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The synergistic antifungal effects of sodium phenylbutyrate combined with azoles against *Candida albicans* via regulating Ras/cAMP/PKA signalling pathway and virulence

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Abstract

The pathogenic fungus *Candida albicans (C. albicans)* is one of the most commonly clinically isolated fungal species and its resistance to the antifungal drug fluconazole is known to be increasing. In this paper, we sought to characterize the effect of sodium phenylbutyrate used alone or in combination with azoles against resistant *C. albicans*. The minimum inhibitory concentrations (MIC) and sessile minimum inhibitory concentrations (sMIC) were determined to explore the synergistic mechanism. The results showed that sodium phenylbutyrate exerted clear antifungal activity and that the combination of sodium phenylbutyrate and azoles functioned synergistically to combat resistant *C. albicans*. In the study of mechanism, we initially found that the combination therapy resulted in the inhibition of hypha growth, the increased penetration of fluconazole through *C. albicans* biofilm, and decreased expression of hyphae-related genes and the upstream regulatory genes (*CYR1* and *TPK2*) of the Ras/cAMP/PKA signalling pathway as determined by reverse transcription (RT)-PCR. In addition, the combination treatment decreased the extracellular phospholipase activities and the expression of aspartyl proteinase genes (*SAP1-SAP3*). The synergistic antifungal effects of the combination of sodium phenylbutyrate and azoles against resistant *C. albicans* was mainly based on the regulation of the Ras/cAMP/PKA signalling pathway, hyphae-related genes, and virulence factors.

Keywords: *Candida albicans*, Sodium phenylbutyrate, Biofilm, Synergism, Virulence

Introduction.

Candida species are opportunistic common fungal pathogens, of which *Candida albicans* (*C. albicans*) is the most common (Pfaller and Diekema 2007). They can cause mucosal (oropharyngeal, vaginal, and urinary tract) infections and, less commonly, skin, nail, and systemic infections. Azole antifungal drugs are the first-line drugs for the treatment of *Candida* infection (Chen et al. 2008). However, given the wide application of antifungal drugs, *Candida* species have gradually become resistant to antifungals, especially fluconazole (FLC) (Arendrup 2013). A number of physiological attributes of yeast are associated with azoles resistance, such as transport alterations (*CDR*, *MDR* and *FLU*), target alterations by mutations and gene upregulation (*ERG*), biofilm formation, and the abnormal expression of heat shock protein. (Li et al. 2015). Biofilm formation is an important mechanism of fungal resistance, and presents a great challenge to clinical treatment (Cowen et al. 2014). Extensive research has investigated the underlying biofilm resistance mechanisms in *C. albicans* cells. Similar to bacterial biofilms, fungal biofilms exhibit up to 20,000-fold increase in antifungal (minimum inhibitory concentrations) MICs compared with planktonic cells (Hawser 1996; Jabra-Rizk et al. 2004). In addition, it has been shown that the cells within a biofilm structure are also less sensitive to the human immune system defenses (Mathe and Van Dijck 2013).

Sodium phenylbutyrate (PBA) is a derivative of the short-chain fatty acid butyrate and is approved for the treatment of urea cycle disorders and progressive familial intrahepatic

cholestasis type 2. PBA exerts pleiotropic functions as a stress inhibitor, ammonia sink, chemical chaperone, and histone deacetylase inhibitor (HDACI) (Jellbauer et al. 2016). HDACIs have great potential as a treatment for cancers and immunological diseases. PBA also exerts beneficial effects against *salmonella enterica* during infection (Jellbauer et al. 2016). It has been shown that the inhibition of HDACs by trichostatin A (TSA) could reduce the growth of *C. albicans* (Li et al. 2015). If PBA has antifungal activity is not clear.

In this paper, we evaluated the *in vitro* activity of PBA used alone and in combination with azoles against resistant *C. albicans* in both planktonic cells and biofilms. In addition, we studied the potential mechanism of action. First, the "Sandwich" model was used to study the penetration of drugs into *C. albicans* biofilm. The expression of hyphae-related genes (*HWP1*, *ALS1 ALS3* and *EFG1*) was determined by using reverse transcription (RT)-PCR. In recent years, studies have reported the Ras/cAMP/PKA signalling pathway plays a decisive role in the regulation of fungal hypha formation (Nobile et al. 2014). Therefore, the expression of genes related to the regulatory factors of the RAS pathway (*RAS1*, *CYR1*, *TPK2*) were determined by using RT-PCR. Phospholipase activity is also considered to be an important virulence factor for many microorganisms (Leidich et al. 1998). In this work, extracellular phospholipase activities was detected by using the egg yolk agar method, and the expression of virulence-related genes (*SAP1- SAP4*) was also determined by using RT-PCR.

Methods

Strains and media: The strains used in this study were two FLC-resistant *C. albicans* strains (CA10, CA16), two FLC-sensitive *C. albicans* strains (CA4, CA8) and a standard strain of *C. albicans*, ATCC 10231, these were collected from the clinical laboratory of Qianfoshan Hospital and obtained from pharmacological institute of Shandong university. The strains were stored at -80°C, and refreshed and incubated on YPD solid medium at least twice at 35°C before each experiment.

Drugs. FLC was purchased from Cheng Chuang Pharmaceutical and dissolved to a concentration of 2560 μg/ml with purified water. Other drugs, including voriconazole (VRC), itraconazole (ITC), and PBA were provided by the Chinese Medicine Institute and stock solutions of 2560 μg/mL were prepared in dimethylsulfoxide (DMSO). DMSO was confirmed not to affect cell viability, with no significant changes in the viability of untreated cells observed in response to 5% DMSO.

Antifungal susceptibility testing

The *in vitro* antifungal activity of PBA and azoles was determined by the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (document M27-A3). For the checkerboard experiments, 100 μl aliquot were plated into a microtiter plate containing RPMI medium with or without different concentrations of PBA, FLC, or their combination. In brief, serial double dilutions of the test drugs were prepared (0.125-64 μg/mL for FLC, 0.007-4 μg/mL for VRC/ITC and 1-64 μg/mL for PBA) and 50 μL of the individual drugs or mixtures was added to each well. Subsequently, 100 μL of *C. albicans* (CA10) cell suspensions (2×10^3 cells/mL) were added to each of the wells. Which were filled to a final volume of 200 μL

with RPMI-1640. The plates were incubated at 35°C for 24 h (Pfaller and Diekema 2012), and the results were determined by measurement of the optical density at 492 nm. The MIC endpoints were defined as the lowest concentration of drug, alone or in combination with other agents, that inhibited the growth of yeast by 80% compared with the control growth rate. All experiments were performed in triplicate. The drug interactions were determined based on the fractional inhibitory concentration index (FICI) model and the percentage of growth difference (ΔE) model (Prichard et al. 1991; Prichard et al. 1993; Prichard and Shipman 1990). FICI values of ≤ 0.5, $0.5 < \text{FICI} \leq 4.0$ and > 4.0 represent synergism, no interaction and antagonism, respecttively (Odds 2003). The ΔE model was defined by the following equation: $\Delta E = E_{\text{predicted}} - E_{\text{measured}}$, with E_{measured} obtained directly from the experimental data. The following equation was derived: $E_{\text{predicted}} = E_A \times E_B$, where E_A and E_B were the experimentally determined percentages of the fungal growth in the presence of each drug alone. Statistically significant interactions of < 100%, 100-200%, > 200% were considered weak, moderate, and strong, as described previously (Shi et al. 2010).

Anti-biofilm susceptibility testing

Biofilms were formed by a previously described method (Gu et al. 2016). Briefly, the biofilms were formed by the addition of 100 µl of the standardized cell suspension (1×10^3 CFU/mL) into 96-well plates at 35°C, with samples taken after four time intervals (4, 8, 12, and 24 h). At each time point, the biofilms were washed three times with sterile phosphate-buffered saline (PBS) to remove the planktonic yeast. FLC and PBA were added to cells in a 96-well plate, to yield final FLC concentration of 1–512 µg/ml and 4–256µg/mL, respectively. The control wells were filled with RPMI 1640 without

antifungal agents. Plates were incubated at 35℃ for 24 h. A colorimetric reduction assay was performed using XTT in according with the protocol of Melo et al. (Melo et al. 2007). The absorbance at 492 nm was measured by using a microtiter plate reader (SPEC TRA MAX190, Thermo lab systems, USA).

Hyphal morphology observation

Candida biofilms were formed as described previously (Chandra et al. 2001). Briefly, *C. albicans* cells were grown overnight at 35°C in YPD solid medium. The cell density was adjusted to 1×10^6 cells/mL to prepare a standardized cell suspension, from which 4000 µL was added to a 6-well tissue culture plate. The wells were treated with FLC (1 µg/mL), PBA (32 µg/mL) and FLC combined with PBA, respectively, and the control wells were filled with 3600 µl RPMI 1640 without antifungal agents. The plate was incubated at 35°C for 24 h, after which the biofilms were washed with sterile phosphate-buffered saline (PBS) to remove the planktonic yeast. Subsequently, the plates were observed by using a phase contrast microscope. The assays were performed on three replicates experiments on different days. The 6-well tissue culture plate biofilms were observed by using an Olympus FSX100 with ×16 and ×10 objectives lenses.

Candida biofilm development and antifungal penetration study evaluated by the "Sandwich" model

The "Sandwich" model was constructed as previously reported (Shi et al. 2010). The schema is showed in **Figure 7.** In brief, the biofilm was developed on a microporous polycarbonate membrane (diameter, 13 mm) rested on YPD plates for 24 h (A). A microporous polycarbonate membrane (diameter, 10mm) was placed on top of the

biofilm and a moistened antibiotic disk was then placed on top of the polycarbonate membrane for another 24 h (B). The entire unit was then transferred to antifungal-laced agar and re-incubated at 35℃ for 24 h. The biofilm system was placed into the YPD plates with different drugs and an isopore filter membrane (0.22 μm) was added to the biofilm system close to one side of the biofilm. A pre-wet blank piece of paper was placed onto the membrane filter to form the sandwich system, which was incubated for 24 h at 35°C. Finally, the blank piece of paper was removed and the biofilm was placed into YPD plates with *C. albicans* and incubatied for 24 h at 35°C. The diameters of the growth inhibition zone was measured by using a Vernier caliper. The concentrations of tested drugs were between 64-1024 μg/mL for FLC and 8-128 μg/mL for PBA.

Phospholipase activity of *C. albicans* treated with PBA and FLC

The phospholipase activity was determined by the measurement of the size of the precipitation zone (P_z) after *C. albicans* growth treated with different drugs on egg yolk agar, as reported previously (Gu et al. 2016). Next, 10-μl aliquots of a suspension (10^7 CFU/mL) were inoculated into the egg yolk agar medium plates. The plates were incubated at 37°C for 72h and the P_z value was measured and expressed as the ratio of the diameter of the colony to the diameter of the Pz. 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 ($P_z \leq 0.69$), as previously described (Gu et al. 2016). Each experiment was performed twice.

RT-PCR.

RT-PCR was performed to determine the expression of hyphae-related genes (*HWP1*, *ALS1* and *ALS3*), virulence-related genes (*SAP1*, *SAP2, SAP3* and *SAP4*) and the RAS pathway regulatory genes (*RAS1*, *CYR1*, *EFG1*). *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: FLC, 1 μg/mL; PBA, 32 μg/mL. Cells that were not treated drugs served as the control samples. Total RNA from CA10 cells was isolated by using RNApure yeast kit (DNase I) (CWBiotech, Beijing, China). The isolated RNA was then treated with the first-strand cDNA synthesis SuperMix kit (CWBiotech) and reverse-transcribed in accordance with the manufacturer's instructions. RT-PCR analysis of a mixture of cDNA, ultra SYBR mixture (with ROX) (CWBiotech), and gene primers was performed in triplicate, with *ACT1* was used as the endogenous control gene (Staniszewska et al. 2015). RT-PCR was performed by using an ABI ViiA 7 (Applied Biosystems) sequence detection system for three separate experiments. An aliquot of 10 μl PCR mixture was used for each experiment, the cycling conditions were 95°C for 10 min, followed by 36 cycles of 95°C for 15 s and 60°C for 1 min, and the results were calculated by using the $2^{-(\Delta\Delta Ct)}$ method (Pfaffl 2001).

Results

MICs of azoles in combination with PBA

The result showed that PBA had antifungal activity with an MIC of 128μg/mL and significantly reduced the MICs of azoles against azole-resistant *C. albicans* strains, the relevant data are shown in **Table 1**. In combination with PBA, the MIC of FLC, ITC and VRC decreased from 512 μg/mL to 1 μg/mL, 8 μg/mL to 0.12 μg/mL, and 8 μg/mL to

0.12 μg/mL, respectively, all had FICI values of <0.5, which showed significant synergism. The results interpreted by Δ*E* method were also shown to have strong synergism (**Figure 1)**. For the azole-sensitive *C. albicans* strains (CA4, CA8), synergism was hardly observed (FICI > 0.5).

sMICs of FLC in combination with PBA

In the sessile MIC (sMIC) assays, we found that the combination of PBA and FLC exerted synergistic effects against planktonic cells and were effective against *C. albicans* biofilm, the results are shown in **Table 2**. Synergistic effects by this combination treatment were identified against *C. albicans* biofilms formed over 4, 8, and 12 h, with FICI < 0.5. However, for mature biofilms (formed over 24 h), low synergism was observed (FICI > 0.5). For the azole-sensitive *C. albicans* strains (CA4, CA8), synergistic effects were also observed against biofilms formed over 4, 8, and 12 h.

Analysis of hyphal morphology

The antibiofilm effect of different drugs was evaluated by the observation of hyphae. As illustrated in **Figure 2,** the control group, which did not receive any drug presented, displayed large amounts of biofilm consisting of an abundance of filamentous and yeast forms. *C. albicans* (CA10) treated with FLC or PBA alone produced biofilms composed of yeast cells and hyphae. Almost no difference was observed in *C. albicans* (CA10) treated with PBA or PBA alone compared with the control. *C. albicans* (CA10) treated with FLC and PBA contained biofilms with no observed microcolonies of yeasts and hyphae.

Effect of PBA on FLC penetrating of the *C. albicans* biofilm

The penetration of FLC through the *C. albicans* biofilm was evaluated by using the Sandwich model. The combined concentration results were 512 μg/mL for FLC and 32 μg/mL for PBA. The diameter of inhibition zone for the group treated with the combination of PBA and FLC (16mm) was larger than that of those treated with FLC alone (10mm). The results showed that PBA could enhance FLC penetrating into the *C. albicans* biofilm.

Phospholipase activity of *C. albicans* treated with PBA and FLC

The extracellular phospholipase activity of *C. albicans* was measured by using the egg yolk agar method. The value of P_z was 0.90±0.03, which indicated that *C. albicans* (CA10) treated FLC (1 μg/mL) and PBA (32 μg/mL) had very low phospholipase activity. In addition, the P_z values of *C. albicans* (CA10) without drugs and treated with only FLC (1 μg/mL) indicated that the phospholipase activity was very high. The value of P_z was 0.70±0.02, in the group treated with PBA (32 μg/mL) alone, which was indicative of high phospholipase activity. These results are shown in **Table 3**.

Expression of hypha-related genes

The RT-PCR assay results showed that treatment with FLC alone downregulated the expression of *ALS1*, *ALS3*, and *HWP1*, and upregulated *EFG1* compared with the control group (*P<0.05) (**Figure 3)**. The combination treatment significantly downregulated the expression of *ALS1*, *ALS3* and *EFG1* compared with the treatment of FLC alone by more than 2-fold, 6-fold, and 3-fold, respectively (**P*<0.05). The combination treatment

downregulated the expression of *HWP1* compared with that of FLC treatment alone, but the difference was not significant.

Expression of genes in the Ras/cAMP/PKA signalling pathway

The results in **Figure 4** show that the expression of *RAS1* was downregulated after challenge with FLC or PBA compared with expression in the control group. (**P*<0.05) After the combination treatment, the expression of *RAS1* was downregulated compared with treatment with FLC alone, but the difference was not significant. The expression of *CYR1* was three-fold higher after FLC challenge than in the control group. (**P*<0.05) The combination treatment significantly downregulated the expression of *CYR1* compared with the treatment with FLC alone by more than 3-fold, (**P*<0.05). The expression of *TPK2* was slightly increased by the FLC challenge compared with the control group. (**P*<0.05) The combination treatment significantly downregulated the expression of *TPK2* compared with treatment with FLC alone by more than 100-fold (*P<0.05).

Expression of virulence factor-related genes

The RT-PCR assays showed that the expression of *SAP1-SAP4* was decreased two-fold by the FLC challenge compared with the control group (*P < 0.05). The combination treatment significantly downregulated the expression of *SAP1*, *SAP2*, and *SAP3* compared with FLC alone by two-fold (**P*<0.05). The combined treated downregulated the expression of *SAP4* compared with the treatment with FLC alone, but the difference was not significant. These results are shown in **Figure 5.**

Discussion

255 *Candida* species are recognized as the major fungal of nosocomial infections (Melo et al.
256 2011). Infections associated with *C. albicans* biofilms represent a serious problem for
257 immune-compromised populations (Ramage et al. 2006). Novel strategies to prevent and
258 overcoming drug resistance are therefore urgently required. In our previous studies, a
259 number of non-antifungal drugs, such as ion channel inhibitors and immune inhibitors,
260 were shown to exert synergistic effects in the treatment of *C. albicans* (Gao et al. 2014;
261 Li et al. 2014; Shi et al. 2010).
262 PBA has been used in pre-clinical studies as a treatment for a variety of diseases,
263 including spinal muscular atrophy, homozygous β-thalassemia, and cancer (Jellbauer et
264 al. 2016). As an HDACI, the combination of PBA with bortezomib exerted synergistic
265 cytotoxic effects on glioblastoma stem cells (Asklund et al. 2012). A number of studies
266 have indicated that HDACIs are effective against *C. albicans*. Smith and Edlind (Smith
267 and Edlind 2002) revealed that TSA could enhance the susceptibility of *C. albicans* to
268 azoles and terbinafine *in vitro* through inhibit the expression of efflux pump genes.
269 Synergistic or enhanced effects were also identified for other HDACIs such as apicidin,
270 MGCD290 and vorinostat (SAHA) (Mai et al. 2007; Pfaller et al. 2009). To the best of
271 our knowledge, this is the first report to describe the antifungal effects of PBA and
272 explore in detail the different potential mechanism
273 The results indicated that PBA not only exerted an antifungal effect when treated alone,
274 with an MIC of 128 μg/mL, but also had strong synergism with azoles against resistant *C.*

275 *albic*ans. PBA reduced the MIC of FLC, ITC and VRC from 512, 8, and 8 μg/mL to 1,
276 0.12, and 0.12 μg/mL, respectively. Furthermore, the combination treatment could also
277 inhibit biofilm formation over 4,8,12 h.
278 A number of studies have reported that the biofilms formation of pathogenic *Candida*
279 species with the expected pathogenic traits (increased resistance to drugs, PMN invasion,
280 and a predominantly filamentous architecture) was dependent on the RAS/cAMP/PKA
281 pathway regulatory factors (Ras1, Cyr1, and Tpk2) and the transcription factor Efg1.
282 Several hypha-specific genes (*ALS1*, *ALS3* and *HWP1*) are primarily regulated by this
283 signalling pathway (Hogan and Muhlschlegel 2011; Nobile and Mitchell 2005; Yi et al.
284 2011). Ras1p is a GTPase that participates in the induction of hyphal formation through
285 the RAS/cAMP/PKA pathway (Feng et al. 1999). Cyr1 integrates environmental signals
286 from a range of sources and is essential for hyphal formation (Rocha et al. 2001). Tpk2
287 can encode PKA and has stimulatory effect on the formation of hyphae (Biswas et al.
288 2007). Efg1, a transcription factor of the RAS/cAMP/PKA pathway, plays an important
289 role in the regulation of the expression of some hypha-specific genes, including *ALS1*,
290 *ALS3* and *HWP1* (Hogan and Sundstrom 2009). Accordingly, *ALS1* and *ALS3* are ALS
291 family genes that play an essential role in the adherence stage of *C. albicans* (Sundstrom
292 2002; Tronchin et al. 2008). *HWP1* is also a unique adherence gene (Nobile et al. 2006).
293 Our results showed that the expression of some hypha-specific genes (*ALS1* and *ALS3*)
294 and a transcription factor gene (*EFG1*) were downregulated when the cells were exposed

295 to the combination of drugs. Further study showed that the Ras/cAMP/PKA pathway
296 genes (*CYR1* and *TPK2*) were also downregulated. As shown in the results presented in
297 **Figure 4**, PBA treatment alone downregulate *CYR1* and *TPK2*, but lower expression was
298 observed when PBA was treated in combination with FLC, conversely, FLC upregulated
299 the expression of *CYR1* and *TPK2*. The expression of *HWP1* and *RAS1* was not
300 significantly altered after the combination treatment compared with the trearment with
301 FLC alone. The above results revealed that the combination of FLC with PBA initially
302 inhibited the expression of *CRY1*, which regulated the Ras/cAMP/PKA signalling
303 pathway and caused changes in hypha-specific genes, as shown in **Figure 6.**
304 In addition, fungal virulence factors have been considered as potential targets for drug
305 development (Ripeau et al. 2002). Secretory aspartyl proteinases (Saps) and
306 phospholipases are enzymes secreted by fungi and may contribute to colonization and
307 infection through the degradation of host tissues (Nailis et al. 2010). Our studies have
308 been performed on two virulence factors: phospholipase activity and the expression of
309 aspartyl proteinase (*SAP*) genes. The combined treatment decreased phospholipase
310 activity and significantly downregulate the expression of *SAP* genes.
311 In conclusion, PBA exerts clear antifungal activity and displayed synergistic activity
312 when combined with azoles for the treatment of resistant *C. albicans*. The synergistic
313 mechanism may be related to an enhancement of the penetration of FLC into the *C.*
314 *albicans* biofilm, which inhibited biofilms formation-regulating genes and attenuated the
315 virulence of *C. albicans*. This surprising discovery provides a reference for PBA, which

316 is used widely as an antifungal infection drug, to be considered for future use in
317 combination with azoles for the treatment of fungal infection. Moreover, PBA has been
318 demonstrated to have a wide variety of effects with different mechanisms, thus, a more
319 in-depth study of these mechanisms is warranted.

320

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Figure 1. Three-dimensional plots of FLC, VRC and ITC combined with PBA against *C. albicans*. PBA+FLC, sodium phenylbutyrate combined with fluconazole; PBA+ITC, sodium phenylbutyrate combined with itraconazole; PBA+VRC, sodium phenylbutyrate combined with voriconazole. Plots were created by using MATLAB. Drug interactions of azoles and PBA are interpreted by the Δ*E* model. The concentrations of azoles and PBA 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.

Figure 2. Hypha morphology observation treated with different drugs.

Control, *C. albicans* (CA10) without drugs; FLC, *C. albicans* (CA10) treated with FLC (1 μg/mL) alone; PBA, *C. albicans* (CA10) treated with PBA (32 μg/mL) alone; FLC+PBA, *C. albicans* (CA10) treated with FLC (1 μg/mL) plus PBA (32 μg/mL); All picture in triplicate experiments.

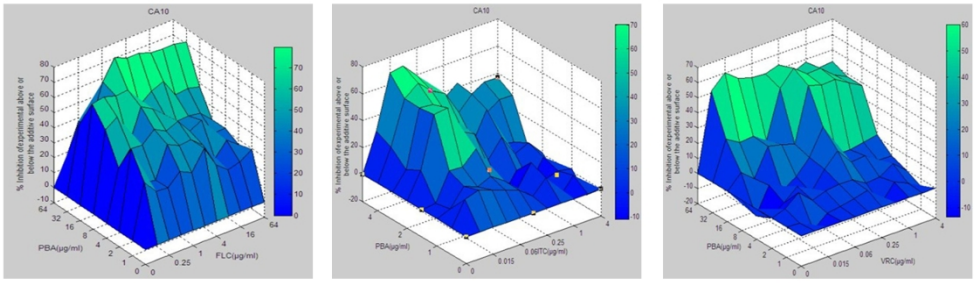
Figure 3. Relative expression levels of *ALS1*, *ALS3*, *HWP1*, and *EFG1* following treatment with FLC and PBA alone or in combination in resistant *C. albicans* (CA10) Cells were treated with FLC at 1μg/mL, PBA at 32μg/mL alone, or in combination. Total RNA was extracted and reverse transcribed to cDNA and was then

used for real-time quantitative PCR to detect the expression levels of *ALS1*, *ALS3*, *HWP1*, and *EFG1*. Values represent the means standard deviations from three replicates. Statistical significances were determined by Student's t-test. *P < 0.05 when compared with the respective controls.

Figure 4. Relative expression levels of *RAS1*, *CYR1* and *TPK2* following treatment with FLC and PBA alone or in combination in resistant *C. albicans* (CA10). Cells were treated with FLC at 1µg/mL, PBA at 32µg/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 *RAS1*, *CYR1* and *TPK2*. Values represent the means standard deviations from three replicates. Statistical significances were determined by Student's t-test. *P < 0.05 when compared with the respective controls.

Figure 5. Relative expression levels of *SAP1*, *SAP2*, *SAP3* and *SAP4* following treatment with FLC and PBA alone or in combination in resistant *C. albicans* (CA10). Cells were treated with FLC at 1µg/mL, PBA at 32µg/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.

510 Statistical significances were determined by Student's t-test. *P < 0.05 when compared
511 with the respective controls.
512
513 **Figure 6.** The synergistic mechanism may be related to reducing *C. albicans* virulence
514 and inhibiting biofilms formation through down-regulating the expression of *CRY1*, thus
515 causing the downstream genes changes.
516
517 **Figure 7. The "Sandwich" model schematic.** Schematic representation of the
518 experimental system used to antifungal penetration through Candida biofilms.
519



PBA+FLC PBA+ITC PBA+VRC

Figure 1. Three-dimensional plots of FLC, VRC and ITC combined with PBA against C. albicans.

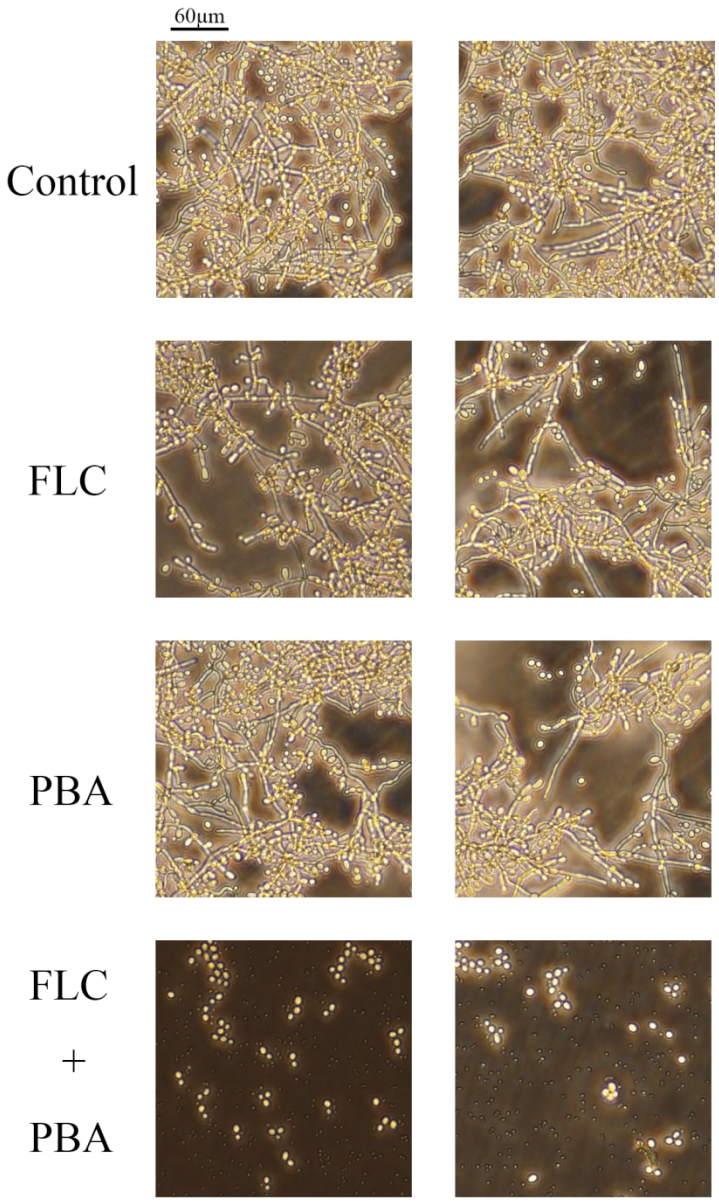


Figure 2. Hypha morphology observation treated with different drugs.

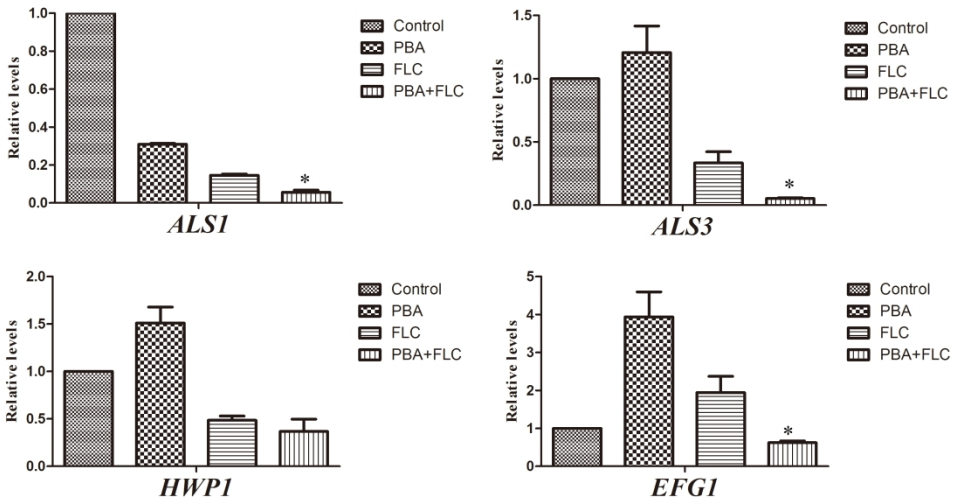


Figure 3. Relative expression levels of ALS1, ALS3, HWP1, and EFG1 following treatment with FLC and PBA alone or in combination in resistant C. albicans (CA10) .

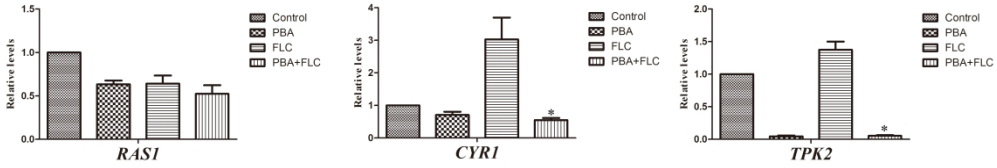


Figure 4. Relative expression levels of RAS1, CYR1 and TPK2 following treatment with FLC and PBA alone or in combination in resistant C. albicans (CA10).

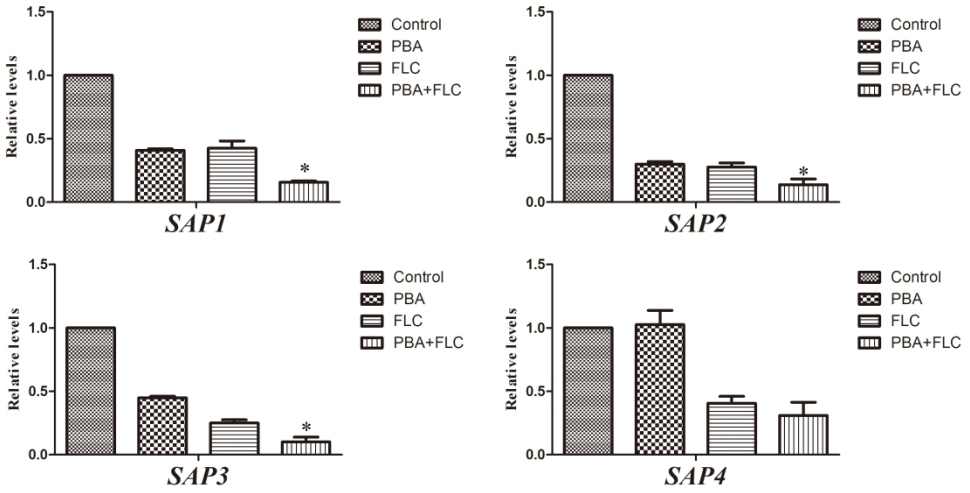


Figure 5. Relative expression levels of SAP1, SAP2, SAP3 and SAP4 following treatment with FLC and PBA alone or in combination in resistant C. albicans (CA10).

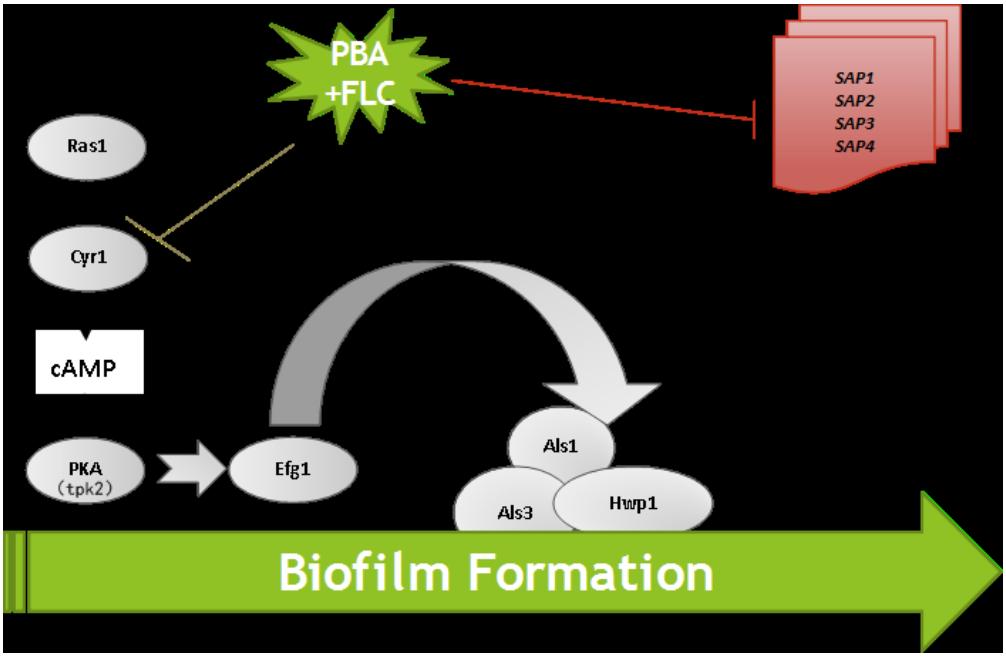


Figure 6. The synergistic mechanism may be related to reducing C. albicans virulence and inhibiting biofilms formation through down-regulating the expression of CRY1, thus causing the downstream genes changes.

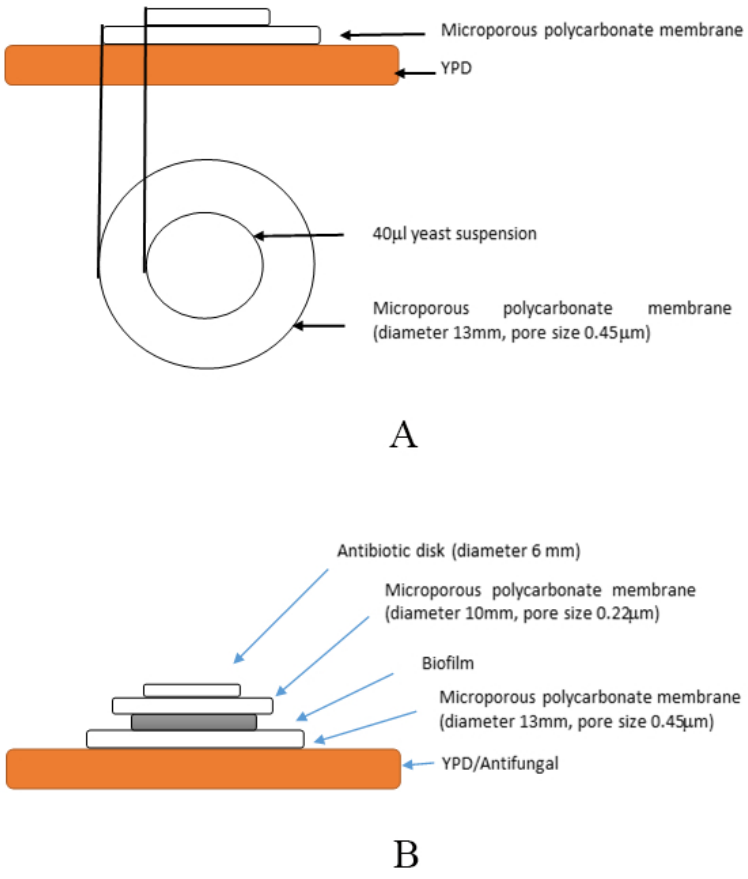


Figure 7. The "Sandwich" model schematic.

Table1. Combined drug effects against *C. albicans* evaluated by the FICI model.

Combination	Strains	MIC(μg/mL)				LA theory	
		MIC_A	C_A	MIC_B	C_B	FICI	IN
FLC+PBA	CA10	512	1	128	32	0.252	SYN
	CA16	512	1	128	32	0.252	SYN
	CA4	0.5	0.5	128	≤2	1.016	NI
	CA8	1	1	128	≤2	1.016	NI
VRC+PBA	CA10	8	0.25	128	32	0.252	SYN
	CA16	8	0.25	128	32	0.252	SYN
	CA4	0.03	0.03	128	≤2	1.016	NI
	CA8	0.5	0.5	128	≤2	1.016	NI
ITC+PBA	CA10	8	0.12	128	32	0.265	SYN
	CA16	8	0.12	128	32	0.265	SYN
	CA4	0.25	0.25	128	≤2	1.016	NI
	CA8	0.5	0.5	128	≤2	1.016	NI

CA, *C. albicans*; FLC, fluconazole; VRC, voriconazole; ITC, itraconazole; MIC_A, the MICs of azole when used alone; MIC_B, the MICs of PBA when used alone; C_A, the MICs of azole when used in combination with PBA; C_B, the MICs of PBA when used in combination with FLC; IN, interpretation; ANT, antagonism; SYN, synergism; NI, no interaction; All data *in vitro* are the averages of triplicate experiments.

Table2. Antifungal effects of FLC alone and in combination with PBA against biofilms of *C. albicans*.

strains	Times (h)	SMIC(μg/mL)				LA theory	
		MIC_A	C_A	MIC_B	C_B	FICI	IN
CA10	4	>512	2	256	32	0.129	SYN
	8	>512	2	256	32	0.129	SYN
	12	>512	2	256	64	0.254	SYN
	24	>512	4	256	128	0.508	NI
CA16	4	>512	4	256	32	0.133	SYN
	8	>512	4	256	32	0.133	SYN
	12	>512	4	256	32	0.133	SYN
	24	>512	4	256	128	0.508	NI
CA4	4	>512	1	256	8	0.033	SYN
	8	>512	1	256	8	0.033	SYN
	12	>512	1	256	16	0.064	SYN
	24	>512	512	256	128	1.5	NI
CA8	4	>512	2	256	16	0.066	SYN
	8	>512	2	256	16	0.066	SYN
	12	>512	2	256	32	0.130	SYN
	24	>512	512	256	128	1.5	NI

Times indicate the incubation periods of biofilm formation. SMICs were read as the lowest concentrations that produced a 80% reduction in growth compared with that of the drug-free control. MIC_A, the MICs of FLC when used alone; MIC_B, the MICs of PBA when used alone; C_A, the MICs of FLC when used in combination with PBA; C_B, the MICs of PBA when used in combination with FLC; IN, interpretation; ANT, antagonism;

SYN, synergism; NI, no interaction; All data *in vitro* are the averages of triplicate experiments.

Table3. Phospholipase activity of *C. albicans* (CA10) treated with drugs.

Groups	Percipitation zone	Phospholipase activity
Control	0.61±0.01	Very high
FLC	0.62±0.01	Very high
PBA	0.70±0.02	high
FLC+PBA	0.90±0.03	Very low

The precipitation zone represents the ratio of the diameter of the colony to the cloudy zone plus colony diameter. P <0.05 compared to the control. Control, *C. albicans* (CA10) without drugs; FLC, *C. albicans* (CA10) treated FLC (1 μg/mL) alone; PBA, *C. albicans* (CA10) treated PBA (32 μg/mL) alone; FLC+PBA, *C. albicans* (CA10) treated FLC (1 μg/mL) with PBA (32 μg/mL); All data *in vitro* are the averages of triplicate experiments.