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The Synergistic Effect of Azoles and Fluoxetine against Resistant *Candida albicans* Strains Is Attributed to Attenuating Fungal Virulence

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This study evaluated the synergistic effects of the selective serotonin reuptake inhibitor, fluoxetine, in combination with azoles against *Candida albicans* both *in vitro* and *in vivo* and explored the underlying mechanism. MICs, sessile MICs, and time-kill curves were determined for resistant *C. albicans*. *Galleria mellonella* was used as a nonvertebrate model for determining the efficacy of the drug combinations against *C. albicans in vivo*. For the mechanism study, gene expression levels of the *SAP* gene family were determined by reverse transcription (RT)-PCR, and extracellular phospholipase activities were detected *in vitro* by the egg yolk agar method. The combinations resulted in synergistic activity against *C. albicans* strains, but the same effect was not found for the non-*albicans Candida* strains. For the biofilms formed over 4, 8, and 12 h, synergism was seen for the combination of fluconazole and fluoxetine. In addition, the time-kill curves confirmed the synergism dynamically. The results of the *G. mellonella* studies agreed with the *in vitro* analysis. In the mechanism study, we observed that fluconazole plus fluoxetine caused downregulation of the gene expression levels of *SAP1* to *SAP4* and weakened the extracellular phospholipase activities of resistant *C. albicans*. The combinations of azoles and fluoxetine showed synergistic effects against resistant *C. albicans* may diminish the virulence properties of *C. albicans*.

The incidence of invasive fungal infections has increased significantly in the last several years because of the wide-spread use of broad-spectrum antibiotics and immunosuppressants and increases in invasive procedures and use of medical implant devices (1–3). *Candida* spp. are some of the most common fungal pathogens in invasive fungal infections (4). Due to the limited number of antifungal agents and their potential toxicity, therapeutic options for fungal infections are exceedingly insufficient. The most commonly used drugs are the azoles, such as fluconazole, itraconazole, and voriconazole. Since azoles (especially fluconazole) have been frequently used in clinical practice because of their greater efficacy and lower toxicity, the emerging resistance to them is becoming a major concern with regard to their clinical application. The development of antifungal agents is not easy; therefore, use of drug combinations is a practical solution.

Selective serotonin reuptake inhibitors (SSRIs) are being used as antidepressants and as the first-line therapy for premenstrual syndrome (5). The antifungal activities of SSRIs were first reported by Lass-Flörl et al. (6). Since then, several studies have been conducted to reveal the antifungal activities of SSRIs (7–11). However, few were carried out to study its anti-biofilm activity and mechanism. Thus, further study of the combination of azoles and SSRIs is of great significance for the problem of increasing drug resistance of *Candida* species.

In this study, we first investigated the *in vitro* activity of one commonly used SSRI, fluoxetine, combined with one of three azoles, fluconazole, itraconazole, or voriconazole, against different *Candida* spp. with antifungal resistance by a microdilution checkerboard method. These drug combinations were also tested against planktonic *C. albicans* to compare the results to those for biofilms. In addition, time-kill curves were performed to investigate the antifungal effects of fluoxetine in combination with fluconazole, itraconazole, or voriconazole against a resistant *C. albicans* strain (CA10) at different time points dynamically. A *Galleria mellonella* infection model was used to study whether the combined treatment had any protective role during *C. albicans* infection *in vivo*. Additionally, fungal burden and histopathology were also evaluated. Secreted aspartyl proteinase (SAP) is encoded by the *SAP* gene family. These genes exhibit differential expression profiles at different stages and sites of infection (12). The gene expression levels of *SAP1* to *SAP4* were determined by reverse transcription (RT)-PCR. Phospholipase activity can destabilize host membranes, lyse cells, and release lipid second messengers (13), which are considered to be important virulence factors for many microorganisms (14) In this work, extracellular phospholipase activities were detected *in vitro* by the egg yolk agar method.

MATERIALS AND METHODS

***Candida* species cultivation.** Eleven resistant isolates of *Candida albicans* ($n = 2$), *Candida glabrata* ($n = 3$), *Candida krusei* ($n = 3$), and *Candida tropicalis* ($n = 3$) were used in this study. Their susceptibilities were de-

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termined according to Clinical and Laboratory Standards Institute document M27-A3 (15, 16) with *Candida parapsilosis* ATCC 22019 as the reference strain. Frozen stocks of isolates were maintained at −80°C until testing. After thawing, the yeast cells were subcultured on yeast-peptone-dextrose (YPD) solid medium (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at least twice at 35°C before each experiment to ensure viability and purity. RPMI 1640 was used as the liquid medium for diluting drugs and strains.

Drugs. All 4 drugs (fluoxetine, fluconazole, itraconazole, and voriconazole) were purchased from Dalian Meilun Biotech Co. Ltd., China. The stock solution was prepared following the manufacturer's instructions. Fluoxetine and fluconazole were dissolved in sterile demineralized water at room temperature to achieve stock solutions of 2,560 μg/ml. Itraconazole and voriconazole were dissolved in dimethyl sulfoxide (DMSO) to form stock solutions of 256 μg/ml. All stock solutions were stored at −20°C. From the stock solutions, 2-fold serial dilutions were prepared.

Determination of MICs of planktonic cells. MICs for the azoles were determined according to the approved CLSI standard reference method (document M27-3) for antifungal susceptibility testing of yeasts by broth diffusion. For the checkerboard assays (17–19), 50 μl of RPMI 1640 medium containing azoles was added to the wells in the 2nd to 11th columns of the microtiter plate, and 50-μl aliquots of RPMI 1640 medium containing fluoxetine with concentrations ranging from 128 to 2 μg/ml were added to the wells in the A to G lines of the microtiter plate. The final concentration of fluconazole ranged from 1 to 512 μg/ml for *C. albicans* with an MIC of 512 μg/ml and from 0.5 to 256 μg/ml for the remaining isolates. The concentrations of itraconazole and voriconazole ranged from 0.08 to 4 μg/ml for all isolates. Next, 100-μl aliquots of *Candida* cell suspensions (1.0×10^3 cells/ml) were added to each well mentioned above. All of the wells on the plate were filled with RPMI 1640 to a final volume of 200 μl. The plate was covered with its lid, sealed with Parafilm and incubated at 35°C for 24 h. Readings were performed with both visual examination and optical density (OD) by determining the absorbance at 492 nm on a microplate reader. MIC endpoints were defined as the MIC_{50} values. All experiments were performed in triplicate. Drug interactions were interpreted by the fractional inhibitory concentration index (FICI) model and the percentage of growth difference (ΔE) model (20–22), respectively. An FICI of ≤ 0.5 represents synergy, an FICI of >4.0 represents antagonism, and a 0.5 < FICI ≤ 4.0 represents no interaction (23). In the ΔE model, the ΔE value was calculated by the data obtained directly from experiments. When the average value of ΔE was positive and the 95% confidence interval (CI) among the three replicates did not include 0, statistically significant (SS) synergy was claimed; when the average value of ΔE was negative and the 95% CI did not include 0, SS antagonism was claimed.

Determination of sessile MICs of biofilms. Biofilms were formed as described by Ramage et al. (24) on 96-well plates in modified cell suspensions of 1×10^3 cells/ml. Results demonstrated that biofilms formed with this cellular density over at least 4 h of cultivation. The biofilms were formed over five time intervals (4, 8, 12, 24, and 48 h) at 37°C by pipetting 100 ml of the standardized cell suspension into selected wells of a 96-well plate. At each time point, the biofilms were washed three times gently with sterile phosphate-buffered saline (PBS) to remove the planktonic yeast. Fluconazole and fluoxetine were then added to the biofilms in serially double-diluted concentrations. The final concentration of fluconazole in wells ranged from 1 to 512 μg/ml for resistant isolate CA10. The final concentration of fluoxetine in wells ranged from 2 to 128 μg/ml. The control wells were filled with RPMI 1640 without antifungal agents. Then the whole system was incubated for a further 48 h at 35°C. A colorimetric reduction assay was carried out with XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] according to the protocol of Melo et al. (25). The absorbance was measured with a microtiter plate reader (Thermo Labsystems Multiskan MK3) at 492 nm. The drug concentration that brought about a reduction in absorption by 50% contrasted to that in the control well was reported as the sessile MIC (SMIC) endpoint. Each test was carried out in duplicate and repeated three times on different days.

Time-kill curve study. Inocula of 5.0×10^3 cells/ml of *C. albicans* (CA10) were used in this experiment. The final concentration was 16 μg/ml for fluconazole, 0.25 μg/ml for itraconazole, and 0.125 μg/ml for voriconazole when combined with fluoxetine (16 μg/ml). A drug-free sample served as a growth control. The XTT test was performed to detect the cell viability after different treatments according to the method described previously (26). Briefly, samples were incubated at 35°C on an orbital shaker and vortexed prior to removal of a sample for the determination of colony counts. At predetermined time points (0, 12, 24, 36, and 48 h after incubation), a 100-μl aliquot from each treatment mixture was transferred to a well of a new 96-well microtiter plate, and then a 100-μl aliquot of XTT-menadione solution was added (XTT was purchased from Sigma). Prior to each assay, XTT was dissolved in a saturated solution at 500 μg/ml in Ringer's lactate. The solution was filter sterilized through a 0.22-μm filter, and 100 mM menadione in acetone was added to a final concentration of 10 μM. The plate was then incubated in the dark for up to 2 h at 35°C. After that, the XTT reduction was assessed by determining the absorbance at 492 nm on a microplate reader (SpectraMax 190; Thermo Lab Systems, USA). All experiments were conducted in triplicate, and the results are reported as mean values (25). Thus, the growth- and metabolism-inhibitory effects of the drugs alone and in combination were observed based on the results of the spectrophotometric analyses.

Survival assay. *Galleria mellonella* survival assays were carried out according to a previously described methodology (27). *Galleria mellonella* larvae (0.25 ± 0.03 g) were placed in petri dishes and incubated at 37°C in the dark the night before the experiments. Larvae with dark spots or apparent melanization were excluded. Yeasts were grown overnight in liquid Sabouraud medium, washed with PBS, and suspended in the same buffer. For survival assays, larvae were inoculated with 1×10^7, 5×10^6, 1×10^6, and 5×10^5cells/larva of *C. albicans*. The inocula were prepared in PBS plus 20 μg/ml of ampicillin to prevent bacterial contamination. Yeast suspensions were injected in the hemocele through the last left proleg of the larvae using a 10-μl syringe (Gaoge, China). The infected larvae were incubated at 37°C, and death was monitored daily for 4 days. Larval death was monitored by visual inspection of their color (brown-dark brown) and by the lack of movement after touching them with forceps. A group of larvae inoculated with PBS-ampicillin were studied in parallel in every infection investigation as controls. For each condition, a total of 20 larvae were used, and each experiment was repeated at least three times.

Efficacy of fluconazole and fluoxetine in *G. mellonella* infected with *C. albicans*. Larval-killing assays were carried out at 37°C as described above, using a dose of 5×10^6 yeast cells/larva. Infected larvae were treated with fluconazole (80, 160, 320, and 640 μg/ml) (Meilun, Dalian, China) and fluoxetine (128 μg/ml) (Meilun). A combination of fluconazole and fluoxetine was also used. In addition, parallel groups of uninfected larvae treated with the same concentrations of drugs were included to eliminate possible drug toxicity and other effects of the drugs as factors contributing to the observed results. Antifungals were administered 2 h postinfection. Survival was monitored every 24 h for 4 days. Each experiment used groups containing 20 larvae, and experiments were repeated twice using larvae from different batches.

Fungal burden determination. The fungal burden was determined (28) by CFU counts at different times after inoculation. For this purpose, four groups of 80 larvae were selected, all of which received 2.5×10^6 cells/larva of the *C. albicans* strain. While one group remained untreated, the others were treated with fluconazole (320 μg/ml), fluoxetine (128 μg/ml), and the combination of fluconazole and fluoxetine. Every 24 h, 5 larvae were taken from each group, washed with ethanol, and cut into small pieces with a scalpel. No discrimination was made between live or dead larvae. The material was suspended in 1 ml of PBS-ampicillin and homogenized gently with a vortex for a few seconds. The homogenate was 10-fold diluted with same buffer, and 5-μl aliquots of the resulting dilu-

tions were inoculated onto Sabouraud agar plates. The plates were incubated for 24 h at 37°C, and CFU were enumerated, with the results expressed as averages and standard deviations.

Histological study of larval tissue. To evaluate the presence of *C. albicans* inside tissues of *G. mellonella*, three larvae from different groups (uninfected, infected, and treated with the fluconazole-fluoxetine combination) were collected on the third day after infection. Larvae were fixed for 24 h in 4% buffered formalin and dehydrated with increasing concentrations of ethanol (70%, 80%, 90%, 96%, and 100%). The samples were then treated with xylene and paraffin embedded. Tissue sections of 8 μm were stained with periodic acid-Schiff (PAS) and observed under an Olympus FSX100 fluorescence microscope with 4.2× and 10× objectives. Samples from noninfected larvae were included as controls.

Statistics. Graphs were created and statistical analyses were performed with GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). Survival curves were analyzed by the Kaplan-Meier method, and fungal burdens were analyzed using a *t* test.

Real-time quantitative PCR. For detecting the expression levels of aspartyl proteinase-related genes (*SAP1*, *SAP2*, *SAP3*, and *SAP4*), *C. albicans* (CA10) cells were grown to mid-log phase in RPMI 1640 medium at 35°C after treatment with drugs alone or in combination at the following final concentrations: fluconazole at 4 μg/ml and fluoxetine at 8 μg/ml. Cultures without drugs served as the controls. Cells were then harvested for RNA extraction. Cell total RNA was isolated using an RNApure yeast kit (DNase I) (CWBiotech, Beijing, China). Then, diluted RNA was treated with a first-strand cDNA synthesis SuperMix kit (CWBiotech) and was reverse transcribed at 42°C for 30 min and 85°C for 5 min according to the manufacturer's instructions. RT-PCR preparations were mixed with cDNA, ultra SYBR mixture (with ROX) (CWBiotech), and gene primers in triplicate. The *ACT1* gene was used as the endogenous control (29). RT-PCRs were carried out with an ABI ViiA 7 (Applied Biosystems) sequence detection system using SYBR green I (CWBiotech) in duplicate for three separate experiments. An aliquot of 25 ml of PCR mix was used for each gene and the cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were analyzed using SPSS statistical software, and significance was defined as a P value of <0.05.

Phospholipase activity of *C. albicans* treated with fluoxetine. The isolates were screened for phospholipase activity by measuring the size of the zone of precipitation after growth on egg yolk agar as described by Price et al. (30) with some modifications. The test medium consisted of 15 g/liter peptone, 3 g/liter beef extract ointment, 5 g/liter NaCl, 15 g/liter agar, 10 g/liter glucose, and 10% sterile egg yolk emulsion. Then 10-μl aliquots of a suspension of 10^7 CFU/ml were inoculated onto the surface of the test medium in duplicates. The plates were incubated at 37°C for 72 h, after which the diameter of the precipitation zone around the colony was measured. Phospholipase activity (P_z) was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone. The phospholipase activity was classified as negative ($P_z = 1$), very low ($P_z = 0.90$ to 0.99), low ($P_z = 0.80$ to 0.89), high ($P_z = 0.70$ to 0.79), and very high ($P_z = 0.69$), as previously reported (31). Each experiment was performed twice.

RESULTS

MICs of azoles in combination with fluoxetine. The combination of fluoxetine and azole agents displayed strong synergistic effects *in vitro*. Table 1 shows the MIC distributions of azoles and fluoxetine alone and in combination against *Candida* spp. Although fluoxetine alone had very limited antifungal activity, it significantly reduced the MICs of azoles against the azole-resistant *C. albicans* strains when used in combination. For the C. *albicans* strains, the MIC of fluconazole decreased from 256 to 2 and 4, the MIC of itraconazole decreased from 4 to 0.125, and that of voriconazole decreased from 4 to 0.06, with FICIs of <0.5. The synergies of the fluconazole-fluoxetine and voriconazole-fluoxetine combinations against C. *albicans* are illustrated in Fig. 1.

Although the combinations displayed strong synergistic effects against *C. albicans*, the same results were not observed when combination therapy was used against non-*albicans Candida*. The MICs of fluconazole reduced 1 or 2 dilutions in the presence of fluoxetine, and the FICI values were 0.625 to 1.5 (shown in Table 1). The combination of fluoxetine and itraconazole or voriconazole showed similar results.

SMICs of fluconazole in combination with fluoxetine. A consistent synergistic effect of fluconazole and fluoxetine against *C. albicans* biofilms formed over 4, 8, and 12 h was observed with an FICI of <0.5. However, as the biofilms matured and their complexity increased, the synergism weakened and was scarcely observed on the biofilms that formed over 24 h (FICI of >0.5). The combined antifungal effects are shown in Table 2.

Time-kill curves. The synergism of the azole-fluoxetine combinations against resistant *C. albicans* was confirmed by time-kill studies with OD values obtained from the XTT reduction assays as the *y* axis and time as the *x* axis (Fig. 2). Little differences were seen among the 4 groups in the first 12 h. After that a growth delay was seen in the azole and azole-fluoxetine groups, and it was more obvious in the combination groups. At 24 and 48 h, the OD values were reduced more than 2-fold in the azole-fluoxetine combination groups than in the azole-alone group, indicating synergistic antifungal effects. The combination of voriconazole and fluoxetine showed the strongest synergism by time-kill curves. The results were consistent with those from the checkerboard microdilution assays.

Survival assay of *G. mellonella* model infected with different yeast concentrations. First, the most suitable concentration of *C. albicans* to cause larvae infection was investigated. The range of inoculation concentrations in the study of Liliana et al. was used (27). Moreover, to confirm that the death was not a consequence of a shock due to large amounts of liquid injected in the larvae, a group of larvae were inoculated with PBS as controls. As expected, larval survival was significantly dependent on the number of yeast cells in the inoculum (Fig. 3). The most reproducible results were found when larvae were infected with 5×10^6 cells/larva. For further survival experiments, inocula of 5×10^6 cells/larva were used.

Activities of fluconazole and fluoxetine in the *G. mellonella* infection model. To determine whether the combination of fluconazole and fluoxetine has a synergistic effect *in vivo*, *G. mellonella* larvae were infected with resistant *C. albicans* (CA10) and treated with different drugs (fluconazole, fluoxetine, and their combination). The results showed that treatment with fluconazole increased survival at the concentration of 320 μg/ml ($P < 0.05$). At higher concentrations (640 μg/ml), there was a decrease in survival, which might be explained by the toxicity of the antifungal at this high concentration. When treated with the combination of fluconazole and fluoxetine, the highest survival rate was also observed in the group with the concentration of 320 μg/ml. Thus, the survival rates of the fluconazole group and the combination group were compared at the same concentration (320 μg/ml). As shown in Fig. 4, the group treated with fluconazole combined with fluoxetine had the highest survival rate.

Fungal burden determination and histopathology. The fungal burden was determined by recovering yeast cells from the larvae infected with *C. albicans* and treated with fluconazole (320

TABLE 1 Combined drug effects against *Candida* spp. evaluated by the FICI model

Drug and strain[a]	MIC (μg/ml) for[b]:				FICI[c]	Effect
	Azole	$Azole_{comb}$	FLUO	$FLUO_{comb}$		
FLC						
CA10	256	2	128	16	0.13	Synergistic
CA16	256	4	128	16	0.14	Synergistic
CG2	32	16	64	32	1	No interaction
CG3	32	16	32	32	1.5	No interaction
CG8	32	16	32	32	1.5	No interaction
CK8	64	32	32	32	1.5	No interaction
CK9	64	16	64	32	0.75	No interaction
CK10	32	16	64	32	1	No interaction
CT10	64	16	64	32	0.75	No interaction
CT11	64	8	64	32	0.625	No interaction
CT18	32	4	64	32	0.625	No interaction
ITC						
CA10	4	0.125	128	16	0.16	Synergistic
CA16	4	0.125	128	8	0.09	Synergistic
CG2	4	0.5	64	32	0.625	No interaction
CG3	2	1	32	16	1	No interaction
CG8	2	0.5	32	32	1.25	No interaction
CK8	2	0.5	32	32	1.25	No interaction
CK9	4	2	64	32	1	No interaction
CK10	1	0.5	64	16	0.75	No interaction
CT10	2	1	64	16	0.75	No interaction
CT11	2	1	64	8	0.625	No interaction
CT18	1	0.5	64	8	0.625	No interaction
VRC						
CA10	4	0.06	128	16	0.14	Synergistic
CA16	2	0.06	128	16	0.16	Synergistic
CG2	1	0.25	64	32	0.75	No interaction
CG3	0.5	0.25	32	16	1	No interaction
CG8	0.5	0.25	32	32	1.5	No interaction
CK8	2	0.5	32	32	1.25	No interaction
CK9	2	0.5	64	32	0.75	No interaction
CK10	1	0.5	64	16	0.75	No interaction
CT10	2	0.5	64	32	0.75	No interaction
CT11	2	0.25	64	32	0.625	No interaction
CT18	1	0.5	64	16	0.75	No interaction

[a] Eleven strains involved 4 kinds of *Candida* spp. CA, *Candida albicans*; CG, *Candida glabrata*; CK, *Candida krusei*; CT, *Candida tropicalis*; FLU, fluconazole; ITC, itraconazole; VRC, voriconazole; FLUO, fluoxetine.

[b] MIC denotes the MIC_{50} of each drug alone or in combination (comb) against the strain and is shown as the median from three independent experiments.

[c] The FICI value is the median from three independent experiments.

μg/ml), fluoxetine (128 μg/ml), or fluconazole-fluoxetine. The CFU of the control group decreased, which may by caused by hemocytes in larvae (Fig. 5). Treatment of larvae infected with *C. albicans* with fluconazole decreased the number of CFU by 2-fold. The combination of fluconazole and fluoxetine reduced the fungal burden by almost 4-fold. The greatest effects were found on the second and third days. The larval burden of the combination group presented an up-trend with time, which may suggest a limited interaction time.

Histopathology studies of larvae infected with *C. albicans* were performed at day 3 postinfection. Yeast cells and filaments were observed in the tissue, primarily in clusters, both in the treated and untreated larvae. However, in the larvae of the untreated, fluconazole, and fluoxetine groups, there were higher numbers of infected areas than for the larvae with the combination treatment. Moreover, the fungi were mainly found in defined structures surrounded by *G. mellonella* cells (Fig. 6).

Effect of fluconazole-fluoxetine on the expression levels of *SAP1* to *SAP4*. The results of RT-PCR assays showed that fluoxetine alone caused downregulation in the expression levels of *SAP1*, *SAP2*, and *SAP4*, and the expression level of *SAP3* had no obvious change. The expression levels of *SAP1* to *SAP4* were decreased with the fluconazole challenge by dozens-fold compared with those of the control group ($P < 0.01$). The combination of fluconazole and fluoxetine significantly downregulated the expression levels of *SAP1*, *SAP2*, and *SAP4* compared with those for the fluconazole challenge alone by a greater than 4-fold, 2-fold, and 8-fold, respectively ($P < 0.01$). The combined group downregulated the expression level of *SAP3* compared with that for the fluconazole-alone groups, but there was no significant difference (Fig. 7).

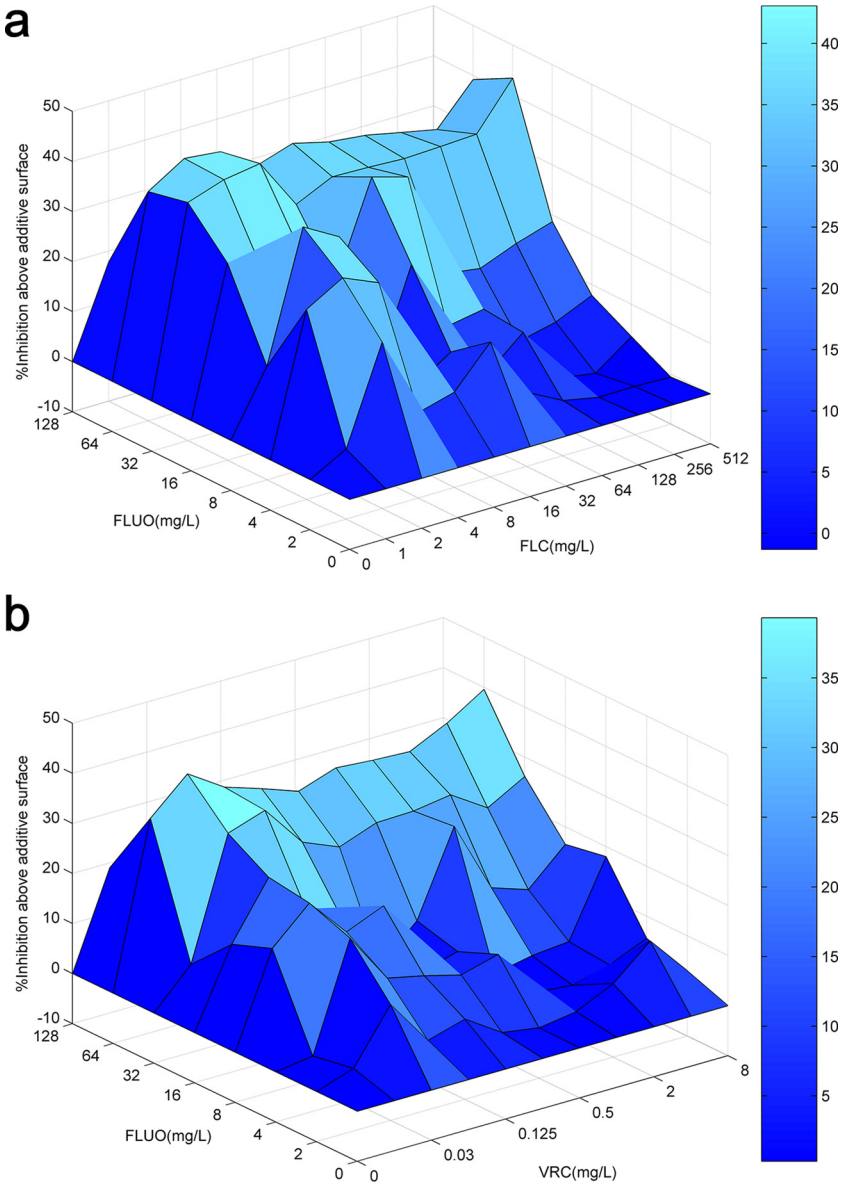

FIG 1 (a and b) Three-dimensional plots of fluconazole (FLC) and voriconazole (VRC) combined with fluoxetine (FLUO) against *Candida albicans* CA10. Plots were created by using MATLAB. Drug interactions of azoles and FLUO are interpreted by the Δ*E* model. The concentrations of azoles and FLUO are depicted on the *x* axis and *y* axis, respectively, and the Δ*E* values are depicted on the *z* axis to construct a three-dimensional (3D) graphic. Peaks above the 0 plane represent synergistic combinations. The color-coding bar on the right indicates that the closer to the top of the bar, the more effective the drug combination.

Effect of fluoxetine on the activity of extracellular phospholipases of resistant *C. albicans*. The extracellular phospholipase activity of *C. albicans* was measured using the egg yolk agar method. The mean extracellular phospholipase activity (P_z) in the control was 0.80 ± 0.04. Treatment with fluoxetine at 16 μg/ml and 32 μg/ml significantly reduced the extracellular phospholipase activity with mean P_z values of 0.71 ± 0.02 ($P < 0.05$) and 0.63 ± 0.04 ($P < 0.01$). No precipitation zone was observed with higher concentrations of fluoxetine. Treatment with fluoxetine significantly decreased extracellular phospholipase activity compared with that of the controls (Table 3).

DISCUSSION

More and more clinical cases of antifungal resistance for the class of azole drugs are being reported (32), and fungal biofilm-associated infections are frequently refractory to conventional therapy (33, 34). It is important to find a synergistic drug combination to reverse this drug resistance.

In our previous studies, a number of non-antifungal drugs such as ion channel inhibitors, antibiotics, and immune inhibitors had an effect on the physiology and viability of fungi (35–38). The antifungal activities of antidepressant drugs were first discovered in 2001 when three patients with chronic vulvovaginal candidiasis (VVC) were treated with sertraline for premenstrual syndrome (6). Since then, several studies have been carried out to explore the effects of SSRIs against fungi. Lass-Flörl et al. showed that sertraline exhibited a clear *in vitro* positive effect against *Candida* species (6). Samanta et al. demonstrated that high-dose sertraline (>200 μg/ml) inhibited the growth of *C. albicans* and *C. tropicalis* (9).

TABLE 2 Combined antifungal effects of fluconazole alone and in combination with fluoxetine against biofilms of resistant *Candida albicans*

Time (h)[a]	SMIC of drug (μg/ml)[b]				FICI[c]	Effect
	FLC	FLC_{comb}	FLUO	$FLUO_{comb}$		
4	512	4–8	128	32	0.25	Synergistic
8	512	4–8	128	32	0.25	Synergistic
12	512	8	128	32	0.27	Synergistic
24	512	32	128	64	0.56	No interaction
48	512	256	128	128	1.5	No interaction

[a] Time indicates the incubation period of biofilm formation.
[b] SMICs were read as the lowest concentrations that produced a 50% reduction in growth compared with that of the drug-free control. FLC, fluconzole; FLUO, fluoxetine.
[c] The FICI value is the median from three independent experiments.

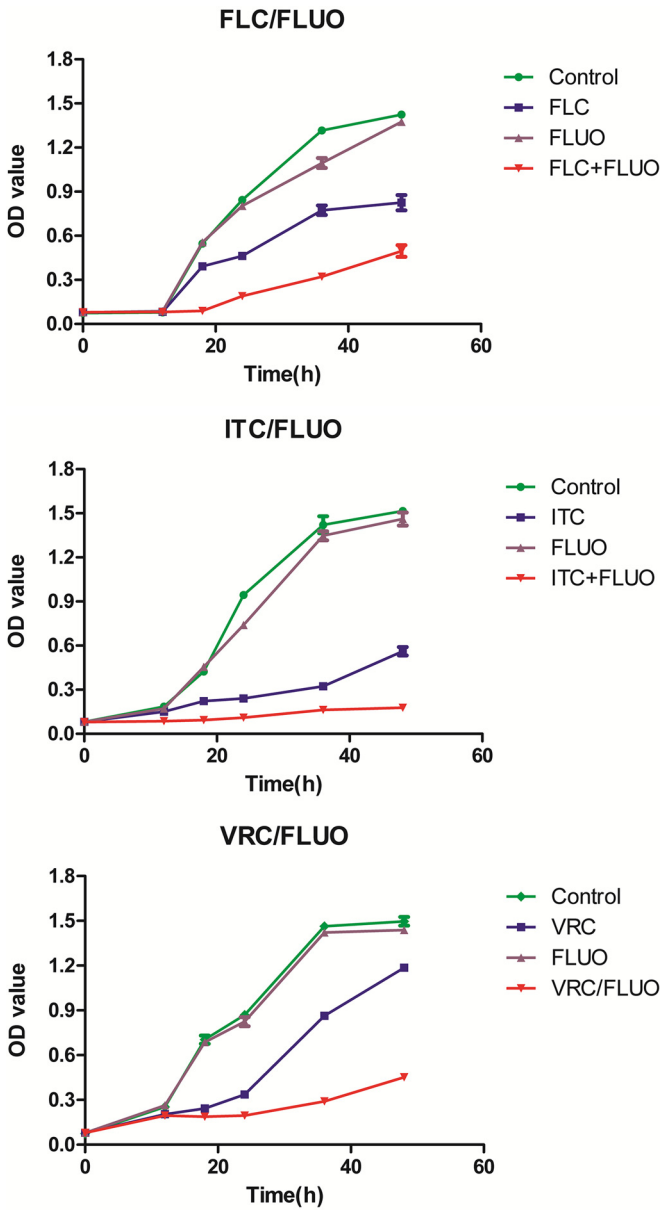

FIG 2 Time-kill curves of fluconazole (FLC)/itraconazole (ITC)/voriconazole (VRC) in combination with fluoxetine (FLUO) against resistant *C. albicans* (CA10). Cells were diluted in RPMI 1640 medium containing FLC, ITC, VRC, FLUO, and combinations of FLUO with each of the four azoles. The concentration of FLUO was 16 μg/ml when combined with FLC (8 μg/ml), ITC (0.25 μg/ml), and VRC (0.125 μg/ml).

Young et al. showed that both *Candida* species and *Aspergillus* species are susceptible to sertraline (10). Two other studies showed that sertraline has antifungal activity against *Aspergillus* species when combined with amphotericin B (7, 8). A recent study demonstrated that the combination of fluconazole and fluoxetine showed synergism against resistant *C. albicans* and non-*albicans Candida* spp. (11). However, that study only determined the MICs of combination therapy. None of the studies mentioned above evaluated antifungal activity by time-kill curves, which may provide a dynamic picture of antifungal action and interaction over time. Furthermore, the antifungal effect against fungal biofilms and the data *in vivo* were lacking, no mechanisms were further studied, and none of the studies evaluated the interaction between fluoxetine and other commonly used azoles. In this study, we evaluated the effect of an azole (fluconazole, itraconazole, or voriconazole) combined with fluoxetine against resistant *Candida albicans* both *in vitro* and *in vivo* and used fluconazole to explore possible mechanisms.

The results of checkerboard tests demonstrated that the SSIR fluoxetine worked synergistically with azoles (fluconazole, itraconazole, and voriconazole) against resistant *C. albicans* strains. A weaker combined antifungal effect of fluconazole-fluoxetine against *C. albicans* biofilms was observed and compared with the results in planktonic cells, and the combined efficacy decreased with a prolonged time of biofilm formation. The time-kill curves confirmed the findings, and these results are in accordance with those of the earlier study on fluoxetine against *Candida* spp. (7, 11).

Furthermore, *in vivo* antifungal activity was determined by using a nonmammalian model, *G. mellonella*. To the best of our knowledge, this study is the first to explore the antifungal effect of the combination of azoles and SSRIs in an invertebrate model. *G. mellonella* allows the use of precise doses of both the pathogen and antimicrobial agents by infection, and there is a correlation between the virulence of a microorganism in *G. mellonella* and in mammalian models (39, 40). With the characteristics of low cost and easy manipulation, *G. mellonella* is gaining wide acceptance as a nonconventional model to study microbial pathogenesis.

The survival assay showed that, at concentrations equivalent to subtherapeutic doses in humans, the combined treatment significantly prolonged the rate of survival of *G. mellonella*, compared with that of larvae treated with fluconazole alone. The efficacies of the antimicrobials on infected larvae closely correlate with the drug susceptibilities of resistant *C. albicans* strain *in vitro*.

In addition, determination of larval burdens postinfection showed that combined antifungal therapy significantly reduced the numbers of *C. albicans* cells detected inside the larvae. Also, the

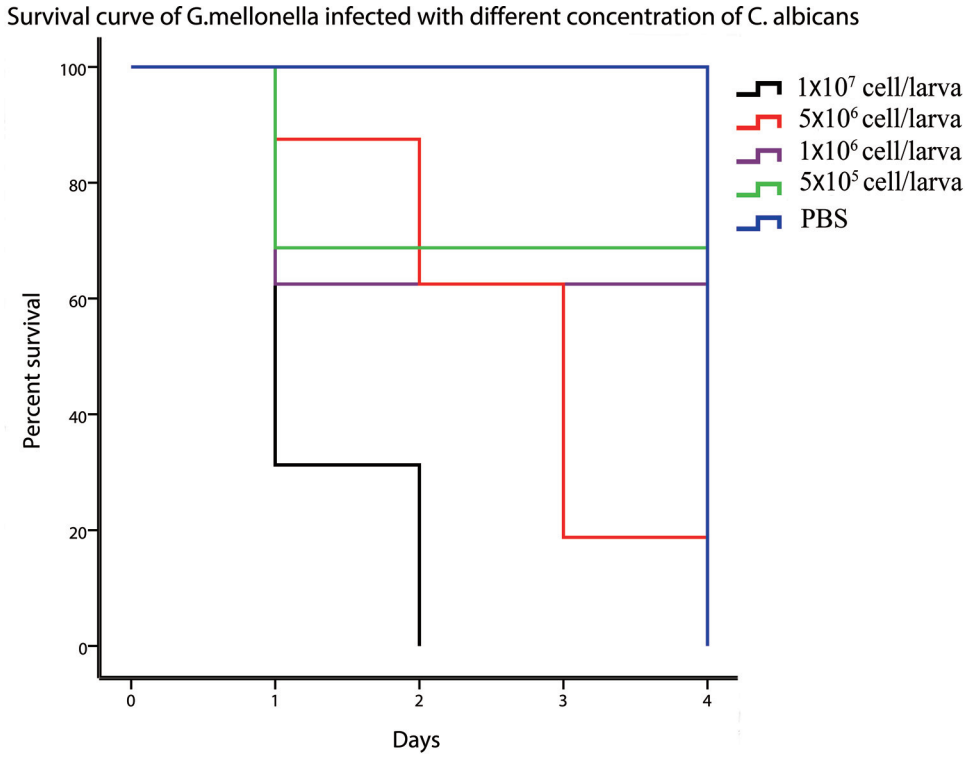

FIG 3 Survival curve of *G. mellonella* infected with different concentrations of resistant *C. albicans* (CA10). The most reproducible results were found when larvae were infected with 5×10^6 *C. albicans* cells/larva.

larval burden of the combination group presented an up-trend with time, which may suggest a limited interaction time. Another point worth noting is that the CFU of the control group also decreased compared to the inoculum injected. This can be explained by the action of hemocytes, which play an important role in the larva's cellular defense against bacterium- and fungus-like phagocytic cells (41, 42).

Taken together, these findings confirm that the *G. mellonella* model may prove useful for evaluating the *in vivo* efficacy of antimicrobial agents. In this work, we have demonstrated that larval killing was significantly dependent on the number of *C. albicans* cells injected. Results revealed a positive correlation between the inoculum number of *C. albicans* cells and the death rate of larvae.

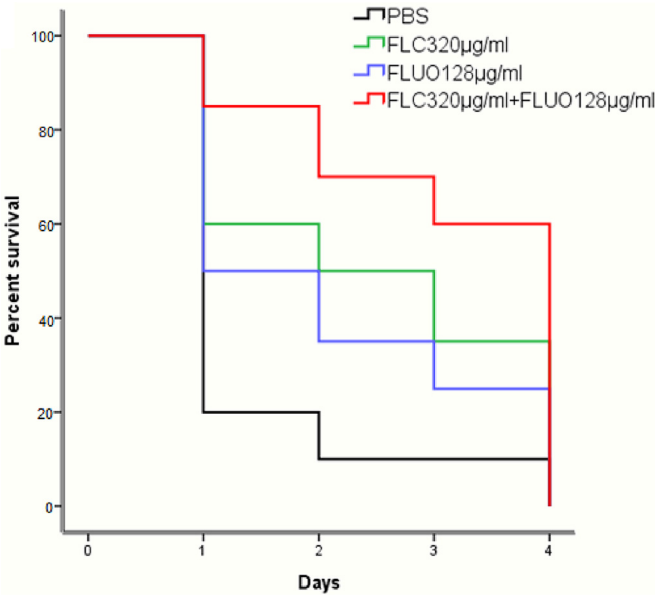

FIG 4 Efficacy of FLC alone or in combination with FLUO during *G. mellonella* infection with resistant *C. albicans*. The concentration of yeast cells was 5×10^6 cells/larva. For comparison purposes, the curves of PBS, 320 μg/ml FLC, 128 μg/ml FLUO, and 128 μg/ml FLUO + 320 μg/ml FLC were extracted. These four curves were put in the same coordinate system to compare the survival rates.

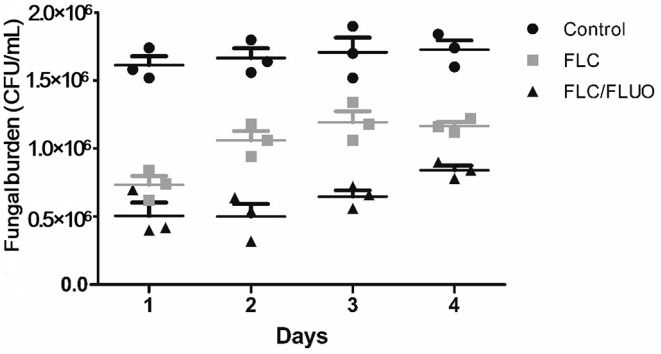

FIG 5 Effect of treatment with FLC and the combination of FLC and FLUO on larval burdens of resistant *C. albicans* (CA10). All larvae were inoculated with 2.5×10^6 cells/larva CA10 and treated with 10 μl of each individual drug or combination at 2 h postinfection. Treatments consisted of PBS, FLC (320 μg/ml) alone, FLUO (128 μg/ml) alone, or a combination of FLC (320 μg/ml) with FLUO (128 μg/ml). For clarity, data for treatment with FLUO are not shown because the data obtained closely followed those shown for the control group.

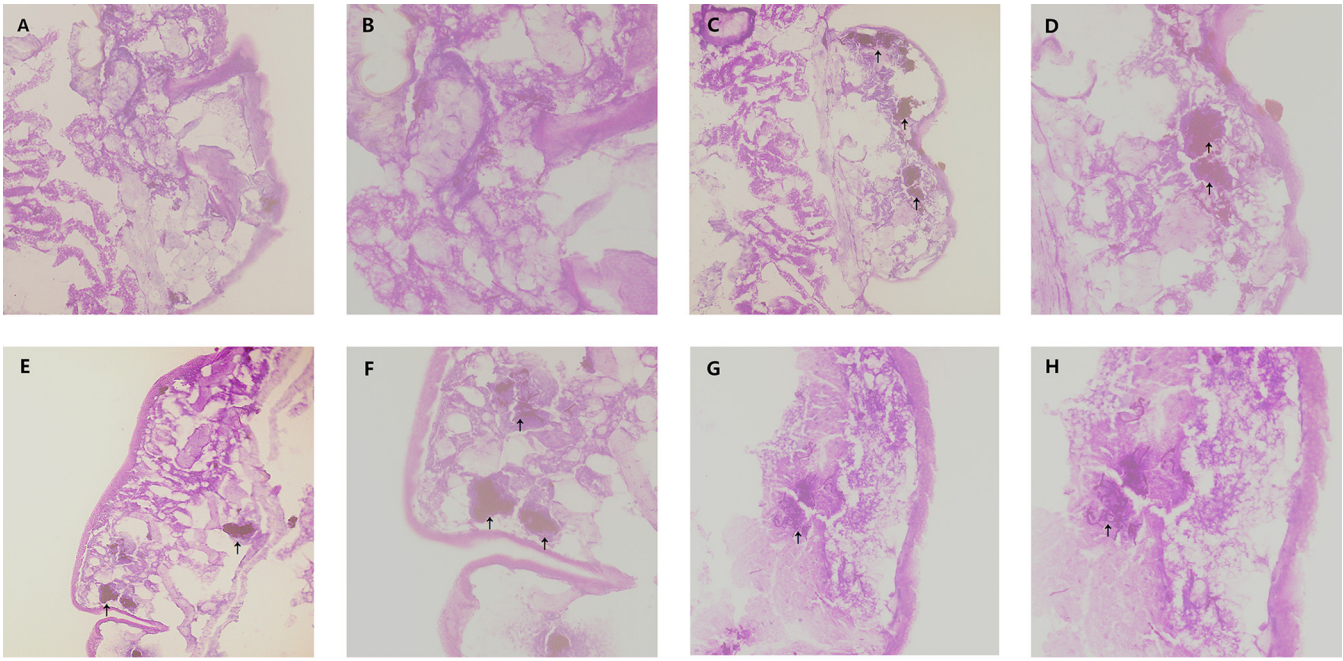

FIG 6 Histopathology of *G. mellonella* infected with *C. albicans* and treated with different agents. *Galleria mellonella* was infected with 5×10^6 cells/larva of resistant *C. albicans* CA10 (C−H). After 72 h of infection, larvae were processed for histopathology as described in Materials and Methods. (A and B) Uninfected controls; (C and D) untreated controls; (E and F) larvae treated with FLC (320 μg/ml); (G and H) larvae treated with the combination of FLC (320 μg/ml) and FLUO (128 μg/ml). Yeast clusters and filaments were observed in the tissue (arrows) of both treated and untreated larvae. Magnification: ×4.2 (A, C, E, and G) or ×10 (B, D, F, and H).

Similar findings were described previously for other pathogens (40, 43, 44).

Microscopic observation indicates the correlation of the virulence with the degree of damage on the histological tissue of *G. mellonella*. Resistant *C. albicans* produced pseudohyphae and severe tissue damage in the larvae with numerous areas of infection; clustered yeast cells were also observed in larvae. With the combined treatment, fewer clustered yeast cells and filaments were observed. Thus, extensive studies have been carried out on two virulence factors: phospholipase activity and expression of aspartyl proteinase (*SAP*) genes.

RT-PCR assays revealed that the combination of fluconazole and fluoxetine can significantly downregulate the expression levels of *SAP* genes compared with those in the control group by up to dozens of times, even at concentrations which are an order of magnitude lower. It is widely recognized that *SAP* gene family members exhibit differential expression profiles at different stages and sites of infection. The most highly expressed secreted proteinase encoded by the *SAP2* gene is capable of digesting human albumin, keratin, and hemoglobin and also has the ability to destroy secreted immunoglobulin A. This may explain why the highest survival rate was observed in *G. mellonella* larvae treated with fluconazole-fluoxetine.

In terms of extracellular phospholipase activity, our findings concur with the results of previous studies which found that another SSRI, sertraline, could decrease phospholipase activity (45). Furthermore, extracellular phospholipase activity, which has been

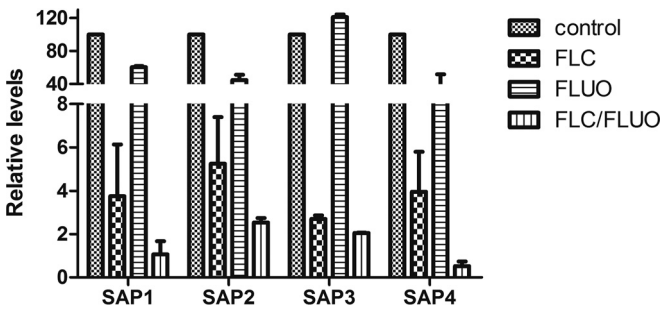

FIG 7 Relative expression levels of *SAP1*, *SAP2*, *SAP3*, and *SAP4* following treatment with fluconazole (FLC) and fluoxetine (FLUO) alone or in combination in resistant *C. albicans* (CA10). Cells were treated with fluconazole at 8 μg/ml, FLUO at 8 μg/ml alone, or in combination. Total RNA was extracted and reverse transcribed to cDNA and was then used for real-time quantitative PCR to detect the expression levels of *SAP1*, *SAP2*, *SAP3*, and *SAP4*. Values represent the means ± standard deviations from three replicates.

TABLE 3 Extracellular phospholipase activity of *C. albicans* treated with fluoxetine

Group (μg/ml)	Precipitation zone[a]	Phospholipase activity
Control	0.80 ± 0.04	
4	0.76 ± 0.04	High
8	0.72 ± 0.04	High
16	0.71 ± 0.02[b]	High
32	0.63 ± 0.04[b]	Very high
64	NZ[c]	
128	NZ[c]	

[a] The precipitation zone represents the ratio of the diameter of the colony to the cloudy zone plus colony diameter.
[b] $P < 0.05$ compared to the control.
[c] NZ, no zone of precipitation.

shown to be predictive for mortality in a murine mouse model of disseminated candidiasis, was also affected by treatment of *Candida* with fluoxetine.

In humans, SSRIs modify the behavior of 5-hydroxytryptamine (5HT) by acting at the 5HT transporter protein (SERT) and block the reuptake process of 5HT (46). Since SERTs are similar to other biogenic amine transporters (46), it is probable that the antifungal activity of fluconazole combined with fluoxetine results from an interaction of fluoxetine with fungal transporter systems. The mechanism by which fluoxetine acts on the biology of fungi remains to be studied.

In conclusion, fluoxetine showed synergism in combination with azoles against resistant *C. albicans* both *in vitro* and *in vivo*. The potential mechanisms were related to downregulation of *SAP* genes with inhibition of extracellular phospholipase activity. With the fact that a positive correlation existed between these factors and the major virulence properties of *Candida*, the synergism of the combination may be explained by a decrease in fungal virulence. The results from this study encourage us to consider future use of a combination of an azole and fluoxetine against fungi, and more animal models and a more in-depth study of the mechanisms are highly warranted.

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