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# Two natural molecules preferentially inhibit azole-resistant *Candida albicans* with *MDR1* hyperactivation

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**[ABSTRACT]** Antifungal drug resistance is a significant clinical problem, and antifungal agents that can evade resistance are urgently needed. In infective niches, resistant organisms often co-existed with sensitive ones, or a subpopulation of antibiotic-susceptible organisms may evolve into resistant ones during antibiotic treatment and eventually dominate the whole population. In this study, we established a co-culture assay in which an azole-resistant *Candida albicans* strain was mixed with a susceptible strain labeled with green fluorescent protein to mimic *in vivo* conditions and screen for antifungal drugs. Fluconazole was used as a positive control to verify the validity of this co-culture assay. Five natural molecules exhibited antifungal activity against both susceptible and resistant *C. albicans*. Two of these compounds, retigeric acid B (RAB) and riccardin D (RD), preferentially inhibited *C. albicans* strains in which the efflux pump *MDR1* was activated. This selectivity was attributed to greater intracellular accumulation of the drugs in the resistant strains. Changes in sterol and lipid compositions were observed in the resistant strains compared to the susceptible strain, and might increase cell permeability to RAB and RD. In addition, RAB and RD interfered with the sterol pathway, further aggregating the decrease in ergosterol in the sterol synthesis pathway in the *MDR1*-activated strains. Our findings here provide an alternative for combating resistant pathogenic fungi.

**[KEY WORDS]** *Candida albicans*; *MDR1*; Azole resistance; Co-culture

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## Introduction

*Candida albicans* is one of the most prevalent human fungal pathogens and causes high morbidity and mortality in immunocompromised patients [1-3]. Azoles such as fluconazole (FLC) are widely used clinically to treat life-threatening invasive fungal infections due to their high efficacy, excellent bioavailability, low toxicity and ease of access [4]. However, the use of azole drugs has promoted the emergence and spread of drug resistance. In infective niches, resistant organisms often co-exist with sensitive ones, or a subpopulation of antibiotic-susceptible organisms may evolve into resistant organisms during FLC treatment and eventually dominate the whole population [5-6]. Therefore, agents that can inhibit or kill both azole-susceptible and azole-resistant organisms, particularly resistant ones, will benefit the treatment of *C. albicans* infections.

Mutations that cause antibiotic resistance in bacteria simultaneously increase sensitivity to other unrelated drugs. This "collateral sensitivity" has been used to develop selective agents or combination strategies to combat resistance problems in bacteria [7-10]. We conjectured that, similar to bacteria, the evolution of azole resistance in *C. albicans* isolates might be penalized by increased sensitivity to other agents. Therefore, we designed a co-culture assay to mimic *in vivo* conditions and screen for antifungal agents. In our assay, an azole-resistant strain and green fluorescent protein-labeled azole-susceptible strain were mixed in equal proportions and exposed to the tested compounds. The simultaneous activity of the tested compounds against the azole-susceptible and azole-resistant strains was evaluated by microscopy.

Natural products have long been regarded as abundant resources for the discovery of novel drugs with a broad spec-

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These authors have no conflict of interest to declare.

trum of biological and pharmacological properties [11]. Our group has focused on obtaining antifungal agents from Chinese liverworts and endolichenic fungi. Five natural molecules that were previously reported to exhibit potent antifungal activity were re-evaluated against azole-susceptible and azole-resistant *Candida* strains using the co-culture screening assay: retigeric acid B (RAB) [12], riccardin D (RD) [13], pyridoxatin (PYR) [14], diorcinol D (DD) [15], and biatriosporin D (BD) [16]. All five compounds inhibited both the susceptible and resistant strains. Moreover, RAB and RD preferentially inhibited *MDR1*-activated strains.

Azole resistance can be caused by different mechanisms, including alterations in the sterol biosynthetic pathway, increased expression of the azole target *ERG11* gene, and overexpression of genes encoding membrane transport proteins [17]. Among them, constitutive overexpression of transporter *MDR1* due to the mutation of its regulator Mrr1 is prevalent in clinical *C. albicans* isolates [18]. Whether the overexpression of *MDR1* affects the ergosterol synthesis pathway remains elusive. In this study, our results revealed that hyperactivation of *MDR1* was associated with changes in cell membrane composition that might enhance the permeability of *C. albicans* cells to RAB and RD and confer selective activity. In addition, RAB and RD interfered with the sterol pathway, which further disrupted the abnormal ergosterol biosynthesis exhibited by *MDR1*-activated strains.

## Materials and Methods

### *Strains and growth conditions*

The susceptible wild-type *C. albicans* strain *TDH3-GFP*-CAI4 (MG1004), eight azole-resistant *C. albicans* strains and their parent strains were used in this study. These strains were stored in physiological saline supplemented with 20% glycerol at –80 °C. Before each experiment, the strains were sub-cultured on yeast-peptone-dextrose (YPD) agar plates twice for 24 h at 30 °C, inoculated in YPD broth, and cultured in an orbital shaker at 30 °C overnight.

### *Chemicals*

RAB was derived from the lichen species *Lobaria kurokawae*. RD, a macrocyclic bisbibenzyl, was previously isolated from the Chinese liverwort *Dumortiera hirsute*. PYR, DD and BD were obtained from three different endolichenic fungi fermentations (Fig. 1). FLC was purchased from the National Institute for the Control of Pharmaceutical Biological Products. The minimum inhibitory concentrations ($MIC_{80}$) of these five compounds against the *C. albicans* strains were measured by a broth microdilution assay according to CLSI guidelines [19] (Table 1).

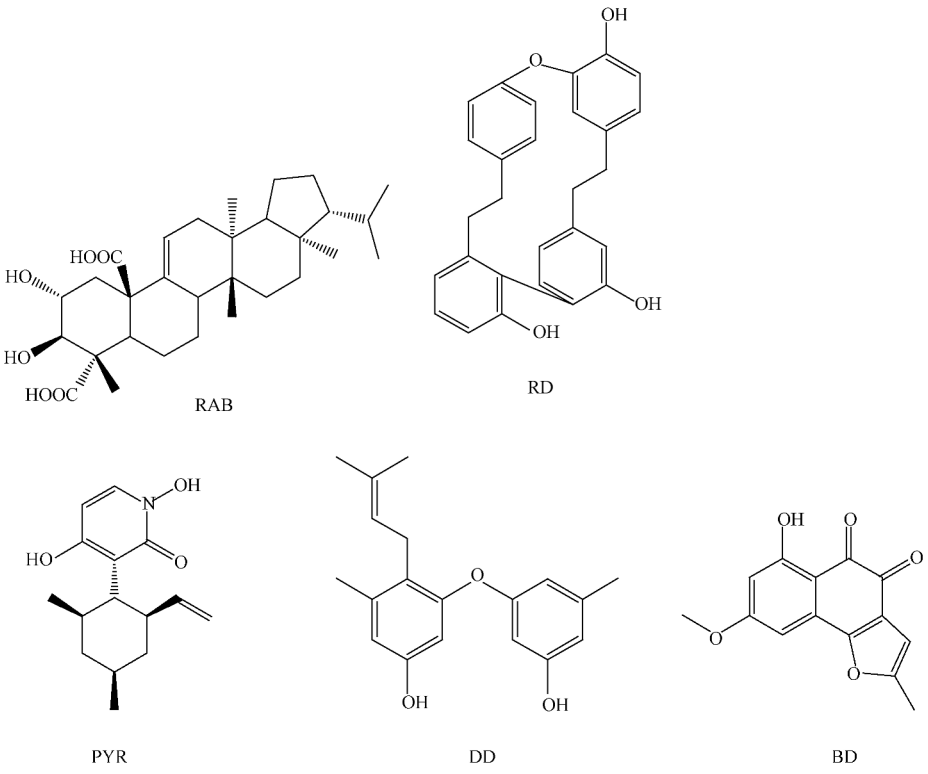


**Fig. 1 Structures of tested compounds**

### *Co-culture assay*

To simulate clinical *C. albicans* infection status, we developed a co-culture screening assay in which the wild-type susceptible strain MG1004 and a resistant strain were mixed in equal proportions. Specifically, initial equal concentrations of $1 \times 10^3$–$2 \times 10^3$ cells/mL of MG1004 and a resistant strain

were mixed in RPMI 1640 medium and deposited into 96-well flat-bottomed microtitration plates. The evaluated antifungal agents were subsequently added at 0.5 ×, 1 × or 2 × MIC followed by culture for 24 h at 35 °C. The cells in each well were photographed with a fluorescence microscope (Olympus IX71, Olympus, Tokyo, Japan). Three visual fields in each well were randomly selected, and the total cell numbers and fluorescent cell numbers were counted. Then, the ratio of the wild-type strain to the azole-resistant strain was calculated based on the average numbers of total cells and fluorescent cells.

**Table 1 The $MIC_{80}$ of tested compounds against *Candida* species**

| Strains | $MIC_{80}$ (μg·mL$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | RAB | RD | PYR | DD | BD | FLC |
| MG1004 | 8 | 16 | 1 | 8 | 16 | 1 |
| YEM13 | 8 | 16 | 1 | 8 | 16 | 64 |
| YEM15 | 8 | 16 | 2 | 8 | 16 | 64 |
| SCMRR1R34MPG2A | 8 | 16 | 1 | 8 | 16 | 8 |
| G5 | 16 | 16 | 2 | 16 | 16 | ＞128 |
| F5 | 8 | 16 | 1 | 8 | 16 | ＞128 |
| Gu5 | 16 | 16 | 1 | 8 | 16 | ＞128 |
| DSY296 | 16 | 16 | 2 | 16 | 16 | ＞128 |
| SCUPC2R14MPG2A | 8 | 16 | 1 | 8 | 16 | ＞128 |

*Growth curves*

The *C. albicans* strains were adjusted to an initial inoculum of 1 × $10^5$ cells/mL in RPMI 1640 medium and incubated in 96-well flat-bottomed microtitration plates at 35 °C without shaking. The growth curves were generated by measuring the $OD_{590}$ values using a BioTek Synergy H1 Microplate Reader at 1-h intervals during 24 h of culture.

*HPLC intracellular accumulation assay*

*C. albicans* MG1004 and three azole-resistant strains with upregulated *MDR1* expression were assayed according to a previously described method [20]. Briefly, cells cultured overnight were diluted to a concentration of 5 × $10^6$ cells/mL in RPMI 1640 medium. The evaluated compound was added to the culture at a final concentration of 1 × MIC. After incubation at 30 °C for 1 h, the cells were collected and lysed by bead beating. 7*β*-Hydroxy-3-oxo-eudesma-4-ene [21] was added to the cell lysates as an internal standard. The supernatants were extracted with ethyl acetate and then evaporated to obtain a dry residue. The dried samples were resuspended in 100% methanol and analyzed by high-performance liquid chromatography (HPLC). Chromatographic separation was achieved on an Agilent XDB-$C_{18}$ 5 μm column (250 mm × 4.6 mm) with a moblie phase of 60% ACN/$H_2O$ (pH 3.0) and a flow rate of 1.0 mL·min$^{-1}$.

*Sterol analysis*

Total intracellular sterols of *C. albicans* were extracted using the alcoholic KOH method [15]. Briefly, overnight-cultured MG1004 and three azole-resistant strains with upregulated *MDR1* expression were inoculated into 20 mL of YPD broth. After incubation of 30 °C for 6 h, the cells were harvested and extracted as previously described [22]. The extracts were analyzed by gas chromatography-mass spectrometer (GC-MS) and quantified with a Shimadzu 2010 GC interfaced with a Shimadzu QP-2010 MS according to a previously described method [23].

The sterol contents of the strains treated with the tested compounds were also measured. *C. albicans* strains were incubated with or without the tested compounds at a dose of 1× MIC, and the total intracellular sterols were extracted and analyzed as described above.

*Lipids analysis*

The lipids of *C. albicans* were extracted by adding boiling isopropanol followed by chloroform: methanol (2 : 1, *V*/*V*) according to a previously described procedure [24]. The solvent mixture was then washed with saturated NaCl solution to separate the chloroform phase. The chloroform phases were combined and dried under a stream of $N_2$. These lipid extracts were then analyzed by GC-MS according to a procedure described by Trinel *et al* [25]. The GC separation was performed on a Shimadzu 2010 GC equipped with a 25 m × 0.32 mm CP-Sil5 CB Low Bleed/MS capillary column with a 0.25-μm film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 280 °C, and the samples were analyzed using the following temperature program: 90 °C for 3 min, followed by an increase at 5 °C·min$^{-1}$ until reaching 260 °C and a hold at 260 °C for 20 min. The column was coupled to a Shimadzu QP-2010 MS. The analysis was performed in the electron impact mode (ionization energy 70 eV, source temperature 150 °C).

*qPCR analysis*

Several genes involved in ergosterol biosynthesis, namely: *ERG1*, *ERG3*, *ERG6*, *ERG9*, and *ERG11*, were selected for transcriptional analysis by quantitative real-time PCR (qPCR) in cells treated with the tested compounds. *C. albicans* MG1004 and the three indicated azole-resistant isolates were diluted to a cell concentration of 5 × $10^6$ cells/mL in RPMI 1640 and

incubated with or without the tested compounds (1 × MIC) at 30 °C for 10 h. Total RNA was isolated using the hot phenol method as previously described [26] and converted to cDNA using a cDNA synthesis kit (TaKaRa Biotechnology, Dalian, China). PCR reactions were performed with SYBR Green master mix (TaKaRa Biotechnology) in an Eppendorf Mastercycler real-time PCR system. *18S* rRNA served as the internal control. The transcript levels of the detected genes were calculated using the formula $2^{-\Delta\Delta^{CT}}$, and the results were represented as the mean ± standard deviations (SD).

*Statistical analysis*

All data were expressed as mean values with the corresponding SD. The statistical significance of differences between the treated and control groups was analyzed by Student's *t*-test (two-tailed, unequal variance). A *P*-value of < 0.05 was considered statistically significant.

## Results

*RAB and RD select against MDR1-activated C. albicans strains in a co-culture assay*

Azole-susceptible *C. albicans* often co-exists with azole-resistant cells in infectious niches of the host. The common mechanisms of azole resistance observed in clinical strains involve the *ERG11* overexpression and the upregulation of genes encoding efflux pumps, such as *CDR1, CDR2,* and *MDR1* [12, 27-28]. The expression levels of *ERG11*, *CDRs* and *MDR1* are regulated by the transcription factors Upc2, Tac1 and Mrr1, respectively. Mutations in these transcription factors are responsible for the constitutively high expression of these transporters in clinical isolates [29]. A gain-of-function mutation (G648D) in *UPC2* contributes to an increase in *ERG11* expression and decreased azole susceptibility in *C. albicans* [30]. The gain-of-function mutations G980E and N977D in *TAC1* constitutively upregulate the expression of *CDR1* and *CDR2* and contribute to azole resistance [29, 31]. *MRR1* containing P683S and G997V mutations constitutively upregulates the expression of *MDR1* and increases azole resistance [12]. Here, we developed a co-culture assay using azole-resistant strains with hyper-expression of *ERG11, CDR1*/*CDR2* or *MDR1* and the GFP-labeled wild-type strain MG1004 to identify agents that can inhibit both azole-susceptible and azole-resistant strains.

We first used FLC to test the validity of this co-culture assay. MG1004 and each azole-resistant strain or its parent strain were equally mixed and exposed to FLC during culture for 24 h (Fig. 2A). We observed that MG1004 maintained its competitive ability against the azole-resistant strains under non-stress conditions. However, upon treatment with FLC (2 μg·mL$^{-1}$), the azole-susceptible strain MG1004 was nearly eliminated, and the azole-resistant strains dominated the population (Fig. 2B, C). This result demonstrates that the co-culture assay can be used to screen for agents that are effective against azole-resistant and azole-susceptible strains simultaneously. We then tested the antifungal activities of the five previously characterized natural agents using this assay. All tested agents exhibited antifungal activity against both azole-susceptible and azole-resistant strains. Although the MIC values of these tested agents did not differ significantly between the azole-susceptible strains and azole-resistant strains, the proportion of *MDR1*-activated azole-resistant organisms was greatly reduced in the presence of RAB or RD (Fig. 2B, C). The proportions of MG1004 and the parent strains of the *MDR1*-activated strains were similar under treatment with the tested compounds. These results suggest that *MDR1* upregulation can increase the antifungal activity of RAB and RD. In the co-cultures of the *CDR*s-activated strains and the susceptible strain, the initial proportions of the strains were maintained under treatment with RAB or RD, suggesting that the presence of the Cdr efflux pumps has a minimal effect on the antifungal activity of RAB and RD. Under treatment with PYR or DD, the proportions of some of the tested azole-resistant strains were greater than 50%, indicating that PYR and DD tend to inhibit the susceptible strain. Among the azole-resistant strains, BD exhibited selective inhibitory activity only against YEM15. RAB and RD, which exhibited selective antifungal activity against the *MDR1*-activated strains, were selected for subsequent study.

*Time-growth curves*

To elucidate the molecular mechanisms underlying this differential antifungal activity, we evaluated the growth rates of the *MDR1*-activated azole-resistant strains and the susceptible strain separately by measuring the $OD_{590}$ growth curves using a microplate reader. The growth profile of the susceptible strain, MG1004, was similar to those of the azole-resistant strains, suggesting that the selective inhibitory effect was not attributable to differential growth rates.

*The intracellular contents of RAB and RD are higher in MDR1-activated strains than in MG1004*

We hypothesized that the selectivity of RAB and RD might be attributable to the differential permeability in *C. albicans* cells. To test this hypothesis, we utilized HPLC to measure the intracellular contents of RAB and RD in MG1004 and three *MDR1*-activated azole-resistant strains according to a previously described method [22]. The intracellular contents of the tested agents were greater in the azole-resistant strains than in MG1004 (Table 2). Specifically, the intracellular accumulation of RAB was, on average, 1.35-fold greater in the three resistant strains than in MG1004, and the accumulation of RD was approximately 1.30-fold greater in the resistant strains than in MG1004 (Fig. 3). These results demonstrated that the selectivity of RAB and RD is at least partially attributable to higher intracellular accumulation although additional factor, such as the cellular targets of these drugs, may also play a role.

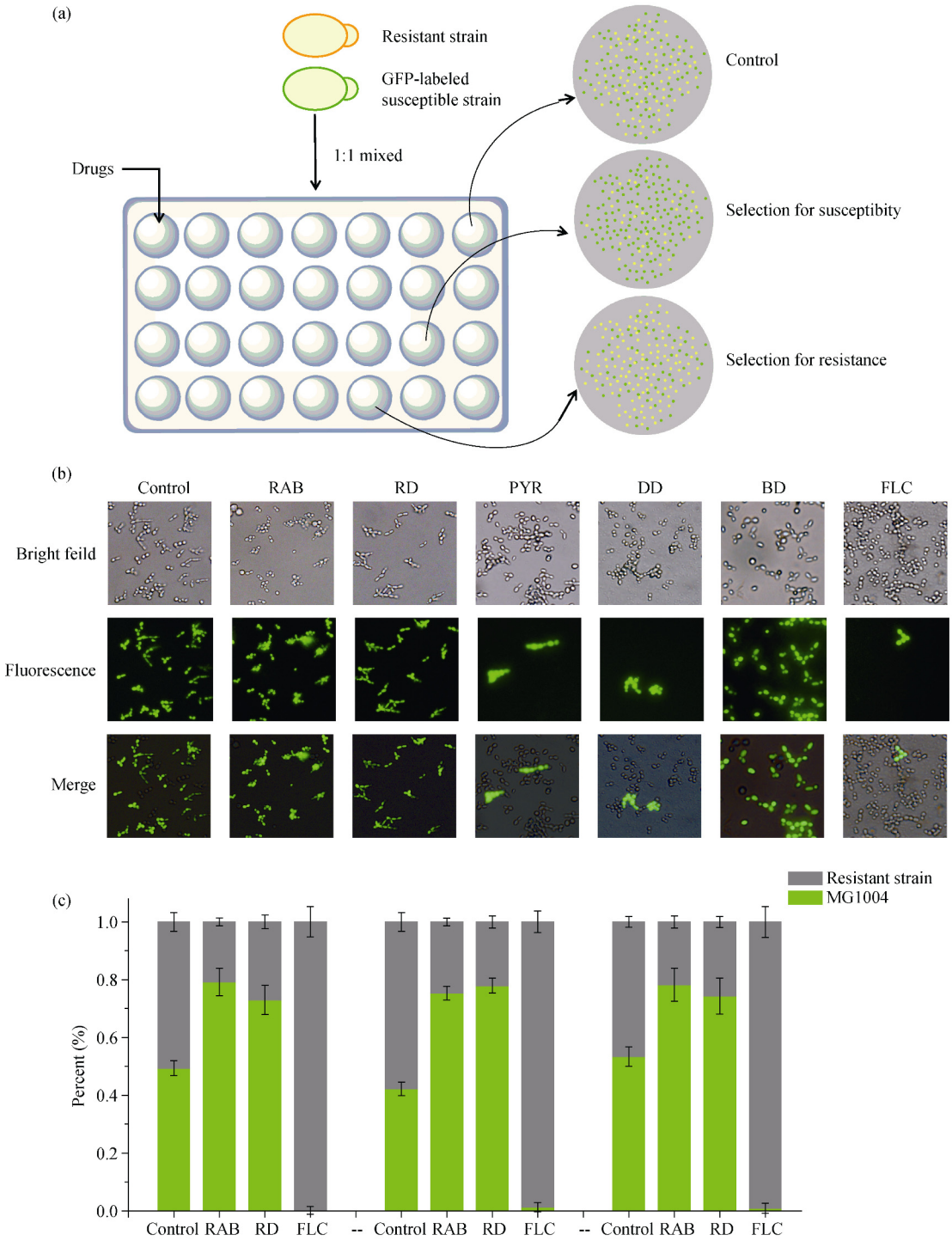


**Fig. 2 Hits that select against azole-resistant strains with *MDR1* hyperactivation using a co-culture assay. (a) Equal amounts of azole-susceptible (shown in green) and azole-resistant strains (shown in yellow) are mixed and incubated in 96-well flat-bottomed microtitration plates with 0.5 ×, 1 × or 2 × fold of MICs of tested antifungal agents. The proportion of fluorescent cells in total *C. albicans* cells reflects the selectivity of evaluated drugs. (b) Azole-resistant strains and MG1004 were co-cultured and photographed after exposed to tested agents for 24 h. FLC (2 μg·mL$^{-1}$) was used as a positive control. The microscopic images of YEM13 and MG1004 co-culture under five tested drugs were shown as an example. The results demonstrated that RAB and RD tend to inhibit YEM13, and BD showed no selectivity between YEM13 and MG1004, whereas other two agents prefer to inhibit the susceptible strain. (c) The proportions of *MDR1*-activated azole-resistant strains and MG1004 were calculated. The majority of FLC-treated population was resistant organisms whereas the proportion of three azole-resistant strains with upregulated *MDR1* expression (YEM13, SCMRR1R34MPG2A, and G5) was reduced to below 50% under the treatment of RAB or RD**

**Table 2 The intracellular contents of tested compounds in *C. albicans* determined by HPLC**

| Intracellular tested agents per milligram of wet organisms (μg·mg$^{-1}$) | MG1004 | YEM13 | SCMRR1R34MPG2A | G5 |
|---|---|---|---|---|
| RAB | 0.416 ± 0.024 | 0.506 ± 0.041 | 0.668 ± 0.031 | 0.618 ± 0.026 |
| RD | 0.068 ± 0.052 | 0.100 ± 0.013 | 0.214 ± 0.009 | 0.242 ± 0.008 |

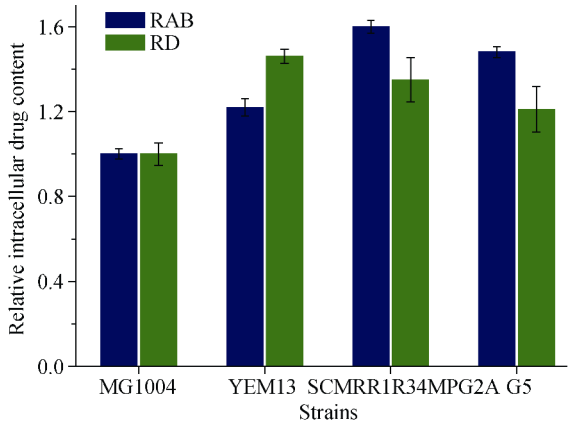


**Fig. 3 The fold increase of intracellular tested compounds in azole-resistant strains compared with susceptible strain MG1004. Three indicated azole-resistant strains and susceptible strain MG1004 were pretreated by 1 × MIC concentration of RAB or RD at 30 °C for 1 h and the intracellular contents of test compounds in *C. albicans* were determined by HPLC. MG1004 served as control. The intracellular accumulation of RAB and RD in the resistant strains showed a 1.20- to 1.50- fold increase compared to MG1004**

*Altered cell membrane compositions may confer differential permeability of RAB and RD in MDR1-activated C. albicans cells*

The plasma membrane is an important barrier that regulates the uptake and expulsion of xenobiotics to maintain intracellular homeostasis [32]. Changes in the sterol and lipid compositions of the plasma membrane may alter the membrane barrier function [33, 34]. We supposed that the high intracellular accumulation of drugs in the *MDR1*-activated strains might be attributable to alterations of cell membrane components and a consequent increase in the permeability of RAB and RD. We therefore analyzed membrane sterol and lipid compositions by GC-MS. Specific changes in sterol content were observed in all of the *MDR1*-activated azole-resistant strains compared with the wild-type strain MG1004 (Table 3), including a significant decrease in ergosterol, a slight decrease in campesterol, and increases in 5, 7, 24(28)-ergostatrienol and lanosterol. The levels of other sterols varied among the different *MDR1*-activated strains.

The lipid composition analysis revealed that the membranes of all *MDR1*-activated azole-resistant strains showed a general redistribution of fatty acid composition compared with MG1004. The *MDR1*-activated strains displayed a decrease in palmitoleic acid ($C_{16:1}$) and increases in hexadecanoic acid ($C_{16:0}$) and 9, 12-octadecadienoic acid ($C_{18:2}$), resulting in a decreased in the $C_{16}/C_{18}$ ratio (Table 3). Fatty acids are an important determinant of the physicochemical properties of membrane lipids [35]. Alterations of membrane lipid composition reduce the plasma membrane permeability of *C. albicans* and confer resistance to azoles [36]. Although we could not confirm that the altered cell membrane compositions resulted in greater uptake of RAB and RD in the *MDR1*-activated strains, common alterations of cell membrane components were observed in the *MDR1*-activated strains. Further investigations of the relationship between cell membrane components and the permeability of RAB and RD may clarify this question.

**Table 3 The compositions of sterols and lipids analyzed by GC-MS**

| | Strains | MG1004 | YEM13 | SCMRR1R34MPG2A | G5 |
|---|---|---|---|---|---|
| Sterol compositions (%) | Ergosterol | 50.42 ± 5.17 | 23.92 ± 2.78 | 23.97 ± 1.63 | 23.45 ± 3.66 |
| | Campesterol | 26.34 ± 3.99 | 15.25 ± 1.56 | 13.88 ± 2.11 | 17.03 ± 2.08 |
| | 5, 7, 24(28)-Ergostatrienol | 10.72 ± 1.28 | 13.64 ± 1.38 | 16.91 ± 2.06 | 18.57 ± 3.76 |
| | Lanosterol | 3.33 ± 0.87 | 5.49 ± 0.97 | 9.10 ± 2.71 | 5.59 ± 0.47 |
| | Other sterols | 9.20 ± 3.90 | 41.70 ± 2.89 | 36.14 ± 3.28 | 35.36 ± 5.02 |
| Lipid compositions (%) | Hexadecanoic acid ($C_{16:0}$) | 4.84 ± 0.24 | 9.06 ± 0.46 | 9.73 ± 0.59 | 11.10 ± 0.97 |
| | Palmitoleic acid ($C_{16:1}$) | 40.03 ± 3.09 | 7.72 ± 1.20 | 7.21 ± 1.47 | 9.03 ± 2.98 |
| | Octadecanethiol ($C_{18:0}$) | 9.45 ± 1.96 | 7.91 ± 2.06 | 7.22 ± 1.63 | 10.75 ± 2.39 |
| | Methyl stearate ($C_{18:0}$) | 1.50 ± 0.18 | 1.57 ± 0.33 | 3.43 ± 0.21 | 3.23 ± 0.57 |
| | Oleic acid ($C_{18:1}$) | 1.06 ± 0.38 | 21.53 ± 0.93 | 1.61 ± 0.81 | 2.61 ± 1.02 |
| | 9-Octadecadienoic acid methyl ester ($C_{18:2}$) | 10.35 ± 2.29 | 22.22 ± 3.01 | 18.71 ± 2.73 | 16.09 ± 0.97 |
| | 9, 12-Octadecadienoic acid ($C_{18:2}$) | 0.97 ± 0.24 | 2.57 ± 0.75 | 3.58 ± 0.88 | 4.85 ± 0.94 |
| | $C_{16}/C_{18}$ | 45 : 23 | 17 : 56 | 16 : 33 | 20 : 37 |

The statistically significant sterol values ($P < 0.05$) were shown in bold.

*The abnormal of ergosterol biosynthesis pathway in MDR1-activated strains contributes to the selective inhibition by RAB and RD*

Because all of the *MDR1*-activated azole-resistant strains exhibited specific changes in membrane sterol content, we performed qPCR to detect the mRNA levels of key genes associated with ergosterol biosynthesis. As shown in Fig. 4A, the expression of *ERG* genes was down-regulated in *MDR1*-activated strains compared with MG1004, indicating that the upregulation of *MDR1* may result in impaired ergosterol biosynthesis. Moreover, RAB or RD treatment greatly reduced the expression of several sterol synthesis-related genes in all tested *C. albicans* strains (Fig. 4B–E), consistent with our previous reports that RAB and RD modulate the ergosterol pathway and reduce ergosterol synthesis to retard *C. albicans* growth [12, 37].

GC-MS analysis revealed that treatment with RAB or RD resulted in a decrease in ergosterol and an increase in lanosterol in *C. albicans* cells. The ergosterol content was 0.317 μg·mg$^{-1}$ of dry weight in untreated YEM13 cells, but decreased to 0.202 μg·mg$^{-1}$ in RAB-treated cells and 0.111 μg·mg$^{-1}$ in RD-treated cells. By contrast, the lanosterol content in YEM13 increased from 0.073 μg·mg$^{-1}$ in untreated cells to 0.091 or 0.093 μg·mg$^{-1}$ in cells treated with RAB or RD, respectively (Table 4). Similar changes in ergosterol and lanosterol content were observed in the other two *MDR1*-activated azole-resistant strains (Table 4). However, the decrease in ergosterol and increase in lanosterol were smaller in MG1004 cells treated with RAB or RD than in the *MDR1*-activated strains treated with the same doses. These results further support the hypothesis that RAB and RD interfere with the sterol biosynthesis pathway and aggravate the degree of sterol composition changes in *MDR1*- activated strains.

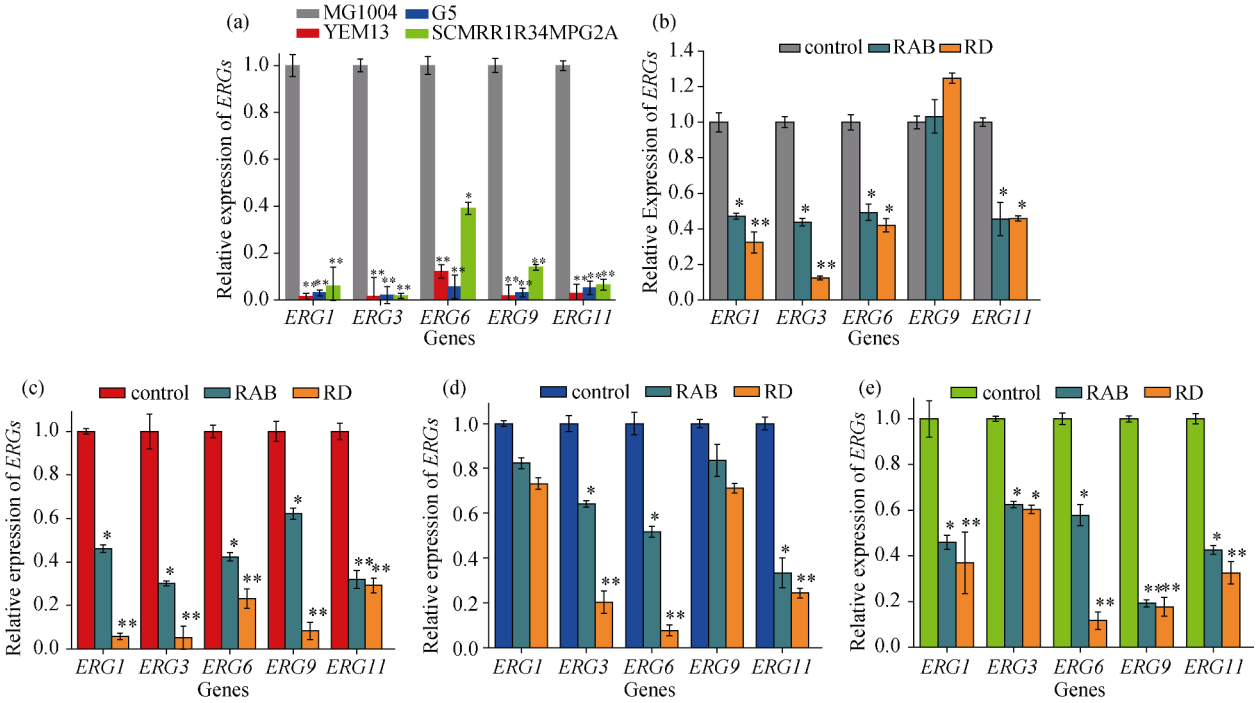


**Fig. 4 The expressions of ergosterol synthesis related genes. (a) *C. albicans* MG1004 and three *MDR1*-activated azole-resistant strains were grown in RPMI 1640 medium for 10 h at 30 °C, The expressions of *ERG* genes was measured using qPCR. The transcriptional expression of each gene was normalized to that in MG1004. (b-e) *C. albicans* MG1004 (b) YEM13 (c), G5 (d), and SCMRR1R34MPG2A (e) were treated with or without RAB or RD (1 × MIC) for 10 h at 30 °C. Transcriptional levels of *ERG* genes were indicated as a fold change relative to that of the untreated group using qPCR assay. Bars represent means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control**

**Table 4 Effect of RAB and RD on sterols synthesis**

| Strains | | MG1004 | YEM13 | SCMRR1R34MPG2A | G5 |
|---|---|---|---|---|---|
| Ergosterol (μg·mg$^{-1}$) | Control | 0.928 ± 0.011 | 0.317 ± 0.019 | 0.337 ± 0.025 | 0.547 ± 0.012 |
| | Treated with RAB | 0.741 ± 0.010 | 0.202 ± 0.008 | 0.27 ± 0.008 | 0.376 ± 0.013 |
| | Treated with RD | 0.703 ± 0.019 | 0.111 ± 0.012 | 0.225 ± 0.021 | 0.233 ± 0.015 |
| Lanosterol (μg·mg$^{-1}$) | Control | 0.039 ± 0.002 | 0.073 ± 0.015 | 0.064 ± 0.027 | 0.102 ± 0.019 |
| | Treated with RAB | 0.043 ± 0.003 | 0.091 ± 0.017 | 0.103 ± 0.025 | 0.181 ± 0.017 |
| | Treated with RD | 0.044 ± 0.002 | 0.093 ± 0.023 | 0.084 ± 0.031 | 0.186 ± 0.021 |

## Discussion

The high frequency of azole resistance in *C. albicans* represents a serious challenge for antifungal treatment and necessitates the continuous development of multiple strategies to evade resistant strains. We developed a co-culture assay to identify antifungal agents that specifically target resistant strains. As expected, FLC exhibited potent inhibitory activity against the azole-susceptible strain, resulting in the emergence of azole-resistant organisms as the majority of the culture. Using this assay, RAB and RD were identified as displaying selective inhibitory activity against three types of *MDR1*-activated strains, and the underlying mechanisms were investigated in three aspects.

HPLC analysis revealed that the intracellular drug contents were higher in the *MDR1*-activated strains than in the wild-type strain MG1004, suggesting that differences in intracellular content may contribute to the observed selective activity. These results are also consistent with our previous findings that RAB and RD inhibit efflux pump activity and significantly reduce the transcriptional expression of *MDR1* [13, 38].

The influx of drugs is significantly restricted by the complex cell membrane [39]. GC-MS analysis showed that all of the *MDR1*-activated strains exhibited specific changes in the content of sterols and fatty acids, important components of *C. albicans* cell membranes. Reductions of ergosterol content can enhance membrane fluidity, which may result in increased influx of drugs [40-41]. Thus, the altered cell membrane composition in the *MDR1*-activated strains may increase the permeability of the cell to RAB and RD.

We previously reported that RAB and RD modulate the ergosterol pathway and reducing ergosterol synthesis [12, 37, 42]. In the present study, we observed that the expression of sterol-related genes was lower in the *MDR1*-activated strains than in MG1004. The expression of ergosterol biosynthesis-related genes in the *MDR1*-activated strains was further down-regulated by RAB and RD; treatment with these agents interfered with the sterol biosynthesis pathway by decreasing ergosterol content and increasing lanosterol accumulation. These results suggest that RAB and RD disrupt the normal sterol biosynthesis pathway and aggregate the disruption of ergosterol biosynthesis in *MDR1*-activated strains, representing another factor conferring the selectivity of RAB and RD.

## Conclusion

In summary, five natural products displayed inhibitory activity against both azole-susceptible and azole-resistant strains. Among these agents, RAB and RD displayed selective inhibitory activity against *MDR1*-activated strains. The increased cell permeability and aggregated disruption of ergosterol biosynthesis contributed to the selective inhibitory activity of RAB and RD. These findings pave the way for developing alternative strategies against resistant pathogenic fungi.

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