Supplementary Materials for Palmarumycin P3 reverses Mrr1-mediated azole resistance by blocking the efflux pump Mdr1

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Materials and methods

Strains and agents

C. albicans G5 (an FLC-resistant clinical isolate from AIDS patient G with G997V/G997V mutation in Mrr1), SCMPG2A (the parent strain of SCMRR1R34MPG2A), SCMRR1R34MPG2A (a constructed *C. albicans* strain with P683S/P683S mutations in Mrr1), Gu5 (an FLC-resistant clinical isolate from AIDS patient Gu with a gain-of-function mutation in Tac1), and DSY296 (an FLC-resistant clinical isolate with N997D/N997D mutations in Tac1) were used in this study (1-4). Before each experiment, *C. albicans* strains were grown in yeast-peptone-dextrose (YPD) medium overnight at 30°C with shaking at 200 rpm.

Microdilution checkerboard assays

The interaction between PP3 and FLC was investigated using broth microdilution checkerboard assays. The initial concentration of the fungal suspension in RPMI 1640 medium was 1×10^3 CFUs/ml, and the final drug concentrations were 8-128 μg/ml for PP3 and 0.25-256 μg/ml for FLC. The MIC value was determined as the lowest concentration of the drug (alone or in combination) that inhibited 80% of growth compared with the control at 48 h. The fractional inhibitory concentration index (FICI) was used to assess the relationship between azole drugs and

PP3. Interactions were considered synergistic (SYN) at FICI values ≤0.50, additive (ADD) at FICI values >0.50 and ≤1, and indifferent (IND) at FICI values >1.

Agar diffusion assay

Overnight cultures were diluted with PBS to 1 × 10^6 CFUs/ml. Aliquots of 200 µl of yeast suspension were spread on YPD agar plates. To evaluate antifungal activity, cellulose disks impregnated with FLC and/or PP3 at the indicated concentrations were placed on the YPD agar plates. After 48 h of incubation at 30 °C, the plates were photographed (5).

Alamar blue assay

Overnight cultured were collected, washed and diluted to a cell density of 1 × 10^3 CFUs/ml in RPMI 1640 medium. Aliquots of 100 µl of the fungal suspension were added to the wells of 96-well flat-bottomed microtitration plates and treated with FLC and/or PP3 at the indicated concentrations. After incubation for 24 h, 10 µl of Alamar blue solution was added to the wells. The subsequent color change was photographed after 2 h of incubation in the dark, and the fluorescence intensity was recorded using a BioTek H1 plate reader ($\lambda_{excitation}$ = 530 nm and $\lambda_{emmision}$ = 590 nm) (5).

Efflux assay

The potential fungal efflux pump inhibition activity of PP3 was investigated using Rh123 and Nile red efflux assays (6). Exponentially grown yeast cells were incubated for 4 h at 30 °C in PBS using a rotating shaker to induce starvation. The energy-depleted cells were then treated with Rh123 (5 µM) or Nile red (5 µM) for 30 minutes, followed by washing twice with PBS to remove the unbound dye. Then, a 200-µl sample was withdrawn and analyzed by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA). The remaining cells were suspended in PBS

containing 40 mM glucose to initiate efflux, and PP3 was added to the suspension at a concentration of 0, 16 or 32 μg/ml. After incubation for an additional 30 min, all samples were harvested and washed. The fluorescence intensity of intracellular Rh123 and Nile red was determined by flow cytometry.

Nile red intracellular accumulation assay

Overnight cultures of G5 cells were diluted to a cell density of 1×10^6 CFUs/ml in PBS and incubated with Nile red (5 μM). The cell suspensions were then treated with PP3 (0, 16, 32 or 64 μg/ml) for 60 min. After washing with PBS, the samples were analyzed by flow cytometry (7).

Assays of intracellular accumulation of a fluorescent fluconazole analogue

The fluorescent fluconazole analogue (FLC-Bodipy) was synthesized by attaching a Bodipy fluorescent group to the FLC skeleton according to a previously reported method (8). In the accumulation assay, overnight cultures of G5 cells were diluted to a cell density of 1×10^6 CFUs/ml in RPMI 1640 medium and treated with FLC-Bodipy (8 μg/ml) and PP3 (0, 16, 32 or 64 μg/ml). After 8 h, samples were harvested, washed and analyzed by flow cytometry.

Transcriptional analysis of *MDR1*.

The effect of PP3 on the mRNA expression of *MDR1* was evaluated by qPCR using the $2^{-\Delta\Delta Ct}$ method as previously described (9). Briefly, overnight cultures of *C. albicans* were diluted to an OD_{600} of 1 in RPMI 1640 medium. The cultures were treated with PP3 (0, 16 or 32 μg/ml) and incubated for 3 h，6 h or 12 h at 30 °C. The cells were subsequently collected by centrifugation, and RNA was isolated using a hot phenol method. cDNA was synthesized using the RT kit (Takara, Dalian, China), and qPCR was performed with an Eppendorf Mastercycler Real-time PCR System. The following primers were used: *MDR1*-F: AGATAATCAAGGTGAACCCAA; *MDR1*-R:

GCTGATCCCATATAAACTGAA; *18S*-F: AATTACCCAATCCCGACAC; *18S*-R: TGCAACAACTTTAATATACGC. Expression was internally normalized to 18*S* RNA and compared with the untreated control.

Computational modeling

The homology model of *C. albicans* Mdr1 was generated using the Swiss-Model server with the Alignment Mode algorithm (10). According to the calculated global model quality estimate (GMQE) values, the known structure of monocarboxylate transporter 1 (PDB: 7CKR) was used as the template structure. Molecular docking was performed using Autodock Vina (11) and the ligand binding site was selected according to the literature (12). The top-scored result was selected as the most favorable binding pose.

***G. mellonella* infection model**

A *G. mellonella* infection model was used to evaluate the *in vivo* efficacy of the combination of PP3 and FLC in treating fungal infections according to a previously described methodology (13). Animals were maintained and treated under the guidelines approved by the Animal Care and Use Committee of Shandong University. A total of one hundred *G. mellonella* larvae weighing 0.28-0.35 g were selected prior to the experiment and randomly divided into five groups: blank, vehicle control, PP3, FLC, and combination. Briefly, the larvae were injected with ~5 × 10^5 CFUs/larva via the last right pro-leg. Two hours after infection, the larvae were injected with 10 μl of the prepared drugs according to the four treatment groups: vehicle control (sterile PBS), PP3 (800 ng/larva), FLC (800 ng/larva), and the combination. The larvae were placed at 35°C in the dark, and worm survival was monitored daily for 4 d.

To determine the fungal burden, three larvae were randomly selected from each group on the

fourth day and homogenized in 5 ml of sterile PBS using a homogenizer. The homogenate was serially diluted 1:10 with sterile PBS, and 10 μl of the suspension at each dilution was spread on solid YPD medium containing ampicillin and kanamycin. After 48 h of growth on the agar plates, the surviving colonies in each group were recorded.

For histological examination, larvae were sacrificed at 2 days after infection, fixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned longitudinally. Specimens were subjected to periodic acid-Schiff (PAS) staining to assess fungal invasion.

Statistical analysis

The experimental data were statistically analyzed using one-way ANOVA test or Student's *t*-test. For survival analysis, the log-rank (Mantel–Cox) test was used to compare differences between groups. Statistical significance was set according to the *P* value: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

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