



Biochanin A as an α -hemolysin inhibitor for combating methicillin-resistant *Staphylococcus aureus* infection

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multidrug-resistant pathogen that poses a significant risk to global health today. In *S. aureus*, α -hemolysin is an important virulence factor as it contributes to the capacity of the bacteria to infect the host. Here, we showed that biochanin A (bioA), an isoflavone present in red clover, cabbage and alfalfa, effectively inhibited hemolytic activity at a dose as low as 32 $\mu\text{g}/\text{mL}$. Further, western blot and RT-qPCR data showed that bioA reduced the production and expression of MRSA hemolysin in a dose-dependent manner. In addition, when different concentrations of bioA were added to a coculture system of A549 cells and *S. aureus*, it could significantly decrease cell injury. Importantly, the in vivo study showed that bioA could protect mice from pneumonia caused by a lethal dose of MRSA, as evidenced by improving their survival and reducing the number of bacterial colonies in lung tissues, the secretion of hemolysin into alveolar lavage fluid and the degree of pulmonary edema. In conclusion, biochanin A protected the host from MRSA infection by inhibiting the expression of the hemolysin of MRSA, which may provide experimental evidence for its development to a potential anti-MRSA drug.

Keywords Methicillin-resistant *Staphylococcus aureus* · Biochanin A · Antivirulence · α -Hemolysin

Introduction

Staphylococcus aureus (*S. aureus*) is a seriously dangerous human and animal pathogen that cause endocarditis, skin and soft tissue infections, bacteremia and pneumonia (Kyaw

et al. 2015; McColl et al. 1998; Ni et al. 2018). The difficulty of treating *S. aureus* infection is due to the emergence of antibiotic-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to most antibiotics currently used (Fridman et al. 2014; Levin-Reisman et al. 2017; Mechler et al. 2015). The World Health Organization (WHO) identified MRSA as a high-priority pathogen (Shrivastava et al. 2018). To cope with the increasingly problematic emergence of antibiotic resistance and the postantibiotic era (Dickey et al. 2017), novel strategies against MRSA are urgently needed.

Pathogens express a variety of virulence factors, which affect the host by destroying the relationship between the host immune system and pathogens. In addition, inhibiting the virulence of pathogens will not place selection pressure on the host, which dramatically reduces the probability of drug resistance. Therefore, screening of antivirulence inhibitors is an ideal strategy to control multidrug resistant pathogens (Casadevall and Pirofski 1999).

S. aureus can produce a large number of cytotoxic molecules, including four hemolysins (α , β , δ and γ). Among them, alpha-hemolysin (Hla) is a pore-forming exotoxin

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that has been widely studied. Hla, a 33 kDa polypeptide, is secreted by 95% of *S. aureus* (2019; Grumann et al. 2014). One of the remarkable characteristics of hemolysin is its ability to dissolve red blood cells. Hla has been classified as a phenol soluble regulatory protein (PSM), and its hemolytic activity is not dependent on any receptor (Fritz et al. 2013; Kolata et al. 2011). As a water-soluble monomer, it can integrate into the cell membrane to form a transmembrane oligomer β -barrel heptamer channel, leading to host cell lysis or death (Dinges et al. 2000; Wu et al. 2019b). Previous studies have shown that the Hla mutant of *S. aureus* has significantly reduced virulence compared with the wild-type strain (Tran et al. 2020). Hla has hemolytic, cytotoxic, dermonecrotic, and other lethal properties and is considered an essential virulence target against *S. aureus* infection.

Natural products from plants and microorganisms are the main sources of antivirulence inhibitors because of their extensive physiological activities and environmental friendliness. A number of natural products have been reported to protect the host against *S. aureus* by inhibiting the production (Jiang et al. 2016; Teng et al. 2017) and self-assembly of Hla (Dong et al. 2013; Marathe et al. 2012). Biochanin A (4'-methoxy-5,7-dihydroxy isoflavone, bioA) (Fig. 1A) is a bioactive isoflavone found in diverse plants (Sarkar et al. 2006). It has been proven to produce marked anti-inflammatory (Kole et al. 2011), antioxidant (Derangula et al. 2020) and antitumor effects (Jain et al. 2015). In addition, bioA has been reported to inhibit the efflux pump of MRSA (Dan et al. 2014) and can be used as an adjuvant in combination with ciprofloxacin against clinical isolates of *S. aureus* (Liu et al. 2011), while its effect on *S. aureus*-related virulence factors has not been reported. In our study, a concentration of bioA at 32 $\mu\text{g}/\text{mL}$ almost completely inhibited hemolysin activity. In addition, bioA also significantly inhibited the hemolytic

activity of MRSA clinical isolates. Subsequently, the protective effect of bioA on *S. aureus* pulmonary infection was confirmed by establishing a mice pneumonia model. In conclusion, our results showed that bioA effectively inhibit the virulence of *S. aureus* Hla in vivo and in vitro, indicating it should be further explored as a drug against MRSA infection.

Materials and methods

Bacterial strains, reagents and growth conditions

S. aureus Newman and *S. aureus* BAA-1717 (USA 300) were purchased from American Type Culture Collection (ATCC, Manassas, V A, United States). Clinically isolated MRSA strain SA28 was obtained within the past 3 years and was identified by 16S RNA and quality control. BioA was obtained from the Rui Fensi Biological Company (Chengdu, China). BioA was dissolved in dimethyl sulfoxide (DMSO, Sigma, United States) to obtain a master stock (20 mg/mL) for standby application. Other chemical reagents were provided by Sangon Biotech (Shanghai, China). *S. aureus* was grown in trypticase soy broth (TSB, Hopebio, Qingdao, China) in an incubator with shaking at 220 rpm at 37 °C.

Susceptibility testing

The minimum inhibitory concentration (MIC) was determined by the microdilution method in 96-well plates according to the NCCLS guidelines as described previously. BioA was serially diluted twofold in 100 μL of cation-adjusted medium (CAMHB) at concentrations ranging from 2 to 512 $\mu\text{g}/\text{mL}$. Then, *S. aureus* USA300 (10^5 CFU/mL) was

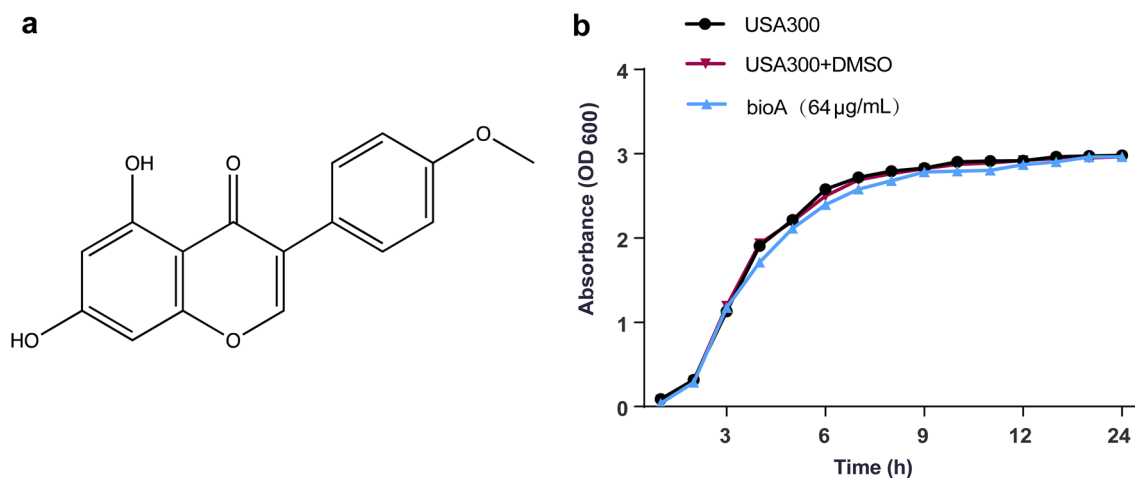


Fig. 1 Chemical structure and growth curve of bioA. **A** Chemical structure of bioA. **B** Growth curves of *S. aureus* USA300 treated with 64 $\mu\text{g}/\text{mL}$ bioA, with DMSO as the positive control (n = 3)

added to a 96-well plate. After incubation at 37 °C for 16 h, the MIC value was determined by reading the absorbance at 600 nm with a microplate reader.

Growth assay

For the growth curve assay, *S. aureus* USA300 was cultivated at 37 °C to an OD value of 0.3 at 600 nm in TSB. Following the addition of bioA at concentration of 64 µg/mL, the bacteria were further cultured at 37 °C with constant shaking for 24 h. *S. aureus* USA300 cultures without added bioA were used as a control group, and *S. aureus* USA300 cultures supplemented with 1% DMSO were used only to verify whether DMSO affects bacterial growth. Subsequently, the OD₆₀₀ value was measured every hour for 24 h, and the curve of the time versus the absorbance was drawn using the statistical program GraphPad Prism version 8.0.

Hemolysis assay

S. aureus Newman, *S. aureus* USA300 and MRSA strain SA28 were grown in TSB in the absence or presence of bioA to the postexponential growth phase, respectively. Bacterial supernatants were collected by centrifugation, and a hemolysis assay of the culture supernatants was conducted based on our previously described method using rabbit red blood cells (Qiu et al. 2010). Briefly, 100 µL supernatant was added to 1 mL phosphate buffered saline (PBS) buffer, followed by the addition of 25 µL defibrinated rabbit blood (Sbjbio, Nanjing, China). It was gently mixed and then incubated at 37 °C for 1 h. Low-speed centrifugation (4000×g, room temperature, 10 min) was used to collect any intact blood cells. The hemolytic activity of the culture supernatant was determined by measuring the absorbance at 543 nm. In addition, PBS was used as a negative control group. The assays were performed in triplicate.

RT-qPCR

A total of 2 mL of *S. aureus* USA300 grown with different concentrations of bioA (0 to 32 µg/mL) was incubated at 37 °C with shaking at 220 rpm for 16 h. *S. aureus* USA300 grown without bioA was treated in the same manner and used as a negative control. Subsequently, each sample was centrifuged at 10,000×g for 10 min to collect the bacteria. Total RNA was extracted using a Beyozol Total RNA Extraction Kit (Biotechnology, Shanghai, China) according to the manufacturer's instructions. Then, 1 µg of the purified RNA was used to generate cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). The *Hla* gene and regulatory gene *RNAlIII* were determined by RT-qPCR using an ABI 7900HT Real-time PCR system. Table 1 shows the oligonucleotide primers, and the RNA transcript levels

were calculated using the $2^{-\Delta\Delta CT}$ method. Each reaction was performed at least in triplicate.

Western blot

To evaluate the effect of bioA on α -toxin expression, bioA (0 to 32 µg/mL) was added to the *S. aureus* USA300 cultures and incubated at 37 °C until the OD₆₀₀ reached 2.5. The α -toxin protein in the supernatant of the bacterial culture was isolated by 12% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane by a transblot semidry system. The nonspecific binding sites of the PVDF membrane were blocked by incubating in 5% bovine serum albumin (BSA) for 2 h at room temperature. The membranes were incubated with rabbit anti- α -toxin polyclonal antibody (diluted 1:8000) (Sigma-Aldrich) for 1 h. After washing with PBST three times, the membranes were incubated with HRP-labeled goat anti-rabbit IgG (Bioworld, China) diluted 1:10,000 in PBST + 1% BSA at 37 °C for 1 h. The blots were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, UK), and the bands were quantified using ImageQuant TL software (GE Healthcare).

Live/dead and cytotoxicity assay

A549 cells (adenocarcinomic human alveolar basal epithelial cells) were seeded in 96-well plates (Nest, United States) at a density of 4×10^5 cells/well and then incubated at 37 °C in a 5% CO₂ incubator for 24 h. After that, the medium was removed gently and the dish was washed with sterile PBS to remove the nonadherent cells. Afterward, 100 µL of *S. aureus* USA300 culture and Dulbecco's modified Eagle's medium (DMEM, 400 µL) containing various concentrations of bioA (0 to 32 µg/mL) were added to the cells. After incubation at 37 °C for 6 h, the medium was collected for LDH assays by an LDH Cytotoxicity Detection kit (Beyotime, Shanghai, China). The amount of released LDH was measured using a microplate reader (Thermo Fisher, USA) at an absorbance of 490 nm.

For the live/dead assay, the dye was added to the cells and incubated in the dark for 30 min. The A549 cells were stained red/green to evaluate cell viability, and the fluorescence was measured from images obtained using fluorescence microscopy (Olympus, Japan).

Murine model of pneumonia

Seven-week-old female C57BL/6J mice (~22 g) were used to establish a pneumonia model as previously described (Brown et al. 2009; Labandeira-Rey et al. 2007). For the survival experiments, a group of 10 mice was infected with a lethal dose of 2×10^8 CFUs (in 30 µL of PBS) of *S. aureus* culture via the intranasal route. After infection

for one hour, the mice were dosed with bioA (50 mg/kg of body weight) via the intraperitoneal route at intervals of 12 h. Similarly, mice were injected with sterile PBS containing 0.5% DMSO as the negative control. The survival of the animals was monitored every 12 h for 96 h postchallenge to calculate their survival rate.

The bacterial count in the lung tissue and pathological correlates of pneumonia were also evaluated, and for this, the mice were infected with 30 μL (1×10^8 CFUs) of *S. aureus* culture. After infection for two days, the mice were euthanized, and the lungs were collected, weighed, and homogenized. After that, the lung tissue homogenate was serially diluted and plated on TSA agar plates and incubated at 37 °C until isolated colonies appeared to be counted.

The level of Hla in the alveolar lavage fluid of mice in each group was determined by ELISA. For histopathological analysis, the lung tissues of mice in each group were aseptically removed and fixed in 10% formalin. After conventional hematoxylin and eosin (H&E) staining, the lung tissue sections were observed and analyzed by light microscopy. The W/D ratio was used to reveal the degree of tissue edema and it was recorded in each case. The wet lung weight was determined 1 min after removal of the surface moisture. The dry lung weight was measured after dehydration at 80 °C (Xia et al. 2016).

Data analysis

Independent samples were statistically analyzed using Student's *t* test, and $P < 0.05$ was considered significant. The data are presented as the mean \pm SD, and all statistical analyses in this study were conducted using the statistical program GraphPad Prism version 8.0. The number of independent replicates for in vitro experiments is three or four replicates and ten replicates for in vivo experiments.

Results

BioA does not affect the growth of *S. aureus*

The MIC of bioA against *S. aureus* USA300 was determined by the microdilution method. The MIC value was 512 $\mu\text{g}/\text{mL}$, which proved that it has no antibacterial activity. In addition, the growth curve experiment further revealed that there was no effect on the growth of *S. aureus* USA300 when cocultured with bioA at a concentration of 64 $\mu\text{g}/\text{mL}$ (Fig. 1B). Thus, bioA can be considered a candidate anti-*S. aureus* virulence inhibitor because it does not have direct anti-*S. aureus* activity.

Effects of bioA on hemolysis of *S. aureus* USA300, Newman and MRSA clinical isolates

The effects of bioA (0 to 32 $\mu\text{g}/\text{mL}$) on the hemolytic activity of *S. aureus* USA300, Newman and MRSA clinical isolates were determined. As shown in Fig. 2A–C, when the medium supernatant of *S. aureus* USA300, Newman and SA28 was co-incubated with rabbit blood cells for 1 h, blood cell rupture and obvious hemolysis were observed. However, no hemolysis was observed when the supernatant of these strains was pretreated with bioA at 32 $\mu\text{g}/\text{mL}$, and the hemolytic inhibitory activities of *S. aureus* USA300, Newman and MRSA clinical isolates were 3.73%, 4.58% and 4.36%, respectively ($P < 0.001$).

BioA downregulates the transcription of *Hla* and *RNAIII*

To further explore the inhibitory mechanism of bioA on hemolysis of *S. aureus*, we evaluated the effect of bioA on the transcription level of *Hla* and its upstream regulatory gene *RNAIII*. As shown in Fig. 3A, when different concentrations of bioA were added to *S. aureus*, the transcriptional levels of *Hla* and *RNAIII* were decreased in a dose-dependent manner. These results suggested that bioA inhibited hemolytic activity by downregulating *Hla* and *RNAIII* transcription.

BioA decreases the secretion of Hla

According to the RT-qPCR results, we further evaluated whether bioA could inhibit the expression of Hla, and western blotting was performed. As shown in Fig. 3B, compared to the WT group (untreated *S. aureus*), the addition of different concentrations of bioA (0, 8, 16 or 32 $\mu\text{g}/\text{mL}$) inhibited the expression of Hla in a dose-dependent manner. This implied that bioA could effectively inhibit the activity of Hla by inhibiting the expression of Hla.

BioA prevents Hla-mediated A549 cell injury

After *S. aureus* infection of A549 cells for 6 h, the survival state of the A549 cells was observed by live/dead cell staining. As shown in Fig. 4A, A549 cells without *S. aureus* showed green fluorescence under the microscope, which proved that there were few injured cells. When *S. aureus* was incubated with A549 cells, many cells were stained red, indicating that *S. aureus* cause the death of A549 cells (Fig. 4B). Remarkably, the addition of various concentrations of bioA (8–32 $\mu\text{g}/\text{mL}$) to the system provided significant protection against A549 injury and death,

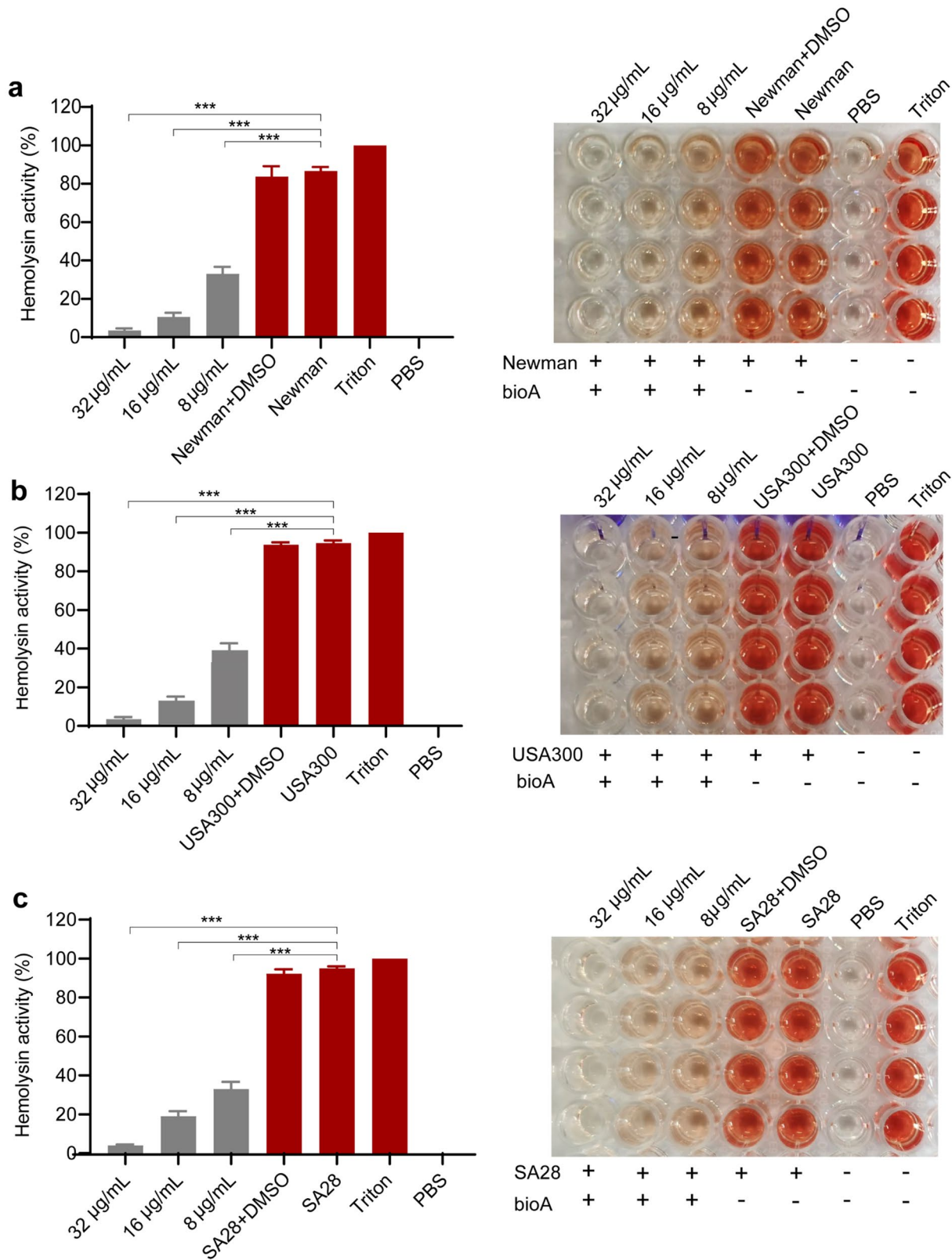


Fig. 2 Effects of bioA on the hemolytic activity of *S. aureus* strains. Effects of bioA on the hemolytic activity of the **A** *S. aureus* Newman, **B** USA300 and **C** clinically isolates of the MRSA strains SA28 (n=4). *** $P < 0.001$ was calculated with the one-way ANOVA

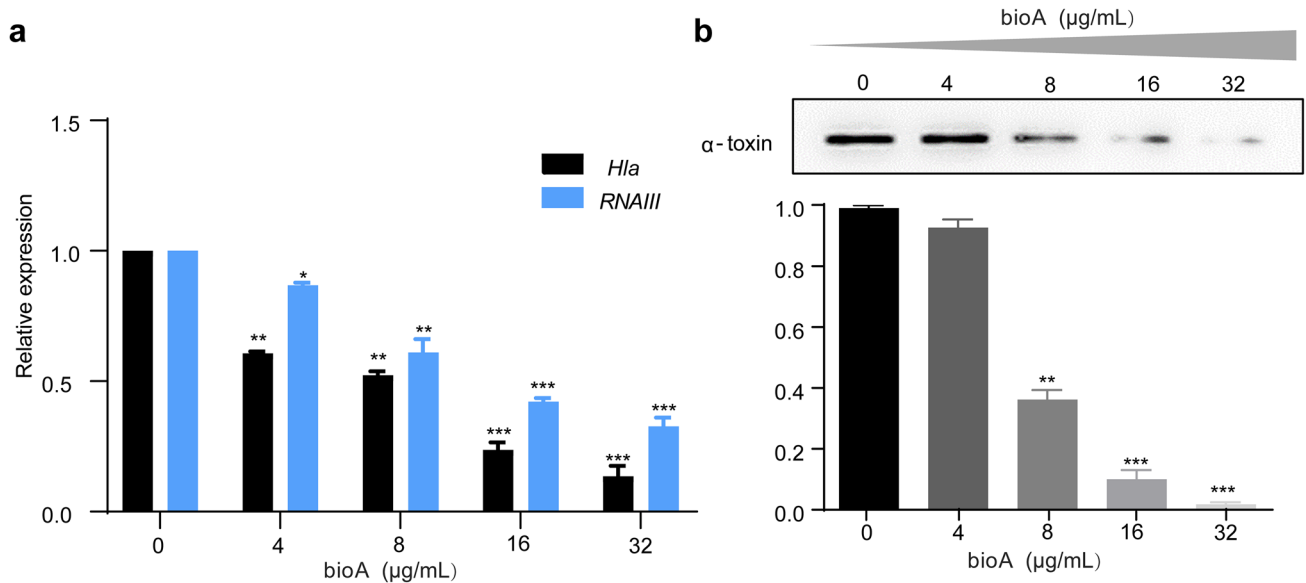


Fig. 3 Determination of bioA affected the expression of Hla and the transcription of the related regulatory gene. **A** The effects of different concentrations of bioA on the transcription of *Hla* and *RNAIII* in *S.*

aureus USA300 (n=3). **B** Western blot analysis of Hla expression by *S. aureus* USA300 treatment with bioA (n=3). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were calculated using one-way ANOVA

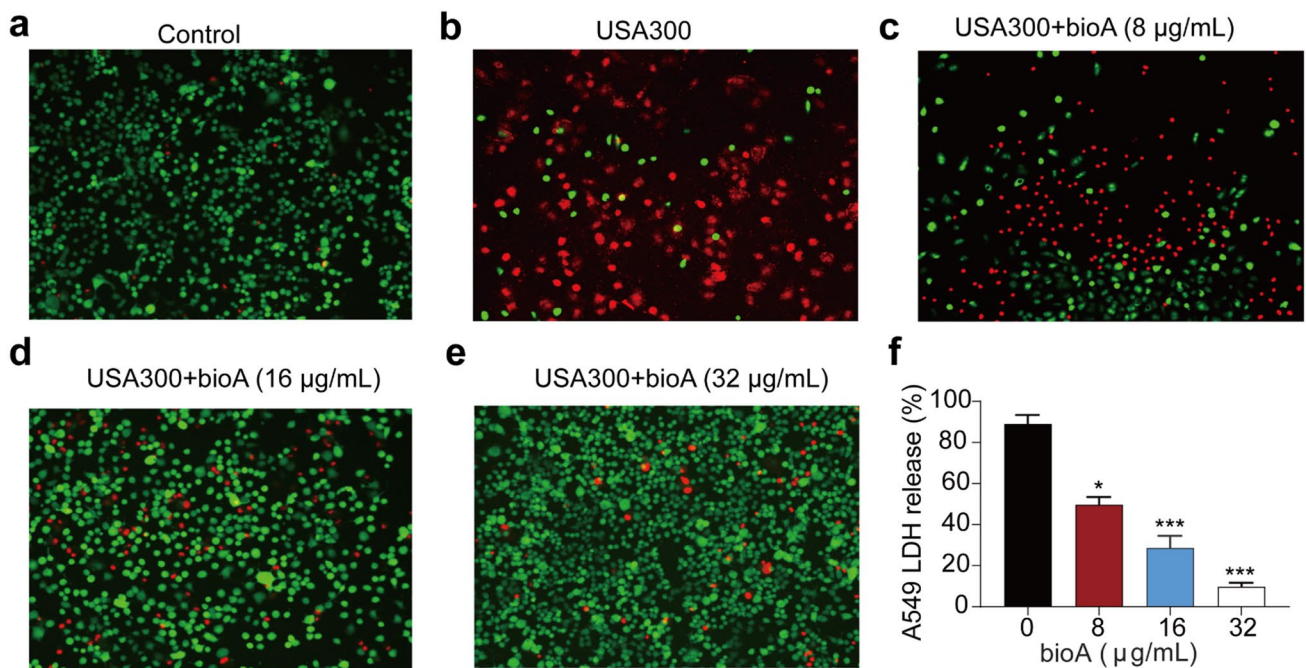


Fig. 4 Effects of bioA on A549 cells injury induced by *S. aureus* Hla. A549 cells were stained with live (green)/dead (red) agent. **A** A549 cells, **B** A549 cells were infected by *S. aureus* USA300. **C–E** A549 cells were infected by USA300 with bioA (8, 16 or 32 µg/mL). **F** The

effect of bioA on *S. aureus* USA300 induced A549 cells cytotoxicity by detecting the release of LDH (n=3). * $P < 0.05$, *** $P < 0.001$ compared with the untreated group

as indicated by a significant reduction in red fluorescence (Fig. 4C–E).

Subsequently, *S. aureus*-induced cytotoxicity was further determined by detecting the release of LDH.

Consistent with the results of the live and dead fluorescent assay, the bioA treatment group inhibited A549 cell death induced by *S. aureus* in a dose-dependent manner. BioA

has a significant protective effect against A549 cell injury induced by *S. aureus*.

BioA alleviates lung injury caused by *S. aureus* infection

To evaluate the therapeutic activity of bioA in the lung model, mice were challenged with *S. aureus* (2×10^8 CFUs) followed by treatment with bioA (50 mg/kg). The untreated mice began to die at 12 h after inoculation with *S. aureus* USA300, and the survival rate was 20% within 72 h. After treatment with 50 mg/kg bioA, the survival rates of the mice were 50% within 72 h (Fig. 5A). Administration of bioA significantly improved the survival rate and prolonged the survival time of the mice ($P < 0.01$).

The W/D ratio was further measured to evaluate the effect of bioA on the degree of pulmonary edema. As shown in Fig. 5B when the mice were infected with *S. aureus* USA300, the pulmonary W/D ratio increased from 4.04 ± 0.18 to 8.62 ± 0.25 . After treatment with bioA, the W/D ratio recovered to 6.30 ± 0.27 , suggesting that bioA significantly reduce the degree of pulmonary edema ($P < 0.01$). In addition, the level of Hla in the alveolar lavage fluid was detected by ELISA. As shown in Fig. 5C, compared with the untreated group, the level of Hla in the alveolar lavage

Table 1 Primers used in this study

Primer name	Sequences (5'–3')
16sRNA-F	TACACACCGCCCGTCACA
16sRNA-R	CTTCGACGGCTAGCTCCAAAT
Hla-F	ACAATTTTAGAGAGCCCAACTGAT
Hla-R	TCCCAATTTTGATTACCACAT
RNAIII-F	AATTAGCAAGTGAGTAACATTGCTAGT
RNAIII-R	GATGTTGTTTACGATAGCTTACATGC

fluid decreased significantly after bioA treatment ($P < 0.01$). Subsequently, we evaluated the colony count in the lung tissue in each group. For the untreated infected mice, the colony count was $9.81 \pm 0.11 \log_{10}$ CFU/g. After treatment with 50 mg/kg bioA, the number of bacteria in the lungs was significantly reduced to $6.54 \pm 0.32 \log_{10}$ CFU/g compared with the untreated group. BioA significantly reduced the number of viable *S. aureus* USA300 in the lungs of the mice (Fig. 5D).

Histopathology was used to examine the changes in the lung tissue of the mice in each group. The lungs of the untreated infected mice showed serious acute injury, which was characterized by congestion and edema in the interstitial area and a large amount of inflammatory cell infiltration. After treatment with 50 mg/kg bioA, the inflammatory

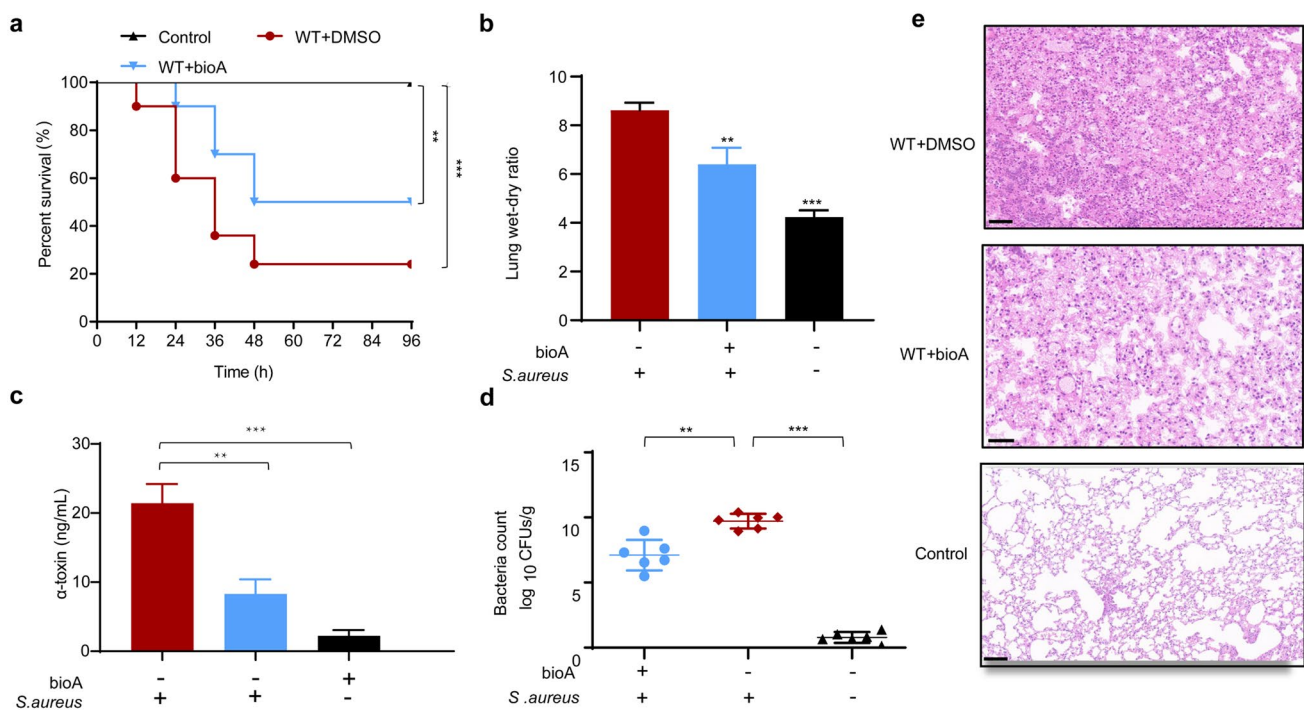


Fig. 5 The therapeutic and protective effects of bioA on mice. **A** Effect of bioA treatment on the survival of mice ($n = 10$) infected with *S. aureus* USA300 at 12 h intervals for 96 h. **B** W/D ratio of lung tissue of mice ($n = 6$) infected with the *S. aureus* USA300 and treated with bioA (50 mg/kg). **C** Hla level in alveolar lavage fluid

of mice ($n = 6$) treated or untreated with bioA after infection with *S. aureus* USA300. **D** Effect of bioA treatment on bacterial load in lungs of mice ($n = 6$). **E** Histopathology of the mouse lung tissue after 48 h of treatment with bioA or DMSO. Lung sections were analyzed by H&E staining. Scale bar, 50 μ m

symptoms and inflammatory cell infiltration were significantly reduced (Fig. 5E). In short, these results indicated that bioA protected mice from a lethal *S. aureus* challenge in vivo.

Discussion

MRSA is a multidrug-resistant pathogen that poses a significant risk to global health today. Antibiotics have always played a leading role in the prevention and therapy of diseases caused by *S. aureus*. However, the resistance of *S. aureus* to antibiotics is increasing rapidly, and the development of new antibiotics is difficult, slow, and far behind the pace of development of drug-resistant strains. Innovative anti-infective drugs are urgently needed to overcome drug resistance. Targeting bacterial virulence is an alternative antimicrobial therapy that provides a promising opportunity to inhibit pathogenesis and will not cause direct death to the target bacteria. Many virulence factors have been proven to be potential targets for drug design and therapeutic intervention. Targeted virulence represents a new model to enable clinicians to prevent and treat current multidrug-resistant pathogens (Diep et al. 2016).

The pathogenicity of *S. aureus* mainly depends on the secretion of a large number of extracellular toxins. In general, infections caused by bacterial toxins are becoming uncontrollable. Hla has been proven to be a significant pathogenic toxin secreted by *S. aureus*. This small β -barrel pore-forming cytotoxin dissolves erythrocytes and leukocytes but not neutrophils by binding to the protein receptor ADAM10, a disintegrin and metalloproteinase (Valeva et al. 1997; Wilke and Bubeck Wardenburg 2010). Therefore, Hla is an ideal target to fight *S. aureus* infection.

The main characteristic of Hla is its ability to dissolve red blood cells, and rabbit blood cells are especially susceptible to this effect, being 100 times higher than that of other mammalian animal cells (Bernheimer and Schwartz 1963; Bhakdi et al. 1984). Based on this, hemolysin inhibitors were screened from an in-house library containing 300 natural small molecular compounds. Many natural products from microorganisms and plants have been reported as candidate antivirulence agents against *S. aureus* and have shown specific activity and high safety (Wu et al. 2019a). We identified the natural compound bioA that could block the hemolytic activity of MSSA, MRSA and MRSA clinical isolates against rabbit blood cells. The supernatant of *S. aureus* treated with bioA (32 $\mu\text{g}/\text{mL}$) almost completely lost hemolytic activity. As a natural flavonoid, bioA has been found in a variety of plants and has a variety of pharmacological functions. Its development cost is much lower than that of antibiotics and vaccines.

To further clarify the mechanism by which bioA inhibits hemolysis, RT-qPCR and western blotting experiments were carried out. BioA decreased the level of *Hla* transcription by downregulating *RNAIII*, which was consistent with the western blot results. BioA significantly inhibited hemolytic activity by affecting the expression of Hla. As one of the major toxins secreted by *S. aureus*, Hla has been proven to affect a variety of human cell types, including epithelial cells, macrophages and endothelial cells (Berube and Bubeck Wardenburg 2013; Powers et al. 2012). In our experiment, we observed that the survival rate of A549 cells was significantly improved after adding bioA. In addition, Hla mutants of *S. aureus* have been shown to significantly reduce the pathogenicity in a variety of models, such as skin and soft tissue infection and pneumonia (Bubeck Wardenburg et al. 2007). BioA has significant therapeutic effects on *S. aureus*-induced pneumonia in mice, including improving the survival rate of the mice, prolonging the survival time, reducing the lung colony number, decreasing the D/W ratio of the lung tissue, reducing the level of Hla in alveolar lavage fluid and alleviating lung tissue damage.

Collectively, in the face of the global antibiotic crisis, there is an urgent need for new clinical treatments. BioA has significant anti-Hla activity in vivo and in vitro and can be further developed against *S. aureus* infection.

Author contributions Material preparation, data collection and analysis were performed by JF, DS, XL, LW, DY and JG. The first draft of the manuscript was written by LW and XW; YZ and HY revised the manuscript; WS, BW conceived and designed the experiments. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declared that they have no conflicts of interest in this work.

Ethical approval The animal work in this report was approved by the Experimental Animal Ethics Committee of Changchun University of Chinese Medicine, and it complied with the regulations on the use of experimental animals.

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