AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

PHARMACOLOGY

Palmarumycin P3 Reverses Mrr1-Mediated Azole Resistance by Blocking the Efflux Pump Mdr1

Minghui Song,[a] Ming Zhang,[b] Jinghui Lu,[a] Fei Xie,[c] Jintong Song,[a] Xiaoyi Luan,[a] Xuben Hou,[d] Hongxiang Lou,[a] Wenqiang Chang[a]

[a]Department of Natural Product Chemistry, Key Laboratory of Chemical Biology, Ministry of Education, School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China

[b]Institute of Medical Science, The Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China

[c]Department of Pharmacy, Qilu Hospital of Shandong University, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China

[d]Department of Medicinal Chemistry, Key Laboratory of Chemical Biology, Ministry of Education, School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan, China

Minghui Song and Ming Zhang contributed equally to this work; author order was determined both alphabetically and in order of increasing seniority.

ABSTRACT Palmarumycin P3 (PP3) reduces fluconazole-induced *MDR1* transcription to reverse azole resistance in clinical *Candida* strains. Here, we demonstrated that PP3 restores the susceptibility to several antifungal drugs for *Candida albicans* strains with gain-of-function mutations in the transcription factor Mrr1. In addition, PP3 inhibits the efflux of Mdr1 substrates by *C. albicans* strains harboring hyperactive *MRR1* alleles. Molecular docking revealed that PP3 is a potential Mdr1 blocker that binds to the substrate binding pocket of Mdr1.

KEYWORDS *Candida albicans*, azole resistance, Mdr1, Mrr1, palmarumycin P3

Candida albicans is one of the most common causes of both superficial and systemic infections, with a mortality rate of ~40% for the latter (1, 2). The limited success of current therapies in reducing the high mortality rate of invasive fungal infections is due in part to azole resistance (3). An important mechanism of azole resistance is upregulation of drug efflux pumps, including members of the ATP binding cassette (ABC) superfamily and major facilitator superfamily (MFS), which are involved in rapid drug extrusion (4). MFS transporters use the proton gradient across the cytoplasmic membrane to supply energy for transport. The MFS transporter Mdr1p, which is encoded by *MDR1*, exports an array of structurally diverse compounds, such as fluconazole (FLC), cerulenin, and brefeldin A (5, 6). In *C. albicans*, *MDR1* overexpression is mainly the result of gain-of-function mutations in the transcription factor Mrr1 (7).

We previously demonstrated that the quinone derivative palmarumycin P3 (PP3) reduces the FLC-induced transcriptional expression of *MDR1* in clinical *C. albicans* strains to reverse azole resistance (8). However, those strains did not harbor mutations in Mrr1 that constitutively induce the expression of *MDR1* (9). In the present study, we found that PP3 also reverses azole resistance in *C. albicans* strains with gain-of-function mutations in Mrr1. In addition, PP3 inhibits the efflux of rhodamine-123 (Rh123) and increases intracellular accumulation of Nile red and a FLC analogue in *C. albicans* strain G5. Molecular docking indicated that PP3 acts as an Mdr1 blocker to prevent the expulsion of substrates, suggesting that PP3 directly inhibits the efflux pump Mdr1 in addition to regulating *MDR1* expression induced by FLC.

PP3 and FLC have synergistic effects against *C. albicans* strains with gain-of-function mutations in Mrr1. PP3 was previously shown to reverse azole resistance in *C. albicans* strains by reducing the transcription of FLC-induced *MDR1* (8). Here, we used another group of *C. albicans* strains with gain-of-function mutations in Mrr1 and Tac1 to further

Address correspondence to Hongxiang Lou, louhongxiang@sdu.edu.cn, or Wenqiang Chang, changwenqiang@sdu.edu.cn.

The authors declare no conflict of interest.

Received 4 November 2021
Returned for modification 30 November 2021
Accepted 11 January 2022
Accepted manuscript posted online 18 January 2022
Published 15 March 2022

TABLE 1 Susceptibility testing of PP3 alone and in combination with FLC against *C. albicans* strains by the checkerboard microdilution assay and drug interaction analysis according to the FICI model

		MIC_{80} (μg/ml)					
		Alone		In combination			
Strain	Characteristics	FLC	PP3	FLC	PP3	FICI	Interpretation[a]
G5	FLC-resistant clinical isolate with *MRR1*^{G997V}/*MRR1*^{G997V} mutation	>256	>128	16	16	0.1875	SYN
SCMPG2A	Control strain for SCMRR1R34MPG2A	1	>128	0.5	16	0.625	ADD
SCMRR1R34MPG2A	Engineered strain with *MRR1*^{P683S}/*MRR1*^{P683S} mutation	16	>128	1	16	0.1875	SYN
Gu5	FLC-resistant clinical isolate with *TAC1*^{G980E}/*TAC1*^{G980E} mutation	>256	>128	128	64	1	ADD
DSY296	FLC-resistant clinical isolate with *TAC1*^{N977D}/*TAC1*^{N977D} mutation	256	>128	128	64	1	ADD

[a]ADD, additive; SYN, synergistic.

explore the ability of PP3 to reverse azole drug resistance, namely, G5, an FLC-resistant clinical isolate from AIDS patient G with a gain-of-function G997V/G997V mutation in Mrr1 (10), SCMRR1R34MPG2A, an engineered *C. albicans* strain with a gain-of-function P683S/P683S mutation in Mrr1 (11), and Gu5 and DSY296, FLC-resistant clinical isolates with gain-of-function mutations in Tac1 (12, 13) (see the supplemental material). Analysis using the broth microdilution checkerboard method revealed synergistic activity of the combination of PP3 and FLC against the *C. albicans* strains with either type of gain-of-function mutation in Mrr1 but not in the strains with gain-of-function mutations in Tac1 (Table 1). The agar plate assay further confirmed that PP3 enhanced the antifungal action of FLC against both Mrr1 gain-of-function mutant strains (Fig. 1A). The alamarBlue assay showed that combined treatment with PP3 and FLC inhibited the growth of *C. albicans* strains SCMRR1R34MPG2A and G5 by more than 90%, whereas single treatment had minimal effects on *C. albicans* growth (Fig. 1B). Strain G5, with hyperactive Mrr1, was further treated with combinations of PP3 and other Mdr1 substrates, including cerulenin and brefeldin A, or other azole antifungal agents, including voriconazole (VRC) and posaconazole (PCZ). Synergistic effects of these combination treatments were also observed according to the fractional inhibitory concentration index (FICI) model (Table 2). These results suggest that PP3 is a specific azole resistance reversal agent for a variety of clinically derived strains with Mdr1-mediated resistance.

PP3 inhibits the activity of the efflux pump Mdr1. To confirm that the reversal of azole resistance by PP3 was mediated by inhibition of the efflux pump Mdr1, we measured the efflux activity of Mdr1. Flow cytometry revealed that 16 or 32 μg/ml PP3 inhibited the efflux of Rh123 by the hyperactive Mrr1 strain G5 (Fig. 2A). In contrast, PP3 treatment even at 32 μg/ml had only a minor effect on the efflux of Rh123 by the Tac1 mutant strain DSY296 (Fig. 2B). Efflux assays using Nile red, another commonly used efflux pump substrate with red fluorescence, showed that *C. albicans* G5 cells treated with PP3 accumulated more Nile red than untreated cells (Fig. 2C), suggesting that PP3 promotes the accumulation of Nile red by inhibiting the efflux activity of Mdr1. PP3 also induced the accumulation of Nile red in G5 cells, compared with cells treated with vehicle (Fig. 2D). Moreover, we synthesized fluorescein-labeled FLC (FLC-Bodipy) by attaching a Bodipy fluorescent group to the FLC skeleton according to a previously reported method (14). As expected, PP3 promoted the intracellular accumulation of FLC-Bodipy in *C. albicans* strain G5, as detected by flow cytometry (Fig. 2E), indicating that the activity of the efflux pump Mdr1 was inhibited by PP3.

PP3 reduces the expression of *MDR1* in Mrr1 mutant strains. To distinguish whether PP3 inhibits the efflux activity of Mdr1 by reducing *MDR1* expression, we performed quantitative real-time PCR (qPCR) to determine the expression level of *MDR1* in *C. albicans* strains with hyperactive Mrr1 under PP3 treatment. Treatment with 16 or 32 μg/ml PP3 for 3 or 6 h decreased the mRNA level of *MDR1* only slightly, compared with the untreated control (Fig. 3). An obvious reduction of *MDR1* expression was observed after 12 h of treatment with PP3 (Fig. 3). However, PP3 inhibited Mdr1-mediated efflux after 30 min of treatment (Fig. 2A), implying that PP3 directly interacts with Mdr1.

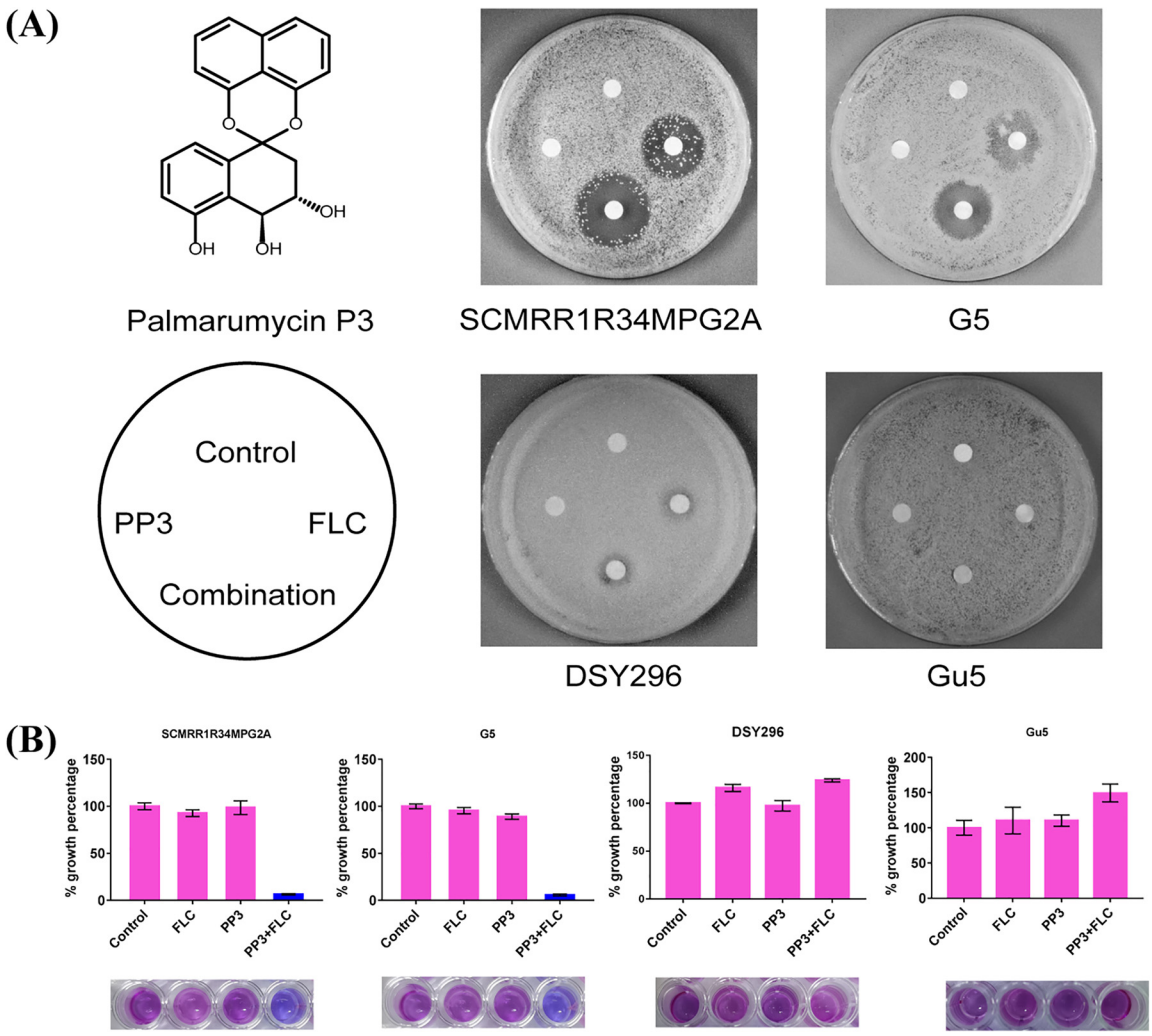

FIG 1 Synergistic effect of PP3 and FLC against Mrr1 mutant strains. (A) Growth inhibition evaluated using the disk diffusion assay. The indicated *C. albicans* strains (2×10^5 CFU) were plated on yeast extract-peptone-dextrose (YPD) agar plates, and cellulose disks impregnated with FLC and/or PP3 were placed on the plates. The following drug doses were used for each strain: 8 μg FLC and/or 16 μg PP3 for SCMRR1R34MPG2A, 32 μg FLC and/or 32 μg PP3 for G5, 32 μg FLC and/or 32 μg PP3 for DSY296, and 32 μg FLC and/or 32 μg PP3 for Gu5. Each plate was incubated at 30°C for 48 h for observation. (B) alamarBlue assay of the growth inhibitory effects of the indicated treatments against *C. albicans*. *C. albicans* cells (1×10^3 CFU/ml) were treated for 24 h with the indicated drugs at the following doses: 1 μg/ml FLC and/or 16 μg/ml PP3 for SCMRR1R34MPG2A, 16 μg/ml FLC and/or 16 μg/ml PP3 for G5, 32 μg/ml FLC and/or 16 μg/ml PP3 for DSY296, and 32 μg/ml FLC and/or 16 μg/ml PP3 for Gu5. Drug treatment was followed by staining with alamarBlue for 2 h in the dark, and the growth percentage was measured using a BioTek Synergy H1 microplate reader (excitation wavelength, 530 nm; emission wavelength, 590 nm).

Molecular docking analysis of the mode of binding of PP3 to Mdr1. To characterize the direct interaction between Mdr1 and PP3 and the mode of binding, we first generated a homology model of *C. albicans* Mdr1 using the SWISS-MODEL server with the Alignment Mode algorithm (15). Based on the calculated global model

TABLE 2 Susceptibility testing of PP3 combined with Mdr1 substrates against *C. albicans* strain G5 by the checkerboard microdilution assay and drug interaction analysis according to the FICI model

	MIC_{80} (μg/ml)					
	Alone		In combination			
Substrate	Substrate	PP3	Substrate	PP3	FICI	Interpretation[a]
Cerulenin	32	>128	8	16	0.375	SYN
Brefeldin A	128	>128	32	16	0.375	SYN
VRC	0.25	>128	0.0625	16	0.375	SYN
PCZ	0.25	>128	0.0625	16	0.375	SYN

[a]SYN, synergistic.

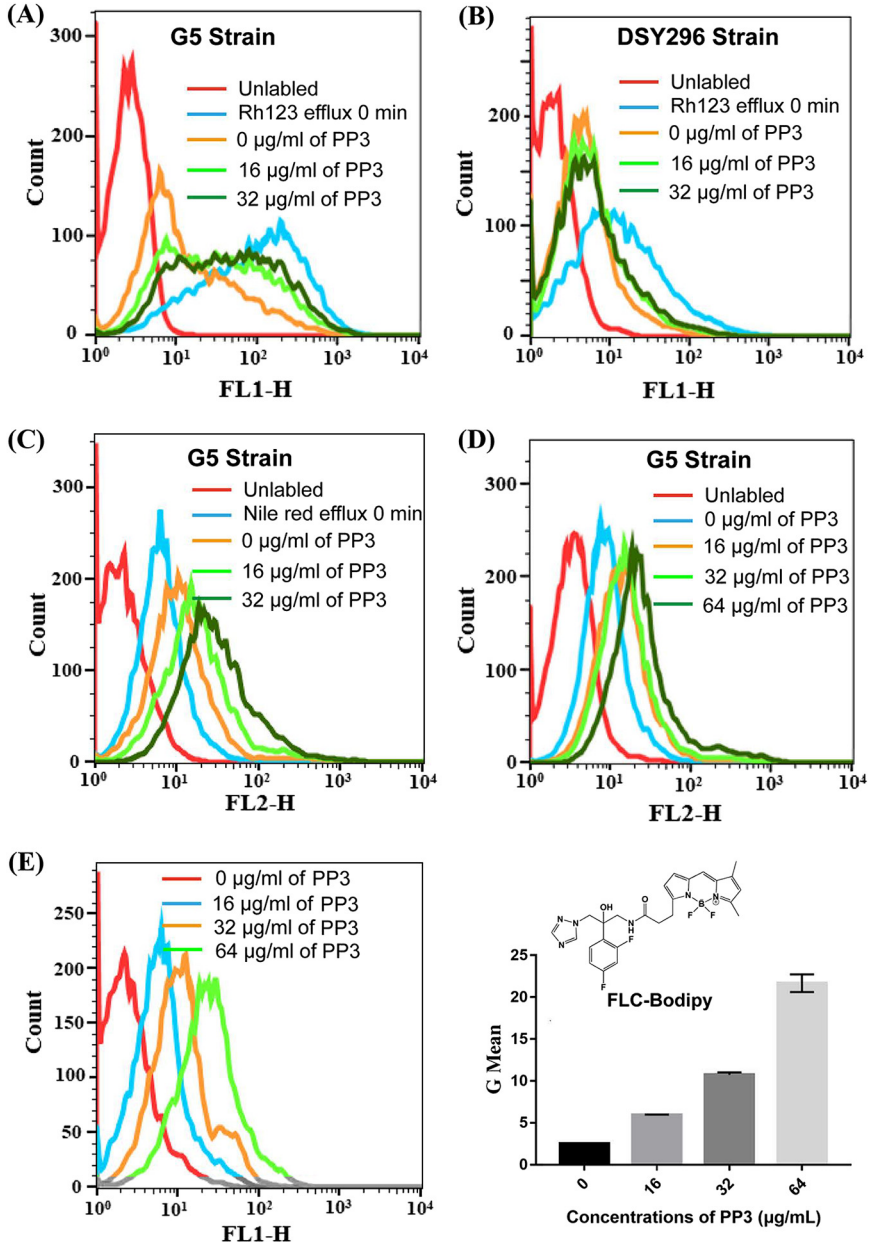

FIG 2 Effects of PP3 on efflux pump activity. (A and B) Rh123 efflux by the *C. albicans* strain G5 with hyperactive Mrr1 (A) and strain DSY296 with hyperactive Tac1 (B), assessed by flow cytometry. Energy-depleted cells were loaded with Rh123 (5 μM) for 30 min. After washing with PBS, the cells were treated with PP3 (0, 16, or 32 μg/ml) for 30 min. Efflux was initiated by adding glucose (40 mM) in all treatment groups. The fluorescence intensity of Rh123 was then monitored by flow cytometry. (C) Efflux of Nile red by *C. albicans* strain G5. Energy-depleted cells were loaded with Nile red (5 μM) for 30 min. After washing with PBS, the cells were treated with PP3 (0, 16, or 32 μg/ml) in PBS for 30 min, followed by the addition of glucose. The fluorescence intensity of Nile red was then monitored by flow cytometry. (D) Intracellular accumulation of Nile red by *C. albicans* strain G5. Cells cultured overnight were incubated with Nile red (5 μM) for 60 min in the presence of PP3 (0, 16, 32, or 64 μg/ml). The fluorescence intensity of Nile red was then monitored by flow cytometry. (E) Efflux activity assessed by measuring the accumulation of a fluorescent FLC analogue. Cells cultured overnight were treated with FLC-Bodipy (8 μg/ml) and PP3 (0, 16, 32, or 64 μg/ml) for 8 h. The fluorescence intensity of FLC-Bodipy was then monitored by flow cytometry (left). The flow cytometry data were analyzed using FlowJo, and the geometric mean value was obtained (right).

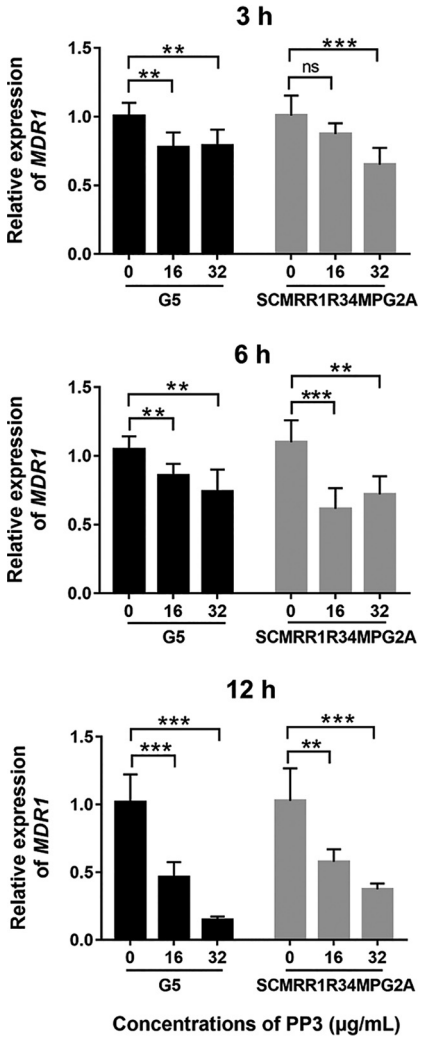

FIG 3 Transcript levels of *MDR1* in response to PP3 treatment. *C. albicans* G5 or SCMRR1R34MPG2A cells were treated with PP3 for 3, 6, or 12 h at 30°C. The relative expression of the *MDR1* genes was determined by qPCR and normalized to that of 18*S* RNA. The bars represent the means ± standard deviations. ns, nonsignificance; **, $P < 0.01$; ***, $P < 0.001$, significance in comparison with the control group.

quality estimate values, the known structure of monocarboxylate transporter 1 (PDB code 7CKR) was used as the template structure, and molecular docking was performed using AutoDock Vina (16, 17). PP3 bound in the previously reported binding pocket for Mdr1 substrates such as FLC, Nile red, cycloheximide, and anisomycin

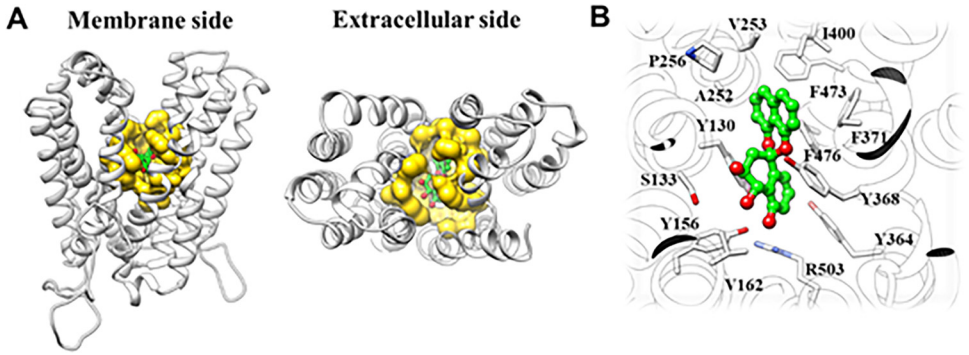

FIG 4 Binding mode of PP3 with Mdr1. (A) Predicted binding mode of PP3 with *C. albicans* Mdr1. The ligand binding pocket is illustrated as the yellow surface. (B) Interactions between PP3 and key residues in Mdr1.

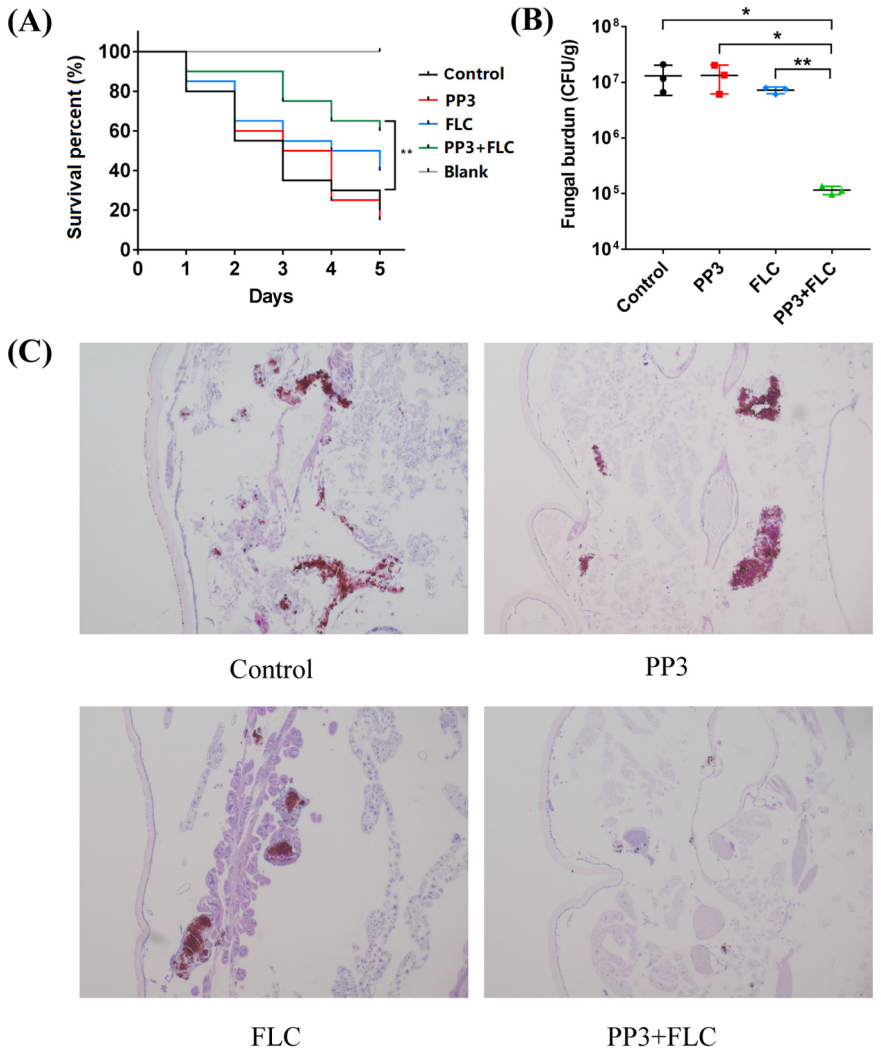

FIG 5 *In vivo* efficacy of the PP3-FLC combination in the *C. albicans*-infected *G. mellonella* model. (A) Survival curves for *C. albicans*-infected *G. mellonella* larvae. Larvae were infected with *C. albicans* G5 (5×10^5 CFU/larva) and then received one of the following treatments: sterile PBS, PP3 (800 ng/larva), FLC (800 ng/larva), or their combination. The survival of infected larvae was monitored for 4 days ($n = 20$ per group). **, $P < 0.01$, compared with the control group. (B) Larval fungal burden after 4 days of infection ($n = 3$ per group). *, $P < 0.05$; **, $P < 0.01$, significant difference between the compared groups. (C) *C. albicans* colonization in infected larvae. Larvae were sacrificed 2 days after infection, fixed in paraformaldehyde, embedded in paraffin wax, sectioned longitudinally, and stained with PAS. The histopathology of infected *G. mellonella* larvae subjected to the different indicated treatments was examined by microscopy.

(Fig. 4), consistent with blocking of efflux function (18). The docking score for the interaction between PP3 and Mdr1 was −10.0 kcal/mol, suggesting a high confidence of docking.

Antifungal activity of FLC combined with PP3 in a *Galleria mellonella* infection model. To assess the efficacy of the combination of FLC and PP3 *in vivo*, we used a *G. mellonella* infection model. The larvae were injected with ~5×10^5 CFU of *C. albicans* G5 cells per larva via the last right proleg and were treated with drug after 2 h of infection. Treatment with PP3 (800 ng per larva) alone had a minimal effect on the survival of *C. albicans*-infected larvae, and individual treatment with FLC (800 ng per larva) only slightly improved the survival rate. In contrast, the combination treatment significantly increased the survival rate (Fig. 5A). *In vivo* efficacy was further confirmed by the results of fungal burden analysis. The combination of FLC and PP3 decreased the fungal burden by ~100-fold in comparison with the control or treatment with PP3 alone and ~62-fold in comparison with treatment with FLC alone (Fig. 5B). Histological examinations using periodic acid-Schiff (PAS) staining revealed a large number of fungal cells in the larvae treated with phosphate-buffered

saline (PBS) or a single treatment, while fungal cells were rarely observed in the larvae treated with the combination of FLC and PP3 (Fig. 5C). The data presented above support the potential application of the combination of FLC and PP3 in the treatment of candidiasis caused by *Candida* strains with Mdr1-mediated azole resistance.

In conclusion, PP3 directly inhibits the efflux pump Mdr1 and regulates *MDR1* expression to inhibit the efflux of Mdr1 substrates and reverse azole resistance. The mechanism by which PP3 reduces *MDR1* expression remains unknown, and further investigations are warranted to determine how PP3 interferes with Mrr1-mediated activation of *MDR1* expression.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

We are grateful to Joachim Morschhäuser of the University of Würzburg for kindly donating the *C. albicans* strains with mutations in Mrr1 and Tac1 used in this study.

This work was supported by the National Natural Science Foundation of China (grants 81773786, 81903674, 81903503, and 81630093), the Natural Science Fund for Excellent Young Scholars of Shandong Province of China (grant ZR2020YQ63), and the Young Scholars Program of Shandong University (grant 2017WLJH41).

M.Z. and W.C. designed the experiments and wrote the manuscript. M.S., M.Z., and J.L. performed microscopy, qPCR, flow cytometry, and animal experiments. F.X., J.S., and X.L. isolated the compound PP3. X.H. performed molecular docking. M.Z. and W.C. analyzed the data. M.S., M.Z., H.L., and W.C. prepared all figures. All authors reviewed the manuscript.

We declare no competing financial interests.

REFERENCES

1. Dadar M, Tiwari R, Karthik K, Chakraborty S, Shahali Y, Dhama K. 2018. *Candida albicans*: biology, molecular characterization, pathogenicity, and advances in diagnosis and control: an update. Microb Pathog 117:128–138. https://doi.org/10.1016/j.micpath.2018.02.028.
2. Gunsalus KT, Tornberg-Belanger SN, Matthan NR, Lichtenstein AH, Kumamoto CA. 2016. Manipulation of host diet to reduce gastrointestinal colonization by the opportunistic pathogen *Candida albicans*. mSphere 1:e00020-15. https://doi.org/10.1128/mSphere.00020-15.
3. Pristov KE, Ghannoum MA. 2019. Resistance of *Candida* to azoles and echinocandins worldwide. Clin Microbiol Infect 25:792–798. https://doi.org/10.1016/j.cmi.2019.03.028.
4. Prasad R, Banerjee A, Shah AH. 2017. Resistance to antifungal therapies. Essays Biochem 61:157–166. https://doi.org/10.1042/EBC20160067.
5. Hiller D, Sanglard D, Morschhauser J. 2006. Overexpression of the *MDR1* gene is sufficient to confer increased resistance to toxic compounds in *Candida albicans*. Antimicrob Agents Chemother 50:1365–1371. https://doi.org/10.1128/AAC.50.4.1365-1371.2006.
6. Pasrija R, Banerjee D, Prasad R. 2007. Structure and function analysis of CaMdr1p, a major facilitator superfamily antifungal efflux transporter protein of *Candida albicans*: identification of amino acid residues critical for drug/H^+ transport. Eukaryot Cell 6:443–453. https://doi.org/10.1128/EC.00315-06.
7. Morschhauser J, Barker KS, Liu TT, Bla BWJ, Homayouni R, Rogers PD. 2007. The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans*. PLoS Pathog 3:e164. https://doi.org/10.1371/journal.ppat.0030164.
8. Xie F, Chang W, Zhang M, Li Y, Li W, Shi H, Zheng S, Lou H. 2016. Quinone derivatives isolated from the endolichenic fungus *Phialocephala fortinii* are Mdr1 modulators that combat azole resistance in *Candida albicans*. Sci Rep 6:33687. https://doi.org/10.1038/srep33687.
9. Shi H, Zhang Y, Zhang M, Chang W, Lou H. 2021. Molecular mechanisms of azole resistance in four clinical *Candida albicans* isolates. Microb Drug Resist 27:1641–1651. https://doi.org/10.1089/mdr.2020.0413.
10. Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M, Morschhauser J. 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob Agents Chemother 42:3065–3072. https://doi.org/10.1128/AAC.42.12.3065.
11. Schubert S, Barker KS, Znaidi S, Schneider S, Dierolf F, Dunkel N, Aid M, Boucher G, Rogers PD, Raymond M, Morschhauser J. 2011. Regulation of efflux pump expression and drug resistance by the transcription factors Mrr1, Upc2, and Cap1 in *Candida albicans*. Antimicrob Agents Chemother 55:2212–2223. https://doi.org/10.1128/AAC.01343-10.
12. Popp C, Hampe I, Hertlein T, Ohlsen K, Rogers PD, Morschhäuser J. 2017. Competitive fitness of fluconazole-resistant clinical *Candida albicans* strains. Antimicrob Agents Chemother 61:e00584-17. https://doi.org/10.1128/AAC.00584-17.
13. Coste A, Turner V, Ischer F, Morschhäuser J, Forche A, Selmecki A, Berman J, Bille J, Sanglard D. 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. Genetics 172:2139–2156. https://doi.org/10.1534/genetics.105.054767.
14. Benhamou RI, Jaber QZ, Herzog IM, Roichman Y, Fridman M. 2018. Fluorescent tracking of the endoplasmic reticulum in live pathogenic fungal cells. ACS Chem Biol 13:3325–3332. https://doi.org/10.1021/acschembio.8b00782.
15. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L, Lepore R, Schwede T. 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 46:W296–W303. https://doi.org/10.1093/nar/gky427.
16. Wang N, Jiang X, Zhang S, Zhu A, Yuan Y, Xu H, Lei J, Yan C. 2021. Structural basis of human monocarboxylate transporter 1 inhibition by anticancer drug candidates. Cell 184:370–383. https://doi.org/10.1016/j.cell.2020.11.043.
17. Trott O, Olson AJ. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 31:455–461. https://doi.org/10.1002/jcc.21334.
18. Redhu AK, Banerjee A, Shah AH, Moreno A, Rawal MK, Nair R, Falson P, Prasad R. 2018. Molecular basis of substrate polyspecificity of the *Candida albicans* Mdr1p multidrug/H^+ antiporter. J Mol Biol 430:682–694. https://doi.org/10.1016/j.jmb.2018.01.005.