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3,5-Dicaffeoylquinic Acid Disperses *Aspergillus Fumigatus* Biofilm and Enhances Fungicidal Efficacy of Voriconazole and Amphotericin B

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ACDE 1 **Jing Luo**
CD 1 **Ke Wang**
BDF 1 **Geng S. Li**
BF 2 **Dan Q. Lei**
B 2 **Yuan J. Huang**
B 2 **Wei D. Li**
AG 1 **Yi Q. Chen**
AEG 1 **Jin L. Kong**

1 Pulmonary and Critical Care Medicine Ward, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, P.R. China
2 Life Sciences institute of Guangxi Medical University, Nanning, Guangxi, P.R. China

Corresponding Authors: Yi Q. Chen, e-mail: chenyq2013@sohu.com, Jin L. Kong, e-mail: kjl071@126.com
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Background: The aim of this study was to evaluate the dispersal effects of 3,5-dicaffeoylquinic acid (3,5-DCQA) against the preformed biofilm of *Aspergillus fumigatus* and to investigate its potential mechanism.

Material/Methods: *Aspergillus fumigatus* biofilms of laboratory strain AF293 and clinical strain GXMU04 were generated in 24- or 96-well polystyrene microtiter plates *in vitro*. Crystal violet assay and XTT reduction assay were performed to evaluate the effects of 3,5-DCQA on biofilm biomass, extracellular matrix, and metabolic activity alteration of cells in biofilms. Real-time PCR was performed to quantify the expression of hydrophobin genes. The cytotoxicity of 3,5-DCQA on human erythrocytes was evaluated by a hemolytic assay.

Results: The results indicated that 3,5-DCQA in subminimum inhibitory concentrations (256 to 1024 mg/L) elicited optimal *A. fumigatus* biofilm dispersion activity and improved the efficacy of VRC and AMB in minimal fungicidal concentrations (MFCs) to combat fungal cells embedded in biofilms. The results of scanning electron microscope (SEM) and confocal laser scanning microscopy (CLSM) revealed 3,5-DCQA facilitated the entry of antifungal agents into the *A. fumigatus* biofilm through eliminating the hydrophobic extracellular matrix (ECM) without affecting fungal growth. Real-time PCR indicated that 3,5-DCQA down-regulated the expression of hydrophobin genes. Hemolytic assay confirmed that 3,5-DCQA exhibited a low cytotoxicity against human erythrocytes.

Conclusions: Subminimum inhibitory concentrations of 3,5-DCQA can disperse A. *fumigatus* biofilm and enhance fungicidal efficacy of VRC and AMB through down-regulating expression of the hydrophobin genes. The study indicated the anti-biofilm potential of 3,5-DCQA for the management of *A. fumigatus* biofilm-associated infection.

MeSH Keywords: **Amphotericin B • *Aspergillus fumigatus* • Biofilms**

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Background

Aspergillus fumigatus is one of the most ubiquitous filament-forming molds, with a worldwide distribution due to the production of conidia with a diameter of 2–3.5 μm [1]. Individuals exposed to airborne conidia of *A. fumigatus* may develop pulmonary diseases such as allergic bronchopulmonary aspergillosis, aspergilloma, and invasive pulmonary aspergillosis as a result of colonization and invasion of the respiratory airway [2]. *A. fumigatus* is now considered as the second most common cause of fungal infection in hospitalized patients after *Candida albicans* [3]. It is the most common species recovered from the respiratory tracts of cystic fibrosis patients in Europe, with a prevalence of up to 80% [4]. It is still a challenge to manage *A. fumigatus* infections as it has a high propensity to develop biofilms, which may be one of the major causes of the severe resistance to a wide variety of clinical antifungal agents such as amphotericin B (AMB) and voriconazole (VRC) [4,5]. Conventional treatment of fungal infectious disease is largely based on agents that kill or inhibit the growth of fungal cells. However, such an approach becomes ineffective against the sessile cells in biofilm. Thus, it is imperative to develop novel drugs that can disperse biofilm and increase penetration of drugs.

3,5-dicaffeoylquinic acid (3,5-DCQA, Figure 1), one of the polyphenolic compounds derived from *Lonicera japonica,* an herb with anti-inflammatory effects, has been commonly used for the management of upper respiratory tract infection in China. Many studies have revealed the pharmaceutical properties of dicaffeoylqunic acids, such as the inhibitory effects on HIV integrase activity [6] and hepatitis B virus replication [7], inhibition of platelet activation and endothelial cell injury [8], as well as the anti-oxidant activity [9]. In the present study, we evaluated the dispersal activity of 3,5-DCQA against the preformed biofilm of *A. fumigatus* and investigated the potential mechanism. The preformed biofilms of *A. fumigatus* were treated with sub-MICs 3,5-DCQA alone or in combination with AMB or VRC. Our results indicated that 3,5-DCQA attenuated the content of extracellular matrix (ECM) of the *A. fumigatus* biofilm, which glued together the hyphae in sessile state and contributed to the penetration of VRC and AMB. In addition, 3,5-DCQA down-regulated the expression of hydrophobin genes, resulting in decrease of mutual adhesion of the hyphae and detachment of biofilm. Our study proves that 3,5-DCQA can interfere with the biofilm of *A. fumigatus* and may be effective for the management of *A. fumigatus*-associated infections.

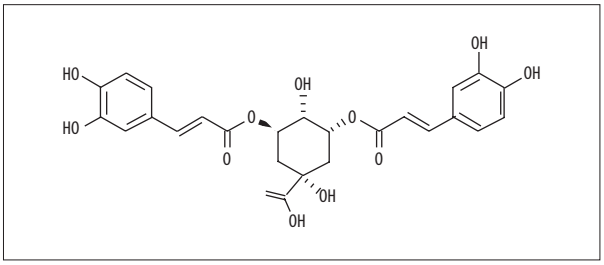

Figure 1. Chemical structure of 3,5-DCQA.

Material and Methods

Strains and agents

A clinical strain, designated as *A. fumigatus* GXMU04, was obtained from the First Affiliated Hospital of Guangxi Medical University (Nanning, China). The laboratory strain *A. fumigatus* AF293 (MYA-4609, CBS 101355) was purchased from the American Type Culture Collection (ATCC). Strains were stored in Sabouraud dextrose broth (Merck Co., Germany) with glycerol at –80°C. Quality control strain *Candida parapsilosis* ATCC22019 used for drug susceptibility testing was provided by the Clinical Microbiology Identification Center of Guangxi Medical University. The isolates were retrieved from the frozen stocks and subcultured on potato dextrose agar (Sigma-Aldrich, USA) slopes at 35°C for 3–5 days to ensure purity and viability. Conidia were harvested from the culture medium using 0.05% Tween 20 with sterile phosphate-buffered saline (PBS, Sigma-Aldrich, USA). Afterwards, the mixture was suspended in morpholinepropanesulfonic acid-buffered RPMI-1640 (pH 6.9–7.1, Sigma-Aldrich, USA) supplemented with L-glutamine, and 165 μM 3-(N-morpholino) propanesulfonic acid (Sigma-Aldrich, USA), followed by 60 s of vortexing. To prepare the inocula, conidial suspensions were adjusted under microscopic enumeration with a hemacytometer to achieve a final concentration of 1×10^5 conidia/ml. VRC (Pfizer Corporation, USA), AMB (Amresco, USA), and 3,5-DCQA standard dry powder purchased from the National Institutes for Food & Drug Control (Beijing, China) with a purity of ≥98% as analyzed by HPLC were freshly dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA). All these stock solutions were sterilized by passing through a 0.22-μm syringe filter (EMD Millipore Corporation, USA) and stored at -80°C until use.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MICs of all tested agents were determined by Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth microdilution method [10]. The final tested drug concentrations were 0.03~16 mg/L for AMB and VRC, and 2~1024 mg/L for 3,5-DCQA. For each *A. fumigatus* isolate, the conidial inoculum was diluted in RPMI 1640 medium until a final concentration of 1×10^4 conidia/ml. The endpoints of MIC were defined as the lowest drug concentrations that caused completely visible inhibition of fungal growth compared with that of the drug-free growth

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control. To determine the MFC, an aliquot (20 µL) of each well that without fungal growth was seeded on Sabouraud dextrose agar (SDA, Sigma-Aldrich, USA) at 37°C for 24~48 h. MFC is considered as the lowest drug concentration in a plate with SDA at which growth was less than 3 CFU. For each test, the experiments were performed at least in triplicate.

Biofilm formation

A. fumigatus biofilms were grown statically at 37°C on 96-well polystyrene microtiter plates (Corning, cat. no. 3599, USA) in aerobic conditions [1]. After 4 h of initial adhesion, the RPMI-1640 media were gently aspirated, and non-adherent cells were removed by washing lightly 3 times with sterile PBS. Afterwards, fresh RPMI-1640 was added and further incubated at 37°C for 24 h to get the preformed *A. fumigatus* biofilms. Upon the removal of non-adhered cells with a pipette, the biofilms were incubated at 37°C in RPMI-1640 supplemented with 3,5-DCQA (64 mg/L~1024 mg/L) or MFC of AMB or VRC, alone or as combinations of 3,5-DCQA with MFC of VRC or AMB for 48 h, and each treatment included 6 wells. Biofilms incubated with RPMI-1640 only served as drug-free controls. Biomass was determined by a crystal violet assay and the metabolic activity of cells in biofilms after drug interference was measured by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay.

Biofilm quantification and metabolic activity evaluation

Quantitation of biomass formation was performed using crystal violet assay as previously described [1]. In brief, after incubating with the tested drugs for 48 h, the medium was gently removed with a pipette and the plates were washed 3 times with sterile PBS to remove the loosely attached fungal cells. After fixation with glutaraldehyde for 20 min and air-drying, the biofilms were staining with 0.5% (wt/vol) crystal violet for 15 min, followed by rinsing with sterile PBS, and destained with 95% ethanol. The absorbance was measured at 590 nm using a spectrophotometer (Thermo Scientific, USA).

The metabolic activity of cells in biofilms after drug interference was measured by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay [1]. Briefly, the supernatant from each well was aspirated and the planktonic cells were removed. XTT (Sigma-Aldrich, USA) was prepared at a concentration of 500 mg/L in PBS, and was filter-sterilized through a 0.22-µm pore filter. Afterwards, a 100-µL aliquot of XTT was added to each well with prewashed biofilm, together with 10 µmol/mL of menadione solution (Sigma-Aldrich, USA) prepared in 100% acetone. Negative-control wells with the same treatment containing no conidia were used to measure the background XTT reduction levels. After incubating in the dark at 37°C for 3 h, the amount of XTT formazan was measured at 490 nm using a microplate reader (Thermo Scientific Multiskan MK3, USA).

Scanning electron microscope (SEM) examination

Initially, 1-mL aliquots of prepared inoculum (1×10^5 conidia/mL) were transferred to a 24-well plate with a coverslip (diameter 13 mm, Nest, China) on the bottom at 37°C for 4 h of adhesion. After aspiration of planktonic cells, the biofilm-coated coverslips were incubated with fresh RPMI1640 medium at 37°C for 24 h to allow biofilms formation. The preformed biofilms were treated with MFC of VRC, or MFC of AMB, or 3,5-DCQA (1024 mg/L), or 3,5-DCQA in combination with VRC or AMB for 48 h. After drug interference, the biofilm-coated coverslips were fixed by 2.5% glutaraldehyde and dehydrated by graded ethanol series (50%, 70%, 80%, 90%, and 100%). Finally, the samples were placed in a vacuum desiccator and coated by gold-sputtering, followed by observation using a SEM (Hitachi SU 8020, Japan) at 20 KV voltage.

Confocal laser scanning microscopy (CLSM) assay

To evaluate the cellular viability of the biofilms, the biofilm-coated coverslips as described above in SEM examination were stained with the LIVE/DEAD™ FungaLight™ Yeast Viability Kit (Invitrogen, USA) [containing 2 dye probes: Propidium iodide (PI) and Syto-9] according to the manufacturer's instructions and the previous description [11]. Images of the stained biofilms were captured using a CLSM system (Nikon A1, Japan) with a 488-nm argon laser, and analyzed using Nikon NIS-Element software (Nikon, Japan).

To observe the potential morphological changes of biofilms after tested agents challenge, we detected the exopolysaccharide (EPS), the major component of the ECM of *A. fumigatus* biofilms, as previously described by Jin et al. [11]. Biofilms treated with tested agents were gently washed 3 times with PBS. Afterwards, the biofilm-coated coverslips were transferred to the wells of a new 24-well plate containing a mixture of *Erythrina cristagalli* (ECA) labeled with fluorescein isothiocyanate (FITC) (Vector Laboratories, USA) and *Canavalia ensiformis A* (ConA) labeled with tetramethyl rhodamine isothiocyanate (TRITC, Molecular Probes, USA), according to the manufacturer's instructions. Images were captured using a CLSM system with a 543-nm argon laser. Three-dimensional reconstruction and thickness measurement were performed using Nikon NIS-Element software (Nikon, Japan). Fluorescent intensity of the images was quantified using Image Pro Plus software (version 6.0, Media Cybernetics, USA). All images were randomly collected at 10 different points from different areas on the surface of the biofilms per sample. The test was performed at least in triplicate.

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Table 1. The MICs and MFCs of the tested agents against *A. fumigatus*.

Tested agents	*A. fumigatus* GXMU04		*A. fumigatus* AF293	
	MIC	MFC	MIC	MFC
VRC	1	32	0.5	2
AMB	2	8	0.25	1
3,5-DCQA	>1024	>1024	>1024	>1024

Real-time PCR assay

Real-time PCR was performed to determine the expression of 6 hydrophobin genes encoding the hydrophobin proteins closely involved in the cellular surface hydrophobicity of hyphae cells [12]. Total RNA was extracted from the fungal tissues using the Fungal RNAout kit (Sangon Biotech, Shanghai, China). First-strand cDNA was synthesized with ReverAid First-Strand cDNA Synthesis Kit (Thermo scientific, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green II (TaKaRa, China) on an ABI 7500 real-time PCR system (Applied Biosystems, UAS) using the specific primers listed in Supplementary Table 1. The amplification of each gene was normalized to that of the 18S rRNA gene levels. The amplification results for real-time PCR was calculated as $2^{(-\Delta\Delta Ct)}$ according to a previous description [13].

Cytotoxic evaluation by hemolysis assay

Cytotoxicity of 3,5-DCQA was evaluated by the red blood cell (RBC) lysis assay [14]. Briefly, RBCs freshly obtained from the healthy individuals were washed 3 times with PBS. The supernatant was removed after centrifugation at 2000 rpm at room temperature for 10 min. The RBC precipitation was prepared in PBS to obtain a 10% RBC suspension. After that, a 1-mL suspension was added into an Eppendorf tube, with 200 µL 3,5-DCQA solutions of different concentrations, or 3,5-DCQA mixed with MFC of VRC or AMB. Subsequently, the mixture was incubated at 37°C for 1 h. References to 100% and 0% hemolysis were made by incubating a 1-mL suspension of red cells with 200 µL Triton X-100 1% (v/v) or 200 µL of sterile PBS, respectively. Subsequently, the tubes were centrifuged at 2000 rpm for 10 min to settle the broken membranes and unbroken RBCs. The liberated hemoglobin in the supernatant was determined using a spectrophotometer at a wavelength of 540 nm. Percentage of hemolysis was calculated according to the formula: Hemolysis=(Absorbance of sample/Absorbance of positive control)×100%. Percentages over 10% were consider positive for hemolysis [15]. The study protocols were approved by the Ethics Committee of Guangxi Medical University (Nanning, China), and the written informed consent was obtained from each blood sample donor.

Statistical analysis

Measurement data are presented as mean ± standard error. Statistical analysis was performed with one-way analysis of variance followed by Dunnett's test. All the data were obtained from 3 independent experiments performed at least in triplicate. $P<0.05$ demonstrated a statistically significant difference.

Results

Antifungal susceptibility results

The MICs and MFCs of the tested agents are shown in Table 1. The MIC and MFC of 3,5-DCQA for *A. fumigatus* GXMU04 and *A. fumigatus* AF293 were more than 1024 mg/L. This revealed that 3,5-DCQA had no direct inhibitory and fungicidal effects on planktonic cells of both strains of *A. fumigatus* at sub-MIC concentrations. In addition, the MIC and MFC of VRC or AMB against the *A. fumigatus* GXMU04 were remarkably higher than those of the *A. fumigatus* AF293.

3,5-DCQA detached the preformed biofilms of *A. fumigatus* and enhanced the fungicidal effects of VRC and AMB on sessile cells in biofilms

After incubating with tested agents, the biomass alterations of preformed biofilms were evaluated by crystal violet assay (Figure 2). The potential of biofilm dispersal induced by MFC of VRC or AMB for *A. fumigatus* GXMU04 and *A. fumigatus* AF293 biofilms showed no significant difference compared with that of the planktonic cells. However, 3,5-DCQA induced reduction of biomass in a dose-dependent manner in both strains. Particularly, the biomass was significantly lower in the presence of 1024 mg/L 3,5-DCQA in both strains compared with the control groups ($P<0.05$). This indicated that 3,5-DCQA (256~1024 mg/L) could eradicate the preformed biofilm of *A. fumigatus*.

The effects of 3,5-DCQA in combination with antifungal agents were estimated by XTT reduction assay. MFC of VRC or AMB showed no effects on the metabolic activity of sessile cells embedded in ECM (Figure 3), even though outstanding fungicidal effects were observed on planktonic cells. Unexpectedly,

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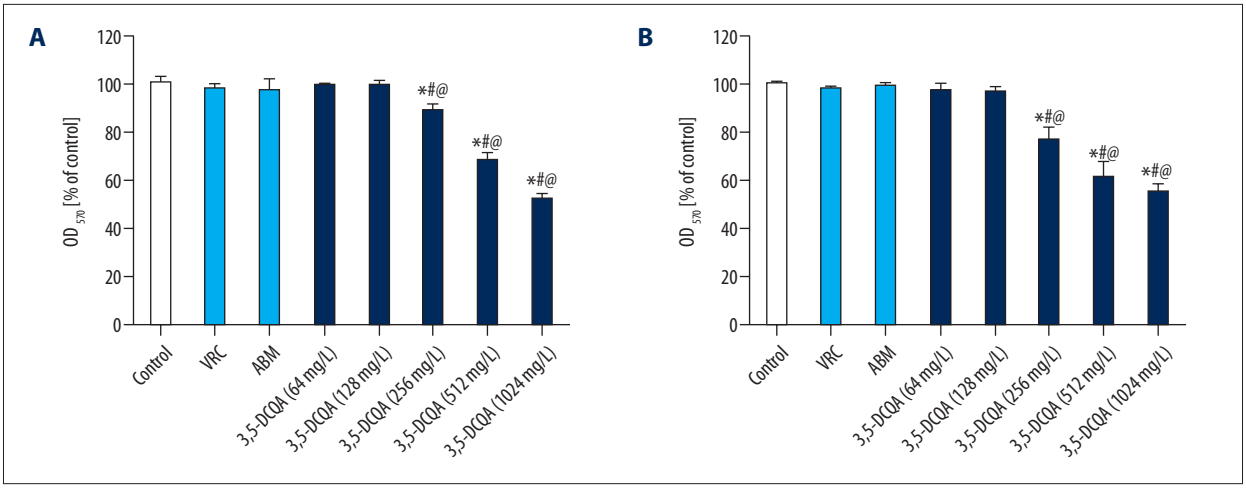

Figure 2. Biomass alterations of *A. fumigatus* GXMU04 (**A**) and *A. fumigatus* AF293 (**B**) using crystal violet assay. Error bars represent standard error. * *P*<0.05 *vs.* drug-free control group; # *P*<0.05 *vs.* the VRC group; @ *P*<0.05 *vs.* the AMB group. VRC – MFC of Voriconazole; AMB – MFC of amphotericin B.

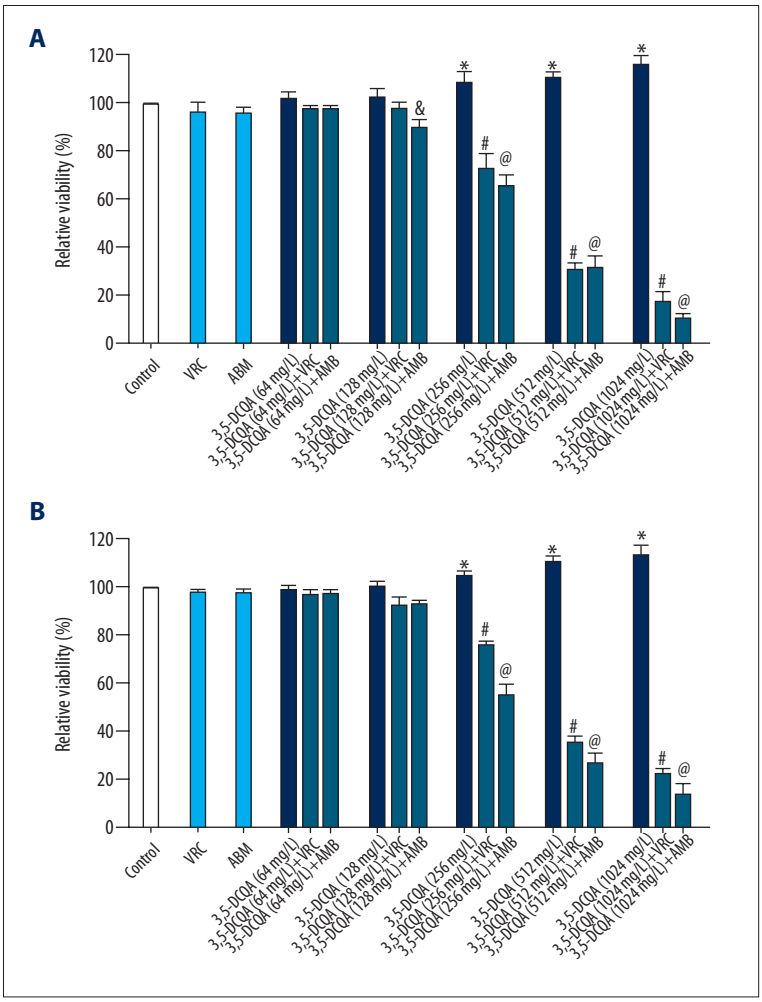

Figure 3. Metabolic activity alteration of cells in *A. fumigatus* GXMU04 (**A**) and *A. fumigatus* AF293 (**B**) of preformed biofilms after exposing to the tested agents evaluated by XTT reduction assay. Error bars represent standard error. * *P*<0.05 *vs.* drug-free control group; # *P*<0.05 *vs.* the VRC group; & *P*<0.05 *vs.* the AMB group; @ *P*<0.05 *vs.* the AMB group. VRC – MFC of Voriconazole; AMB – MFC of amphotericin B.

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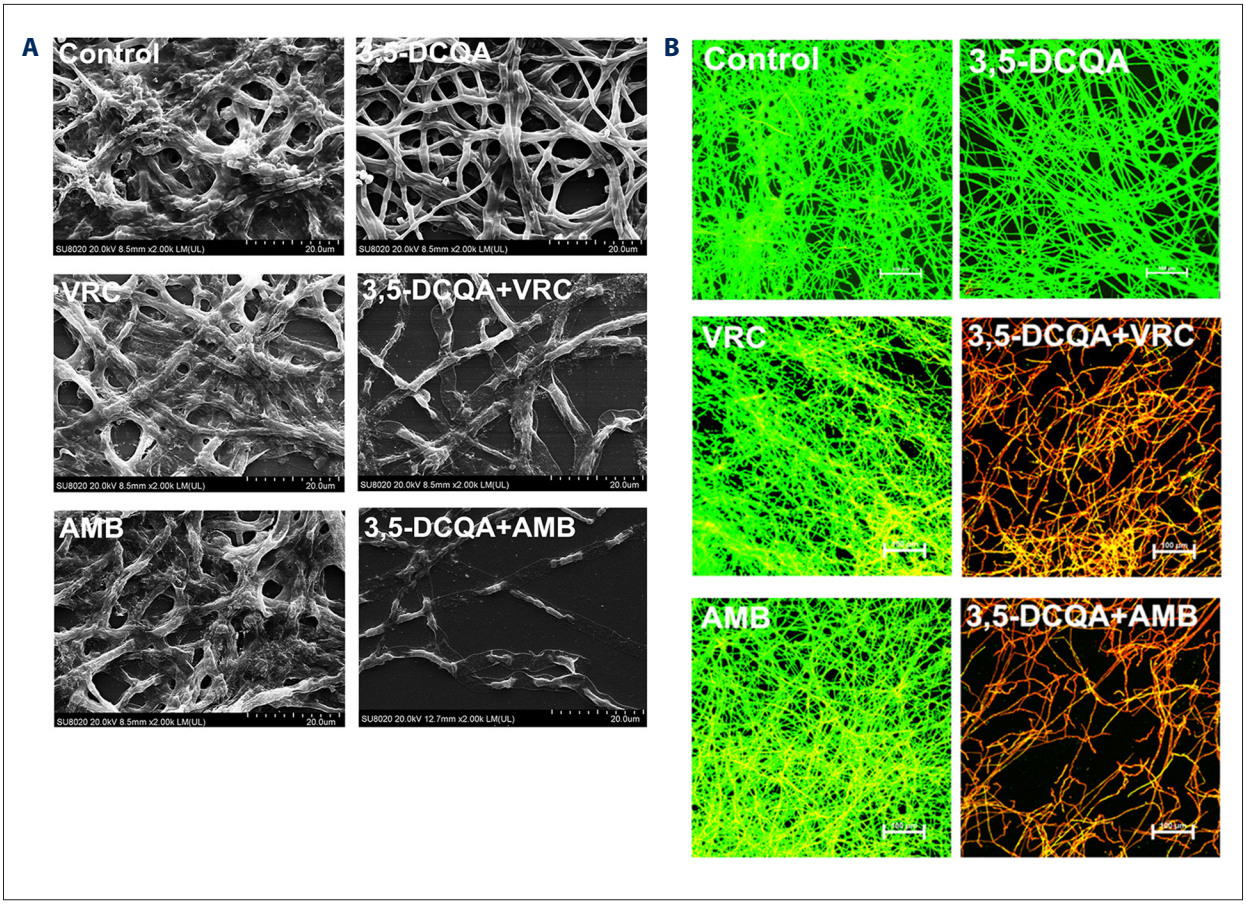

Figure 4. SEM (**A**) images and CLSM (**B**) view of preformed *A. fumigatus* GXMU04 biofilms stained with the combination of PI and Syto-9 in control group, MFC of VRC or AMB, 3,5-DCQA (1024 mg/L), and 3,5-DCQA in combination with VRC or AMB.

the cellular viability was significantly increased in the biofilms treated with 3,5-DCQA alone, particularly of the sub-MICs (256~1024 mg/L). Nevertheless, attenuation of viability was observed in the cells exposed to higher concentrations of 3,5-DCQA (256~1024 mg/L) in combination with VRC. Similarly, the cellular viability was decreased in the biofilm exposed to 3,5-DCQA (128~1024mg/L for *A. fumigatus* GXMU04 and 256~1024mg/L for *A. fumigatus* AF293) in combination with AMB. These results indicated that 3,5-DCQA contributed to the antifungal effects of VRC or AMB.

Three-dimensional (3D) structural analysis by SEM (Figure 4A) indicated the biofilm was composed of a highly dense network of cross-linked, parallel-packed hyphae, and the visual field was occupied by a large amount of sticky and cloudy ECM. Compared with the thick biofilm with hyphae crisscrossing in the control samples, the thickness of the biofilm or hyphae in the ECM was not affected by MFC of VRC or AMB. In contrast, *A. fumigatus* biofilm was disrupted by 3,5-DCQA and the outline of hyphae was exposed due to the reduction of mucoid-like ECM; however, cell density was not remarkably affected. After exposure to 3,5-DCQA in combination with VRC or AMB, the amount of hyphae and ECM was obviously decreased, together with rupture and disintegration of the cell wall, which finally resulted in leakage of cytoplasm.

In the CLSM assay, viable cells with intact membranes were stained green, whereas those with damaged membranes were stained red (Figure 4B). Similar with that of the control group, green fluorescence was obviously detected in biofilms treated with 3,5-DCQA, MFC of VRC or AMB, respectively. For the biofilms treated by VRC or AMB, some hyphae in the superficial surface were stained red, which finally resulted in formation of yellow zones with the 2 colors merged together on the images. Nevertheless, hyphae of biofilm were mostly stained with red fluorescence in the presence of 3,5-DCQA in combination with VRC or AMB.

3,5-DCQA attenuated the production of EPS

As seen in Figure 5, sessile cells were encased in a diffuse mass of EPS visualized in a green (ECA positive) color or acquired a yellow stain (positive with both ECA and ConA). Compared with the control group, biofilms treated by VRC or AMB alone

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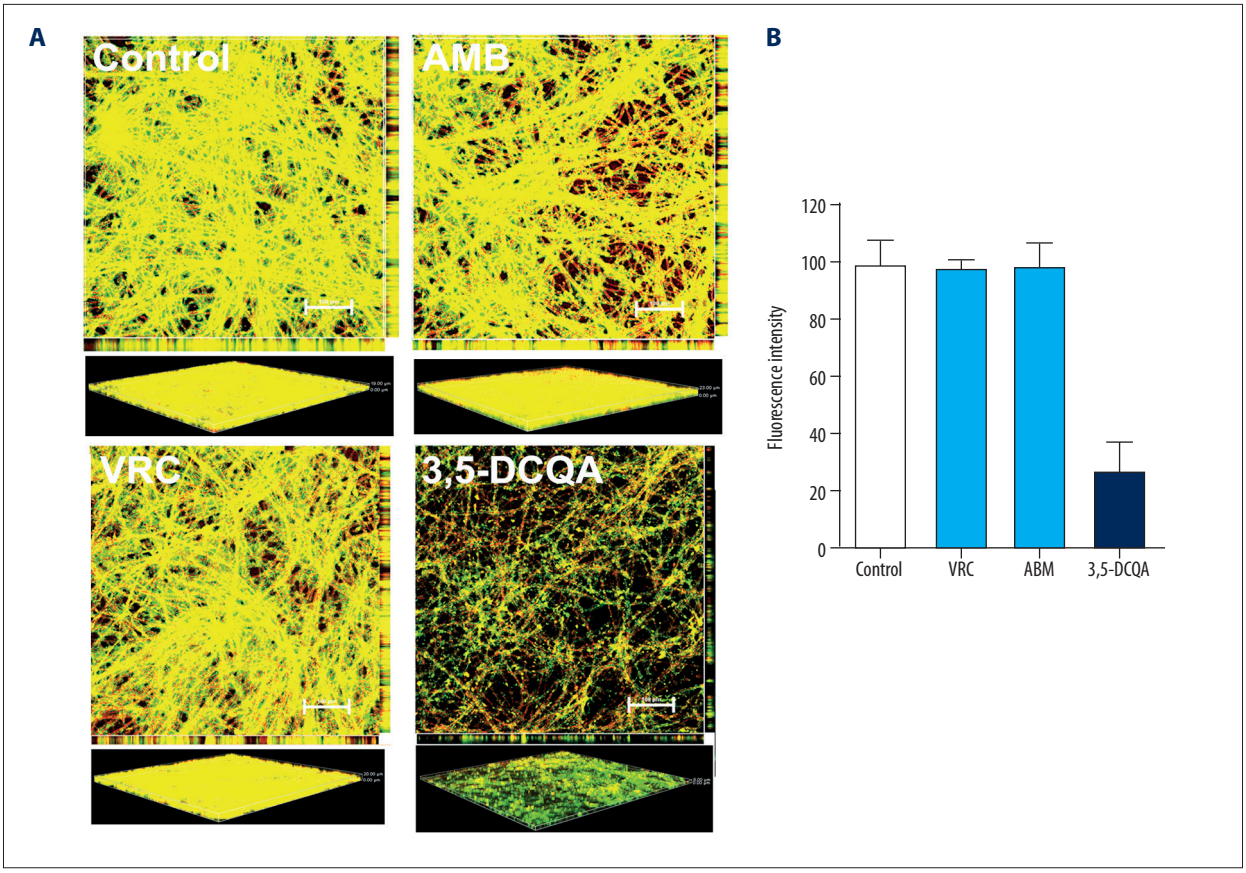

Fiugre 5. (**A**) CLSM Images of preformed A. fumigatus GXMU04 biofilm stained with the combination of ECA and ConA in control group, VRC or AMB alone of MFC, 3,5-DCQA (1024 mg/L), and 3,5-DCQA in combination with VRC or AMB. Scale bars indicates 100 μm. Three-dimensional reconstructions were shown in the bottom views. (**B**) Fluorescence intensity of CLSM images in each group were also quantified by Image Pro Plus software.

Table 2. The thickness of the 3D reconstruction of *A. fumigatus* GXMU04 biofilms under CLSM.

Group	Control	VRC	AMB	3,5-DCQA
Thickness (μm)	20.0±1.5	19.8±1.7	19.5±2.1	8.5±1.0*

* $P<0.05$ *vs.* drug-free control group.

caused no difference of EPS production and thickness of the biofilm (Table 2). On the contrary, a sharp decrease of ECM and biofilm thickness was observed in preformed biofilm treated with 3,5-DCQA.

3,5-DCQA altered *A. fumigatus* hydrophobin gene expression

To investigate the biofilm dispersal mechanism of 3,5-DCQA, the expression of 6 known hydrophobin genes (*RODA*, *RODB*, *RODC*, *RODD*, *RODE*, and *RODF*) was determined using real-time RT-PCR. These genes had been proved to be overexpressed in sessile biofilm cells compared with their planktonic states, and the expression level of RODA was the most obvious (Supplementary Figure 1). All of the 6 target genes in the biofilms were significantly down-regulated after incubating with 1024 mg/L 3,5-DCQA. Moreover, the inhibition of the gene that encoded *RODA* hydrophobin was the most obvious in both strains. However, the expression of these genes showed no significant difference in the biofilms treated by VRC or AMB compared with the drug-free control (Figure 6).

3,5-DCQA showed extremely low toxicity to blood cells

The mechanical stability of the erythrocyte membrane is a good indicator for screening cytotoxicity. In our study, human erythrocytes incubated with 3,5-DCQA showed no hemolytic activity at 1024 mg/L, the highest sub-MIC concentration used

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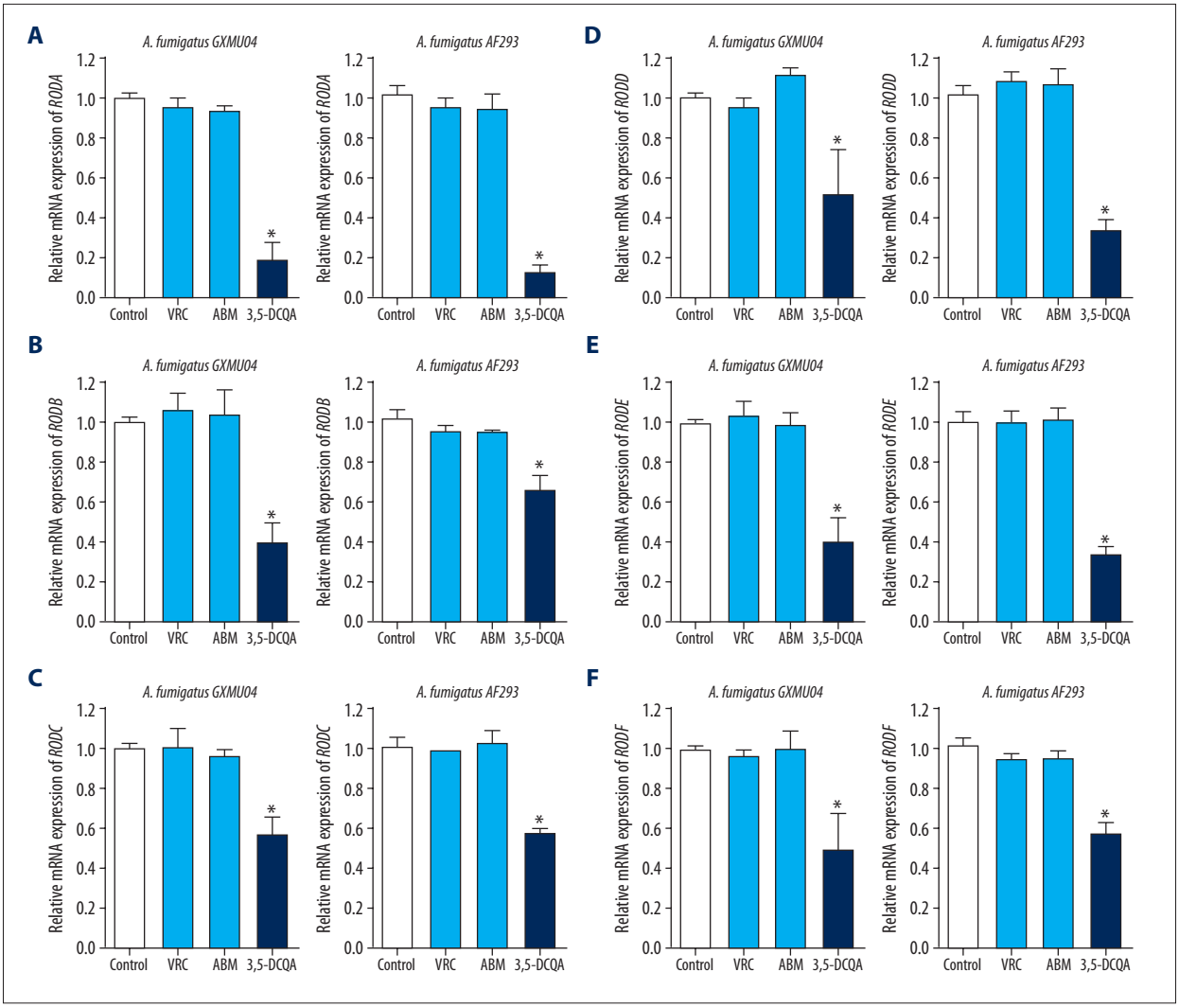

Figure 6. (**A–F**) Expression of hydrophobin genes (*RODA-F*) in the biofilms of *A. fumigatus* GXMU04 and *A. fumigatus* AF293 after exposing to MFC of AMB or VRC, and 3,5-DCQA at 1024 mg/L. The 18srRNA gene served as the reference gene. * $P<0.05$ *vs.* the drug-free control group.

Table 3. Percentage of hemolysis in the presence of different concentrations of 3,5-DCQA or in combination with VRC or AMB.

Drugs	3,5-DCQA					
	0 mg/L	64 mg/L	128 mg/L	256 mg/L	512 mg/L	1024 mg/L
0 mg/L	–	5.24±1.55	5.96±2.11	5.71±0.48	7.47±1.35	7.79±1.01
VRC (32 mg/L)	3.55±1.93	4.14±2.06	3.96±0.86	5.90±0.68	5.08±2.84	8.06±0.97
AMB (8 mg/L)	2.92±0.74	5.19±1.56	6.37±1.37	5.27±1.58	6.58±0.55	7.66±0.94

in this study. In addition, no hemolytic effect was observed in human erythrocytes incubated with 3,5-DCQA at 1024 mg/L in combination with MFC of VRC or AMB, respectively (Table 3).

Discussion

A. fumigatus sessile cells are more resistant to antifungal agents compared with the planktonic ones. This is confirmed by the fact that the formation of biofilms contributes to the development of persistent, chronic, and recurrent infections in clinical

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settings [2]. The structure of biofilm is complex, with ECM being considered as the dominant part of *A. fumigatus* biofilms and acting as a cohesive linkage bonding hyphae into a contiguous sheath [16]. Thus, the most appropriate antifungal drugs for the management of *A. fumigatus* biofilm-associated clinical infection may be those with effective fungicidal property and those with strong capacity to penetrate the ECM. Our results showed 3,5-DCQA could eradicate the hydrophobic ECM of biofilm through breaking down the hydrophobic interaction and modulating the adhesion ability of cells in biofilm, which may lead to an increase of ECM penetration of antifungal agents.

This is the first report indicating that sub-MIC of 3,5-DCQA has a significant anti-biofilm effect against *A. fumigatus*. In this study, clinically isolated *A. fumigatus* GXMU04 was selected as the tested strain because our preliminary study confirmed that *A. fumigatus* GXMU04 showed the strongest and the most stable abilities to form biofilms. According to the biomass quantitation assay, the biofilm-forming ability of *A. fumigatus* GXMU04 was superior to that of *A. fumigatus* AF293. Our results also indicated that MFC of VRC and AMB had no effects on the preformed biomass and metabolic activity of cells in biofilms of both strains. However, outstanding fungicidal effects were observed on planktonic cells exposed to these agents. This is consistent with a previous study [1], in which the biofilm-forming fungi showed an up to 1000-fold resistance to antifungal agents compared to planktonic free-floating cells. This level will often exceed the highest deliverable doses, which makes efficient treatment impossible. As killing or inhibiting the growth of fungal cells is less effective, the use of anti-biofilm agents to modulate the resistance and enhance the efficacy of fungicidal agents becomes attractive. Although 3,5-DCQA showed no fungicidal effects on planktonic cells, it induced the reduction of total biomass of biofilms in a dose-dependent manner, especially a decrease of about 50% in the presence of 1024 mg/L 3,5-DCQA. Unlike the previous study, in which the metabolic activity was decreased in fungal cells embedded in the thick ECM of mature biofilms [17], our results revealed an increase of metabolic activity of biofilms cells after exposure to 3,5-DCQA in contrast to the reduction of biomass quantitation at the concentration range from 256 mg/L to 1024 mg/L. We speculate that the increase of the metabolic activity induced by 3,5-DCQA may be associated with the dispersal of the biofilm and detachment of the sessile cells into planktonic cells. To further confirm this speculation, 3,5-DCQA of serially increasing concentrations were tested in combination with MFC of VRC or AMB against *A. fumigatus* mature biofilms. A synergic effect was observed between 3,5-DCQA and VRC or AMB on killing the cells embedded in the ECM as revealed by the percentage of metabolic activity alteration measured by XTT reduction assay. Similarly, in a previous study [18], a decrease of the metabolic activity in *A. fumigatus* biofilms was observed in cells exposed to alginate lyase-antifungal combinations compared with those exposed to the antifungals alone. These results support the assumptions that 3,5-DCQA may disperse *A. fumigatus* biofilms through reducing the ECM production, and facilitating the entry of fungicidal drugs into the biofilms.

Our SEM results were similar to the quantitation results. The ECM in a sticky and cloudy pattern closely coating the hyphae cells was markedly reduced after the preformed biofilms were treated with 3,5-DCQA, and the outlines of hyphae were distinct. No obvious changes were observed in the morphologies of preformed biofilms incubated with VRC or AMB alone. Nevertheless, a remarkable decrease was noticed in the density of cells and thickness of ECM in biofilms treated with 3,5-DCQA in combination with VRC or AMB, together with the presence of shrivelled and disintegrated hyphae. Similarly, CLSM images revealed no difference in the cellular viability after treating with VRC and AMB alone, which is probably due to ECM protection. The hyphae remained viable even in the presence of 3,5-DCQA, which resulted in the eradication the ECM of biofilm. Nevertheless, 3,5-DCQA in combination with VRC or AMB showed a fungicidal effect and eradicated the formation of ECM as verified by the sharp decrease of hyphae density and viability. These findings suggest that the loss of ECM, following 3,5-DCQA treatment, could better enhance the penetration of antifungal agent through the ECM. Therefore, 3,5-DCQA in combination with antifungal agents may be an effective strategy to improve the management of *A. fumigatus* biofilm-associated infections.

To confirm the effects of 3,5-DCQA on ECM of *A. fumigatus* biofilm, the biofilms were stained with TRITC-labeled ConA and FITC-labeled ECA to visualize any possible alteration of ECM. CLSM reconstruction and the fluorescence-based quantitation assay indicated no alternation in the ECM in the biofilms exposed to VRC or AMB due to their limited capacity to eliminate the ECM and penetrate into the deeper layer of the biofilms. Nevertheless, significant changes were observed in the ECM of the biofilms treated with 3,5-DCQA.

Hyphae are wrapped by the hydrophobic ECM of *A. fumigatus* biofilms, and the hydrophobic interactions are generally considered to play an important role in the adherence of *A. fumigatus* hyphae [2]. Hydrophobins, one of the major components of *A. fumigatus* ECM, are responsible for conferring a hydrophobic character to fungal morphotypes due to the highly conserved cysteine bridges [19]. Several hydrophobin genes (e.g., *RODA*, *RODB,* and *RODD)* are up-regulated in *A. fumigatus* sessile cells compared to the planktonic cells, implying a possible role in biofilm formation. Thus, it is reasonable to speculate that the biofilm dispersal effect of 3,5-DCQA is associated with its anti-hydrophobic interaction and modulation of the adhesion ability. In the present study, RT-qPCR

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was performed to determine the expression of the 6 hydrophobin genes (*RODA-F*). The results revealed that 3,5-DCQA induced down-regulation of these genes, then contributed to the reduction of hydrophobins synthesis. Therefore, the loss of hydrophobicity may result in the reduction of hyphal adhesion and hydrophobic ECM production in *A. fumigatus* biofilms. It seems it is possible for antifungal agents to facilitate the entry of fungicidal drugs into the deeper layers of the biofilm and effectively inactivate the sessile cells.

Given the biofilm dispersal activity of the 3,5-DCQA, it can be used as an agent for managing biofilm-related diseases, or as a sensitizer for antifungal agents. In this study, the cytotoxicity was determined using *in vitro* experiments. Our data revealed that 3,5-DCQA showed no hemolytic effects on human erythrocytes, even in combination with VRC or AMB. 3,5-DCQA is commonly found in vegetables, tea, and coffee, and in traditional medical infusions or decoctions, with no described toxic properties [20]. These prove the safety of 3,5-DCQA as an antifungal agent.

Conclusions

3,5-DCQA is effective against adaptive resistance to antifungal agents of *A. fumigatus* biofilms. This is proved by the fact that 3,5-DCQA in combination with MFC of VRC or AMB could disperse the biofilms, but single administration of VRC or AMB could not. CLSM and SEM indicated that 3,5-DCQA facilitated the entry of VRC or AMB into the *A. fumigatus* biofilm through eliminating the ECM without affecting fungal growth. 3,5-DCQA resulted in a decrease of cellular surface hydrophobicity and down-regulation the expression of hydrophobin genes. The concentration of 3,5-DCQA (256~1024 mg/L) used for disrupting the *A. fumigatus* biofilm in the current study is a bit higher than the conventional dose of antifungal agents; however, the *in vitro* results do not entirely reflect the practical situation for the action of 3,5-DCQA *in vivo*, as the immunological factors of host, pharmacokinetics-pharmacodynamics of drugs, and many other complicated factors may be involved in this process. Further *in vivo* studies are needed to discover the therapeutic roles of 3,5-DCQA against biofilm-associated infection. The anti-biofilm potential of 3,5-DCQA, as the present study indicates, may be effectively used for dealing with the *A. fumigatus* biofilm formed on the surface of medical implants such tracheal, central vein, and ureter catheters. Moreover, 3,5-DCQA can be used as a drug for external use for treating of local or superficial chronic infection caused by *A. fumigatus* biofilm.

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Conflicts of interest

None.

Supplementary Files

Supplementary Table 1. List of primers used in this study.

Target genes	Primer sequence
18s rRNA	Forward: CTTAAATAGCCCGGTCCGCATT Reverse: CATCACAGACCTGTTATTGCCG
RODA	Forward: AACCAGAAGTGCAAGCAGAACA Reverse: GCA AGG AAGACCCAGTCCAA
RODB	Forward: GACACCACCGCCTTCAACTAC Reverse: AGCCAGAGAGGATACCAACACC
RODC	Forward: TCCCTCCCTCCAGGTCATT Reverse: TGACGACCCGATTACAGCAC
RODD	Forward: TCCCTCTTATGCCTTCTCTCGT Reverse: GATTTCGTTCATTCCTCTCCTTTC
RODE	Forward: ATGCCGATGCCTTGAACC Reverse: CGTTAGCAAAAGACACGCAAA A
RODF	Forward: CATTCTCCCAGGCATCCAAA Reverse: CTGAGTGTTGGCGAGGGTAGA

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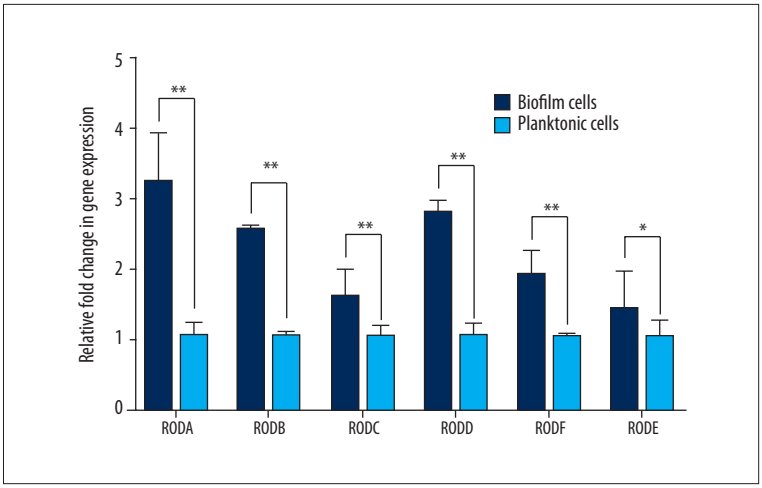

Supplemenatry Figure 1. The expression level of hydrophobin genes (*RODA-F*) in the biofilms compared with that in the planktonic cells of *A. fumigatus* GXMU04. * $P<0.05$ *vs.* the planktonic cells.

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