL 358	>512	32 (32)	1 (1–2)	2 (1–4)	0.06 (0.03–0.13)	SYN	1288.2 (51)	−26.6 (8)	SYN
YL 360	256 (256–512)	32 (32)	2 (1–4)	2 (1–4)	0.07 (0.04–0.13)	SYN	883.1 (37)	−12.2 (5)	SYN
YL 363	512 (512)	32 (16–32)	0.25 (0.13–0.5)	2 (1–4)	0.06 (0.03–0.13)	SYN	910.3 (28)	−27.6 (9)	SYN
YL 366	256 (64–256)	32 (32)	2 (1–4)	2 (1–4)	0.07 (0.04–0.13)	SYN	790.6 (33)	−28.5 (11)	SYN
YL 370	>512	32 (32)	1 (1)	2 (1–4)	0.06 (0.03–0.13)	SYN	922.7 (43)	−16.4 (6)	SYN
YL 371	512 (256–512)	32 (32)	2 (1–4)	1 (1–2)	0.03 (0.03–0.06)	SYN	1026.5 (58)	−6.6 (2)	SYN
Clinical isolates of FLC-S *Candida albicans*									
YL 378	8 (4–8)	512 (256–512)	8 (2–8)	2 (1–4)	1 (0.5–1)	IND	108.8 (17)	−67 (9)	SYN
YL 380	8 (4–8)	256 (128–512)	8 (2–8)	1 (0.5–2)	1 (0.5–1)	IND	38.2 (21)	−85.5 (15)	IND
YL 381	8 (4–8)	512 (256–512)	8 (2–8)	1 (0.5–2)	1 (0.5–1)	IND	15.6 (5)	−36.2 (14)	IND
YL 383	8 (2–8)	256 (128–512)	8 (2–8)	4 (2–4)	1.02 (0.52–1.02)	IND	40.8.2 (14)	−55.6 (20)	IND
YL 388	4 (2–8)	512 (256–512)	4 (2–4)	2 (1–4)	1 (0.5–1)	IND	29.2 (14)	−45.2 (33)	IND
YL 391	8 (2–8)	512 (256–512)	4 (2–8)	1 (2–4)	0.25 (0.25–0.5)	SYN	92.2 (50)	−52.4 (9)	SYN
YL 392	8 (4–8)	256 (128–512)	8 (2–8)	0.5 (0.5–1)	1 (0.5–1)	IND	112.6 (24)	−42.5 (10)	IND
YL 393	4 (2–8)	128 (128–256)	4 (2–4)	4 (2–8)	1.03 (0.50–1.03)	IND	15.6 (4)	−86.2 (7)	IND
YL 398	2 (2–4)	128 (64–128)	2 (1–4)	0.5 (0.5–1)	1 (0.5–1)	IND	75.8 (20)	−80.3 (8)	IND
YL 410	2 (2–4)	256 (128–512)	1 (1–2)	1 (0.5–2)	0.25 (0.25–0.5)	SYN	121.6 (10)	−18.2 (3)	SYN
YL 423	4 (2–8)	128 (64–128)	4 (2–4)	1 (0.5–2)	1 (0.5–1.02)	IND	55.4 (4)	−90.8 (9)	IND
YL 433	8 (4–8)	256 (128–256)	8 (2–8)	1 (0.5–2)	1 (0.5–1.01)	IND	38.6 (5)	−7.6 (3)	IND
ATCC 10231	2 (1–4)	32	0.25 (0.125–0.5)	32 (16–32)	1.13 (0.75–1.25)	IND	62.8 (6)	−17 (5)	IND

PAB, pseudolaric acid B; MIC, minimum inhibitory concentration; FLC, fluconazole; FICI, fractional inhibitory concentration index; LA, Loewe additivity; BI, bliss independence; INT, interpretation; SYN, synergism; ANT, antagonism; IND, indifference.

respective increases or decreases of ≥ 2 $\log_{10}$ cfu ml^{-1} in antifungal activity produced by the combination treatment compared with the more active agent alone after 24 h. A change of <2 $\log_{10}$ cfu ml^{-1} was considered indifferent.[20] Any decrease in the viable counts of the starting inoculum was considered 'killing'. Killing of >99.9% (3 logs) of the starting inoculum was defined as a fungicidal effect.[19] Statistical analysis was performed using one-way analysis of variance (ANOVA), in SPSS 13.0 (SPSS Inc., Chicago, IL, USA) for Windows.

Results

Antifungal activities and interactions of drugs

The antifungal activities of the drug alone was assessed. For 22 clinical isolates of FLC-R *C. albicans*, the MIC values were ranged from 128 to ≥ 512 μg ml^{-1} for FLC and 16 to 64 μg ml^{-1} for PAB treatment. For 12 clinical isolates of FLC-S *C. albicans*, the MIC values ranged from 2 to 8 μg ml^{-1} for FLC and 128 to 512 μg ml^{-1} for PAB treatment (Table 1). In this experiment, the MIC value of FLC against ATCC 10231 was 2 μg ml^{-1} while the MIC of PAB against ATCC 10231 was 32 μg ml^{-1}. These results showed that PAB have better potential *in vitro* antifungal activity against FLC-R clinical isolates than against FLC-S clinical isolates.

The results of *in vitro* interaction between FLC and PAB against the *C. albicans* strains are shown in Table 1. There was good agreement between the FICI and BI models for the FLC/PAB combination treatment. For the 22 FLC-R strains tested, the interaction between FLC and PAB was SYN in all FLC-R strains using the FICI method, with FICI values ranging from 0.02 to 0.13. Using the BI method, all FLC-R strains showed very high percentages of SYN interactions, ranging from 250.5% to 1336.4% (Table 1). For 13 of the FLC-S strains tested, including *C. albicans* ATCC 10231, the FLC/PAB combination treatment displayed SYN or indifference with FICI values ranging from 0.25 to 1.25 using the FICI method, while the BI method indicated that the strain also showed either SYN or indifference interactions. We did not observe ANT interactions between FLC and PAB in either FLC-R or FLC-S *C. albicans*. The results above showed that there was good SYN antifungal effects against FLC-R clinical isolates when PAB was combined with FLC.

Time–kill curves

Further time–kill studies were conducted using FLC and PAB against one chosen clinical isolate FLC-R *C. albicans*

YL313 and one FLC-S *C. albicans* YL381. For FLC-R strain tested, time–kill curves verified synergism for the FLC/PAB combination [Fig. 1, resistant strain (R)]. The antifungal effect of PAB at 16 μg ml^{-1} was more marked against *C. albicans* YL313 than FLC at 256 μg ml^{-1}. Given an initial inoculum density of 10^5 cfu ml^{-1}, combination therapy yielded a 2.06 $\log_{10}$ cfu ml^{-1} decrease compared with 16 μg ml^{-1} PAB after 24 h of incubation. The fungistatic activity of FLC was dramatically enhanced by the addition of PAB. In *C. albicans* YL313, the combination of FLC and PAB was fungicidal after 36 h of incubation (>99.9% decrease in viable counts). However, for FLC-S strain tested, an increases of 0.83 $\log_{10}$ cfu ml^{-1} in antifungal activity was produced by FLC/PAB combination treatment compared with the more active agent alone after 24 h. According to the result, indifference was observed for drug combinations against the FLC-S strain YL381 [Fig. 1, susceptible strain (S)].

Discussion

Previous reports revealed that the aqueous extract of *P. kaempferi* Gord significantly thickened the hypha of *Trichophyton rubrum* when observed under a transmission electron microscope, and in addition, the internal substances of the cytoplasm and organelles were degraded, the empty cavity appeared and an irregular membraneous structure resided in the fungal cell.[21] It was also shown that PAB is effective at inhibiting tumour growth targeting microtubules.[22] From the data presented above in this study, we found that PAB alone has moderate and high MIC values against the FLC-R and FLC-S *C. albicans* strains tested respectively. In addition, PAB showed more SYN activity when administered with FLC against FLC-R strains than against FLC-S strains. To our surprise, treatment with PAB alone is more effective against FLC-R strains than against FLC-S strains, while the phenomenon is different from the synergist compounds to FLC such as tacrolimus (FK506), cyclosporin A, amiodarone, ibuprofen and retigeric acid B reported previously.[13,14,23–25] Notably, the synergistic effect of FLC in combination with PAB is better than FLC in combination with other reported chemicals.[13,14,23–25] In our studies, compared with the FICI, the BI model not only allows for objective statistical criterion, but also fits all the experimental concentrations to construct a 3D graph in order to visualise the nature and intensity of drug combinations without arbitrarily choosing an end point.[13] Compared with the fully parametric and semi-parametric response surface approaches,[26] the BI model is not dependent on
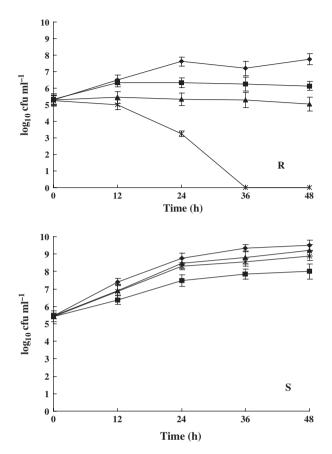
Figure 1 Time–kill curves of fluconazole (FLC) and pseudolaric acid B (PAB) alone and in combination against clinical azole-resistant strain (R) YL 313 and clinical azole-susceptible strain (S) YL 381. The strains at a starting inoculum density of 10^5 cfu ml^{-1} were exposed to *in vivo*-achievable concentrations of 256 μg ml^{-1} FLC, 16 μg ml^{-1} PAB and 256 μg ml^{-1} FLC + 16 μg ml^{-1} PAB for R strain and 4 μg ml^{-1} FLC, 256 μg ml^{-1} PAB and 4 μg ml^{-1} FLC + 256 μg ml^{-1} PAB for S strain respectively. At 0, 12, 24, 36, and 48 h, aliquots were removed from each test tube to examine the cell viability. ♦, Growth control; ■, FLC; ▲, PAB; *, FLC + PAB. The experiments were performed three times. Data are expressed as mean ± standard deviation.

the data analysis program or the sigmoid dose–response, and thus does not fail to fit the data. Furthermore, the BI model showed an excellent reproducibility for the 77 combinations calculated for each strain. We also suggest the use of the BI model to assess the interaction between two drugs for its peerless advantages.[23] In general, our results indicated that there was a good agreement between the FICI and the BI models.

We verified the interactions in chequerboard microdilution using the time-killing test. Time–kill curves can provide growth kinetic information and give a more detailed picture of the effect of drug combinations on cell viability. This method is able to detect differences in the rate and extent of antifungal activity over time.[7] Our results showed that the combination of PAB and FLC exhibited synergism or indifference against FLC-R or FLC-S *C. albicans* respectively. In this experiment, there was a good agreement between the conclusions drawn from the FICI method and the time–kill curves for the strains tested.

In conclusion, results in this study showed that the combination of FLC and PAB exhibited a good synergism against azole-R isolates of *C. albicans* using two non-parametric model approaches and that these results were verified by the time-killing test in some strains. However, the potential of using this combination therapy *in vivo* requires further investigation and further analysis is necessary to determine the underlying mechanism of this SYN interaction between FLC and PAB.

Acknowledgments

References

1 Sims CR, Ostrosky ZL, Rex JH. Invasive candidiasis in immunocompromised hospitalized patients. *Arch Med Res* 2005; **36**: 660–71.

2 Gafter-Gvili A, Vidal L, Goldberg E, Leibovici L, Paul M. Treatment of invasive candidal infections: systematic review and meta-analysis. *Mayo Clin Proc* 2008; **83**: 1011–21.

3 Georgopapadakou NH, Tkacz JS. The fungal cell wall as a drug target. *Trends Microbiol* 1995; **3**: 98–104.

4 Rex JH, Walsh TJ, Sobel J *et al.* Practice guidelines for the treatment of candidiasis. *Clin Infect Dis* 2000; **30**: 662–78.

5 Masia Canuto M, Gutierrez-Rodero F. Antifungal drug resistance to azoles and polyenes. *Lancet Infect Dis* 2002; **2**: 550–63.

6 Priya U, Jeniel N, Joseph H, David A. Synergistic effect of calcineurin inhibitors and fluconazole against *Candida albicans* biofilms. *Antimicrob Agents Chemother* 2008; **52**: 1127–32.

7 Lewis RE, Diekema DJ, Messer SA, Pfaller MA, Klepser ME. Comparison of Etest, chequerboard dilution and time-kill studies for the detection of synergy or antagonism between antifungal agents tested against *Candida* species. *J Antimicrob Chemother* 2002; **49**: 345–51.

8 Yang SP, Wang Y, Wu Y, Yue JM. Five new diterpenoids from *Pseudolarix kaempferi*. *J Nat Prod* 2002; **65**: 1041–44.

9 Erguang L, Alice MC, Charles DH. Antifungal evaluation of pseudolaric acid B, a major constituent of *Pseudolarix kaempferi*. *J Nat Prod* 1995; **58**: 57–67.

10 Quan H, Cao YY, Xu Z *et al.* Potent in vitro synergism of fluconazole and berberine chloride against clinical isolates of *Candida albicans* resistant to fluconazole. *Antimicrob Agents Chemother* 2006; **50**: 1096–99.

11 Nooney L, Matthews RC, Burnie JP. Evaluation of Mycograb, amphotericin B, caspofungin, and fluconazole in combination against *Cryptococcus neoformans* by checkerboard and time-kill methodologies. *Diagn Microbiol Infect Dis* 2005; **51**: 19–29.

12 National Committee for Clinical and Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved standard. M27-A.* Wayne, PA: National Committee for Clinical and Laboratory Standards, 1997

13 Sun S, Li Y, Guo Q, Shi C, Yu J, Ma L. *In vitro* interactions between tacrolimus and azoles against *Candida albicans* determined by different methods. *Antimicrob Agents Chemother* 2008; **52**: 409–17.

14 Li Y, Sun S, Guo Q *et al.* In vitro interaction between azoles and cyclosporin A against clinical isolates of *Candida albicans* determined by the chequerboard method and time-kill curves. *J Antimicrob Chemother* 2008; **61**: 577–85.

15 Meletiadis J, Mouton JW, Meis JF, Verweij PE. In vitro drug interaction modeling of combinations of azoles with terbinafine against clinical *Scedosporium prolificans* isolates. *Antimicrob Agents Chemother* 2003; **47**: 106–17.

16 Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother* 2003; **52**: 1.

17 Prichard MN, Prichard LE, Shipman CJ. Strategic design and three-dimensional analysis of antiviral drug combinations. *Antimicrob Agents Chemother* 1993; **37**: 540–45.

18 Klepser ME, Wolfe EJ, Jones RN, Nightingale CH, Pfaller MA. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B tested against *Candida albicans*. *Antimicrob Agents Chemother* 1997; **41**: 1392–95.

19 Marchetti O, Moreillon P, Glauser MP, Bille J, Sanglard D. Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrob Agents Chemother* 2000; **44**: 2373–81.

20 Nash JD, Burgess DS, Talbert RL. Effect of fluvastatin and pravastatin, HMG-CoA reductase inhibitors, on fluconazole activity against *Candida albicans*. *J Med Microbiol* 2002; **51**: 105–9.

21 Tang JH, Zhang XX, Liu W, Wang GL. Study on fungistasis action and ultrastructure of *Pseudolarix kaempferi* Gord. *J Liaoning Normal Univ (Nat Sci Edn)* 2005; **28**: 339–41.

22 Vincent KWW, Pauline C, Stephen SMC *et al.* Pseudolaric acid B, a novel microtubule-destabilizing agent that circumvents multidrug resistance phenotype and exhibits antitumor activity in vivo. *Clin Cancer Res* 2005; **11**: 6002–11.

23 Guo Q, Sun S, Yu J, Li Y, Cao L. Synergistic activity of azoles with amiodarone against clinically resistant *Candida albicans* tested by chequerboard and time-kill methods. *J Med Microbiol* 2008; **57**: 457–62.

24 Arai R, Sugita T, Nishikawa A. Reassessment of the *in vitro* synergistic effect of fluconazole with the non-steroidal anti-inflammatory agent ibuprofen against *Candida albicans*. *Mycoses* 2005; **48**: 38–41.

25 Sun L, Sun S, Cheng A, Wu X, Zhang Y, Lou H. In vitro activities of retigeric acid B alone and in combination with azole antifungal agents against *Candida albicans*. *Antimicrob Agents Chemother* 2009; **53**: 1586–91.

26 Afeltra J, Vitale RG, Mouton JW, Verweij PE. Potent synergistic in vitro interaction between nonantimicrobial membrane-active compounds and itraconazole against clinical isolates of *Aspergillus fumigatus* resistant to itraconazole. *Antimicrob Agents Chemother* 2004; **48**: 1335–43.