

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

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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

Introduction

Staphylococcus aureus is a Gram-positive opportunistic human pathogen that causes many community-acquired, nosocomial, and biofilm-related infections worldwide.¹ Implanted medical devices (eg, joint prostheses, fluid shunts, and pacemakers) and catheters (eg, intravenous, urinary, and dialysis catheters) have revolutionized modern healthcare and are widely used in clinical practice. However, they are often associated with chronic and recurrent infections caused by *S. aureus* biofilms that form on the device surface.² Biofilms are produced upon switching the bacterial growth mode from planktonic to sessile, the process of its formation comprises 3 main stages: attachment, maturation, and detachment.³ In the detachment phase, cell clusters or a single cell separates from the biofilm, spreading to and colonizing other parts of the host.^{4,5} Bacterial biofilms are key players in the development of highly refractory to

antibacterial agents because of the complex structure of the extracellular polymeric substances (EPS) matrix.⁶ Biofilm formation can lead to the persistence and recalcitrant of *S. aureus* and is a major clinical challenge.⁷ 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.^{8,9}

In several decades, although many non-growth-altering biofilm inhibitors and dispersal agents have been described, there are few biofilm-eradicating agents.¹⁰ 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.^{11,12} Previous studies have also shown that chromone derivatives possess a broad range of biological activities¹³ depending on the substitution pattern of the chromone scaffold.¹⁴ Maleimide is an important structural parent nucleus of a class of bioactive molecules and functional materials from Marine natural alkaloids.¹⁵ 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'Etoile, 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

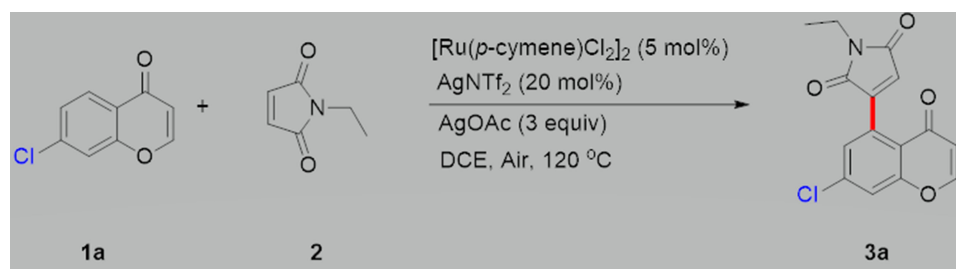


Figure 1 Procedure for synthesis of CM3a. 1a: chromone. 2: maleimides. 3a: CM3a.

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.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.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.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).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 10⁵, 10⁴, and 10³ 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).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 $\mu\text{g/mL}$; $n \approx 10$ 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 (elution: 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, CDCl_3) 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. ^{13}C [^1H] NMR (126 MHz, CDCl_3) 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*: [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{15}\text{H}_{11}\text{ClNO}_4$: 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 $\mu\text{g/mL}$ (about 26.4 μM). Subinhibitory concentrations of CM3a—ie, 1/4 MIC (2 $\mu\text{g/mL}$), 1/8 MIC (1 $\mu\text{g/mL}$), 1/16 MIC (0.5 $\mu\text{g/mL}$), and 1/32 MIC (0.25 $\mu\text{g/mL}$)—had no effect on the growth of *S. aureus* SA113 and

JP21. At 1/2 MIC (4 $\mu\text{g/mL}$), 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 $2 \times \text{MIC}$ (16 $\mu\text{g/mL}$), the OD₆₀₀ of the biofilms decreased from ~ 3 to ~ 1 . At CM3a concentrations of $4 \times \text{MIC}$ (32 $\mu\text{g/mL}$), $8 \times \text{MIC}$ (64 $\mu\text{g/mL}$), $16 \times \text{MIC}$ (128 $\mu\text{g/mL}$), and $32 \times \text{MIC}$ (256 $\mu\text{g/mL}$), 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 $4 \times \text{MIC}$ and $8 \times \text{MIC}$ (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 $2 \times \text{MIC}$ (16 $\mu\text{g/mL}$) and almost disappeared at $4 \times \text{MIC}$ (32 $\mu\text{g/mL}$) (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 $\log_{10}(\text{c.f.u. values})$

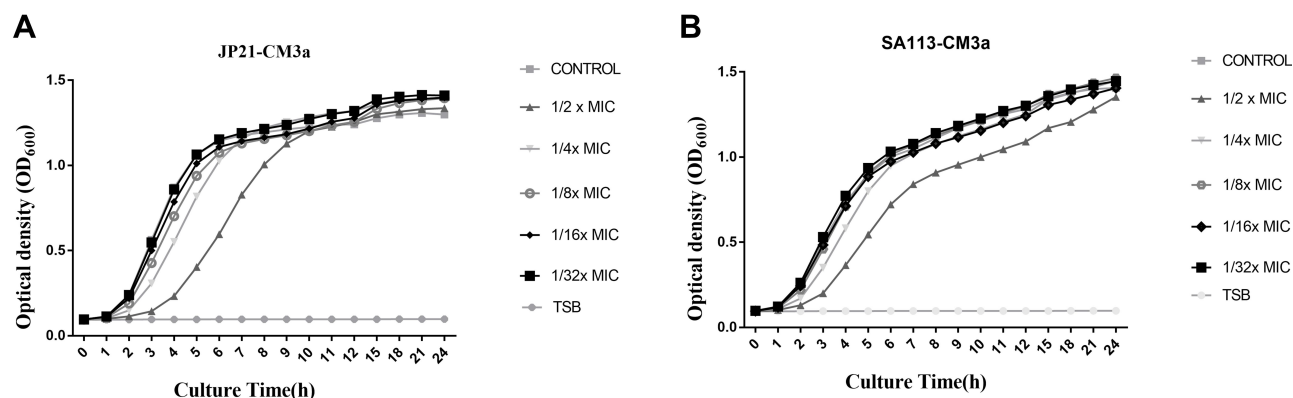


Figure 2 Growth assay for *S. aureus* strains treated with subinhibitory concentrations of CM3a. (A and B) Strains JP21 (A) and SA113 (B) were cultured with 4, 2, 1, 0.5, and 0.25 $\mu\text{g/mL}$ CM3a or without CM3a for 24 h.

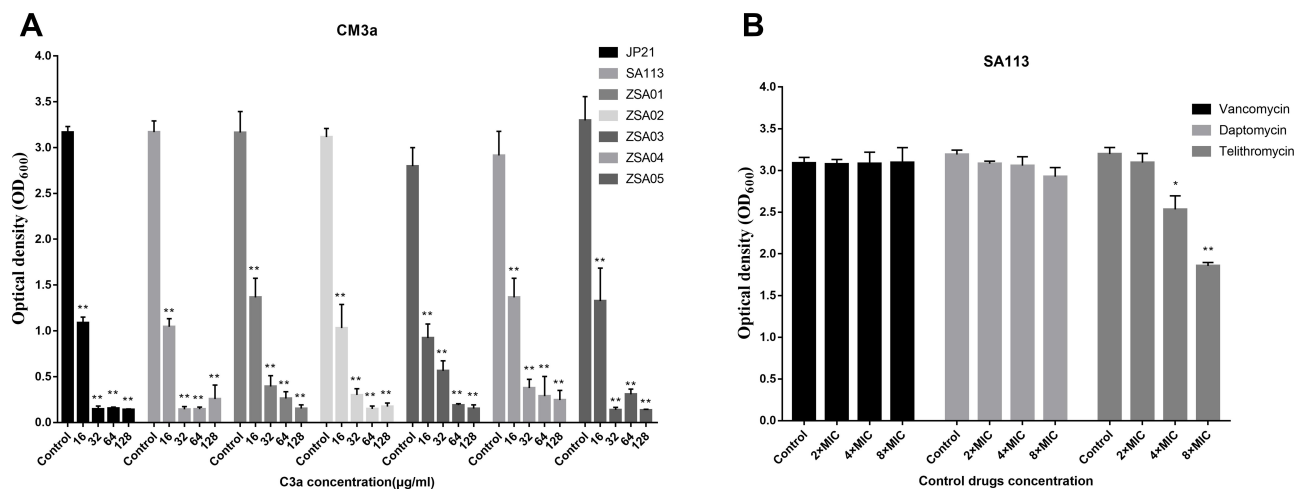


Figure 3 (A) Eradication of *S. aureus* (JP21, SA113, ZSA01, ZSA02, ZSA03, ZSA04, and ZSA05) biofilms by CM3a (16–128 µg/mL). (B) Eradication of *S. aureus* SA113 biofilms by vancomycin, telithromycin, and daptomycin. Each experiment was repeated 3 times, and data represent mean±standard deviation. **P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

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 10^5 times lower than in the control sample; when the CM3a concentration was $\geq 4\times$ MIC (32 µg/mL), the survival number of the remaining bacteria was about 10^8 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 recalcitrant than planktonic bacteria to conventional antibiotics.²¹ The presence of persister cells can

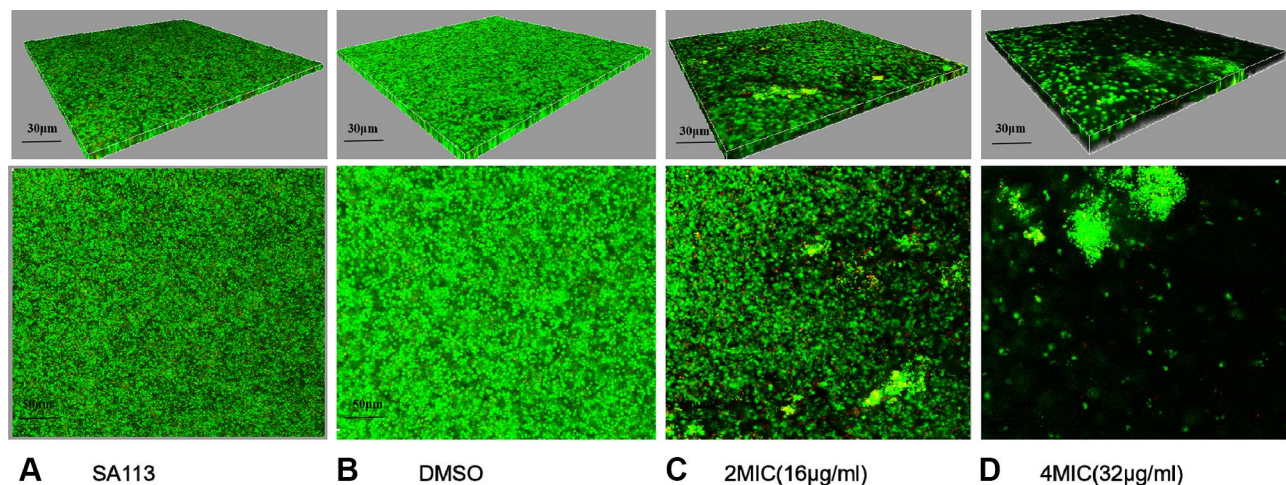


Figure 4 Biofilm formation after treatment with CM3a was observed by laser scanning confocal microscopy after the LIVE/DEAD assay. (A) Strain SA113 without treatment. (B) SA113 treated with dimethylsulfoxide (DMSO). (C) SA113 treated with CM3a at 2 times the MIC (2×MIC). (D) SA113 treated with CM3a at 4 times the MIC (4×MIC).

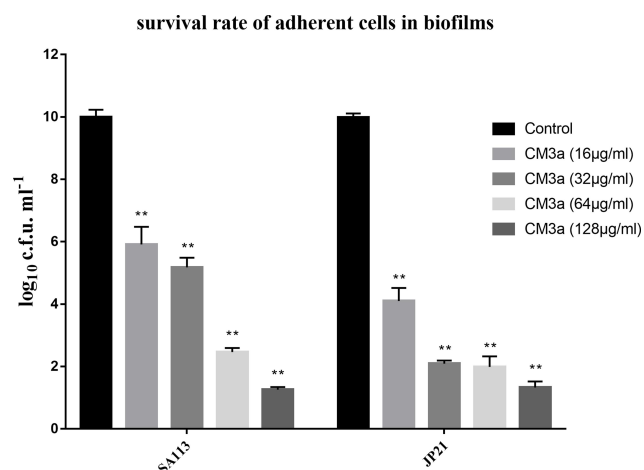


Figure 5 The survival rate of adherent cells in biofilms after treatment with CM3a (16–128 µg/mL). Each experiment was repeated 3 times, and data represent mean \pm standard deviation. ** $P < 0.01$.

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

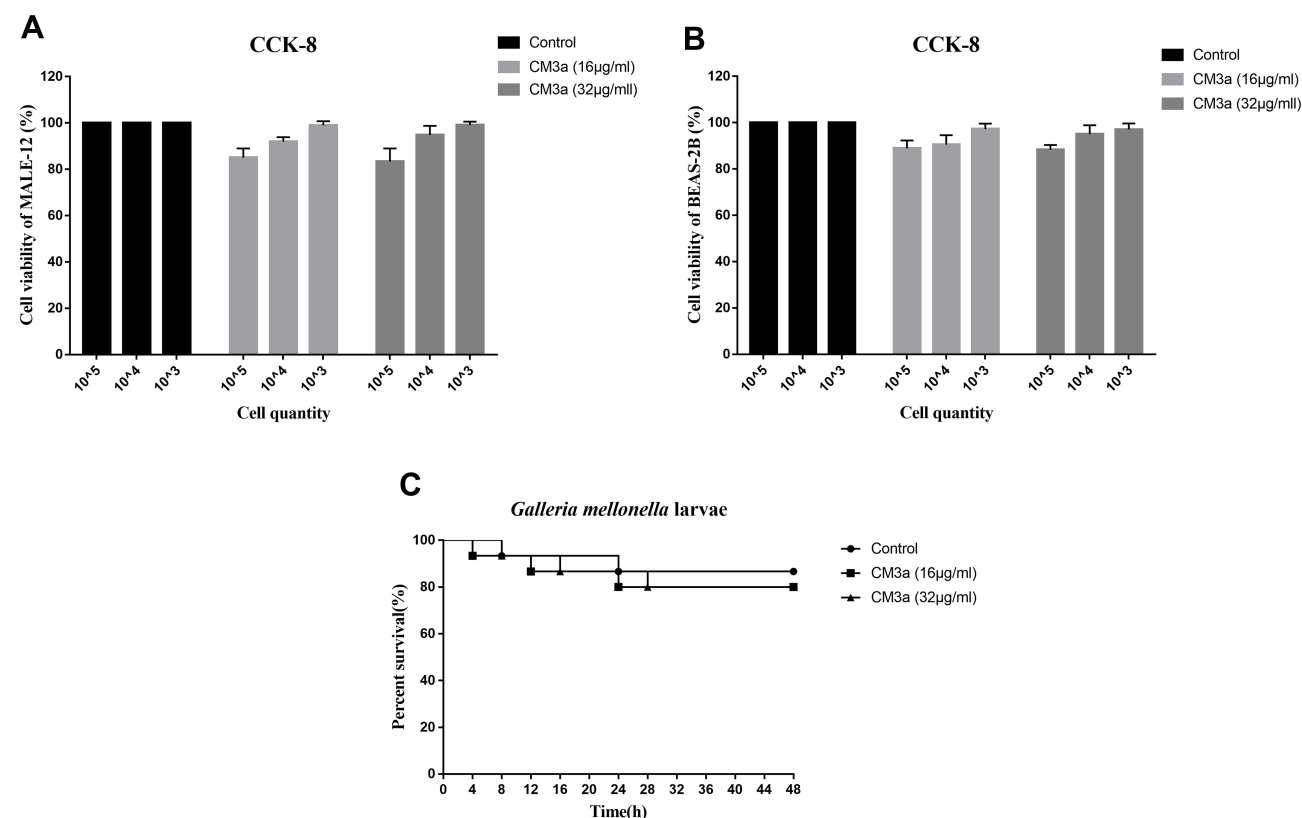


Figure 6 Toxicity of CM3a. (A and B) Viability of mouse alveolar epithelial cells MALE-12 (A) and human bronchial epithelial cell BEAS-2B (B) with or without CM3a treatment (16 and 32 µg/mL) as determined with the CCK-8 assay. (C) Survival of *G. mellonella* larvae following injection of CM3a (16 and 32 µg/mL) or PBS.

rate of the remaining live bacteria was about 105 times lower than in the control sample at CM3a concentrations $\geq 32 \mu\text{g/mL}$ (about $106 \mu\text{M}$). CM3a also promoted biofilm detachment and disintegration.

Chromone 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 chromone 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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