Chromone Derivatives CM3a Potently Eradicate Staphylococcus aureus Biofilms by Inhibiting Cell Adherence

Introduction: The ability of Staphylococcus aureus to form biofilms is associated with high mortality and treatment costs. Established biofilms cannot be eradicated by many conventional antibiotics due to the development of antibiotic tolerance by S. aureus. Here we report the synthesis and biological characterization of novel small-molecule compounds with antibiofilm activity. Chromone 5-maleimide substitution compounds (CM3a) showed favorable antibacterial activity against S. aureus.

Methods: CM3a with antibacterial activity was synthesized and screened. The minimum inhibitory concentration (MIC) of CM3a were determined by the broth microdilution method. Biofilm eradication assay and colony count methods were used to investigate the effect of CM3a on S. aureus biofilm disruption and killing. Changes in biofilm architecture when subjected to CM3a, were visualized using confocal laser scanning microscopy (CLSM). CCK-8 assay and survival rate of Galleria mellonella larvae were used to test the toxicity of CM3a.

Results: The minimum inhibitory concentration (MIC) of CM3a against S. aureus was about 26.4 μM. Biofilm staining and laser scanning confocal microscopy analysis showed that CM3a eradicated S. aureus biofilms by reducing the viability of the constituent bacterial cells. On the other hand, CM3a showed negligible toxicity against mouse alveolar epithelial cells and Galleria mellonella larvae.

Conclusion: Chromone derivatives CM3a has therapeutic potential as a safe and effective compound for the treatment of S. aureus infection.

Keywords: chromone derivative, maleimide, Staphylococcus aureus, biofilm, eradicate
antibacterial agents because of the complex structure of the extracellular polymeric substances (EPS) matrix. Biofilm formation can lead to the persistence and recalcitrant of S. aureus and is a major clinical challenge. Currently employed antibiotics mainly target microbial growth mechanisms and cell division, while bacterial biofilms resist clearance by antibacterial agents and host defense molecules. Thus, bacterial biofilms increase the risks of morbidity and mortality in patients, as well as treatment costs.

In several decades, although many non-growth-altering biofilm inhibitors and dispersal agents have been described, there are few biofilm-eradicating agents. It is impendency to develop new biofilm eradication agents and complementary antimicrobial strategies with multiple therapeutic applications to address persistent bacterial infections and reduce patient mortality and treatment costs.

Chromone is a natural compound present in the diet of humans and animals that has low toxicity to mammalian cells. Previous studies have also shown that chromone derivatives possess a broad range of biological activities depending on the substitution pattern of the chromone scaffold. Maleimide is an important structural parent nucleus of a class of bioactive molecules and functional materials from Marine natural alkaloids. In this study, we synthesized chromone 5-maleimide substitution compounds and assessed their therapeutic potential by characterizing their capacity to eradicate S. aureus biofilm.

**Materials and Methods**

**Procedure for Synthesis**

Chromone 1a (0.2 mmol, 1 equiv) and maleimide 2 (0.5 mmol, 2.5 equiv) were combined in a 12-mL screw-capped tube with 2 mL of 1.2-dichloroethane (0.1 M), [Ru(p-cymene)Cl2]2 (0.01 mmol, 0.05 equiv), AgNTf2 (0.04 mmol, 0.2 equiv), and AgOAc (0.6 mmol, 3 equiv) were added to the reaction mixture, which was heated to 120°C in a heating mantle with stirring for 2 h. After the reaction was completed, the reaction mixture was directly loaded onto a silica gel column and purified with a petroleum ether/EtOAc solution to obtain the 3a product (≥83% yield; >95% purity; Figure 1). The full name of CM3a is 3-(7-chloro-4-oxo-4H-chrome-5-yl)-1-ethyl-1H-pyrrole-2,5-dione.

**Bacterial Strains and Cells**

S. aureus strains JP21, ZSA01, ZSA02, ZSA03, ZSA04, and ZSA05 were provided by the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). S. aureus strain SA113 was a gift from the Department of Infectious Diseases and the Key Laboratory of Endogenous Infection, Shenzhen Nanshan People’s Hospital (Shenzhen, China). S. aureus isolates were identified using a VITEK-2 automated system (bioMérieux, Marcy l’Étoile, France) according to the manufacturer’s instructions. As all S. aureus strains used in this study are routinely encountered during laboratory procedures at the hospital, the Ethics Committee of Shanghai Pulmonary Hospital of Tongji University School of Medicine waived the requirement for ethics approval.

The mouse alveolar epithelial cells MALE-12 and human bronchial epithelial cell BEAS-2B was a gift from the Department of Anesthesiology, Shanghai Pulmonary Hospital, Tongji University School of Medicine (Shanghai, China).

**Biofilm Formation**

Overnight cultures of S. aureus strains JP21, SA113, ZSA01, ZSA02, ZSA03, ZSA04, and ZSA05 were diluted 1:200 in tryptic soy broth (TSB) containing 0.5% glucose (Sigma-Aldrich, St. Louis, MO, USA) and dispensed into 96-well microfilter plates (BD Biosciences, Franklin Lakes, NJ, USA). After 24 h of static culture at 37°C, the wells were washed 3 times with 50 mM phosphate-buffered saline (PBS [pH 7.2]) to remove unattached bacterial cells. Biofilms were stabilized by fixation with methanol (99.5%) for 15 min and stained with 1% crystal
violet for 15 min. The wells were gently washed 3 times with distilled water to remove floating cells. After drying, 33% glacial acetic acid was added to the well to release the biofilms into the solution, and the optical density at 600 nm (OD600) was measured.\(^\text{16}\)

**Determination of Minimum Inhibitory Concentration (MIC)**

CM3a MICs for *S. aureus* strains JP21, SA113, ZSA01, ZSA02, ZSA03, ZSA04, and ZSA05 were determined by the broth microdilution method. Samples (100 μL) were added to a 96-well microfilter plate containing CM3a (1–512 μg/mL) and 100 μL of cation-adjusted Mueller–Hinton broth (CAMHB). After 24 h of static cultivation at 37°C, OD600 was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). The MIC was defined as the lowest concentration that completely inhibited *S. aureus* growth.

The MICs of vancomycin, telithromycin, daptomycin for SA113 were determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.\(^\text{17}\)

**Growth Assay**

*S. aureus* strains were grown in MHB for 3 h, then diluted 1:200 with CAMHB; 100-μL aliquots were added to a 96-well microfilter plate. A 100-μL volume of CM3a was added to each well to obtain final concentrations of 1/32, 1/16, 1/8, and 1/4 MIC. The fully automated Bioscreen C microbial growth curve analyzer (Growth Curves USA, Piscataway, NJ, USA) was used to measure OD600 every 30 min for 24 h and a growth curve was generated from the measured values.

**CM3a Eradicate the Established Biofilms of *S. aureus***

Overnight cultures of *S. aureus* were diluted 1:200 in 200 μL of TSB with 0.5% glucose (TSBG) and inoculated into a 96-well microfilter plate. After 24 h of static incubation at 37°C, mature biofilms formed and the supernatant was discarded. The plate was washed with PBS to remove floating cells, and fresh TSBG containing CM3a was added to the wells; TSBG without CM3a served as the control. After static incubation for 48 h with daily medium replacement, the remaining biofilms were stained with crystal violet. Vancomycin, telithromycin, and daptomycin were used as control antibacterial agents for SA113 biofilms.

**Detecting the Adherent Cells in the Established Biofilms**

*S. aureus* SA113 and JP21 were inoculated into 12 polystyrene microtiter plates with TSBG and mature biofilms formed after static incubation for 24 h. After discarding the supernatant and washing the plates 3 times, fresh TSBG containing CM3a was added to the wells; TSBG without CM3a served as an untreated control. After 48 h of static incubation with daily medium replacement, the adherent cells remaining in the established biofilms were collected by scratching the wall of the wells with a cell scraper after discarding the supernatant. Ultrasonic Bacteria Dispersion Counter (TB healthcare, Guangdong, China) was used to disperse bacteria of collected biofilms. The colony-forming units (CFU) of live bacteria were counted after diluting and plating the cells.\(^\text{18}\)

**Laser Scanning Confocal Microscopy**

*S. aureus* strains were statically incubated in a 20-mm glass-bottomed cell culture dish (NEST, Wuxi, China) for 48 h. The intermediate steps were the same as those described in Section 2.6. SYTO-9 (300 μL, 0.02%; Thermo Fisher Scientific, Waltham, MA, USA) and propidium iodide (300 μL, 0.067%; Thermo Fisher Scientific) were added to the cell culture dish for 30 min in the dark to stain the cells. The samples were imaged by laser scanning confocal microscopy (TCS SP5; Leica, Wetzlar, Germany) using a 63×1.4-numerical aperture oil immersion objective lens. Images were reconstructed into a 3-dimensional (3D) model using Imaris v7.4.2 software (Bitplane, Belfast, UK).\(^\text{19}\)

**Assessment of CM3a Cytotoxicity**

Cell Counting Kit (CCK)-8 (Beyotime, Shanghai, China) was used according to the manufacturer’s instructions to assess the viability of mouse alveolar epithelial cells MALE-12 and human bronchial epithelial cell BEAS-2B treated with CM3a. Briefly, the cells were seeded in a 96-well plate at 10^3, 10^4, and 10^5 cells/well. After incubation for 48 h in a serum-free medium containing CM3a or PBS, the cells were incubated with 20 μL CCK-8 solution for 1 h at 37°C. OD450 was measured with a Biotek Synergy 2 microplate reader (Biotek, Winooski, VT, USA).\(^\text{20}\)

**Evaluation of CM3a Toxicity to *Galleria mellonella* Larvae**

As the larvae of the greater wax moth *G. mellonella* turn from white to black when they die, they are well-suited for
testing drug toxicity. We injected the larvae with PBS or CM3a (16 and 32 μg/mL; n=10 per group). The viability of the larvae was recorded after 48 h. The experiment was performed in triplicate.

Statistical Analysis
Data were analyzed with Student’s t-test and by 1-way factorial analysis of variance. Data analyses were performed using SPSS v19 software (IBM, Armonk, NY, USA). P values <0.05 were regarded as statistically significant.

Results
Characterization of Product CM3a
CM3a was a white solid (50.3 mg; >95% purity) after purification by chromatography (eluion: 10% EtOAc in petroleum ether) with a melting point of 205°C–206°C. The compound was characterized by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS), yielding the following values:1H NMR (400 MHz, CDCl3) δ 7.81 (d, J = 6.0 Hz, 1H), 7.59 (d, J = 1.9 Hz, 1H), 7.22 (d, J = 1.9 Hz, 1H), 6.46 (s, 1H), 6.28 (d, J = 6.0 Hz, 1H), 3.64 (q, J = 7.2 Hz, 2H), 1.25 (t, J = 7.2 Hz, 3H) ppm. 13C[1H] NMR (126 MHz, CDCl3) δ 176.2, 170.0, 168.8, 157.2, 154.8, 148.0, 139.2, 131.0, 127.4, 125.2, 122.0, 120.2, 114.0, 33.3, 13.9 ppm. HRMS (ESI-TOF) m/z: [M +H]+ calcd for C15H14ClNO4; 304.0377, found: 304.0370.

CM3a Inhibits the Growth of S. aureus
The MIC value of CM3a against all S. aureus strains tested in our study was 8 μg/mL (about 26.4 μM). Subinhibitory concentrations of CM3a—i.e., 1/4 MIC (2 μg/mL), 1/8 MIC (1 μg/mL), 1/16 MIC (0.5 μg/mL), and 1/32 MIC (0.25 μg/mL)—had no effect on the growth of S. aureus SA113 and JP21. At 1/2 MIC (4 μg/mL), both strains grew more slowly than cells in the control group after 10 h (Figure 2).

CM3a Eradicating the Established Biofilms of S. aureus
We selected 7 S. aureus strains that formed robust biofilms to evaluate the biofilm-inhibitory activity of CM3a. At 2×MIC (16 μg/mL), the OD600 of the biofilms decreased from ~3 to ~1. At CM3a concentrations of 4×MIC (32 μg/mL), 8×MIC (64 μg/mL), 16×MIC (128 μg/mL), and 32×MIC (256 μg/mL), biofilm formation was almost completely inhibited (OD600<0.3), showing statistically significant differences relative to the control group (Figure 3A).

The MIC Values of Vancomycin, telithromycin, and daptomycin against SA113 were 1, 2, and 0.25 separately. Vancomycin, telithromycin, and daptomycin were used as control drugs to treat the biofilm of SA113. Vancomycin and daptomycin have almost no effect on the biofilm of SA113, and telithromycin has a certain effect on the concentrations of 4×MIC and 8×MIC (Figure 3B).

To confirm these findings, we examined the state of the biofilms after CM3a treatment with the LIVE/DEAD assay followed by laser scanning confocal microscopy. The biofilms were sparse and weak at 2× MIC (16 μg/mL) and almost disappeared at 4× MIC (32 μg/mL) (Figure 4).

CM3a Kills Adherent Cells in S. aureus Biofilms
Adherent (live) cells remaining in S. aureus biofilms following CM3a treatment were counted. CM3a was highly effective in inhibiting the growth of adherent cells in the established biofilm. Due to the quite remarkable difference between the c. f. u. values, we used the log10(c.f.u. values)

![Figure 2](image-url) Growth assay for S. aureus strains treated with subinhibitory concentrations of CM3a. (A and B) Strains JP21 (A) and SA113 (B) were cultured with 4, 2, 1, 0.5, and 0.25 μg/mL CM3a or without CM3a for 24 h.
algorithm to draw a bar graph and compare the number of remaining live bacteria. At 2× MIC (16 μg/mL) of CM3a, the number of live bacteria (CFU/mL) was around 10⁵ times lower than in the control sample; when the CM3a concentration was ≥4× MIC (32 μg/mL), the survival number of the remaining bacteria was about 10⁸ times lower than in the control group, living bacteria are almost gone (Figure 5).

**CM3a Has Low Toxicity in Mammalian Cells and Insect Larvae**

We assessed the cytotoxicity of CM3a in mouse alveolar epithelial cells MALE-12 and human bronchial epithelial cell BEAS-2B cultured with different concentrations of CM3a (16 and 32 μg/mL) with the CCK-8 assay. There were no differences in survival rates between the treatment groups (>80%) and the control group (Figure 6A and B). We also injected G. mellonella larvae with PBS or CM3a (16 and 32 μg/mL) and evaluated their viability after 48 h. Consistent with the results of the CCK-8 assay, there were no differences in survival rates between the treatment groups and the control group (Figure 6C).

**Discussion**

The ability of S. aureus to develop robust biofilm on implanted medical devices is a major barrier to successful treatment, as biofilm bacteria are 10–1000 times more recalcitrance than planktonic bacteria to conventional antibiotics.²¹ The presence of persister cells can

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**Figure 3** (A) Eradication of S. aureus (JP21, SA113, ZSA01, ZSA02, ZSA03, ZSA04, and ZSA05) biofilms by CM3a (16–128 μg/mL). (B) Eradication of S. aureus SA113 biofilms by vancomycin, telithromycin, and daptomycin. Each experiment was repeated 3 times, and data represent mean±standard deviation. **P<0.01, *P<0.05.

**Figure 4** Biofilm formation after treatment with CM3a was observed by laser scanning confocal microscopy after the LIVE/DEAD assay. (A) Strain SA113 without treatment. (B) SA113 treated with dimethylsulfoxide (DMSO). (C) SA113 treated with CM3a at 2 times the MIC (2×MIC). (D) SA113 treated with CM3a at 4 times the MIC (4×MIC).
disseminate and enter the circulation to colonize other organs and cause the recalcitrance and relapse of persistent bacterial infections.\(^2\,^22\) Many conventional antibiotics and new drugs in development stages can only inhibit the biofilm during the biofilm formation process but cannot eradicate the mature biofilm. Additionally, some antibiotics may thicken biofilms throughout treatment, making them more difficult to clear.\(^23\,^24\) Therefore, new drugs are needed that can eradicate difficult-to-treat biofilms.

Chromone has a unique structure that is suited to the synthesis of new drugs.\(^25\) Chromone has been recognized as a privileged structure for the invention and development of new drugs.\(^26\) Lots of research studies prove that the Medicinal properties exhibited by chromone derivatives are antibacterial, antifungal, antioxidant, antimalarial, neuroprotective, and HIV inhibitory potential.\(^13\,^27\) Maleimides are the base compounds for drug conjugation to antibodies, peptides, and other targeting units through their reaction with thiol groups, and have the advantages of rapid kinetics, quantitative conversion, and high specificity.\(^15\) In our study, the chromone 5-maleimide substitution compound CM3a showed strong antibacterial activity toward S. aureus, with a MIC of 26.4 μM. In the presence of 4 μg/ mL CM3a, the growth of S. aureus was slowed by 10 h. CM3a effectively eradicated S. aureus biofilms and effectively killed the constituent bacteria: the survival

**Figure 5** The survival rate of adherent cells in biofilms after treatment with CM3a (16–128 μg/mL). Each experiment was repeated 3 times, and data represent mean ± standard deviation. **P<0.01.**

**Figure 6** Toxicity of CM3a. (A and B) Viability of mouse alveolar epithelial cells MALE-12 (A) and human bronchial epithelial cell BEAS-2B (B) with or without CM3a treatment (16 and 32 μg/mL) as determined with the CCK-8 assay. (C) Survival of G. mellonella larvae following injection of CM3a (16 and 32 μg/mL) or PBS.
rate of the remaining live bacteria was about 105 times lower than in the control sample at CM3a concentrations ≧ 32 μg/mL (about 106 μM). CM3a also promoted biofilm detachment and disintegration.

Chromosome is known to have minimal toxicity to mammalian cells. We found that CM3a had low toxicity to mouse alveolar epithelial cells MALE-12 and human bronchial epithelial cell BEAS-2B, and did not cause the death of G. mellonella larvae. It is worth mentioning that we first synthesized the chromosome derivatives without Cl, but the solubility was poor. After modification with Cl or Br, the solubility was greatly increased, and the 2 derivatives had similar antibacterial activity and capacity to eradicate S. aureus biofilms. Although further study is required to clarify the mechanism of action of CM3a in the destruction of biofilms, our findings show that CM3a is a promising new drug for eradicating mature S. aureus biofilms and thereby improving the outcomes of patients with implanted medical devices or catheters.

Ethical Approval and Consent to Participate
The Ethics Committee of the Shanghai Pulmonary Hospital of Tongji University School of Medicine approved our study. The use of all cell lines in this study was approved by the Ethics Committee of the Shanghai Pulmonary Hospital of Tongji University School of Medicine.

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Author Contributions
All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure
The authors declare that there are no conflicts of interest.

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