Azoles resistance reversal by oridonin in *Candida albicans*

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Research

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Abstract

Background

Candida albicans (*C. albicans*) is a yeast causing hazardous fungal infections with high mortality, especially accompanied by resistance to azole drugs (fluconazole, itraconazole and voriconazole). To overcome the azoles resistance of *C. albicans*, we explored the Oridonin (ORI) with three azole drugs mainly focused on the synergistic activity. In this study, *C. albicans* strains were obtained from cancer patients, and the reversal of drug resistance of azole-resistant *C. albicans* was further studied.

Methods

The synergistic antifungal activities of ORI and azoles were measured by checkerboard microdilution and time-kill assays. The resistance reversal mechanisms, inhibition of drug efflux and induction of apoptosis, were investigated by flow cytometry after Annexin V-FITC/PI co-staining. The expression levels of efflux pump related genes *CDR1* and *CDR2* were quantitatively detected by qRT-PCR.

Results

The azole-resistant isolates identified by checkerboard microdilution method and time-kill curves. The efflux pump inhibition assay with ORI showed that the MIC of fluconazole (128-fold), itraconazole (64-fold) and voriconazole (250-fold) decreased significantly. The upregulation of genes coding for *CDR1* and *CDR2* were confirmed by qPCR with respect to the housekeeping gene *ACT1* in the resistant strain. The sensitizing effect of ORI on fluconazole in the treatment of *C. albicans* also includes promoting apoptosis. We demonstrated that the combination of azoles with ORI exerted potent synergism and further displayed that ORI could promote the sensitization to azoles for azoles-resistant *C. albicans*.

Conclusions

We speculate that the resistance to azoles depends on the overexpression of efflux pump and its related genes *CDR1* and *CDR2*, which reduces the accessibility of antifungal agents to *C. albicans*. The discovery that ORI can effectively inhibit drug efflux and promote apoptosis may provide new insights and therapeutic strategies for overcoming the increasing azole resistance in *C. albicans* infections.

Background

Fungal invasive infections of humans are now referred to as “hidden killers” [1]. Candidemia was cited as the fourth most prevalent nosocomial bloodstream infection in the United States and has a highly attributable mortality rate, causes prolonged hospitalization and rising health care costs [2, 3]. Due to low immunity, tumor patients have an increased risk of nosocomial infection. Infection may affect anti-tumor

therapies and even lead to death. Invasive candidiasis may be a presenting symptom of cancer and a predictor of increased cancer risk in later years [4]. More attention should be paid to fungal infection in tumor patients [5].

Azole has been widely applied clinically in the treatment for fungal infection and is still the first line treatment for Candida infection. However, the resistance to azoles in *C. albicans* is constantly emerging [6]. This makes infections caused by azole-resistant *C. albicans* often recalcitrant to conventional antifungal therapy. *C. albicans* can become resistant to azoles by increasing the number of efflux pumps in the cell, as described above [7, 8]. Efflux pumps are membrane-associated transporters that work by preventing the intracellular accumulation of drug, thereby avoiding toxic levels that would kill the cell [7, 8]. Due to this overexpression of efflux pumps, cross-resistance between azoles is often seen in *C. albicans*, both in vitro and clinically [9]. Up-regulation of *CDR1* and *CDR2* was mainly responsible for the resistance of CA-R [10].

Although amphotericin B and echinocandins are two antifungal compounds which only display effective antifungal activity against azole-resistant *C. albicans*, their high cost and toxicity limit their clinical application. Therefore, it is imperative to find novel treatment strategies to overcome the problem. Interest is increasing in the study of the antifungal effects of azoles combined with one non-antifungal drug [11–13]. Using traditional Chinese medicines or their extracts to reverse the resistance of *C. albicans* to azoles has emerged to be one of the most promising means. Oridonin (ORI) has a molecular formula of $C_{20}H_{28}O_6$ (Fig. 1), an ent-kaurane diterpenoid identified from the Chinese medicinal herb *Rabdosia rubescens*. It was reported that ORI has multiple health-promoting effects, including antioxidant, anti-inflammatory, and antitumor effects [14–16]. There is little evidence showing that ORI can effectively treat infections caused by azole-resistant *C. albicans*, and the relevant mechanisms need to be further clarified. In this study, we investigated the effects of ORI combinations of azoles against azoles-resistant *C. albicans*, and characterized the resistance reversal mechanisms in vitro.

Methods

Strains and chemicals.

Candidemia was defined by a Candida species-positive culture of blood from a patient with cancer (e.g., leukemia, lymphoma, multiple myeloma, or solid tumor) who presented with fever. According to the routine method, *C. albicans* were identified and screened by Candida CHROMagar medium after cultured according to the routine method. The *C. albicans* strains used in this work (CA2489, CA3208, CA10 and CA136) were clinical isolates from Shandong Tumor Hospital and Shandong Provincial Qianfoshan Hospital. *C. albicans* strains were grown routinely on yeast–peptone–dextrose (YPD) agar medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 2% (w/v) agar at 35℃. RPMI 1640 medium (pH 7.0) with L-glutamine and without sodium bicarbonate was purchased from Gibco and buffered with MOPS (Sigma). Their susceptibilities were determined according to CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS) M27-A3 document with *C. albicans* ATCC 10231 as

reference strain [17]. The break points at 24 and 48 h are described as follows: as for fluconazole (FLC), minimum inhibitory concentration (MIC) ≤ 8 μg/ml (susceptible, S), MIC = 16–32 μg/ml (susceptible dose dependent, SDD), MIC ≥ 64 μg/ml (resistant, R); as for itraconazole (ITR), MIC ≤ 0.12 μg/ml (S), MIC = 0.25/0.5 μg/ml (SDD), MIC ≥ 1 μg/ml (R); as for voriconazole (VOR), MIC ≤ 1 μg/ml (S), MIC = 2 μg/ml (intermediate, I), MIC ≥ 4 μg/ml (R) [18]. FLC, ITR and VOR were kindly provided by Cheng Chuang Pharmaceutical Co., Ltd., China; ORI (chemical structure shown in Fig. 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Stock solution of FLC was prepared in sterile distilled water. ITR, VOR and ORI were dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. DMSO concentration was kept below 0.01% in all the cell cultures, and did not exert any detectable effect on cell growth or cell death.

Checkerboard assay.

In order to determine possible synergistic interactions between the azoles and ORI against all *Candida* strains, checkerboard analysis was used and fractional inhibitory concentration index (FICI) values were calculated. The FICI was calculated by the formula FICI = $FICI_A$ + $FICI_{B,}$ where $FICI_A$ is calculated as MIC_A alone/MIC_A combination and $FICI_B$ is calculated as MIC_B alone/MIC_B combination. The interpretation of the FICI was as Odds suggested: the value of FICI ≤ 0.5 should be considered synergy, 0.5 < FICI ≤ 4 should be considered no interaction, and FICI > 4 antagonism [19]. Serial 2-fold dilutions were performed in RPMI 1640 medium and 50 μl of each drug dilution was added to each well of a round-bottomed 96-well plate. The final concentration of FLC, ITR, VOR and ORI ranged from 1 to 512 μg/ml, 0.016 to 8 μg/ml, 0.016 to 8 μg/ml and 2 to 128 μg/ml, respectively. The Candida cells in exponential phase of growth were harvested and suspended in sterilized phosphate buffer saline (PBS; pH 7.2). The concentrations of the Candida suspensions were measured by a hemocytometer (Shanghai Qiujing Biochemical Reagent Instrument Co. Ltd., China), followed by serial dilutions. A volume of 100 μl of the inoculum was added to the polystyrene plates and the final size of the inoculum was 2×10^3 CFU/ml for all stains. The plates were incubated at 35 °C. After 24 h and 48 h, 100 μl of the reagent containing 0.5 mg/ml XTT and 10 μmol/L menadione was added, followed by 2 h incubation in the dark at 35 °C. Then the colorimetric changes were measured at 492 nm with a microtiter plate reader (Bio-Rad, USA). The minimum inhibition concentration (MIC) is defined as the lowest drug concentration that caused an 80% reduction in optical density compared with that of drug-free control well. All the experiments were repeated three times.

Plotting of time-kill curve.

To investigate the effect of concentration and exposure time on the antifungal activities of the azoles with or without ORI, a time-killing test was performed against azole-resistant *C. albicans*, at the starting inoculum of 10^4 CFU/ml. Time-killing studies were conducted in eight groups: (1) drug-free control; (2) azole (FLC/ITR/VOR) alone; (3) ORI (8 μg/ml); (4) ORI (16 μg/ml); (5) ORI (32 μg/ml); (6) azole + ORI (8 μg/ml); (7) azole + ORI (16 μg/ml); (8) azole + ORI (32 μg/ml) FLC, ITR and VOR were used at the concentration of 8, 0.125 and 0.125 μg/ml, respectively. At predetermined time points (0, 6, 12, 24, and

48 h after incubation at 35 °C), an aliquot (100 μl) was aspirated from each group and transferred to a well of a 96-well plate. Then 100 μl of the reagent containing 0.5 mg/ml XTT and 10 μmol/L menadione was added, followed by 2 h incubation in the dark at 35 °C. After 2 h, the colorimetric changes were measured at 492 nm with a microtiter plate reader (Bio-Rad, USA). All experiments were conducted in triplicate, and the results were reported as mean values ± standard deviation (SD). The OD value for each incubation time point was plotted as the vertical ordinate.

Yeast apoptosis assays.

In yeast, phosphatidylserine is predominantly located on the inner leaflet of the lipid bilayer on the cytoplasmic membrane and is translocated to the outer leaflet during apoptosis [20]. The apoptotic marker, phosphatidylserine externalization, was analysed via staining with FITC-labelled annexin V and PI with the FITC-annexin V apoptosis detection kit (Solarbio Science and Technology Co., Ltd.). *C. albicans* cells were cultured overnight in YPD liquid medium and collected after 18 h. The exponentially growing yeasts were adjusted to $5{\cdot}10^6$ CFU/ml with PBS, and diluted to $4{\cdot}10^5$ CFU/ml in RPMI 1640 medium. FLC, ORI and combination of both were respectively added to cultures of *C. albicans*. Cell cultures without drug treatment served as controls. Cells were incubated for 10 h at 35 °C, and afterwards were collected by centrifugation and washed with cold PBS. Then cells in each group were incubated for 15 minutes at room temperature in dark in an annexin-binding buffer containing 5μ l Annexin V-FITC and 5μ l PI, respectively. Samples were then detected with FACS Calibur flow cytometer (Becton Dickinson).

Flow cytometric analysis of the efflux of rhodamine 6G (rh6G).

Rh6G (Sigma) can be absorbed into yeast cells and the efflux of rh6G uses the same membrane transporter as FLC in yeasts [21]. The intracellular rh6G concentration can be used to investigate the drug efflux mechanism in azole-resistant *C. albicans* [22]. The rh6G efflux was investigated by a flow cytometer (Becton Dickinson FACS Calibur) at 525 nm with the logarithmic-phase *C. albicans* cells (5×10^6 CFU/ml). *C. albicans* cells were firstly incubated at 35 °C at 120 rpm in glucose-free PBS buffer containing 10 μM rh6G. When rh6G absorbed into the cell reached equilibrium, uptake of rh6G was stopped by cooling the tubes on ice. The reaction mixture was washed three times with cold PBS buffer to remove rh6G, and then the fluorescence of the cells was determined. After removing the excess rh6G, 8 μg/ml ORI was added to detect efflux. The cells were subjected to a second incubation in PBS buffer containing 5% glucose. Cell cultures in the absence of ORI served as controls. At 90 min after the second incubation, the fluorescence of the cells was measured. Ten thousand cells with similar size and complexity were selected for evaluation in every assay. Experiments were replicated three times. Raw data were analyzed and plotted with GraphPad Prism 5.

Determination of possible resistance mechanisms to FLC by qPCR.

Total RNA was isolated from *C. albicans* planktonic cells with an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. All RNA samples were treated with DNase I, RNase-free (Thermo Scientific) to prevent contamination with genomic DNA. The cDNA of each strain was synthesized using

the Maxima First-Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) according to the manufacturer's instructions. The PCR primers used to amplify and identify the *C. albicans CDR1, CDR2* and *ACT1* primer sequences (5' to 3') were as follows [23]: CDR1-F ACTCCTGCTACCGTGTTGTTATTG,CDR1-R ACCTGGACCACTTGGAACATAT TG, CDR2-F CTGTTACAACCACTATTGCTACTG, CDR2-R TACCTTGGACAACTGTGCTTC, ACT1-F TAAAGAGAAACCAGCGTA, ACT1-R CTCTTCTGGTAGAACCAC. All primers were synthesized by Sangon Biotech. The cDNA samples were mixed with Maxima SYBR Green/ ROX qPCR Master Mix (2 ×) (Thermo Scientific) and qPCR was performed using a 7500HT Fast Real-Time PCR System (Thermo Scientific). The cycling conditions were as follows: 1 cycle of 10 min at 95 °C; followed by 40 cycles of 15 sec at 95 °C, and 1 min at 58 °C for all genes. After amplification, a melting curve was analyzed to ensure the absence of primer dimers; the dissociation cycle was 15 sec at 95 °C 15 sec at 58 °C, and 15 sec at 95 °C. The level of gene expression was calculated using the $2^{-\Delta\Delta}CT$ method with respect to the housekeeping gene *ACT1*. Samples were compared with the control, which was represented by CA10 cultivated without FLC and normalized to 1. Each experiment was repeated at least three times with three parallel samples in each experiment. Values are mean from three separate experiments. Bars are standard deviations. ANOVA (IBM SPSS Statistics version 23, Armonk, NY) was used for intergroup comparisons. Individual comparisons were done using the Tukey HSD analysis. Differences were considered statistically significant at $p < 0.05$ (*), strongly significant at $p < 0.01$ (**), and extremely significant at $p < 0.001$ (***).

Results

ORI reverse azoles resistance in C. albicans.

The MIC distributions of ORI and azoles alone and in combination against *C. albicans* were shown in Table 1 and Table 2. ORI acted synergistically with azoles against resistant *C. albicans* at 24 h and 48 h. However, the synergistic effect on sensitive strains (CA2489 and CA3208) was not obvious. Although ORI alone had very limited antifungal activity, significant decrease in MICs of the three azoles could be observed when resistant strains were treated in combination with various concentrations of ORI. The MICs for FLC, ITR and VOR alone were respectively > 512, > 8 and > 8 μg/ml against *C. albicans* (CA10 and CA136). The data indicated that there were *C. albicans* resistance toward azoles. After adding ORI to the culture system, the concentration of FLC, ITR and VOR to azoles resistance *C. albicans* were decreased dramatically. When the concentration of combined ORI was 8 μg/ml, the MIC of FLC, ITR and VOR decreased by 128 times, 64 times and 250 times respectively at 24 h, and the FICI values were all less than 0.1. In order to visually present the strong synergism between the azoles and ORI against CA10, the results were illustrated in Fig. 2 as three-dimension graphics. Although the FICI values for the susceptible strains were > 0.5, the MIC of each antifungal agent was reduced 2-fold when it was in combination with ORI. The trend of CA136 was consistent with CA10, and the image was not shown. In the experiment, it was found that the drug-resistant strain CA10 had good repeatability and stability in the treatment of fluconazole, and its mechanism was studied later. The MIC of the quality control strain, ATCC 10231, fell within the normal range.

Table 1
Combined drug effects evaluated by the FICI model against *C. albicans* at 24 h

Strain[a]	MIC (μg/ml)[b]				FICI model[c]	
	alone		in combination			
	Azole	ORI	Azole	ORI	FICI value	INT
FLC						
CA2489	1	> 512	0.5	2	< 0.504	NI
CA3208	2	> 512	1	2	< 0.504	NI
CA10	> 512	> 512	4	8	< 0.023	SYN
CA136	> 512	> 512	8	8	< 0.031	SYN
ITR						
CA2489	0.063	> 512	0.063	2	< 1.004	NI
CA3208	0.125	> 512	0.063	2	< 0.504	NI
CA10	> 8	> 512	0.032	8	< 0.020	SYN
CA136	> 8	> 512	0.125	8	< 0.031	SYN
VOR						
CA2489	0.008	> 512	0.004	2	< 0.504	NI
CA3208	0.016	> 512	0.008	2	< 0.504	NI
CA10	> 8	> 512	0.032	8	< 0.012	SYN
CA136	> 8	> 512	0.032	8	< 0.020	SYN

[a] CA, *C.albicans*.

[b] MIC denotes the $MIC_{80\%}$ of each drug alone or in combination (comb) against *C. albicans* at 24 h, and is shown as the median of three independent experiments. FLC, fluconazole; ITR, itraconazole;VOR, voriconazole;ORI, oridonin.

[c] FICI values are shown as the median of three independent experiments. INT, interpretation; NI, no interaction; SYN, synergism.

Table 2
Combined drug effects evaluated by the FICI model against *C. albicans* at 48 h

Strain[a]	MIC (μg/ml) [b]				FICI model[c]	
	alone		in combination			
	Azole	ORI	Azole	ORI	FICI value[α]	INT
FLC						
CA2489	2	> 512	1	2	< 0.504	NI
CA3208	4	> 512	2	2	< 0.504	NI
CA10	> 512	> 512	8	8	< 0.031	SYN
CA136	> 512	> 512	8	16	< 0.047	SYN
ITR						
CA2489	0.125	> 512	0.063	2	< 0.504	NI
CA3208	0.25	> 512	0.125	2	< 0.504	NI
CA10	> 8	> 512	0.063	8	< 0.023	SYN
CA136	> 8	> 512	0.125	16	< 0.047	SYN
VOR						
CA2489	0.016	> 512	0.008	2	< 0.504	NI
CA3208	0.032	> 512	0.016	2	< 0.504	NI
CA10	> 8	> 512	0.063	8	< 0.023	SYN
CA136	> 8	> 512	0.063	8	< 0.023	SYN

[a] CA, *C.albicans*.

[b] MIC denotes the $MIC_{80\%}$ of each drug alone or in combination (comb) against *C. albicans* at 48 h, and is shown as the median of three independent experiments. FLC, fluconazole; ITR, itraconazole;VOR, voriconazole;ORI, oridonin.

[c] FICI values are shown as the median of three independent experiments. INT, interpretation; NI, no interaction; SYN, synergism.

Association between the degree of reversal activity and FLC susceptibility.

Determination of ORI and azoles by checkerboard microdilution method and time-kill curves showed concentration-dependent synergistic effects in all *C.albicans*. The intensity and nature of interactions between ORI and azoles against CA10 were shown in Fig. 3. The antifungal effect of ORI hardly changed

by increasing concentration, and the curves of ORI alone almost stayed close to the curve of drug-free group. When the antifungal agent was used alone, either azole assumed weak antifungal effect against the drug-resistant *C.albicans* CA10. However, the antifungal effects of azoles against CA10 cells were dramatically enhanced by addition of ORI (4 μg/ml, 8 μg/ml, 16 μg/ml), and the discernible improvement in the extent of fungistatic activity was noted as the amount of ORI in solution was increased. When the concentration of ORI was 16 μg/ml, the sensitization exerted by ORI was the most potent and the curves were almost straight lines, suggesting that the drug combinations composed of 16 μg/ml ORI and 8 μg/ml FLC, or 0.125 μg/ml ITR, or 0.125 μg/ml VOR almost completely inhibited the growth of the CA10 cells. Sensitized concentrations of ORI resulting in inhibitory ratios below 10% ranged from 0 to 1 μg/ml, and the IC 80 was 8 μg/ml (Fig. 2), so this concentration was used in subsequent experiments.

Determination of changes in expression of CDR1 **and** CDR2 **genes by qPCR**.

Quantitative detection of the expression of efflux pump related genes *CDR1* and *CDR2* by qRT-PCR. Figure 4 summarizes the results of qPCR, which express the relative changes in the regulation of the selected gene relative to the housekeeping gene *ACT1*. The results showed that the expression levels of *CDR1* and *CDR2* genes of FLC-resistant strain increased significantly after being cultured overnight with FLC ($p < 0.001$). Compared with FLC alone, the addition of ORI significantly decreased the gene expression level of *CDR1* and *CDR2* ($p < 0.001$). ORI alone showed a slight upregulation of the *CDR1* ($p = 0.017$) and *CDR2* genes ($p = 0.455$). The expression level of *CDR2* was the most upregulated, which was 3.73 (FLC alone) and 1.06 (with ORI) times higher than that of the control (Fig. 3).

Inhibition of C. albicans **drug resistance through the modulation of efflux pumps**.

The efflux of rh6G in CA10 cells was evaluated by flow cytometry and the results were shown in Fig. 5. The intracellular concentration of rh6G reached equilibrium at approximately 90 min after the first incubation, and then the efflux of rh6G was measured after a second incubation for 90 minutes in the glucose-supplemented medium. The fluorescence intensity in the control group decreased approximately by half of the balanced fluorescence intensity, while the fluorescence intensity in the ORI-added group still retained at relatively high levels. The average relative fluorescence intensity in the ORI-added group was 1.65-fold higher than that in the control group ($p < 0.05$). These results demonstrated a decreased efflux of rh6G in the ORI-treated *C. albicans* cells.

Combination of FLC and ORI induced apoptosis in C. albicans.

The apoptotic and living cells are distinguished by double staining with annexin V-FITC and PI. The results were shown in Fig. 6. Apoptosis was barely detectable in untreated control cells (0%, right quadrant in Fig. 6a), ORI-treated cells (0.14%, right quadrant in Fig. 6b) and FLC-treated cells (0.92%, right quadrant in Fig. 6c). However, the number of early and late apoptotic cells increased in cells treated with ORI and FLC (62.3%, right quadrant in Fig. 6d). In detail, *C. albicans* cells showed an AnnexinV-FITC$^+$/P$^-$ phenotype (6.09%) and AnnexinV-FITC$^+$/PI$^+$ phenotype (56.21%). These results indicated that the sensitizing effect of ORI on FLC was observed through induction apoptosis of *C. albicans*.

Discussions

The patients suffering from cancer prone to *C. albicans* infection are due to decreased immunity caused by radiotherapy, chemotherapy, and long-term use of corticosteroids and broad-spectrum antibiotics [14]. In solid cancer patients, *C. albicans* candidemia accounted for 32.8% [14]. *C. albicans* was more frequently associated with solid tumors of the gastrointestinal and genitourinary tracts and breast patients [24]. It is efficacious for azole antifungal agents to prevent and treat the infections caused by *C. albicans*. However, the ever-increasing azole resistance in *C. albicans* emerges. The development of strategies to combat azole-resistances in *C. albicans* can be greatly facilitated by studying drug combinations of azoles and antifungal sensitizers. Combined use of drugs can improve the curative effect and reduce the adverse reactions and economic costs. The focus of research and development of antifungal synergist began to shift to traditional Chinese medicine extracts, which are potential sources for new drugs. In this work, a FLC-resistant clinical isolate of *C. albicans* was studied. The first goal was to explore a potential mechanism of azole resistance.

In this study, the activities of three azole drugs (fluconazole, itrconazole and voriconazole) were examined against *C. albicans* strains with different susceptibilities in presence of ORI, a traditional Chinese medicine extract. ORI has potential as anti-tumor, anti-microbial, anti-inflammatory, and anti-oxidant agent [14, 15, 25]. Chequerboard analysis revealed synergistic activity for FLC, ITR and VOR with ORI against resistant *C. albicans* (FICI < 0.1) at 24 and 48 h. Positive interactions in checkerboard microdilution were confirmed. The synergy of ORI with azoles was further confirmed by the dynamic experiments. The time-kill curve can portray a more detailed picture of the kinetic effects of drug combinations on cell viability over time. In the present study, there was an excellent agreement between the FICI method and the time-kill curves. The combination of ORI and azoles recorded significant synergistic interaction against drug-resistant *C. albicans*.

Azoles exert their action by inhibiting the enzyme lanosterol 14-demethylase in yeasts and molds and thus interfere with the biosynthesis of ergosterol in the fungal cell membrane. The upregulation of the *CDR1* and *CDR2* genes after previous exposure to FLC is a well-known phenomenon in clinical isolates [23]. As expected, only slight upregulation of *CDR1* and *CDR2* genes was observed in the standard strain. Compared with the culture without FLC, the expression of the same gene in FLC resistant strains cultured with FLC was significantly increased. Encouragingly, ORI can significantly down-regulate the expression of this fluconazole-resistant gene. The efflux of FLC leads to the reduction of intracellular FLC concentrations in *C. albicans*. Analysis of rh6G's flow cytometer measurements confirmed that ORI reduced the efflux of FLC, which may be related to the inhibition of ORI on the efflux pump function on the cell membrane. It is well acknowledged that interruption of membrane integrity is an important reason why apoptosis of late stages occurs in yeast cells [26]. Therefore, inhibition of the efflux of FLC by ORI may bring about the occurrence of cell apoptosis in *C. albicans*, and these may together contribute the synergy against resistant *C. albicans*.

It has been reported that some Chinese medicine exert antifungal activity by inducing apoptosis in *C. albicans* [27–29], and the induction of cell apoptosis has become an important antifungal pathway [30]. However, the reversal of azole-resistance in *C. albicans* by ORI has never been described. Apoptosis is a highly regulated cellular suicide programme crucial for metazoan development. As eukaryotic cells, *C. albicans* have an asymmetric distribution of phospholipids within the cytoplasmic membrane, with 90% of phosphatidylserines oriented toward the cytoplasm [31]. In this study, the reversal mechanism of ORI in FLC-resistant *C. albicans* was determined by comparing the cells apoptosis. During apoptosis, phosphatidylserine is externalized from the inner to the outer layer [32]. Annexin V is a phospholipid-binding protein with high affinity for phosphatidylserine, and PI is amembrane-impermeant fluorescent dye which stains DNA. Thus, we investigated this specific apoptotic hallmark using AnnexinV/PI double staining. In FLC-resisitant *C. albicans*, the combination of ORI and FLC induces early (annexinV-FITC$^+$/PI$^-$, 6.09%) and late apoptosis (annexinV-FITC$^+$/PI$^+$, 56.21%). Although there is almost no apoptotic population on ORI/ FLC alone, apoptosis pathway is switched on in the majority of yeast cells when they are used together.

Conclusions

Taken as a whole, the combination of ORI and azoles exerts synergistic effects against resistant *C. albicans*. The mechanism of ORI reversing FLC resistance is that it affects the expression level of efflux-related genes, inhibits drug efflux, and induces apoptosis of *C. albicans* after entering cells. Nevertheless, the precise synergistic mechanisms require further investigation owing to the unclear resistance mechanism in the resistant *C. albicans* strain. This study provides new information about the synergistic antifungal effects and mechanism of this drug combination, as well as insight into antifungal agent discovery.

Abbreviations

C. Albicans: Candida albicans; ORI: Oridonin; FLC: fluconazole; ITR: itraconazole; VOR: voriconazole; VOR: fractional inhibitory concentration index; MIC: minimum inhibition concentration; rh6G: rhodamine 6G

Declarations

Ethics approval and consent to participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article related to micro-organisms.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The interest declare that they have no competing interests.

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Authors' contributions

Wenna Shi conceived and planned the project. Haisheng Chen and Hui Li completed all the experiments. Chuanjie Song, Zuoliang Peng and Hui Li analyzed and interpreted the collected data. Wenna Shi and Cunxian Duan wrote the manuscript. All authors read and approve the final manuscript.

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Figures

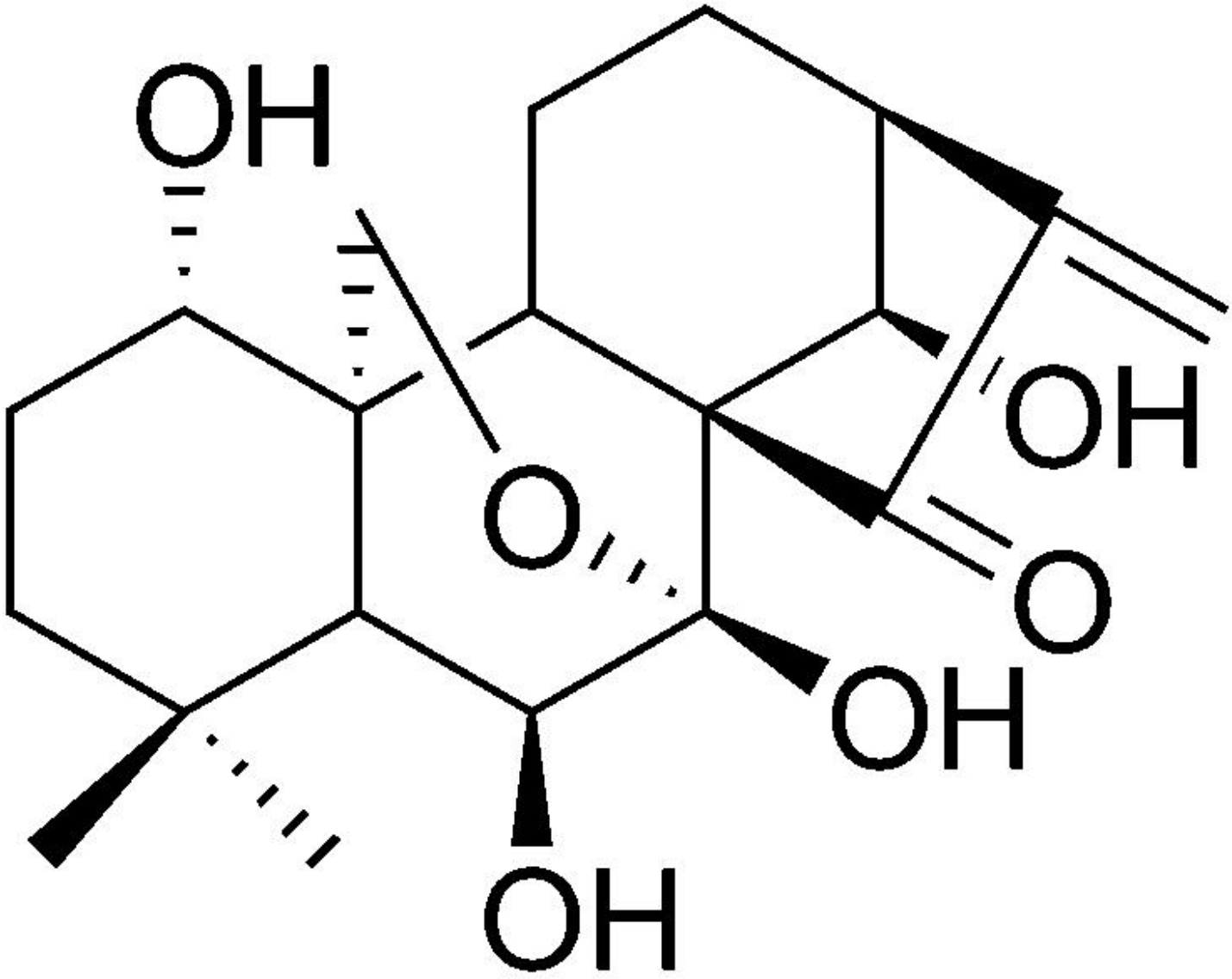


Figure 1

Chemical structure of oridonin.

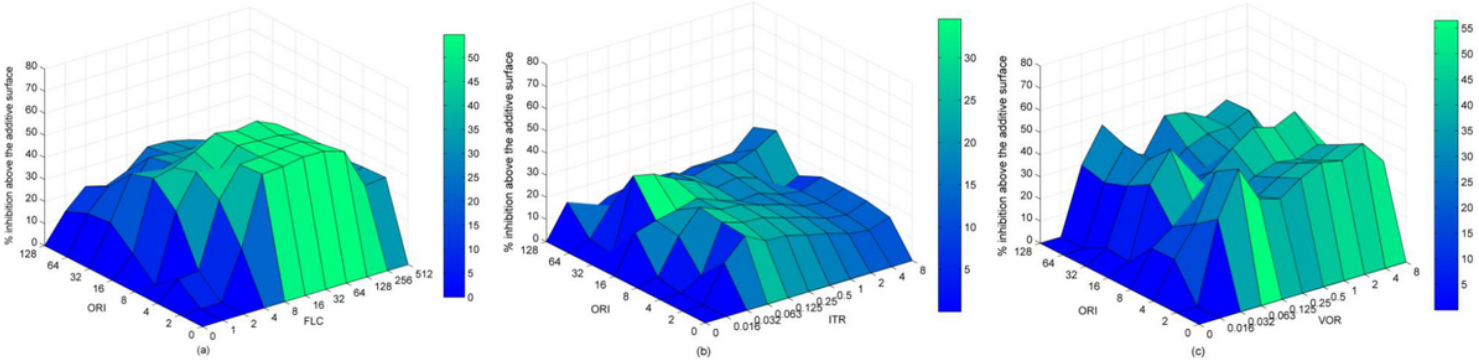
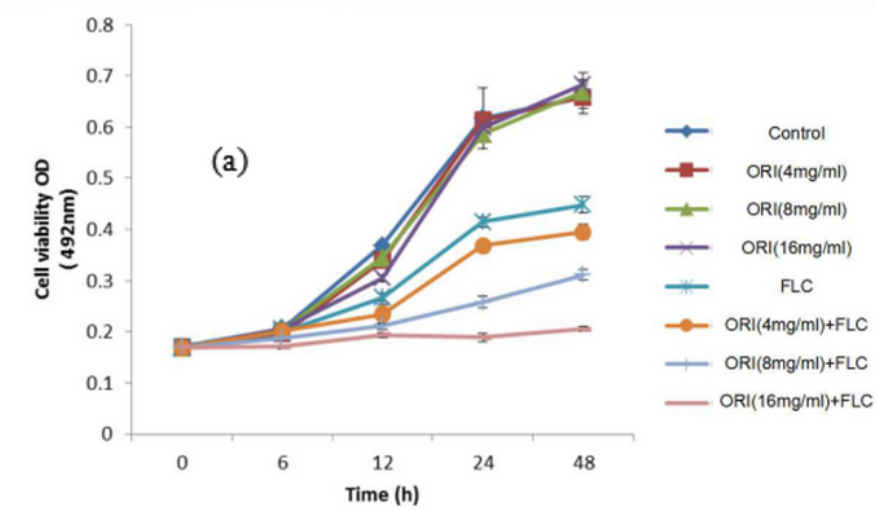
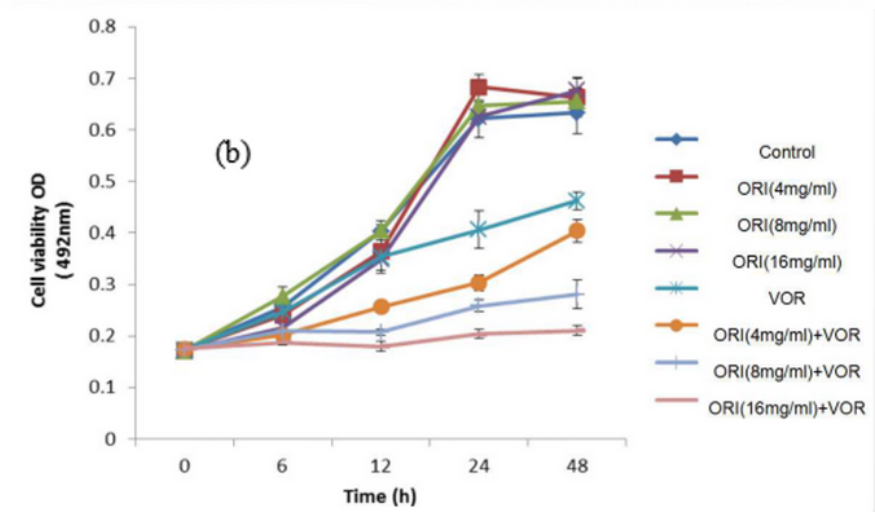


Figure 2

Three-dimensional plots of FLC(a)/ITR(b)/VOR(c) combined with ORI against CA10 using MATLAB program. Drug interaction of azole and ORI can be interpreted by ΔE model and the ΔE value is defined as follows: ΔE =Eazole×EORI-Ecomb, where Eazole and EORI are growth percentages of azole and ORI used alone against CA10, and Ecomb is growth percentage of azole and ORI in combination against CA10. The ΔE values can be depicted on the z axis to construct a 3D graphic. Peaks above the 0 plane represent synergistic combinations. The color-coding bar on the right denotes that the closer to the top of the bar, the more effective the drug combination.





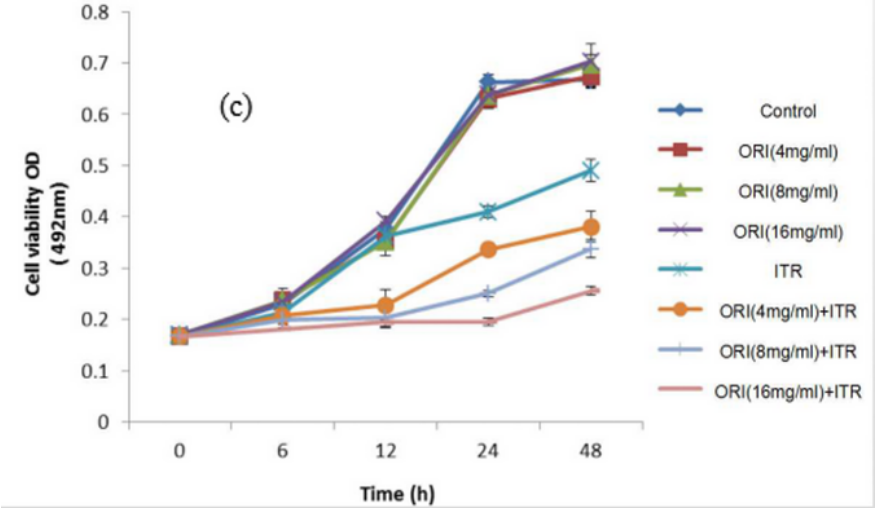


Figure 3

Representative time-kill curves of ORI (4μg/ml, 8μg/ml, 16μg/ml) and FLC(a)/ITR(b)/VOR(c) alone or in combination against CA10. The cells were incubated at 35 °C in RPMI 1640 medium in the presence or absence of ORI and FLC (8 μg/ml)/ITR (0.125 μg/ml)/VOR (0.125 μg/ml). XTT-assay was carried out at the predetermined time points (0, 6, 12, 24 and 48 h) after drug treatment. Data were mean values±standard deviation (SD) of three independent experiments.

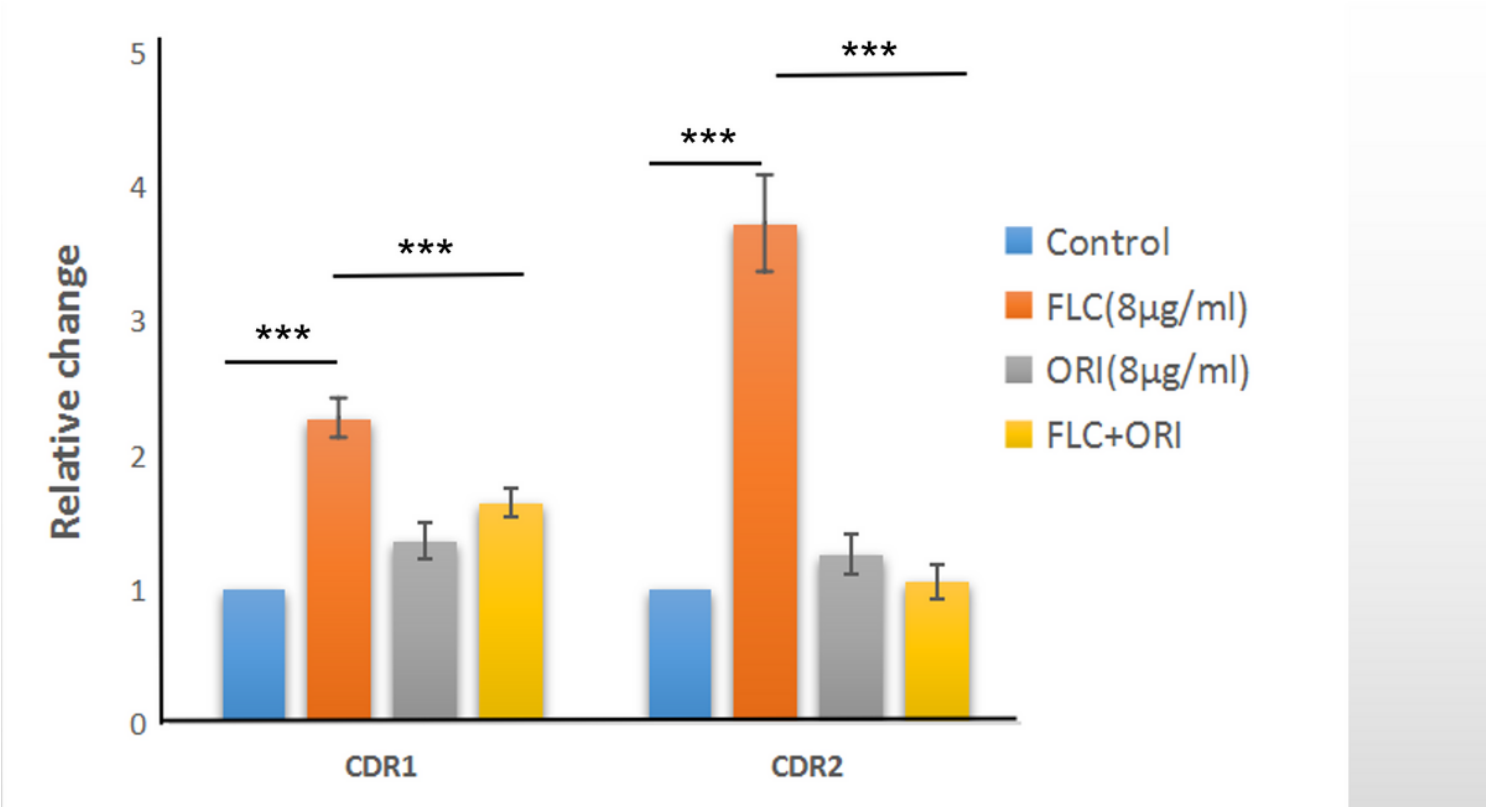


Figure 4

The fold changes of CDR 1 and CDR 2 by qRT-PCR after the treatments of no drugs (control), 8 μg/ml FLC, 8 μg/ml ORI, and 8 μg/ml FLC + 8 μg/ml ORI on CA10. The Tukey HSD analysis was used to calculate the differences between groups. *** $p < 0.001$, compared with the control.

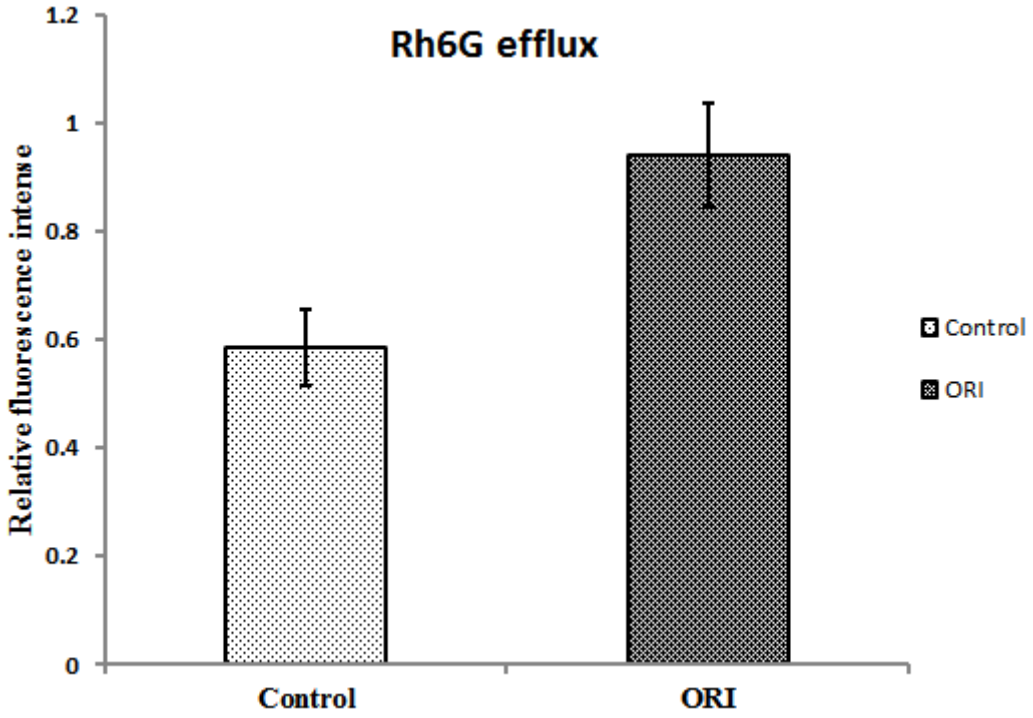


Figure 5

Phosphatidylserine externalization C. albicans, induced by FLC and / or ORI. Cells treated with 8μg/ml FLC and/or 8μg/ml ORI were incubated for 10 h at 35℃,followed by annexin V-FITC/PI assay. (a) Nontreated cells, (b) ORI-treated cells, (c) FLC-treated cells and (d) FLC and ORI-treated cells.

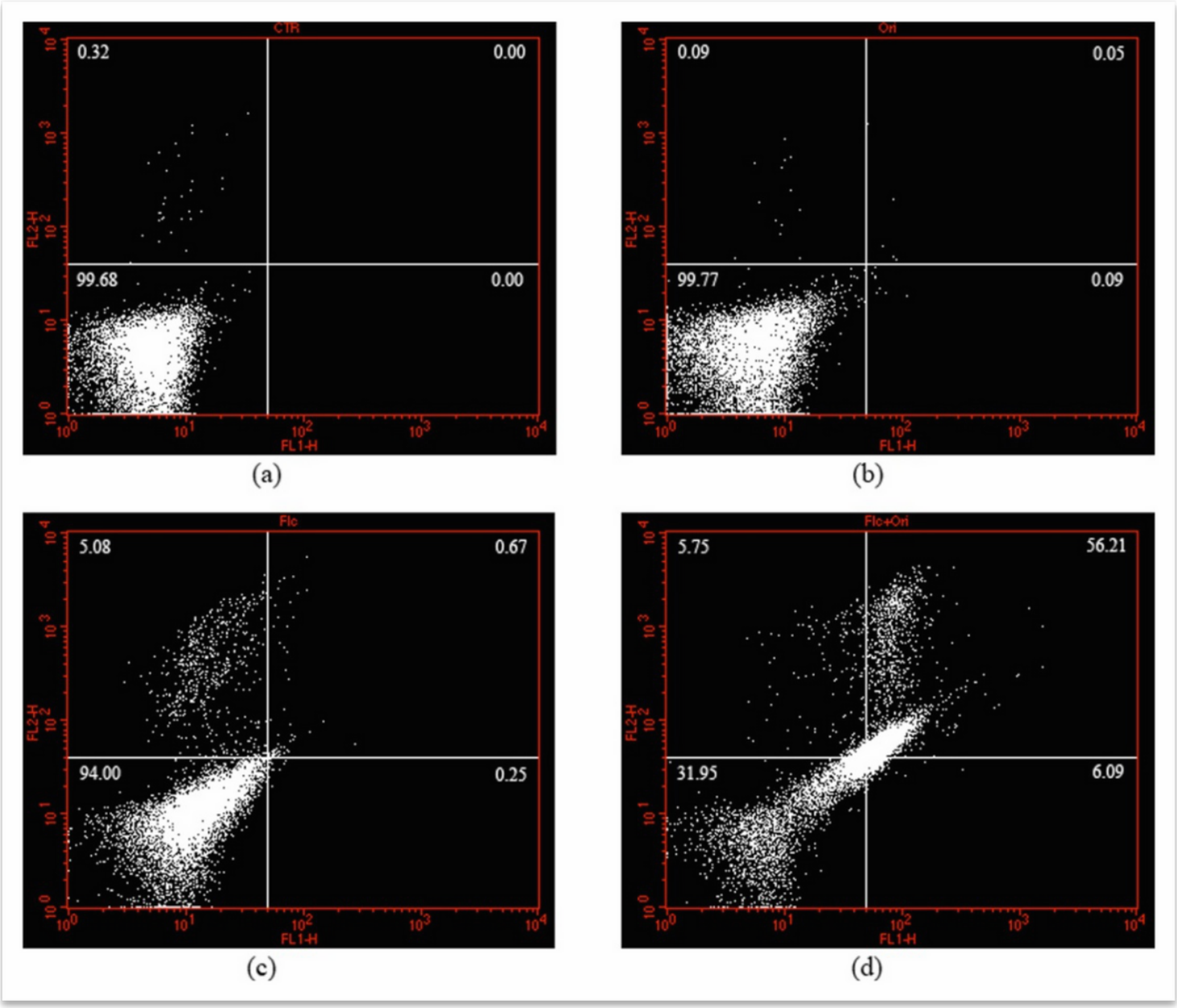


Figure 6

Effects of ORI on rh6G efflux in azole-resistant C. albicans. The CA10 cells were incubated in PBS buffer with 10 μM rh6G (first incubation). Uptake of fluorochrome was quantified when rh6G absorbed into the cell reached equilibrium. Then efflux was evaluated by quantifying the residual fluorescence of the cells at 90 min after the second incubation in glucose-supplemented PBS buffer in the presence or absence of ORI (8μg/ml). Data were compared with the balanced fluorescence intensity and were mean and SEM of two independent experiments.