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α-Cyperone Alleviates Lung Cell Injury Caused by *Staphylococcus aureus via* Attenuation of α-Hemolysin Expression

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In this study, we aimed to evaluate the effect of α-cyperone on *S. aureus*. We used a hemolysin test to examine the hemolytic activity in supernatants of *S. aureus* cultured with increasing concentrations of α-cyperone. In addition, we evaluated the production of α-hemolysin (Hla) by Western blotting. Real-time RT-PCR was performed to test the expression of *hla* (the gene encoding Hla) and *agr* (accessory gene regulator). Furthermore, we investigated the protective effect of α-cyperone on Hla-induced injury of A549 lung cells by live/dead and cytotoxicity assays. We showed that in the presence of subinhibitory concentrations of α-cyperone, Hla production was markedly inhibited. Moreover, α-cyperone protected lung cells from Hla-induced injury. These findings indicate that α-cyperone is a promising inhibitor of Hla production by *S. aureus* and protects lung cells from this bacterium. Thus, α-cyperone may provide the basis for a new strategy to combat *S. aureus* pneumonia.

Keywords: *Staphylococcus aureus*, α-cyperone, α-hemolysin, antivirulence

Staphylococcus aureus is a major pathogen that causes a broad range of local and systemic infections, ranging from minor skin infection to life-threatening septicemia, endocarditis, and pneumonia in both communities and healthcare settings [19]. Among the invasive diseases associated with *S. aureus*, respiratory tract infection ranks second and is reported to cause higher mortality than any other nosocomial infection, ranging from 24% to 50% [13, 30]. The related medical costs of *S. aureus* infection per patient are as high as $35,000 and represent a great medical burden on society [30]. Moreover, the emergence of methicillin-resistant *S. aureus* (MRSA) makes the situation more serious and makes the control and treatment of *S. aureus* pneumonia even more difficult [15]. Along with the multidrug resistance phenomenon, the lagging development of new antimicrobial drugs calls for immediate alternative therapeutic solutions. Recently, therapeutic strategies targeting bacterial virulence have attracted great interest because they would not impose immediate selective pressure on pathogenic bacteria, which would otherwise quickly lead to drug resistance [6, 27] .

S. aureus expresses a wide variety of virulence factors, including exotoxin, surface-associated proteins, and enzymes, which contribute to the pathogenesis of its infections [19]. It has been widely accepted that no *S. aureus* virulence factor alone can lead to disease, although reports have revealed that different virulence factors represent the primary causative agent of different staphylococcal diseases. However, compared with wild-type bacteria, there was a significant decrease in mortality and morbidity with Hla-deficient strains in a mouse pneumonia model [4]. Hla, secreted by most *S. aureus* strains as a water-soluble 33.2 kDa monomer, is cytolytic to a variety of mammalian cells, such as monocytes, lymphocytes, endothelial cells, and particularly erythrocytes. Encoded by *hla*, it is secreted in the late exponential phase of growth under the control of the accessory gene regulator (*agr*) [8, 32]. Upon binding to susceptible cell membranes, the protein first oligomerizes to form a 232.4 kDa channel-forming heptamer.

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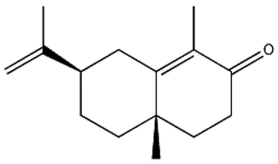

Fig. 1. Chemical structure of α-cyperone.

After oligomerization, the heptamer forms a 1 to 2 nm pore, which is the pathway for the leakage of ions, water, and small molecules out of and into the cell to induce cell injury and death [1]. Owing to its lytic property, Hla causes several types of infection. Hla mutants show less virulence in animal models of intraperitoneal, intramammary, and corneal infection in addition to pneumonia [3, 5, 23]. Therefore, the current focus on Hla in the development of potential alternative therapeutic agents for the treatment of *S. aure*us-induced infection is promising.

Cyperus rotundus (Cyperaceae) is a traditional Chinese medicinal herb used widely as an antidiarrheal, antidiabetic, anti-inflammatory, antidepressant, and analgesic [9, 10, 28]. It has been reported that *C. rotundus* oil exhibits considerable biological properties, such as antigenotoxic, antimutagenic, and antibacterial effects [11]. Previous studies showed that α-cyperone (Fig. 1), a sesquiterpene compound, is the most abundant compound of *C. rotundus* oil, representing 25.23% of the total oil [12]. We hypothesize that α-cyperone may disrupt the pathogenesis of *S. aureus*. In the present study, we investigated the effect of α-cyperone on *S. aureus* Hla expression and determined its influence on Hla-induced lung cell injury.

Materials and Methods

Bacterial Strains, Culture, and Reagents

The *S. aureus* strains used in the study are listed in Table 1. For hemolysin, Western-blot, and real-time RT-PCR assays, strains were grown at 37°C in tryptic soy broth (TSB) to an optical density at 600 nm of 2.0, 2.5, 2.5, 2.5, and 2.5 for strains wood 46, BAA-1717, ATCC 29213, 8325-4, and DU 1090, respectively. For cytotoxicity studies, *S. aureus* 8325-4 and DU 1090 were cultured in TBS at 37°C to an OD_{600nm} of 0.5. Culture aliquots (50 ml) were centrifuged and washed in phosphate-buffered saline (PBS) prior to resuspension. Then, 5 ml of culture prepared as described above was resuspended in 10 ml of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen). A 100 μl suspension was used per assay well.

α-Cyperone was commercially obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). α-Cyperone was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

Susceptibility Testing

The minimal inhibitory concentrations (MICs) of α-cyperone for *S. aureus* strains wood 46, BAA-1717, ATCC 29213, 8325-4, and DU 1090 were determined by the broth dilution method according to the Clinical and Laboratory Standards Institute performance standards for antimicrobial testing [7].

Growth Curve Assay

S. aureus strain 8325-4 was cultured in TBS at 37°C to an $OD_{600\ nm}$ of 0.3, and 100 ml volumes were aliquoted into five 250 ml Erlenmeyer flasks. Four cultures containing graded subinhibitory concentrations of α-cyperone and one culture without α-cyperone used as a control were grown with constant shaking (200 rpm) at 37°C under aerobic conditions. OD_{600nm} values of the cultures at 30 min intervals were used to monitor cell growth.

Hemolysis Assay

Hemolysis activity was assessed as previously described with rabbit red blood cells [29]. Briefly, 0.5 ml bacterial samples were centrifuged (5,500 ×*g*, 4°C, 1 min), and then 100 μl of the supernatants was added to 1 ml of PBS. Twenty-five microliters of defibrinated rabbit erythrocytes was then added and the samples were incubated for 20 min at 37°C. The samples were then centrifuged at 5,500 ×*g* for 1 min. The absorbance of the supernatants was determined at $OD_{543\ nm}$.

Western Blot Assay

For the Western blot assay, culture supernatants were collected and boiled in Laemmli sample buffer, and then prepared for analysis by sodium dodecyl sulfate (SDS)-polyacrylamide (12%) gel electrophoresis [17]. Proteins were blotted on polyvinylidene fluoride membranes (Wako Pure Chemical Industries, Ltd). After blocking in 5% bovine serum for 2 h, the membranes were stained with anti-Hla antibody (Sigma-Aldrich; diluted 1:8,000) for 1 h, followed by incubation

Table 1. Strains used in this study and their associated MICs of α-cyperone.

S. aureus strain	Description	Source	MIC (μg/ml)[a]
			α-Cyperone
ATCC 29213	β-Lactamase-producing, oxacillin-susceptible, Hla-producing strain	ATCC	256
ATCC 10832	Wood 46, a natural isolate that produces high levels of Hla	ATCC	256
BAA-1717	USA300-HOU-MR, isolated from adolescent patient with severe sepsis syndrome in Texas Children's Hospital, Hla-producing strain	ATCC	256
8325-4	High-level Hla-producing strain derived from NCTC 8325	Timothy J. Foster	256
DU 1090	Hla-negative mutant of *S. aureus* 8325-4, prepared by insertion of a transposon in the *hla* gene	Timothy J. Foster	256

[a]The MICs of α-cyperone for *S. aureus* strains are representative of three independent experiments.

Table 2. Primers used in real-time RT-PCR.

Primer	Sequence	Location within gene
16S rRNA gene-forward	5'-GCTGCCCTTTGTATTGTC-3'	287–305
16S rRNA gene-reverse	5'-AGATGTTGGGTTAAGTCCC-3'	446–465
agrA-forward	5'-TGATAATCCTTATGAGGTGCTT-3'	111–133
agrA-reverse	5'-CACTGTGACTCGTAACGAAAA-3'	253–274
hla-forward	5'-TTGGTGCAAATGTTTC-3'	485–501
hla-reverse	5'-TCACTTTCCAGCCTACT-3'	569–586

with horseradish peroxidase-conjugated anti-rabbit antiserum (Sigma-Aldrich; diluted 1:4,000) for 0.5 h. The blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare).

Real-Time RT-PCR

S. aureus 8325-4 was cultured *in vitro* in TBS with or without subinhibitory concentrations of α-cyperone to an OD_{600} of 2.5. The RNA was isolated as previously described [31]. Following centrifugation (5,000 ×*g*, 4°C, 5 min), cells were harvested and resuspended in TES buffer containing 100 μg/ml lysostaphin (Sigma-Aldrich). The samples were incubated at room temperature for 10 min and then applied to a Qiagen RNeasy Maxi column for isolation of total bacterial RNA according to the manufacturer's protocol. To remove contaminating DNA, treatment with RNase-free DNase I (Qiagen) was performed. The quality, integrity, and concentration of the purified RNA were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. The primer pairs used for real-time RT-PCR are listed in Table 2. RNA was reverse transcribed into cDNA using a Takara RNA PCR kit (AMV) Ver. 3.0 (Takara) as described by the manufacturer. cDNA was stored at −20°C until needed. PCR was performed in 25 μl volumes using SYBR Premix Ex TaqTM (Takara) according to the manufacturer's directions. PCR amplification was performed using the 7000 Sequence Detection System (Applied Biosystems). All PCR reactions were done in triplicate and the 16S rRNA gene served as an endogenous control to normalize the changes in transcription levels between samples.

Live/Dead and Cytotoxicity Assays

A549 human lung epithelial cells (ATCC CCL 185) were cultured in DMEM with 10% fetal bovine serum (Invitrogen). Cells were plated in 96-well plates at a density of 2.0×10^4 cells per well. For both assays, A549 cells were incubated in triplicate with 100 μl of staphylococcal suspension prepared as described above with indicated concentrations of α-cyperone for 6 h at 37°C. After incubation, cells were treated with either live/dead (green/red) reagent (Invitrogen) or by detecting the lactate dehydrogenase (LDH) activity using a Cytotoxicity Detection Kit (LDH) (Roche) at the recommendation of the manufacturer. Microscopic images of stained cells were obtained using a Confocal Laser Scanning Microscope 1 (Nikon). LDH activity was measured on a microplate reader (Tecan).

Statistical Analysis

The experimental data were analyzed with SPSS 12.0 statistical software. An independent Student's t-test was used to determine statistical significance and a p value less than 0.05 was considered to be statistically significant.

RESULTS

Influence of α-Cyperone on *S. aureus* Growth

As shown in Table 1, the MIC of α-cyperone for all of the *S. aureus* strains tested was 256 μg/ml. The growth curves for *S. aureus* cultured with 2 to 16 μg/ml of α-cyperone are presented in Fig. 2. The data indicate that these concentrations of α-cyperone have no influence on *S. aureus* growth.

α-Cyperone Decreases Hla Production by *S. aureus* Strains

A hemolysis assay was performed using rabbit erythrocytes to determine the hemolytic activity of *S. aureus* culture supernatants in the presence of subinhibitory concentrations of α-cyperone. As shown in Table 3, the drug-free culture supernatants served as 100% hemolysis controls. When cultures were treated with 16 μg/ml of α-cyperone, the hemolytic activity of the supernatants was 6.3%, 4.4%, 12.6%, and 6.1% for *S. aureus* ATCC 29213, BAA-1717, Wood 46, and 8325-4, respectively. Apparently, α-cyperone inhibited the hemolytic activity of *S. aureus* culture supernatants in a concentration-dependent manner (2 to

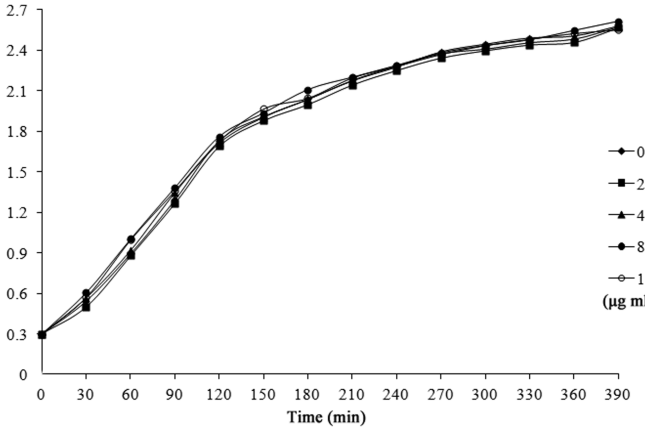

Fig. 2. Growth curve for *S. aureus* 8325-4 cultured with or without α-cyperone.

◆, ■, ▲, ●, and ○ represent *S. aureus* 8325-4 grown in TSB with 0, 2, 4, 8, and 16 μg/ml of α-cyperone, respectively. The data shown are representative of three independent experiments.

Table 3. Hemolytic activity of Hla produced by *S. aureus* co-cultured with subinhibitory concentrations of α-cyperone.

Strain	Hemolysis (%) of rabbit erythrocytes by culture supernatant[a]				
	0	2 μg/ml	4 μg/ml	8 μg/ml	16 μg/ml
ATCC 29213	100	86.6 ± 1.5**	70.7 ± 2.2**	24.8 ± 0.4**	6.3 ± 0.5**
ATCC 10832	100	82.3 ± 3.9**	74.3 ± 1.3**	56.3 ± 6.5**	12.6 ± 0.7**
BAA-1717	100	74.7 ± 4.2*	44.1 ± 3.1**	5.9 ± 5.1**	4.4 ± 1.1**
8325-4	100	83.4 ± 1.4**	56.4 ± 0.7**	50.4 ± 1.3**	6.1 ± 0.5**

[a]Drug-free culture supernatants served as 100% hemolysis controls.
Values represent the mean and standard error of three independent experiments.
*indicates $p<0.05$ and **indicates $p<0.01$ compared with the α-cyperone-free culture.

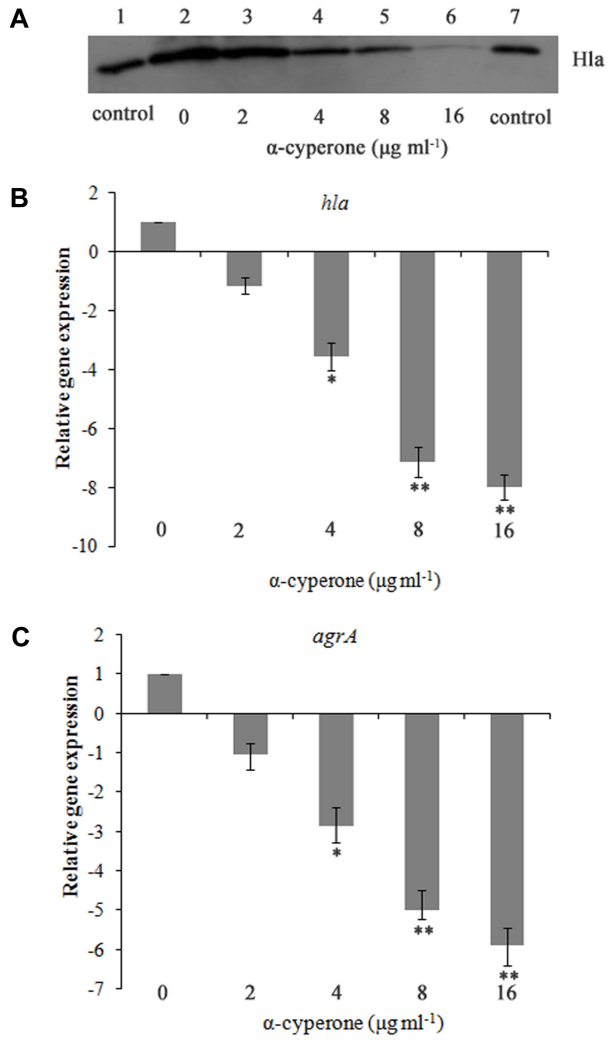

Fig. 3. The influence of α-cyperone on *S. aureus* Hla secretion. (**A**) Western blot of Hla. Supernatants of *S. aureus* 8325-4 cultured with graded concentrations of α-cyperone were subjected to SDS-PAGE. After proteins were transferred to polyvinylidene fluoride membranes, Hla was detected with specific rabbit antibodies. Ten nanograms of purified Hla was used as the control (lanes 1, 7). Data are representative of three independent experiments. (**B**) Relative expression of *hla* in *S. aureus* 8325-4 after growth with increasing concentrations of α-cyperone; (**C**) relative expression of *agrA*. Values in **B** and **C** represent the mean and standard error of three independent experiments. *indicates $p<0.05$ and **indicates $p<0.01$ compared with the α-cyperone-free culture.

16 μg/ml). The Hla-defective mutant, DU 1090, cannot cause lysis of rabbit erythrocytes (data not shown). We then determined the effect of subinhibitory concentrations of α-cyperone on the secretion of Hla by *S. aureus* by Western blot analysis. Notably, following treatment with 16 μg/ml of α-cyperone, Hla in the bacterial culture supernatants was almost undetectable (Fig. 3A). Remarkably, α-cyperone reduced the production of Hla in a dose-dependent manner. The result was consistent with that of the hemolysis assay. Thus, α-Cyperone can reduce the hemolytic activity of *S. aureus* by reducing the production of Hla. Furthermore, we used real-time RT-PCR to investigate the effect of α-cyperone on transcription of *hla* and *agrA* in *S. aureus* 8325-4. α-cyperone diminished the transcription of *hla* and *agrA* in a dose-dependent manner (Fig. 3B and 3C). When *S. aureus* 8325-4 was cultured with 16 μg/ml of α-cyperone, the *hla* and *agrA* transcription levels were decreased 8.0- and 5.8-fold, respectively. The production of Hla in *S. aureus* was positively controlled by the Agr regulatory system. Consequently, based on the observation that α-cyperone inhibits the transcription of *agrA*, it may be inferred that the mode of action by which α-cyperone reduces the production of Hla may be partially due to the inhibition of the Agr locus.

Effect of α-Cyperone on Hla-Mediated A549 Human Alveolar Epithelial Cell Injury

Hla has been proven to be an essential factor for the induction of human alveolar epithelial cell (A549) injury and death by *S. aureus* [4]. A549 cells were co-cultured with *S. aureus* 8325-4 in the presence of increasing concentrations of α-cyperone, and then cell viability was observed using live/dead (green/red) reagent (Fig. 4). Uninfected A549 cells retained the green fluorophore (Fig. 4a). Co-culture of A549 with *S. aureus* 8325-4 resulted in cell death (Fig. 4b), as demonstrated by an increased number of red fluorescent cells. As expected, in the presence of 2 to 16 μg/ml of α-cyperone, the number of dead cells was decreased in a dose-dependent manner (Fig. 4c to 4f). Furthermore, we also investigated the effect of *S. aureus* DU 1090, a Hla-deficient mutant of *S. aureus* 8325-4, on cell death. As shown in Fig. 4g, the strain did

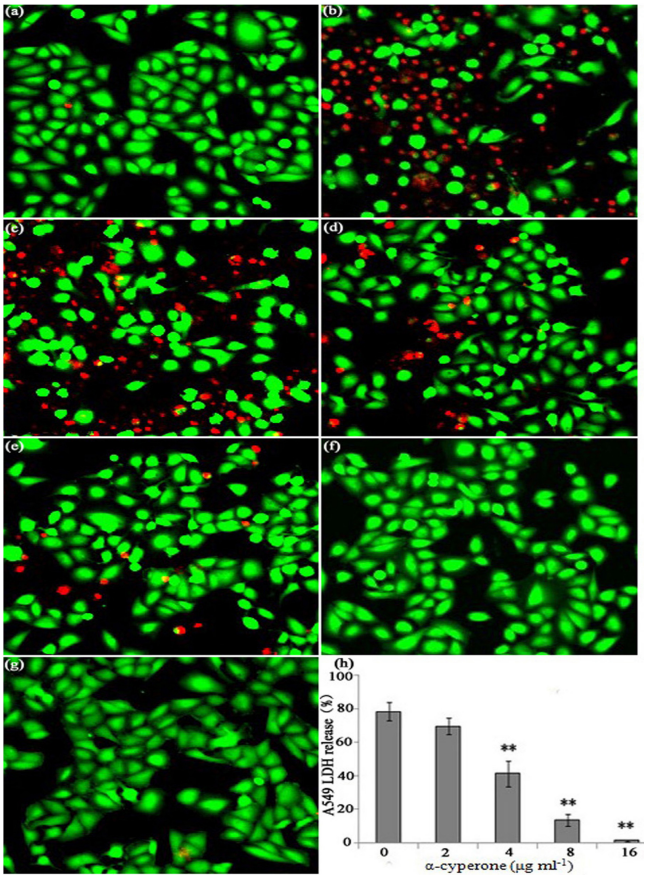

Fig. 4. α-Cyperone protects A549 human alveolar epithelial cells from *S. aureus*-induced damage.

Live/dead reagent-stained A549 alveolar epithelial cells were imaged using confocal laser scanning microscopy after infection with *S. aureus* 8325-4 or DU 1090 (100×). Live cells are stained with green fluorophore, while the presence of red fluorophore indicates dead cells. Uninfected A549 cells (**a**); *S. aureus* 8325-4-infected cells in the absence (**b**) or presence of 2 to 16 μg/ml of α-cyperone (**c** to **f**); DU 1090-infected cells (**g**). LDH release by A549 cells (**h**). The data shown in panels **a** to **g** are representative of three independent tests. Values in panel **h** represent the mean and standard error of three independent experiments. *indicates $p<0.05$ and **indicates $p<0.01$ compared with the α-cyperone-free culture.

not lead to significant injury and death of A549 cells, indicating that Hla plays a crucial role in *S. aureus* lung injury, as previously reported.

We also quantified cell death by detecting LDH release. LDH can be released to the culture supernatant through damaged cell membranes, thus indicating cell death. In the drug-free culture supernatants, the death rate was 78.3%. In the presence of 2 to 16 μg ml^{-1} of α-cyperone, the degree of LDH release indicated that cell death was diminished to 69.5%, 41.3%, 13.9%, and 1.6%, respectively (Fig. 4h). As indicated above, 16 μg ml^{-1} of α-cyperone did not inhibit *S. aureus* growth; thus, the reduction in cell injury was not due to the decrease in *S. aureus* CFUs. The live/dead and LDH assay results indicate that α-cyperone prevents A549 cells from dying in a dose-dependent manner.

Discussion

S. aureus is a leading bacterial pathogen of hospital-associated pneumonia, causing high morbidity and mortality [13, 14]. Moreover, community-associated pneumonia caused by *S. aureus* is becoming a major problem in public health [20]. With the continuous emergence of MRSA, current antibiotic options have become limited and can no longer meet the need for *S. aureus* infection treatment. New therapeutic strategies and antimicrobial agents for the prevention and treatment of this fatal infection are greatly needed. In this study, we show that α-cyperone decreases Hla production and prevents Hla-mediated human alveolar epithelial cell injury (A549). We have previously reported that many natural compounds, which protect against Hla-induced lung cell damage, may protect against *S. aureus* pneumonia [24, 33]. Thus, α-cyperone may also have the potential to be used to fight *S. aureus* pneumonia, although further studies should be performed to assess the *in vivo* pharmacokinetic and toxicological characteristics, as well as *in vivo* performance in the treatment of diseases. Furthermore, the structure of α-cyperone may rationally serve as the lead structure for the development of drugs aimed at staphylococcal Hla.

S. aureus expresses numerous surface proteins and exotoxins, including protein A, Panton–Valentine leukocidin (PVL), and Hla, which contribute to the pathogenesis of pneumonia [35]. Strategies targeting bacterial virulence factors are now attracting interest as treatments for *S. aureus* infections [6, 27]. Previous studies have suggested that PVL is the main cause of *S. aureus* pneumonia [2, 16, 18]. However, recent reports have revealed that Hla is the essential virulence factor causing the infection rather than PVL in a mouse pneumonia model [4, 34]. In the process of inducing pneumonia, the toxin may bind to the lung epithelial cells to disrupt the integrity of cellular membranes, increasing the permeability of the cell and eliciting the expression of cytokines, such as interleukin-1β, thereby promoting severe lung inflammation and injury to the air–blood barrier [21].

Strategies aimed at attenuating the detrimental function of Hla in an animal model of pneumonia have proven to be successful. Wardenburg and colleagues reported that anti-Hla antibodies significantly protect against *S. aureus* pneumonia in a mouse model [26, 36]. They also found that a modified β-cyclodextrin compound, which may block the heptameric pore, can protect lungs from *S. aureus* challenge [25]. Even though there is no ideal antibiotic treatment available, antimicrobial agents are still the leading solution for *S. aureus* infection. Traditional strategies are aimed at killing bacteria or preventing their growth, causing the rapid development of drug resistance. In contrast, antivirulence strategies show no direct effect in killing the bacteria. The combination of traditional

antimicrobial drugs and anti-Hla complexes would allow lower doses of each agent to be used, putting reduced survival pressure on the pathogenic strains. Thus, combination therapy may be a promising solution in dealing with the increasing problem represented by MRSA pneumonia. Previous studies have demonstrated that subinhibitory concentrations of β-lactams, the primary option for sensitive *S. aureus* strains, can dramatically increase Hla production [22]. According to our study, a specific concentration of α-cyperone can dramatically attenuate Hla production and have a protective effect on A549 lung cells. Therefore, the combination of α-cyperone and β-lactams may be a potent treatment for *S. aureus* pneumonia.

Acknowledgments

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