

In vitro activity of chlorogenic acid against *Aspergillus fumigatus* biofilm and gliotoxin production

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Abstract. *Aspergillus (A.) fumigatus*, one of the most common causes of life-threatening fungal infections in immunocompromised patients, shows resistance to antifungal agents as has a high propensity to forming a biofilm. The present study aimed to investigate the effects of chlorogenic acid (CRA) on *A. fumigatus* biofilm formation and integrity. Confocal laser scanning microscopy was performed to determine the inhibitory effects of CRA against *A. fumigatus* biofilm formation. Transmission electron microscopy was performed to investigate the ultrastructural changes of *A. fumigatus* biofilm after CRA exposure. High-performance liquid chromatography and reverse-transcription quantitative PCR were performed to determine the expression of gliotoxin production in biofilm culture. The results showed that CRA at sub-minimum inhibitory concentrations inhibited *A. fumigatus* biofilm formation. In addition, CRA could decreased the gliotoxin production in the biofilm culture supernatant through inhibiting the expression of master genes involved in gliotoxin biosynthesis. The present study provided useful information for the development of novel strategies to reduce the incidence of *A. fumigatus* biofilm-associated diseases.

Introduction

Aspergillus (A.) fumigatus, a saprophytic fungus commonly distributed in soil and compost piles, is one of the major causes for the life-threatening fungal infections in immunocompromised patients (1), particularly those with human immunodeficiency infection or hematonosis, and those

undergoing organ transplantation (2,3). It is now well acknowledged as an organism growing as a multicellular community, in which the hyphae are bonded together by a hydrophobic extracellular matrix (ECM) (4).

Increasing evidence has revealed that biofilm formation-associated drug resistance is closely linked to the poor outcome of severe invasive pulmonary aspergillosis and allergic bronchopulmonary aspergillosis. To date, the management of *A. fumigatus* biofilm-associated infections has remained a challenge worldwide, as the biofilms are resistant to a variety of clinical antifungal agents, including amphotericin B (AMB), voriconazole (VRC) and caspofungin (CAS) (4-6). Compared with planktonic cells, biofilm cells have unique phenotypic traits, particularly the notorious resistance to antifungal agents and the host's immune defense. Therefore, it is required to identify novel strategies for the management of *A. fumigatus*-associated infections through dispersing the biofilm. However, the major components and molecular mechanisms underlying biofilm formation in *A. fumigatus*-mediated invasive disease have remained elusive. Studies have reported that gliotoxin, one of the most well-known secondary metabolites of *A. fumigatus*, has important roles not only in fungal pathogenesis and the host's immune response, but also in modulating *A. fumigatus* biofilm formation (7,8), which may represent an approach for developing novel methods to disperse the biofilm of *A. fumigatus*.

The present study, showed that chlorogenic acid (CRA; Fig. 1), a major component derived from *Lonicera japonica* commonly used in the Chinese Pharmacopoeia, decreased gliotoxin production through inhibiting the expression of master genes involved in gliotoxin biosynthesis.

Materials and methods

Fungal strains, culture and agents. The *A. fumigatus* strain GXMU04 was obtained from the Clinical Microbial Identification Center of the First Affiliated Hospital of Guangxi Medical University (Nanning, China). The strains were isolated from a patient with pulmonary tuberculosis concurrent with invasive pulmonary aspergillosis. The *A. fumigatus* strain AF293 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). *Candida parapsilosis* ATCC22019 (ATCC) was used as a quality control strain. All strains were stored in Sabouraud dextrose broth (Sigma-Aldrich; Merck

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Millipore, Darmstadt, Germany) supplemented with glycerol at -80°C prior to usage.

Conidia were retrieved from frozen stocks and subcultured on potato dextrose agar slopes at 35°C for 3–5 days to ensure purity and viability. Subsequently, the isolates were harvested using 0.05% Tween-20 with sterile phosphate-buffered saline (PBS; Sigma-Aldrich, Merck Millipore). The mixture was then suspended in RPMI-1640 (Sigma-Aldrich, Merck Millipore) supplemented with L-glutamine and $165\ \mu\text{M}$ 3-(*N*-morpholino) propanesulfonic acid (Sigma-Aldrich, Merck Millipore), followed by vortexing for 30 sec. Prior to use, conidial suspensions were adjusted under microscopic enumeration with a cell counting hemocytometer to a final concentration of 1×10^5 conidia/ml to prepare the inocula.

CRA standard dry powder was synthesized by the National Institutes for Food & Drug Control (Beijing, China) with a purity of $\geq 98\%$ based on high-performance liquid chromatography (HPLC). AMB was from Sigma-Aldrich (Merck Millipore). All of the tested agents were freshly dissolved in dimethyl sulfoxide (Sigma-Aldrich, Merck Millipore) and sterilized by passing through a syringe filter ($0.45\ \mu\text{m}$ pore size; 25 mm diameter, Merck Millipore).

Antifungal susceptibility test. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the tested agents (CRA and AMB) were determined by using a broth microdilution method following the Clinical and Laboratory Standards Institute M38-A2 guidelines (9). In brief, the initial concentration of conidial suspensions in RPMI-1640 medium was 5×10^3 colony-forming units (CFU)/ml. The final concentrations were $2\text{--}1,024\ \mu\text{g/ml}$ for CRA and $0.03\text{--}16\ \mu\text{g/ml}$ for AMB. The MIC was considered to be the lowest concentration at which no gross fungal growth appeared. The MFC was determined by transferring an aliquot ($20\ \mu\text{l}$) of each sample treated with concentrations higher than the MIC onto an antifungal agent-free Sabouraud-dextrose agar plate, followed by incubation at 35°C for 48 h. MFC was defined as the lowest concentration of test agent at which fungal growth was less than three CFU.

Biofilm formation assay. The effects of CRA against the formation of *A. fumigatus* biofilm were determined according to a previously described method (6). In brief, a suspension of *A. fumigatus* (1×10^5 conidia/ml) was grown statically at 37°C on 96-well polystyrene microtiter plates (Corning, Inc., Corning, NY, USA) under aerobic conditions. After incubation for 4 h for initial adhesion, the media was gently aspirated and the non-adherent cells were removed followed by addition of fresh RPMI-1640 to the adherent cells. Subsequently, the plates were incubated with CRA ($64\text{--}1,024\ \mu\text{g/ml}$) at 37°C for 24 h.

To determine the effects of CRA on the integrity of mature biofilms, *A. fumigatus* biofilms were cultured at 37°C for 24 h. The culture supernatant was discarded, replaced with RPMI-1640 media supplemented with CRA ($64, 128, 256, 512$ or $1,024\ \mu\text{g/ml}$) and incubated at 37°C for an additional 24 h.

A semi-quantitative measurement of biofilm formation was performed by using a crystal violet assay (6). Twenty-four hours after treatment, the supernatant was aspirated from each well and the non-adherent cells were removed by three washes with sterile PBS. The plates were air-dried and the biofilms

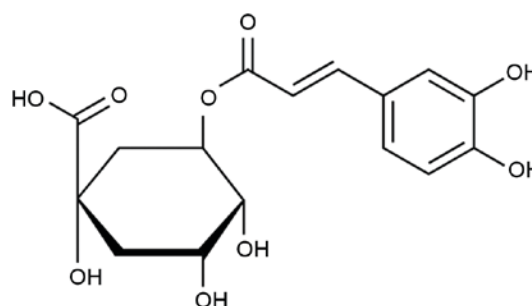


Figure 1. Chemical structure of chlorogenic acid. Molecular weight: 354.31 g/mol.

were stained with $100\ \mu\text{l}$ 0.5% (w/v) crystal violet solution for 15 min. Excess stain was removed by rinsing the biofilms using running water. Subsequently, $100\ \mu\text{l}$ 95% ethanol was added to each well to dissolve the stain attached to the fungus. Finally, the absorbance value was measured at 590 nm using a spectrophotometer (Multiskan MK3, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Biofilm biomass was measured as described by Li *et al.* (10). Glass coverslips (diameter, 13 mm; Nest Biotechnology Co., Wuxi, China) were pre-treated with fetal bovine serum (Sigma-Aldrich; Merck Millipore) overnight and washed with sterile PBS before inoculation. *A. fumigatus* suspension (1×10^5 conidia) was added to a sterile 24-well plate (Corning, Inc.) with one prepared coverslip in each well. After adhesion, non-adherent cells were removed by gently washing the coverslip with sterile PBS. Subsequently, the coverslips were transferred to a fresh 24-well plate containing 1 ml RPMI-1640 medium. For the CRA treatment group, CRA was added to the medium. The plate was incubated at 37°C for an additional 24 h to allow for the biofilm to form. The coverslips with attached biofilms were removed from the wells and dried overnight at 70°C . The total biomass of each biofilm sample was calculated by deducting the net weight of the coverslip.

Confocal laser scanning microscopy (CLSM) assay. To determine the inhibitory effects of CRA against *A. fumigatus* biofilm formation, CLSM samples were prepared as described in the biomass evaluation section. The coverslips of the CRA and control groups were transferred to a fresh 24-well plate after 24 h of cultivation. The samples were incubated in the dark for 90 min with Canavalin A (ConA; $250\ \mu\text{g/ml}$; Molecular Probes, Eugene, OR) and Erythrina crista-galli (ECA; $200\ \mu\text{g/ml}$; Vector Laboratories, Burlingame, CA, USA). ConA shows marked affinity for mannose and glucose residues in the ECM and emits red fluorescence. The excitation wavelength was set at 543 nm and the filter set LP560 was used to capture the signal from tetramethyl rhodamine isothiocyanate. ECA specifically binds to galactose and galactoside residues in the ECM on cell surfaces or between fungal cells and emits green fluorescence (11). The excitation wavelength was 488 nm and the emitting light was collected by the filter set BP515-530 to capture the signal of fluorescein isothiocyanate. After incubation, the coverslips were gently rinsed with PBS and biofilms were observed using a Nikon A1 CLSM system (Nikon, Tokyo, Japan). Three-dimensional reconstruction was performed by Nikon NIS-Element software (Nikon, Version 3.20).

Transmission electron microscopy (TEM) assay. TEM was performed to investigate the ultrastructural changes of *A. fumigatus* biofilm after exposure to CRA. In brief, 4 ml *A. fumigatus* suspension (4×10^5 conidia) was added to a 25 mm² cell culture flask with a vented cap, followed by adhesion for 4 h. Subsequently, the supernatant containing non-adherent cells was removed, followed by incubation at 37°C for 24 h after adding fresh RPMI-1640 medium. For the CRA treatment group, CRA was added to fresh RPMI-1640 medium added after 4 h of adhesion. Biofilms were washed in PBS and fixed in a solution of 2.5% glutaraldehyde (Solarbio, Beijing, China) and 4% formaldehyde (Biosharp, Hefei, China) in 0.1 M sodium cacodylate buffer (pH 7.2, Sigma-Aldrich; Merck Millipore). Subsequently, the mixture was fixed for 2 h in the same buffer containing 1% osmium tetroxide (pH 7.2, Sigma-Aldrich, Merck Millipore), and then embedded in Spurr's resin (Polysciences, Warrington, USA). Ultrathin sections were stained with 4% uranyl acetate (Polysciences) and 2.6% lead citrate (Polysciences). Finally, the images were observed under a Hitachi 7650 TEM (Hitachi, Tokyo, Japan) at 80 kV.

Analysis of gliotoxins by HPLC. To determine the potential mechanism by which CRA induces decreases of biofilm formation, the expression of gliotoxins, a component with important roles in *A. fumigatus* biofilm formation, was determined using HPLC. *A. fumigatus* biofilm was cultured and treated as for TEM described above. Supernatants of control biofilms or CRA-treated groups were extracted three times with 100 ml ethyl acetate. The combined organic phases were dried over sodium sulfate, followed by evaporation of the solvent and dissolving of the residue in 800 μ l methanol (concentrated sample). Subsequently, the samples were analyzed using a LC-20AB HPLC with a diode array detector (Shimadzu, Kyoto, Japan). Gliotoxin standard (Sigma-Aldrich, Merck Millipore) was used to establish the calibration curve. For HPLC measurement, 20 μ l of the concentrated sample was injected [Column: Nucleosil 100 (250x4.6 mm, Macherey-nagel Co., Düren, Germany), stationary phase, C₁₈ (5 μ M); mobile phases: A, H₂O with 0.1% v/v TFA and B, ACN; isocratic elution: 30% v/v B for 30 min and 100% B for 10 min; flow rate, 1 ml/min]. The retention time of gliotoxin was reported to be 6.4 min (12).

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to determine the expression of gliotoxin biosynthetic genes (*GliK*, *GliP*, *GliT* and *GliZ*). Total RNA was extracted from the fungal tissue using Fungal RNAout kit (Tiangen Biotech Co., Beijing, China) with strict adhesion to the manufacturer's instructions. First-strand complementary (c)DNA was synthesized using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. PCR amplification was then performed with SYBR[®] Premix Ex Taq[™] II kit (Takara Bio, Inc., Otsu, Japan) according to manufacturer protocol on the ABI 7500 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.), and using the specific primers listed in Table I. PCR conditions consisted of denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Gene amplification was normalized to that of the 18S rRNA gene. The gene expression was calculated according to the method used in a previous study (13).

Table I. Primers for polymerase chain reaction used in the present study.

Target gene	Primer sequences 5'-3'
18S ribosomal RNA	Forward: CGGCCCTTAAATAGCCCCGGTC Reverse: ACCCCCCTGAGCCAGTCCG
<i>GliK</i>	Forward: ATGCCTCGATCCTCGACAAG Reverse: CGAGATGAGGCCAGGTAG
<i>GliP</i>	Forward: CCGATGATGAAGAGACCACA Reverse: AAGAGCAGCAAGGGAGTTTC
<i>GliT</i>	Forward: ACGGAGGGCTTTTTGGTGT Reverse: CTTTAACGGCGTGGCACAAT
<i>GliZ</i>	Forward: CAGACCCTCAGCAGCCTAAC Reverse: GGACAGTGGGAACAGTGGTA

Hemolysis assay. To analyze the potential cytotoxicity of CRA, a red blood cell (RBC) lysis assay was performed as previously described (14). In brief, RBCs freshly isolated from healthy individuals were washed with PBS thrice. The supernatants were discarded by centrifugation at 380 x g for 10 min at room temperature. The precipitate was diluted in PBS (1X) to prepare a 10% RBC suspension. Subsequently, 1 ml suspension was added to an Eppendorf tube, together with 200 μ l CRA solution (64-1,024 μ g/ml) or AMB at the MFC (8 μ g/ml). Subsequently, the mixture was incubated at 37°C for 1 h. References for 100 and 0% hemolysis were made by incubating a 1-ml suspension of RBCs with 200 μ l Triton X-100 1% (v/v) or 200 μ l PBS, respectively. The tubes were centrifuged at 380 x g for 10 min to settle the broken membranes and unbroken RBCs. The liberated hemoglobin in the supernatant was determined using the Multiskan MK3 spectrophotometer at a wavelength of 540 nm. The percentage of haemolysis was calculated according to the following formula: Percentage of haemolysis = (Absorbance of the test sample - Absorbance of PBS solvent control) / (Absorbance of positive control - Absorbance of PBS solvent control) x 100%. Haemolysis of >10% was considered as hemolytic cytotoxicity. The written informed consent was obtained from each blood sample donor. The study protocols were approved by the Ethical Committee of Guangxi Medical University (Nanning, China).

Statistical analysis. All experiments were performed in triplicate and repeated at least three times. Values are expressed as the mean \pm standard deviation. Statistical significance between groups was determined by a two-tailed analysis of variance and Dunnet's multiple comparisons test using GraphPad Prism software version 6.0 (GraphPad Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

CRA does not directly affect the growth of planktonic cells. The MIC and MFC of CRA against the clinical isolate *A. fumigatus* GXMU04 and the standard strain *A. fumigatus* AF293 were >1,024 μ g/ml, indicating that CRA had no direct inhibitory effect on the growth of planktonic cells. The MIC

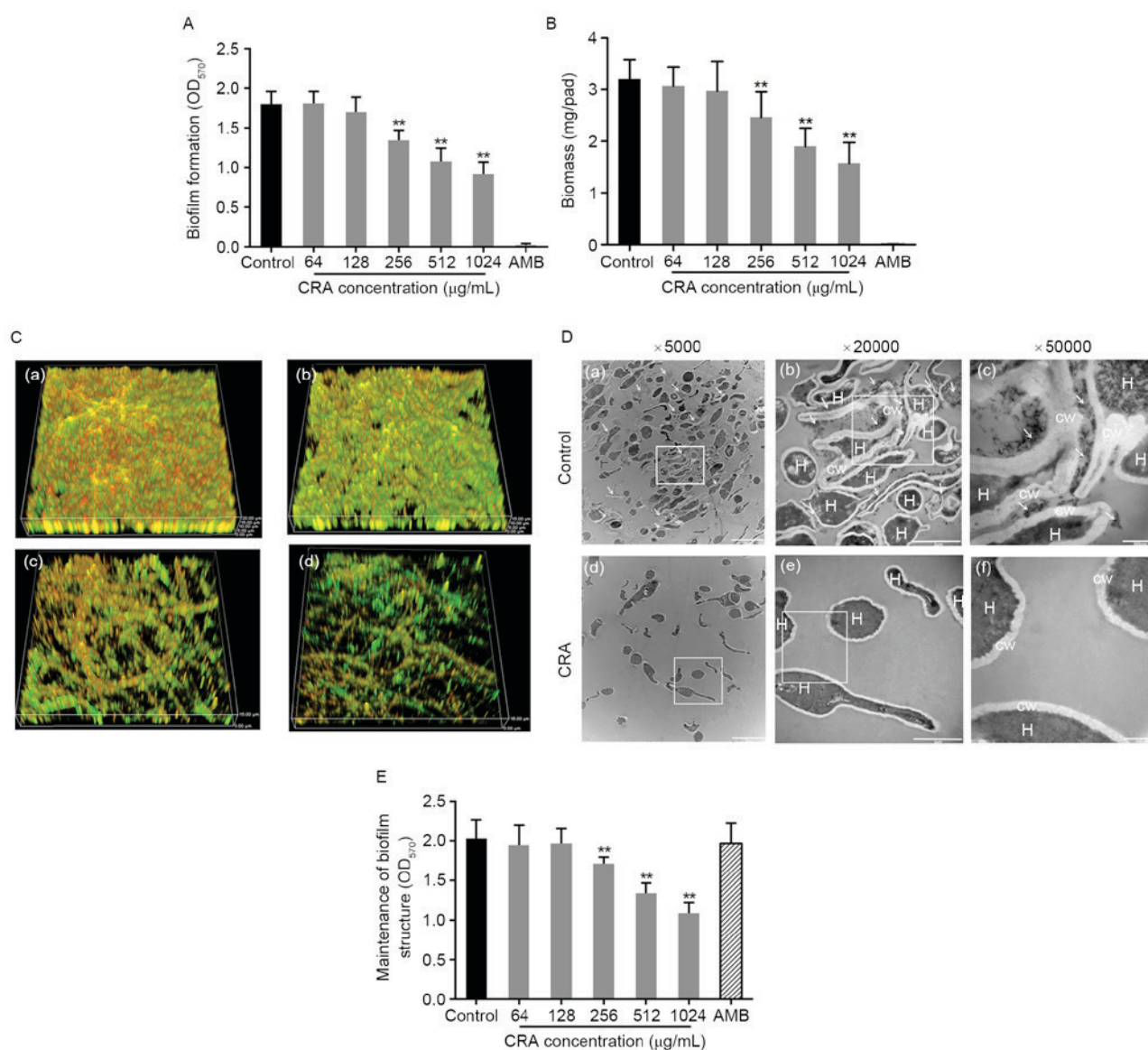


Figure 2. CRA inhibits *A. fumigatus* biofilm formation *in vitro*. (A) Effects of CRA on *A. fumigatus* biofilm formation. (B) Effect of CRA on the formation of *A. fumigatus* biofilms determined by dry weight method. The experiments were performed in triplicate with *A. fumigatus* GXMU04 and *A. fumigatus* AF293, respectively. Similar results were obtained for both strains. Values are present as the mean \pm standard deviation from three repeats of three independent experiments with the strains *A. fumigatus* GXMU04. (C) Effects of CRA on biofilm formation of *A. fumigatus* GXMU04, shown in confocal laser scanning microscopy reconstruction images. (a) Control; (b) 256 μ g/ml CRA; (c) 512 μ g/ml CRA; (d) 1,024 μ g/ml CRA. (D) Effect of CRA (1,024 μ g/ml) against *A. fumigatus* GXMU04 biofilm formation, shown in transmission electron microscopy images [top images: Control; bottom images: CRA treatment (1,024 μ g/ml). Magnification: (a,d) $\times 5,000$, (b,e) $\times 20,000$ and (c,f) $\times 50,000$; magnified windows are indicated with scale bars]. White arrows indicate extracellular matrix. Similar morphological observations were also made with the *A. fumigatus* AF293 strain (data not shown). (E) Effects of CRA on the maintenance of mature *A. fumigatus* biofilms. Biofilm formation was evaluated using a crystal violet assay. The experiments were performed in triplicate with *A. fumigatus* GXMU04 and *A. fumigatus* AF293, respectively. Similar results were obtained for both strains. Values are expressed present as the mean \pm standard deviation from three repeats of three independent experiments with the strains *A. fumigatus* GXMU04. * $P < 0.05$, ** $P < 0.01$, compared to the control group. AMB, amphotericin B; OD, optical density; H, hyphae cells; cw, cell wall; CRA, chlorogenic acid; *A. fumigatus*, *Aspergillus fumigatus*.

and MFC of AMB against *A. fumigatus* GXMU04 were 2 and 8 μ g/ml, respectively, and those against *A. fumigatus* AF293 were 0.25 and 2 μ g/ml, respectively.

CRA inhibits the formation of A. fumigatus biofilms by decreasing ECM production. CRA affected the formation of *A. fumigatus* biofilm in a dose-dependent manner (Fig. 2A and B). To further investigate the effects of CRA on formation of biofilm, the morphology of *A. fumigatus* biofilm was observed using CLSM and TEM. Two lectins (ECA and ConA) were used to identify the presence of extracellular polysaccharides in

the biofilm as they specifically bind to specific polysaccharide residues in the ECM. As shown in Fig. 2C, ECM formation was decreased in the CRA treated groups compared with that in mature *A. fumigatus* biofilms in the control group. Furthermore, absence of ECM around the hyphae was observed after CRA treatment (Fig. 2D).

CRA but not AMB affects the integrity of mature A. fumigatus biofilms. The present study further determined the effects of CRA and AMB on the maintenance of mature *A. fumigatus* biofilm, which revealed that CRA (256–1,024 μ g/ml) markedly

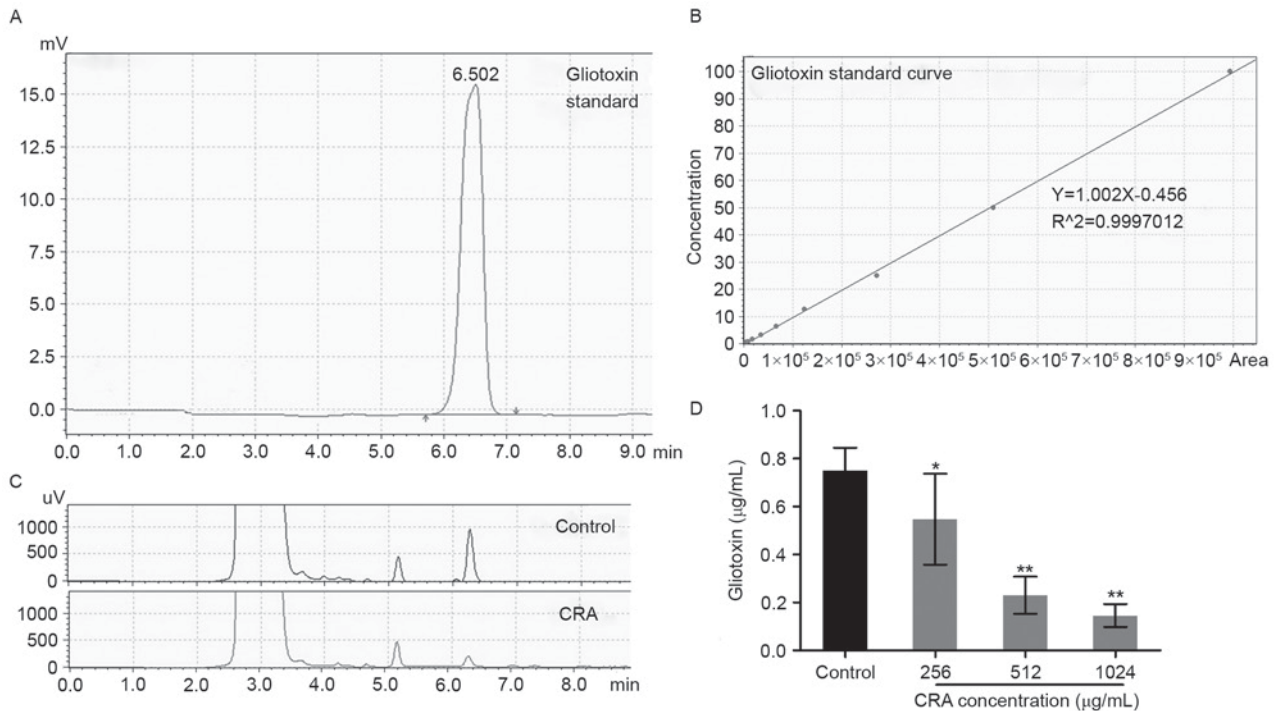


Figure 3. Detection of gliotoxin in culture supernatants detected by HPLC. (A) Gliotoxin had a retention time of 6.5 min as revealed by injection of a gliotoxin standard. (B) Various 2-fold diluted concentrations of standard gliotoxin were injected to obtain the standard calibration curve. (C) Chromatographic peak of gliotoxin in the supernatants of *A. fumigatus* cultures with biofilm harvested after optional treatment with CRA for 24 h were detected by HPLC. (D) Gliotoxin concentration in the culture supernatants. The experiments were performed in triplicate with *A. fumigatus* GXMU04 and *A. fumigatus* AF293, respectively. Similar results were obtained for both strains. Values are expressed as the mean \pm standard deviation from three repeats of three independent experiments for *A. fumigatus* GXMU04. * $P < 0.05$, ** $P < 0.01$, compared to the drug-free control group. HPLC, high-performance liquid chromatography; CRA, chlorogenic acid; *A. fumigatus*, *Aspergillus fumigatus*.

decreased the biofilm formation, while AMB showed no effects on its formation (Fig. 2E).

CRA decreases gliotoxin production by *A. fumigatus*. The present study then determined the production of gliotoxin, a well-known secondary metabolite closely associated with *A. fumigatus* biofilm formation. HPLC was performed to determine the synthesis of gliotoxin in the supernatant of *A. fumigatus* cultures treated with CRA (256-1,024 $\mu\text{g/ml}$) and drug free control, which was quantified using an external standard calibration curve method. The results revealed that treatment with CRA (512 or 1,024 $\mu\text{g/ml}$) resulted in decreased synthesis of gliotoxin by the biofilms compared with that of the control group ($P < 0.05$; Fig. 3).

CRA reduces the expression of gliotoxin biosynthetic genes by *A. fumigatus*. RT-qPCR was performed to determine the expression of genes encoding gliotoxin after CRA treatment. Four genes predominantly involved in gliotoxin biosynthesis, including *GliK*, *GliP*, *GliT* and *GliZ*, were selected for RT-qPCR analysis. As shown in Fig. 4, a significant downregulation was observed in *GliK* (0.63-fold; $P < 0.01$), *GliP* (0.84-fold; $P < 0.05$), *GliT* (0.26-fold; $P < 0.01$), and *GliZ* (0.50-fold; $P < 0.01$) after incubation with 1,024 $\mu\text{g/ml}$ CRA for 24 h. These results indicated that CRA altered the expression of gliotoxin biosynthetic genes *in vitro*.

CRA is nontoxic to erythrocytes. The mechanical stability of the erythrocyte membrane is a good indicator for screening

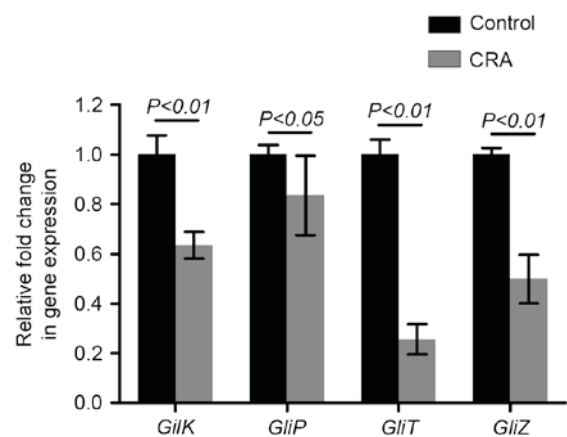


Figure 4. Expression changes of certain genes with significance in gliotoxin biosynthesis after treatment with 1,024 $\mu\text{g/ml}$ CRA compared with the control group. The experiments were performed in triplicate with *A. fumigatus* GXMU04 and *A. fumigatus* AF293, respectively. Similar results were obtained for both strains. Values are expressed as the mean fold changes \pm standard deviation three independent experiments performed in triplicate with *A. fumigatus* GXMU 04. Gli, gliotoxin; CRA, chlorogenic acid; *A. fumigatus*, *Aspergillus fumigatus*.

for cytotoxicity. In the present study, incubation of human erythrocytes with CRA showed no hemolytic activity even at the highest sub-MIC concentration (Fig. 5). In addition, no apparent hemolysis was observed in the human erythrocytes incubated with AMB at the MFC. This indicated that CRA induced had low toxicity in erythrocytes.

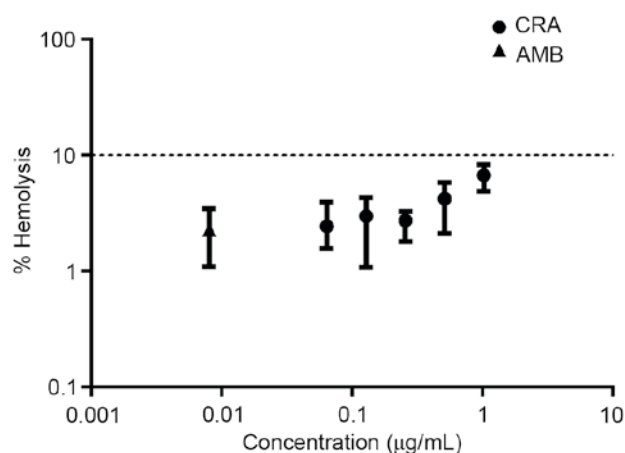


Figure 5. Hemolytic activity of CRA at sub-minimum inhibitory concentrations or AMB at the minimum fungicidal concentration towards human erythrocytes. Values from three repeats are shown as mean percentages \pm standard deviation in the graph with log x and y axes. AMB, amphotericin B; CRA, chlorogenic acid; *A. fumigatus*, *Aspergillus fumigatus*.

CRA pre-treatment enhances the anti-fungal effects of AMB.

In the present study, marked changes were observed in the structure of *A. fumigatus* biofilms upon CRA pre-treatment (1,024 µg/ml) followed by AMB under SEM. After exposure to CRA, the formation of ECM was markedly decreased and a defined layer of hyphae was observed (Fig. 6A, b) compared with the non-treatment group (Fig. 6A, a). However, in the AMB group, the biofilm showed a highly dense network of cross-linked, parallel-packed hyphae covered with thick ECM (Fig. 6A, c). Furthermore, marked disintegration was observed in the hyphae cells of the biofilms subjected to CRA pre-treatment and AMB at its MFC (Fig. 6A, d). Meanwhile, only few shriveled filamentous forms remained on the coverslip. However,

for the biofilms exposed to AMB only, minimal changes were observed in the structure of the biofilms. For the cell viability, CLSM indicated that the viability and growth of hyphae were not affected by CRA alone at sub-MICs (Fig. 6B, b), which was similar with the non-treatment group (Fig. 6B, a). Meanwhile, for the cells exposed to AMB at its MFC alone, only part of the hyphae on the superficial biofilm were affected (Fig. 6B, c). However, AMB treatment caused a significant decrease of cellular viability in the biofilms pre-treated with CRA (Fig. 6B, d). Taken together, pre-treatment with CRA contributed to the increased fungicidal effects of AMB in *A. fumigatus* GXMU04 biofilms.

Discussion

Treatment of *A. fumigatus*-associated infection remains a challenge due to the drug resistance induced by formation of biofilms. The present study confirmed that CRA decreased the formation of *A. fumigatus* biofilm and impaired the integrity of the mature biofilm in a dose-dependent manner. Moreover, CRA inhibited the production of gliotoxin and the expression of gliotoxin biosynthetic genes. Notably, similar results and trends were observed for both the clinical strain *A. fumigatus* GXMU04 and the laboratory strain *A. fumigatus* AF293 when treated with CRA, in the quantitative experiments (crystal violet assay, dry weight assay, gene expression and gliotoxin production) or morphological observation experiments (SEM, CLSM or TEM). Therefore, the major data presented in the results section were obtained from the clinical strain *A. fumigatus* GXMU04, because this strain was isolated from the patient in clinics and more representative for this study. As CRA showed low toxicity in the hemolysis assay, it may be applicable in the management of *A. fumigatus*-associated infection in clinical practice.

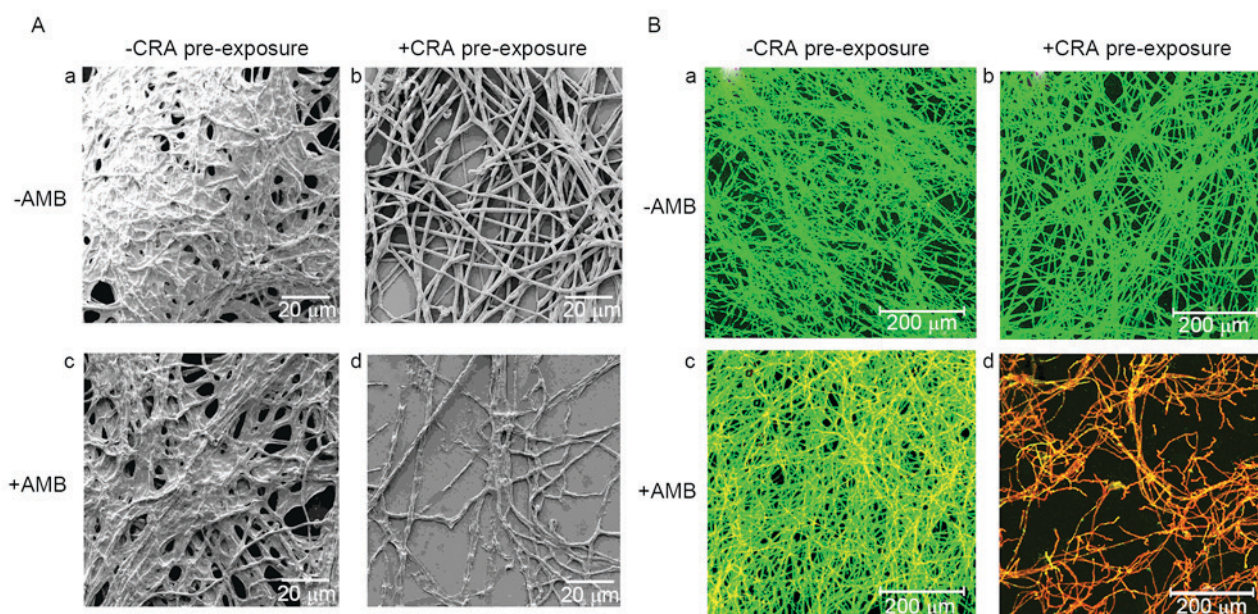


Figure 6. Pre-incubation with CRA enhances the fungicidal effects of AMB to fungal cells in *A. fumigatus* GXMU04 biofilms. Biofilms were incubated with AMB (control) or CRA for 24 h, followed by (A) staining with propidium iodide and SYTO-9 and analysis by scanning electron microscopy (scale bar, 20 µm) or (B) fluorescence staining of damaged membranes and analysis by confocal laser scanning microscopy (scale bar, 200 µm). (a) Control biofilm (b) CRA pre-incubated biofilm untreated with AMB; (c) control biofilm treated with AMB; (d) CRA pre-incubated biofilm treated with AMB. Similar results were also obtained using the *A. fumigatus* AF293 strain (data not shown). AMB, amphotericin B; CRA, chlorogenic acid; *A. fumigatus*, *Aspergillus fumigatus*.

To the best of our knowledge, the present study was the first to indicate that CRA had a significant anti-biofilm effect against *A. fumigatus*. It is well known that antifungal agents (e.g. AMB, VRC and CAS) are not effective for the management of *A. fumigatus* biofilm-associated infection (6,15). The antifungal resistance of *A. fumigatus* biofilms was reported to be closely associated with the ECM acting as a barrier to prevent drug diffusion. The findings of the present study showed that CRA at sub-MICs not only inhibited the formation of *A. fumigatus* biofilms, but also dispersed the structure of mature biofilms in a dose-dependent manner. In addition, CRA decreased the production of ECM, based on which it enhanced the antifungal effects of AMB on fungal cells in biofilms.

The process of *A. fumigatus* biofilm development includes the following steps: Resting conidia adhesion, hyphae germination, biofilm growth and maturation (15). Gliotoxin, one of the mycotoxins produced by *A. fumigatus*, has been proved to contribute to biofilm development, ECM production and maturation (5,16). For instance, a marked increase in gliotoxin production has been observed in *A. fumigatus* cultures grown as biofilms (15). In addition, redox-active secondary metabolites such as gliotoxin also act as a key signaling molecules for sporulation in filamentous fungi, including *A. fumigatus*, and may take part in biofilm development (17). Furthermore, Bugli *et al* (7) confirmed that increased production of gliotoxin was associated with the formation of *A. fumigatus* biofilm. Therefore, gliotoxin represents a potential target for novel anti-biofilm drugs. The present study determined the levels of gliotoxin in the supernatant of *A. fumigatus* biofilms, revealing that CRA decreased gliotoxin production in a dose-dependent manner. In a previous study, disruption of the gliotoxin biosynthetic gene cluster resulted in a marked decrease in gliotoxin production (12). On this basis, it may be hypothesized that the inhibition of *A. fumigatus* biofilm formation by CRA may be associated with the decrease of gliotoxin production. To confirm this, the expression of four major genes encoding gliotoxin in *A. fumigatus* as biofilms grown for 24 h was detected by RT-qPCR. The expression of four gliotoxin biosynthetic genes was downregulated in CRA-treated biofilms compared with that in the control. In particular, the expression of the *GliT* gene was significantly downregulated by CRA. This result was also confirmed by a 31-fold increase of *GliT* in biofilm-grown cells in a previous gene microarray assay (12). These results indicated that CRA may reduce gliotoxin production by downregulating the expression of gliotoxin biosynthetic genes, which may finally inhibit the formation of *A. fumigatus* biofilms. In addition, gliotoxin also acts as a virulent factor affecting the contribution of neutrophils to the host defense via the induction of neutrophil apoptosis (18,19). Furthermore, the inhibitory effects of CRA on gliotoxin production indicated that it may reduce the invasiveness of *A. fumigatus*.

Considering the effects of CRA on biofilm formation and gliotoxin activity, it may act as an agent used for the management of *A. fumigatus* biofilm-associated infection. In the present study, the cytotoxicity of CRA was determined, and the results showed that CRA had no hemolytic effects on human RBCs. In addition, CRA is commonly found in vegetables, tea and coffee and used as an active component in

Traditional Chinese Medicine with no described toxic properties (20). All of these facts prove the safety of CRA in the antifungal field.

Combination or sequential therapy may be an alternative to monotherapy (e.g. with AMB, VRC or CAS) for patients with invasive infections due to resistant organisms and for in certain patients who failed to respond to standard treatment. For instance, pentraxin 3 in combination with AMB inhibited the growth of *A. fumigatus* *in vitro* (21). However, on certain occasions, adverse effects may occur during sequential antifungal therapy. For instance, Rajendran *et al* (5) showed that pre-incubation of *A. fumigatus* germings with VRC at a sub-MIC led to an increase of ECM production and tolerance to AMB by two- to four-fold. Therefore, it is urgent to develop novel sequential antifungal therapies with satisfactory antifungal properties. In the present study, CRA pre-treatment with subsequent AMB treatment not only inhibited germination and viability of hyphae, but also markedly reduced ECM. Therefore, CRA pre-treatment followed by antifungal agents may be an effective therapeutic strategy to improve the management of *A. fumigatus* biofilm-associated infections.

In conclusion, CRA inhibited the formation of *A. fumigatus* biofilm. In addition, CRA reduced gliotoxin production by decreasing the expression of gliotoxin synthetic genes in *A. fumigatus*. These results supported the potential of CRA as a natural agent for the treatment of *A. fumigatus* infections. Pre-treatment with CRA markedly increased the fungicidal effects of AMB on *A. fumigatus*, suggesting that this combination treatment may represent a novel strategy to enhance the efficacy of anti-fungal treatments by reducing biofilm-associated drug resistance.

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