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Research article

A mechanism study on the synergistic effects of rifapentine and fluconazole against fluconazole-resistant *Candida albicans in vitro*

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ABSTRACT

Candida albicans (*C. albicans*) is one of the most common clinical isolates of systemic fungal infection. Long-term and inappropriate use of antifungal drugs can cause fungal resistance, which poses a great challenge to the clinical treatment of fungal infections. The combination of antifungal drugs and non-antifungal drugs to overcome the problem of fungal resistance has become a research hotspot in recent years. Our previous study found that the combination of rifapentine (RFT) and fluconazole (FLC) has a significant synergistic against FLC-resistant *C. albicans*. The present study aimed to further verify the synergistic effect between FLC and RFT against the FLC-resistant *C. albicans* 100, and explore the underlying mechanism. The growth curve and spot assay test not only showed the synergistic effect of FLC and RFT on FLC-resistant *C. albicans in vitro* but exhibited a dose-dependent effect on RFT, indicating that RFT may play a principal role in the synergic effect of the two drugs. Flow cytometry showed that the combined use of RFT and FLC arrested cells in the G2/M phase, inhibiting the normal division and proliferation of FLC-resistant *C. albicans*. Transmission electron microscopy (TEM) demonstrated that FLC at a low concentration could still cause a certain degree of damage to the cell membrane in the FLC-resistant *C. albicans*, as represented by irregular morphologic changes and some defects observed in the cell membrane. When FLC was used in combination with RFT, the nuclear membrane was dissolved and the nucleus was condensed into a mass. Detection of the intracellular drug concentration of fungi revealed that the intracellular concentration of RFT was 31–195 fold that of RFT alone when it was concomitantly used with FLC. This indicated that FLC could significantly increase the concentration of RFT in cells, which may be due to the damage caused to the fungal cell membrane by FLC. In short, the present study revealed a synergistic mechanism in the combined use of RFT and FLC, which may provide a novel strategy for the clinical treatment of FLC-resistant *C. albicans*.

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1. Introduction

As a conditional pathogenic fungus, *Candida albicans* (*C. albicans*) is a yeast-like fungus colonizing in the skin, oral mucosa, upper respiratory tract, intestinal tract, and vagina of people. With the widespread use of broad-spectrum antibiotics, glucocorticoids, immunosuppressants, and anti-tumor drugs in recent years, as well as large numbers of interventional diagnoses, treatments, and transplantation procedures, the incidence of clinical invasive *Candida* infections has been increasing yearly [1]. *C. albicans* remains one of the most common *Candida* species, although several other *Candida* species have been discovered or become important pathogens [2–4]. As two multi-center prospective observational studies, China Hospital Invasive Fungal Surveillance Net (CHIF-NET) and Invasive Candidiasis in Intensive Care Units in China (China-SCAN) both showed that *C. albicans* is the main pathogen of invasive candidiasis, accounting for 32.9–40.1% of all infection [5,6]. The clinical symptoms of invasive candidiasis are often insidious, but the fatality rate is high. Surveillance data from the US Centers for Disease Control and Prevention (CDC) showed that candidiasis-related mortality accounts for approximately 30% of all in-hospital deaths [2]. Fluconazole (FLC) is the drug of choice for the treatment of *C. albicans* infection owing to its good oral absorption, high bioavailability, and minor adverse reactions. However, with the long-term and inappropriate use of FLC, the number of resistant cases leading to treatment failure has gradually increased [7]. Current studies have discovered that the drug resistance mechanisms of *C. albicans* to azole drugs mainly include the overexpression of membrane efflux pump gene, change in the drug target enzymes, biofilm formation, calcineurin signaling pathway activation, zinc transcription factor mutation, and change in cell membrane permeability [8–10].

In the early 1970s, researchers began to explore the synergistic effects of rifamycin antibiotics combined with antifungal agents. Rifamycins are broad-spectrum antibiotics produced by *Amycolatopsis mediterranei*, mainly used to resist *Mycobacterium tuberculosis* and *Mycobacterium leprae* [11,12]. Clinically, it is often used in combination with other anti-tuberculosis drugs to treat various types of tuberculosis. Rifampin (RFP) is the most widely used classic rifamycin, and both rifabutin and RFT are newly synthetic rifamycins. Beggs et al. [13] reported that the combined use of RFP and amphotericin B (AMB) had a synergistic effect against 40 *Candida* species, such as *C. albicans, C. parapsilosis, C. stellatoidea,* and other clinically isolated strains by checkerboard microdilution method. *Candida* biofilm formation is known to be closely related to drug resistance [14]. explored the effect of RFP or AMB alone and their combination effect on *C. albicans* biofilm formation and found that the minimum biofilm clearance concentration of AMB was above 10 mg/mL. When RFP was combined with AMB, the minimum concentration of biofilm clearance was reduced to 0.04 mg/mL, indicating that the RFP and AMB combination produced a synergistic effect on *Candida* species biofilm formation *in vitro*. In addition, it has been found that RFP can significantly enhance the *in vitro* activity of AMB on biofilm formation of *non-albicans Candida* species (*C. parapsilosis, C. krusei and C. glabrata*) [15]. Rifabutin, another rifamycin antibiotic, can enhance the *in vitro* antifungal activity of AMB against several clinically isolated Aspergillus and Fusarium strains [16]. All the above results demonstrate that rifamycin antibiotics can enhance the antifungal activity of antifungal agents, but the underlying synergistic mechanism remains unclear. As the concentration of rifampicin required to achieve the synergistic effect *in vitro* and *in vivo* often exceeds the safe concentration in humans, further *in vivo* studies are of low significance.

Rifapentine (RFT) is a novel semi-synthetic rifamycin antibiotic with a similar chemical structure and antibacterial mechanism compared with other rifamycins. Its antibacterial effect is mainly through inhibiting the synthesis of bacterial RNA and protein by competitive binding of DNA-dependent RNA polymerase β subunits and blocking the transcription of mRNA, ultimately leading to bacterial death [17,18]. Compared with other rifamycins, the protein binding rate of RFT is higher. It can be accumulated in cells and its intracellular concentration can reach 5 times that of the extracellular concentration [19]. Compared with RFP, RFT has been increasingly used in the treatment of tuberculosis owing to its high bioavailability, longer half-life, less damage to liver function, and less gastrointestinal tract irritation. It has become a common first-line drug in the treatment of tuberculosis [20–22]. However, few studies on the synergistic antifungal effects of RFT have been reported in the currently available literature.

Our previous research found that there was no anti-*C. albicans* activity when RFT was used alone, with the minimal inhibitory concentration (MIC) against 9 FLC-resistant and 3 FLC-sensitive strains above 1024 μg/mL. When RFT was used in combination with FLC, no synergistic effect was observed in FLC-sensitive *C. albicans* (FICI = 1), but a significant synergistic effect was observed in several FLC-resistant *C. albicans*. When the two drugs were combined, the MIC_{80} of RFT decreased from above 1024 μg/mL to 32–64 μg/mL, and that of FLC decreased from 64 to 256 μg/mL to 1–8 μg/mL. The fractional inhibitory concentration index (FICI) range was 0.020–0.047 [23]. The present study aimed to further verify the synergistic effect of RFT and FLC against the FLC-resistant *C. albicans*, and explore the underlying synergistic mechanism of the two drugs combination.

2. Materials and methods

2.1. Strains and antifungal agents

FLC-resistant *C. albicans* 100 was provided by the Mycology Laboratory of Changhai Hospital and kept in the New Drug Research Center of the Naval Medical University College of Pharmacy ($MIC_{80} > 256$ μg/mL). Our previous research has revealed the synergistic effect of RFT and FLC against several clinically isolated strains of FLC-resistant *C. albicans*, especially the FLC-resistant *C. albicans* 100 (the MIC_{80} of FLC decreased from above 256 μg/mL to 2 μg/mL). Thus, the FLC-resistant *C. albicans* 100 was selected as the experimental strain in our subsequent experiments.

RFT (S0118A, Meilun Biotechnology Co., Ltd., Dalian, China) was prepared in dimethyl sulfoxide (DMSO, Sigma, America) and stored at −20 °C in the dark. FLC injection (A075102, Pfizer, America) was 2 mg/mL and stored at 4 °C.

2.2. Growth curves

The single colony of FLC-resistant *C. albicans* 100 selected from the SDA plate was inoculated into 1 mL YEPD medium under shaking (200 rpm) at 30 °C. The strain in the late-exponential growth phase was adjusted to 1×10^3–5×10^3 cells/mL in RPMI 1640 medium. The fungal suspension was treated with or without RFP and/or FLC with the concentrations where indicated. The same volume of normal saline was added to the control group. A 100 μl fungal suspension was collected from each group at 0, 2, 4, 6, 8, 10, 12, 24 and 36 h, and added to the 96-well plate. The OD_{630} value was detected with a Multiskan MK3 microplate reader (Labsystems, Vantaa, Finland). Three independent experiments were performed.

2.3. Spot assay

SDA plates containing different drugs were prepared. The final concentrations of RFT were 15.63, 31.25, and 125 μg/mL, and the final concentrations of FLC were 4 and 16 μg/mL. The exponential phase fungal suspension was adjusted to 10-fold dilutions at five concentration gradients of 10^5 cells/mL, 10^4 cells/mL, 10^3 cells/mL, 10^2 cells/mL, and 10 cells/mL with normal saline. 5 μl of the above suspensions were spotted onto the SDA plates containing the indicated drugs, which were then incubated at 35 °C for 48 h and photographed.

2.4. Flow cytometry

Activation and preparation procedures of the fungal suspensions were the same as described before. They were adjusted to 10^6 cells/mL in RPMI 1640 medium with or without 125 μg/mL RFT and/or 4 μg/mL FLC. These fungal suspensions were cultured under shaking (200 rpm) at 30 °C for 24 h under dark conditions. The samples were centrifuged at 5000 g for 5 min, washed with PBS three times, and fixed with 70% ethanol in a refrigerator at 4 °C overnight. The three samples were centrifuged and cleaned again three times. The number of cells was adjusted to about 1×10^6 cells/mL, and the cells were fully mixed with RNAse in a water bath at 37 °C for 1 h. Then, RNA was removed and propidium iodide (PI) solution was added for staining. After tranquilization in the dark for 30 min, each sample was transferred into the sampling tube, from which 2000 cells were obtained. Single parameter DNA content in the sample was analyzed by FACS Calibur (Becton, Dickinson, San Jose, CA) and FL2 at an excitation wavelength of 488 nm and detection wavelength of 560–580 nm. The experiment was repeated three times.

2.5. Transmission electron microscopy (TEM)

The fungal suspension was adjusted to 10^6 cells/mL with the treatment of 125 μg/mL RFT and/or 4 μg/mL FLC. The strain suspension without the drugs was set as the control group. The samples were cultured under shaking (200 rpm) at 30 °C for 8 h under dark conditions. Then, the samples in each group were centrifuged at 5000 g for 5 min, washed with PBS 3 times, fixed with 4% paraformaldehyde fixative, mixed evenly, and stored in the refrigerator at 4 °C overnight. The samples were rinsed with PBS 3 times, fixed with 1% osmium solution for 2–3 h, rinsed with PBS again for 3 times, added with ethanol and acetone in a certain concentration for gradient dehydration, embedded in Spurr's resin, sliced into ultrathin sections, double stained with 3% uranium acetate and lead citrate, and finally observed by TEM (JEM-1200EX, Japan).

2.6. Determination of the intracellular drug concentration

The fungal suspension was adjusted to 5×10^6 cells/mL in RPMI1640 medium. According to the MIC_{80} of the FLC-resistant *C. albicans* 100 (RFT: 32 μg/mL, FCZ: 2 μg/mL), the concentrations of RFT were set at 8.32 and 128 μg/mL and the concentrations of FLC were set at 0.5, 2 and 8 μg/mL. The fungal suspensions with or without the treatment of FLC or RFT were cultured under shaking (200 rpm) at 30 °C for 8 h under dark conditions. After culture, fungal samples in each group were centrifuged at 4000 rpm for 5 min and washed with deionized water 4 times. 500 mg of precisely weighed wet fungi, glass beads, and ceramic beads were added together and crushed in Precellys 24 biological sample homogenizer (Bertin, France) under the following conditions: 6500 rpm, 30 s × 3 times, 30 s interval, 3 cycles, 5 min interval, and ice bath during cycle interval. The samples were centrifuged at 500 rpm for 2 min. The upper layer of fungal lysate was mixed with the internal standard solution NaOH, and dichloromethane (CH_2Cl_2). Finally, the above mixture was vortexed for 5 min and centrifuged at 12 000 rpm for 10 min. The CH_2Cl_2 phase of the lower liquid was taken, added into a centrifugal tube, and concentrated in a vacuum until it was dried (heat time: 50 min, run time: 200 min at 35 °C). The sample was then redissolved with 80 μl mobile phase [acetonitrile:0.1% formic acid = 40:60 (V/V)] and added to each dried sample tube. All the liquid in each sample tube was taken out and centrifuged at 21 000 rpm for 10 min, and then 10 μl of the supernatant solution was added into a liquid chromatograph triple quadrupole mass spectrometer (LC-MS/MS, Agilent, America) for concentration determination.

2.7. Statistical analysis

All data were analyzed by SPSS22.0 software (IBM, the USA). The counting data are expressed as a percentage (%), and the comparison between the two groups was performed by $\chi 2$ tests. The measurement data of normal distribution are expressed as the mean and standard deviation (SD). A one-way ANOVA test was used for comparison between multiple groups. A one-way ANOVA LSD test was used for homogeneity, and a one-way ANOVA Dunnett T3 test was used for heterogeneity. Values of $p < 0.05$ were considered

statistically significant.

3. Results

3.1. RFT plays a principal antifungal role in the synergistic effect of FLC and RFT

As shown in Fig. 1A, when 125 μg/mL of RFT was used alone, the growth curve of the FLC-resistant *C. albicans* 100 was slightly different from that of the control group, indicating that RFT had a low inhibitory effect on fungal growth. When 64 μg/mL of FLC was used alone, the growth curve of FLC-resistant *C. albicans* 100 was separated from that of the control group to a certain extent, indicating that FLC had a certain fungistatic effect on fungal growth. When 125 μg/mL RFT was used concomitantly with FLC, the growth of FLC-resistant *C. albicans* 100 was significantly inhibited after 6 h ($p < 0.05$). There was no significant dose-dependent inhibition of the combination of 125 μg/mL of RFT and different concentrations (4, 16, and 64 μg/mL) of FLC on fungal growth ($p > 0.05$). When different concentrations (15.63, 31.25, and 125 μg/mL) of RFT were combined with 4 μg/mL FLC (Fig. 1B), the inhibitory effect on strain growth displayed a significant dose-dependent manner ($p \leq 0.001$), indicating that RFT played a principal role against the FLC-resistant *C. albicans* 100 in the combination of the two drugs. In our subsequent experiments, we followed the drug concentration and the time points in this growth curve experiment.

To further confirm the effects of single or combined use of different concentrations of drugs on the FLC-resistant *C. albicans* 100, a spot assay was performed (Fig. 2). RFT alone at either 15.63, 31.25, or 125 μg/mL had little effect on the growth of fungal colonies, and FLC alone at either 4 or 16 μg/mL had an insignificant inhibitory effect on colony growth. The combined use of RFT and FLC exhibited significant inhibitory effects on fungal colonies, and this inhibitory effect was the most pronounced when a concentration of 125 μg/mL RFT was combined with either 4 μg/mL or 16 μg/mL FLC, which was consistent with the result of the growth curve experiment. Taken together, these results suggest that the combination of RFT and FLC inhibited the fungal growth, in which RFT played a principal role.

3.2. The combination of FLC and RFT induces G2/M phase arrest

The effect of combined treatment with RFT and FLC on the fungal cell cycle was assayed by flow cytometry. The percentage of cell count in G0/G1 phase in the control group, RFT-alone group, FLC-alone group, and the combination group was 82.36 ± 1.25%, 70.52 ± 3.11%, 60.45 ± 1.07% and 8.11 ± 0.22% respectively; the percentage of cell count in S phase was 6.03 ± 0.92%, 12.15 ± 1.92%, 15.19 ± 1.23% and 7.79 ± 1.45% respectively; and the percentage of G2/M phase cell count was 11.61 ± 0.86%, 17.33 ± 1.32%, 24.36 ± 0.40% and 86.26 ± 0.94% respectively (Fig. 3). Compared with the control group, the proportion of cells in the G2/M phase

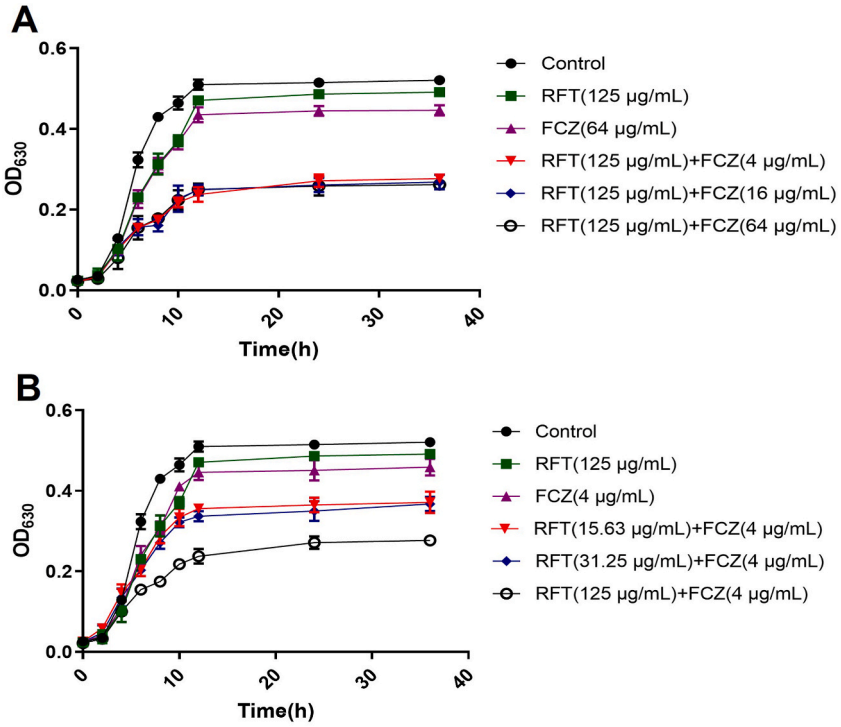

Fig. 1. Growth curve of the FLC-resistant *C. albicans* 100 exposed to different concentrations of drugs in RPMI 1640 culture at different time points. The results are shown as the mean ± SD values of three independent experiments.

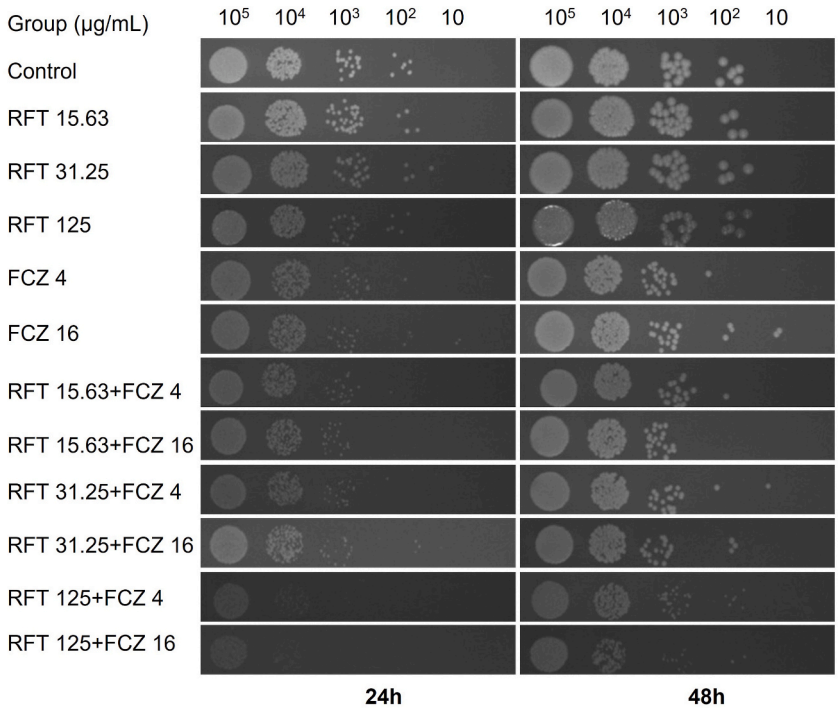

Fig. 2. Spot assay shows the combined inhibition of RFT and FLC. The FLC-resistant FLC-resistant *C. albicans* 100 was spotted on the SDA plates containing the indicated drugs and incubated for 48 h.

was significantly increased in the combination group ($p < 0.001$), while the proportion of G0/G1 phase cells was significantly decreased ($p < 0.001$). The proportion of cells in the G0/G1 and S phase in the combination group was significantly lower than that in either RFT-along or FLC-alone group ($p < 0.05$), and the proportion of cells in the G2/M phase was also significantly increased ($p < 0.001$). These results showed that the combination of the two drugs blocked the cell cycle in the G2/M phase, thus inhibiting the normal division and proliferation of the FLC-resistant *C. albicans* 100.

Destruction of the cell membrane by FLC contributes to its synergism with RFT against FLC-resistant *C. albicans* 100.

The morphology and structural changes of the FLC-resistant *C. albicans* 100 after treatment with RFT and/or FLC were observed by TEM. As shown in Fig. 4, the untreated fungal cells were round or oval with visible buds, smooth cell walls, continuous cell membranes, normal cytoplasm, and nuclear nuclei. Cells treated with RFT-alone showed no significant morphological difference compared with those in the control group. When FLC was used alone, the cell membrane detached from the cell wall, and several spots of high density were observed in the low electron density layer near the cell wall. However, there was no significant damage to the nucleus or cytoplasm. When RFT and FLC were combined, most cells underwent significant structural changes, including swelling and separation of the outer cell wall layer, discontinuous cell membrane, extensive cytoplasm disintegration, aggregation of membranous structures, nuclear shrinkage, and granular nuclear changes. These observations demonstrate that the fungal structure was damaged after a combination of treatment with RFT and FLC.

3.3. FLC plays an important role in increasing the intracellular RFT concentration

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) was used to determine the intracellular drug concentration in the FLC-resistant *C. albicans* 100 after 24h treatment with RFT-alone, FLC-alone, or a combination of RFT and FLC. When different concentrations of RFT (8, 32, and 128 μg/mL) were combined with 2 μg/mL FLC, the intracellular concentration of RFT in the FLC-resistant *C. albicans* 100 increased by 31–195 times compared with that in the RFT-alone group ($p < 0.001$) (Fig. 5A). When different concentrations of FLC (0.5, 2, and 8 μg/mL) were combined with 32 μg/mL RFT, the concentration of intracellular RFT in FLC (2 and 8 μg/mL) combined with 32 μg/mL RFT was higher than that in 0.5 μg/mL FLC combined with RFT ($p < 0.001$) (Fig. 5B). In addition, the intracellular concentration of RFT increased with the increase of the FLC concentration when the two drugs were used in combination. These findings indicate that FLC not only promoted the internalization of RFT into the *C. albicans* strain but exhibited a dose-dependence tendency. As shown in Fig. 5C, the intracellular concentration in the group of 8 or 32 μg/mL RFT in combination with 2 μg/mL FLC was still significantly higher than that in the 128 μg/mL high concentration of RFT-alone group. In addition, the effect of FLC combined with different concentrations of RFT on the FLC concentration in *C. albicans* 100 was also observed (Fig. 5D). When 2 μg/mL of FLC was combined with low concentrations of RFT (8 and 32 μg/mL), the intracellular FLC concentration was lower than that in the FLC-alone group. However, when FLC was combined with a high concentration of RFT (128 μg/mL), the intracellular FLC

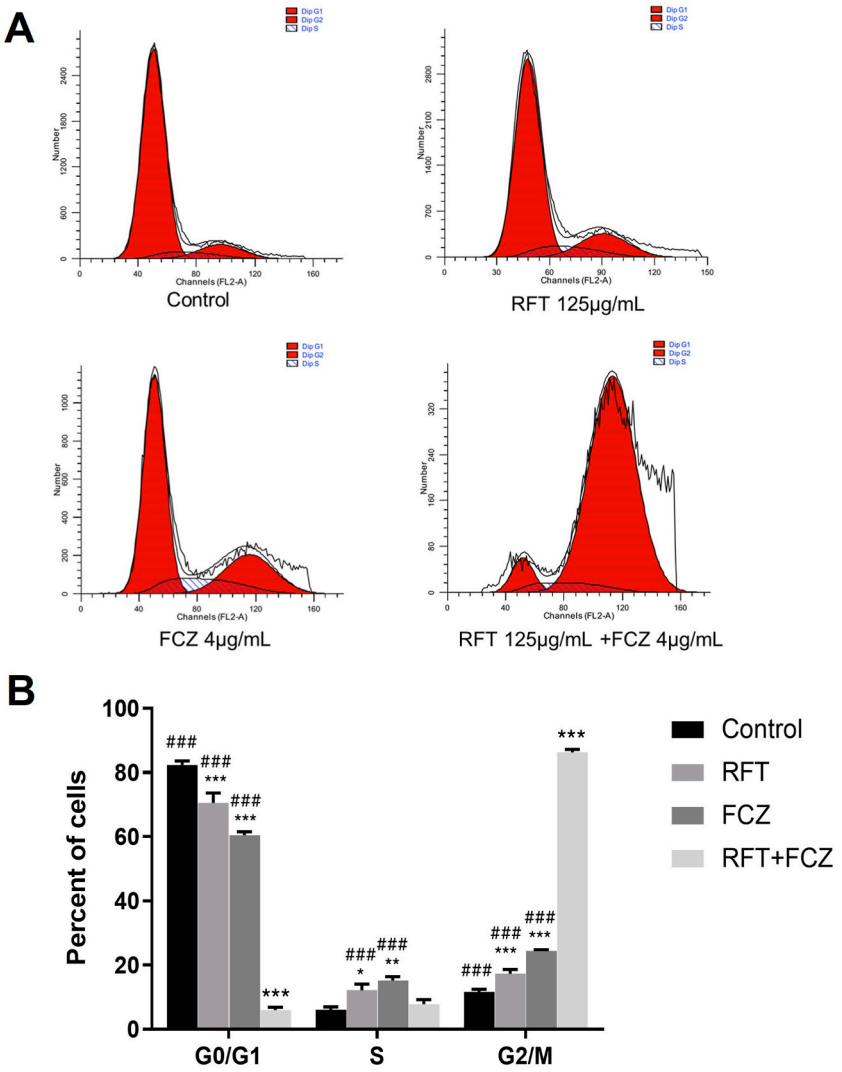

Fig. 3. Cell cycle analysis by flow cytometry. **(A)** The DNA concentration in the FLC-resistant *C. albicans* 100 was treated with RFT or FLC alone and in combination. **(B)** Histogram indicating the percentage of fungal cells treated for 24 h with different drugs in cycle progress. Results are shown as the mean ± SD; *$p < 0.05$ *vs.* control group,**$p < 0.01$ *vs.* control group, ***$p < 0.001$ *vs.* control group , ###$p < 0.001$ *vs.* RFT + FLC group.

concentration was higher than that of FLC alone ($p < 0.05$). In conclusion, a low-concentration RFT could reduce the intracellular FLC concentration to a certain extent, and a high-concentration RFT could increase the intracellular FLC concentration in the FLC-resistant *C. albicans* 100.

4. Discussion

Antifungal resistance of *Candida* species is a serious threat to public health. More than 34 000 hospitalized patients developed resistance to antifungal drugs. About 1700 deaths were due to drug resistance every year according to the 2019 AR Threats Report released by the US Centers for Disease Control and Prevention (CDC). Fluconazole and other azole drugs are commonly used in the clinical treatment and prevention of *Candida* infections. However, with the long-term use of azole drugs, the resistance of *C. albicans* to them has become very common [24,25]. Fungal resistance could be solved by the combined use of two or more antifungal agents, increasing the dosage of antifungal agents, developing new fungistatic agents, and combining antifungal agents with non-antifungal agents [26–28]. However, the types of antifungal agents commercially available are limited, and the combination of two or more antifungal agents can easily develop cross-resistance or even antagonism of fungi [29]. Increasing the drug concentration will not only increase the adverse effects but also enhance the resistance of fungi [30]. The development of new fungistatic agents is costly and time-consuming, which cannot catch up with the rate of fungal drug resistance [31]. Antifungal agents combined with non-antifungal agents can enhance antifungal activity and reduce fungal resistance and adverse reactions [28,32,33]. Therefore, the combination of

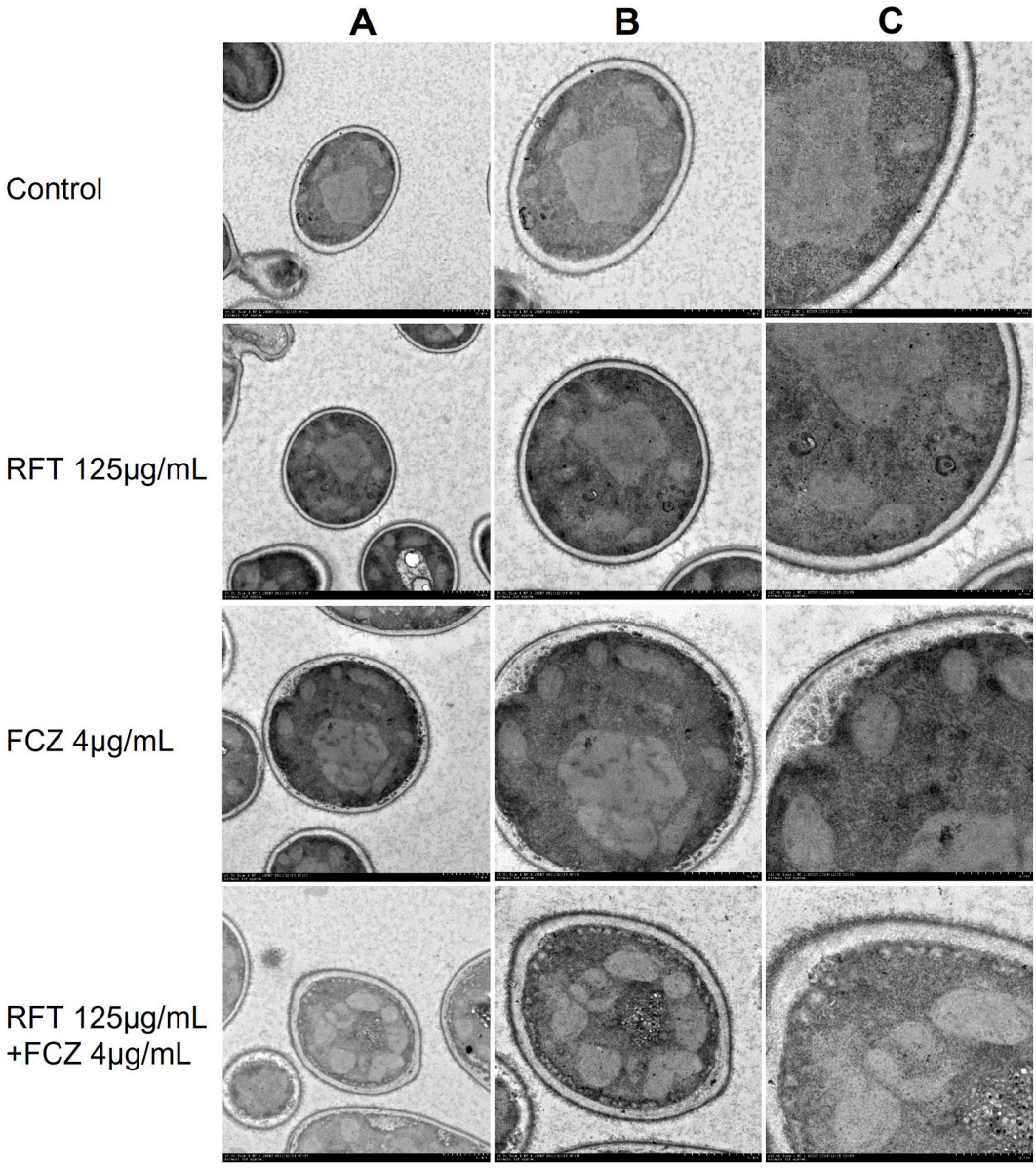

Fig. 4. Transmission electron microscopic images of the FLC-resistant *C. albicans* 100 after 8-h incubation of the indicated drugs. A: 3000 × ; B: 5000 × ; C: 10 000 ×.

antifungal and non-antifungal drugs has become a hot research topic in recent years.

Some researchers have found that the combination of FLC and tetracycline antibiotic minocycline produced a synergistic effect on FLC-resistant *C. albicans* [34]. Biofilm formation in *C. albicans* is one of the factors leading to drug resistance to FLC. *In vitro* studies have proved that the combination of FLC and doxycycline produced a significant synergistic effect in combating *C. albicans* biofilm formation [35]. It has also been proved that calcineurin inhibitors, such as tacrolimus and cyclosporine A, can significantly enhance the antifungal activity of FLC against FLC-resistant *C. albicans* and inhibition of biofilm formation [36,37]. Zhang et al. [38] found that ribavirin, commonly used as a clinical antiviral drug, showed a significant synergistic effect when combined with FLC against FLC-resistant *C. albicans* and *C. albicans* biofilms, and ribavirin enhanced the antifungal activity of FLC *in vivo*. Similar studies have found that the antimalarial drug chloroquine [39], non-steroidal anti-inflammatory drug ibuprofen [40], amlodipine and other four calcium channel blockers [41], lipid-lowering drug atorvastatin [42], anti-arrhythmia drug amiodarone [43], proton pump inhibitor omeprazole [44], phlegm-reducing drug ambroxol hydrochloride [45], and antipsychotic drug aripiprazole [46] can enhance FLC efficacy against drug-resistant *C. albicans* or *C. albicans* biofilm formation *in vitro* and *in vitro*. In addition, several studies have proved that a variety of traditional Chinese medicines and their extracts, such as berberine [47], baicalein [48], flavonoids [49], and oridonin [50] not only own independent antifungal activities but also show a good synergistic effect against FLC-resistant *C. albicans* when they

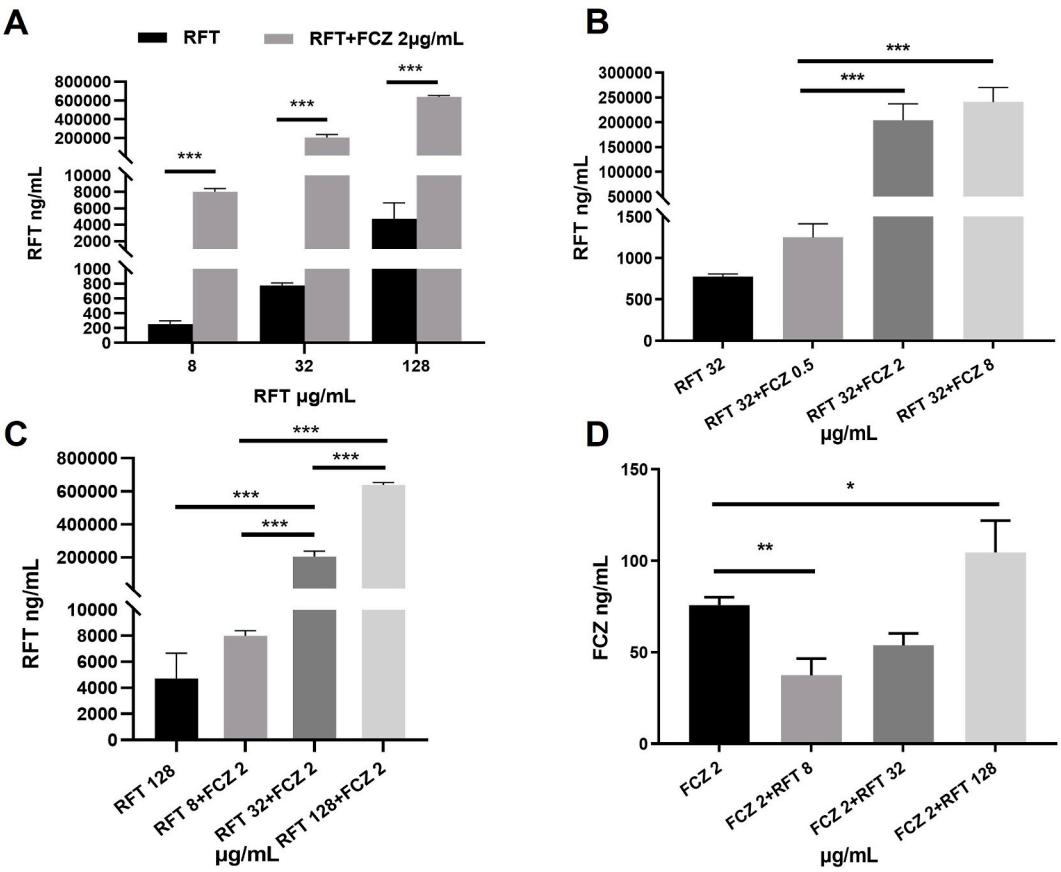

Fig. 5. Intracellular RFT or FLC accumulation in the FLC-resistant *C. albicans* 100. 500.0 mg wet strain was collected after 24h treatment with different concentrations of RFT and/or FLC. **(A)** The intracellular RFT concentration at different concentrations of RFT (8, 32, and 128 μg/mL) was used alone or combined with 128 μg/mL FLC. **(B)** The concentration of intracellular RFT at different concentrations of FLC (0.5, 2, and 8 μg/mL) combined with 32 μg/mL RFT. **(C)** Comparison of the intracellular RFT concentration between the high concentration of 128 μg/mL RFT alone and low concentrations of RFT (8 and 32 μg/mL) combined with 2 μg/mL FLC. **(D)** The intracellular FLC concentration at different concentrations of RFT (8, 32, and 128 μg/mL) was used with 128 μg/mL FLC. The data are shown as the mean + SD from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

are used in combination with FLC.

To the best of our knowledge, this is the first study to evaluate the effect of FLC in combination with RFT against FLC-resistant *C. albicans*. It was found in our previous studies that RFT alone had no anti-*Candida* activity by the checkerboard microdilution method, but it significantly enhanced the activity of FLC against FLC-resistant *C. albicans in vitro*, and decreased the MIC_{80} of FLC from above 64–256 μg/mL to 1–8 μg/mL in several FLC-resistant *C. albicans* clinical isolates [23]. The growth curve and spot assay of the present study showed that the combination of the two drugs inhibited the growth of the strain and this synergistic effect was dose-dependent on RFT, indicating that RFT may play a principal role when the two drugs were used in combination.

Flow cytometry can quickly distinguish the phases of the cell cycle and evaluate the effects of various factors on the cell cycle [51]. *C. albicans* is a eukaryotic organism, and its mitosis includes G1, S, G2, and M phases. Flow cytometry can quickly calculate the percentage of sample cells in the G0/G1 phase (diploid DNA), S phase (between diploid DNA and tetraploid DNA), and G2/M phase (tetraploid DNA) according to the distribution of DNA in each phase of the cell cycle, to understand the division and proliferation of fungal cells [52]. When fungal cells are disturbed by external factors, the normal process of the cell cycle may be affected. Several studies have shown that berberine [53], doxycycline [54], plagiochin E [55], pseudolaric acid A [56] and rubus chingii extract [57] combined with FLC can arrest the cell cycle of FCL-resistant *C. albicans* or *C. albicans* biofilm formation. Cell cycle analysis of our study showed that FLC combined with RFT significantly increased the percentage of cells in G2/M and decreased the percentage of cells in G0/G1, indicating that the combination of FLC and RFT resulted in cell cycle arrest in the G2/M phase, thus inhibiting the normal division and proliferation of the fungal cell. In eukaryotic cells, there are two main checkpoints of the cell cycle (G1 to S phase transition and at the G2 to M phase transition), and the cell cycle stagnation in the G2/M phase is one of the apoptosis signs. When the damaged cells enter mitosis, they will be stopped at the G2/M cell cycle checkpoint, and the cells will start self-examination, thus providing enough time for the repair of cell damage and preventing the cells from entering mitosis before they are repaired. If the cell damage is repaired, cells would continue to enter mitosis, otherwise, apoptosis would happen [58]. In the cell cycle, the G2 phase is the

pre-mitotic gap between the completion of DNA replication and the completion of mitosis, during which the synthesis of RNA and certain proteins is the main material preparation for the cell to enter mitosis. If the synthesis of RNA and protein is inhibited, cells will arrest at G2/M and cannot enter mitosis [59].

The anti-bacterial target of RFT is the β subunit of DNA-dependent RNA polymerase, which inhibits the transcription process of RNA and thus prevents the synthesis of bacterial RNA and protein [18]. Therefore, we suspected that intracellular RFT inhibited the activity of FLC-resistant *C. albicans* 100 through a mechanism similar to an antibacterial agent, i.e., RFT could bind to DNA-dependent RNA polymerase of fungus, thus terminating RNA synthesis, blocking the normal process of cell cycle and preventing fungal cells from dividing and proliferating normally. Wu et al. [55] found that Plagiochin E had an anti-*C. albicans* effect and could block the cell cycle of *C. albicans* at G2/M. In their further study, it was found that Plagiochin E down-regulated the expressions of *CDC28*, *CLB2,* and *CLB4*, which might be related to the G2/M cell cycle arrest induced by Plagiochin E. Li et al. [53] observed the synergistic effect of berberine and FLC against FLC-resistant *C. albicans* through the effect on cell cycle. Their study found that the combination of BBR and FLC caused DNA damage and cell cycle arrest in the S phase. Since the S phase is the DNA replication stage of fungal cells, they suspected that the combination of the two drugs would cause damage to the DNA of FLC-resistant *C. albicans* and then arrest the cell cycle in the S phase. Clancy et al. [16] found that AMB had a synergistic effect against aspergillus *in vitro* when combined with rifabutin, a bacterial RNA polymerase inhibitor, while it had no antifungal activity when used alone. In addition, they found that the combination of the two drugs inhibited the synthesis of *Aspergillus fumigatus* RNA, which was consistent with rifabutin's anti-bacterial mechanism. However, prokaryotes only have one RNA polymerase, and the transcription process is relatively simple, while eukaryotes have three different RNA polymerases. Therefore, it is still unclear which RNA polymerase is affected by rifamycins to exert the synergistic antifungal effect. The normal division and proliferation of cells is an extremely complex and precise process, and eukaryotic cells can make the cell cycle start and end correctly, depending on the intracellular cycle regulation system [60]. At present, it is still not clear through which target in the RFT case in which the cell cycle of FLC-resistant *C. albicans* 100 in the G2/M phase is blocked, and further research is needed.

TEM is a high-resolution electron microscopic observation device used to observe the ultrastructure of cells, such as cell walls, cell membranes, cytoplasm, and nucleus. It is of great significance to study cell damage caused by various treatments at cellular and subcellular levels [61]. FLC exerted its antifungal effect by reducing the ergosterol synthesis, inhibiting fungal cytochrome P450-dependent lanosterol 14-α-demethylase activity, and finally causing damage to the structure and function of the fungal cell membrane [62]. The integrity of the cellular structure of fungi is necessary for normal proliferation. In this study, the morphology and structure of the FLC-resistant *C. albicans* 100 did not change significantly when RFT was used alone, indicating that the strain was almost not damaged by RFT. Although the FLC-resistant *C. albicans* 100 is highly resistant to FLC with MIC_{80} above 256 μg/mL, FLC at a low concentration (4 μg/mL) still damaged the cell membrane to a certain extent. The combination of FLC and RFT caused severe damage to the cells, especially to cytoplasm and nucleus. It can be inferred that by causing cell membrane damage, FLC increased the intracellular concentration of RFT, resulting in an enhanced effect against FLC-resistant *C. albicans*.

To verify the above hypothesis, we determined the intracellular concentration of RFT and FLC by LC-MS/MS. It was found that when RFT was used together with FLC, the intracellular concentration of RFT was 31–195 times higher than that of RFT alone. We also found that the intracellular concentration of RFT was significantly higher when a low-concentration RFT was combined with FLC than a high-concentration RFT used alone. This further demonstrated that FLC significantly promoted the intracellular entry of RFT. In addition, we also found that the intracellular concentration of RFT increased with the increase of FLC concentration when the two drugs were used in combination. Therefore, FLC could promote the enrichment of RFT in fungal cells in a FLC dose-dependent manner. This may be because the higher the FLC concentration, the more damage to the fungal membrane, and the easier entry of RFT into fungal cells. Finally, we also analyzed the effect of the combination of the two drugs on the FLC concentration in the FLC-resistant *C. albicans* 100. It was found that the intracellular FLC concentration decreased when FLC was combined with a low-concentration RFT compared with FLC alone. However, when it was combined with a high concentration of RFT, the intracellular FLC concentration was higher than that of fluconazole alone. This phenomenon may be related to the mechanism of resistance to FLC in *C. albicans*, and the combination of the two drugs with different concentrations produced different degrees of damage to the FLC-resistant *C. albicans* 100. The change in the intracellular FLC concentration was far less obvious than that of RFT when the two drugs were combined. Therefore, we speculated that FLC increased the intracellular RFT concentration by causing damage to the cell membrane and that RFT might play a principal antifungal role in the FLC-resistant *C. albicans* isolate. However, it is still unclear how RFT exerts its antifungal effect, which needs further study.

In conclusion, we found that the combination of FRT and FLC was superior to that of either agent alone against FLC-resistant *C. albicans in vitro*. When the two drugs were used in combination, the cell cycle was arrested in the G2/M phase, and the normal division and proliferation of FLC-resistant *C. albicans* 100 were inhibited. A low concentration of FLC could still cause certain damage to the cell membrane of FLC-resistant *C. albicans* 100. FLC may enhance the cellular penetration of RFT by disrupting the cell membrane of FLC-resistant *C. albicans*. In this study, we preliminarily explore the synergistic antifungal effect of RFT and FLC and the results may provide novel ideas for a better understanding of the mechanism underlying the synergistic effect of the different drug combinations. However, given the unknown target of the drug combination and the limitation of *in vitro* experiments, further experimental studies are still needed.

Additional information

No additional information is available for this paper.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Yulian Wang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft. **Yufei He:** Data curation, Formal analysis. **Tongkai Cai:** Data curation, Formal analysis, Supervision, Validation. **Zhongwei Lei:** Data curation, Formal analysis. **Wenzhi Lei:** Supervision, Writing – review & editing. **Yongbing Cao:** Conceptualization, Supervision, Writing – review & editing. **Jianhua Wu:** Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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