Sclareol Protects Staphylococcus aureus-Induced Lung Cell Injury via Inhibiting Alpha-Hemolysin Expression

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Introduction

Staphylococcus aureus is a gram-positive pathogen that causes a wide range of infections, ranging from minor skin infections to serious diseases such as endocarditis, pneumonia, and toxic shock syndrome in humans and animals [1]. Since methicillin-resistant S. aureus (MRSA) was first reported in 1961 [2], the prevalence of MRSA has been increasing worldwide. USA300, the first described strain of community-associated MRSA (CA-MRSA), has spread throughout the USA. Over the last two decades, infections with USA300 have been reported in an increasing number of countries [3–5]. USA300 is the most widespread MRSA strain and USA300 infections have threatened global public health. Infections caused by MRSA often fail to respond to antibiotic treatment, thus making them harder to cure. Consequently, new agents are urgently required for the treatment of S. aureus infections, especially MRSA infections.

Antibiotics are the commonly prescribed drugs for S. aureus infections. However, the development of bacterial resistance has been linked to antibiotic use. Because traditional antibiotics are used to control bacterial infections by killing or restraining the multiplication of pathogens, this increases the selective pressure on these pathogenic bacteria, leading to bacterial resistance [6]. So far, some studies have found that the pathogenicity of S. aureus is closely related to the secretion of virulence factors [1]. Alpha-hemolysin (Hla) is a major cytotoxin secreted by S. aureus. USA300 has been shown to highly express Hla and is highly virulent [7, 8]. Hla binds to target host cell membranes, altering cellular permeability and causing leakage of the cytoplasm, activation of stress-signaling pathways, and cell death [9]. Hla has been shown to play a
secretion in evaluated the effect of sclareol on the inhibition of Hla

3,4,4a,6,7,8–hexahydro-1H-naphthalene-2-ol) is a fragrant
target for the development of antivirulence drugs against
S. aureus infection.

According to previous studies, some natural products have been shown to inhibit the production and activity of S. aureus Hla, and to relieve symptoms of S. aureus pneumonia in a mouse model [11–14]. Sclareol ((1R,2R,4aS,8aS)–1–[(3R)-3-hydroxy-3-methylpent-4-enyl], 3,4,4a,6,7,8-hexahydro-1H-naphthalene-2-ol) is a fragrant chemical compound (Fig. 1) found in Salvia sclarea [15]. It is classified as a bicyclic diterpene alcohol that is primarily used in cosmetics, such as decorative cosmetics, fine fragrances, shampoos, and toilet soaps [16]. Sclareol has potent pharmacological activities, including antimicrobial [15], anti-inflammatory and antitumor activities [17]. Miski et al. [15] reported that sclareol has limited activity against S. aureus. However, no studies have focused on the effects of sclareol on Hla secretion in S. aureus. In this study, we evaluated the effect of sclareol on the inhibition of Hla secretion in S. aureus USA300, using the hemolysis assay, western blotting, RT-PCR, and cell experiments.

Materials and Methods

Bacterial Strains, Cell Line, and Reagents

The CA-MRSA S. aureus strain BAA-1717 (USA300), a Hla-producing strain, used in this study was purchased from the American Type Culture Collection (ATCC). The human alveolar epithelial cell line (A549, ATCC CCL 185) was also obtained from ATCC. Sclareol (purity ≥ 98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (China). The chemical structure of sclareol is presented in Fig. 1. Sclareol was dissolved in dimethyl sulfoxide (DMSO, ≥99.5%; Sigma-Aldrich, USA) to make a stock solution (40.96 mg/ml). Defibrinated rabbit blood was purchased from Zheng Zhou Jiu Long Biological Products Co. Ltd. (China), and no animals were directly used in the experiments.

Preparation of Bacterial Cultures

For in vitro hemolysis assays, western blotting, and real-time PCR, S. aureus USA300 was cultured with the addition of varying amounts of sclareol in tryptic soy broth (TSB; Oxoid, UK) at 37°C until the cultures reached the post-exponential phase (OD600nm = 2.5). For cytotoxicity assays, S. aureus USA300 was cultured at 37°C in TSB to OD600nm = 0.5. Five milliliters of bacterial culture was pelleted (1 min, 1,000 × g, 4°C) and washed with sterile phosphate-buffered saline (PBS). The pellet was then resuspended in 10 ml of Dulbecco’s modified Eagle’s medium (DMEM).

Susceptibility Testing

The broth microdilution method was used to determine the minimal inhibitory concentration (MIC) of sclareol against S. aureus USA300 and was performed according to the Clinical and Laboratory Standards Institute [18]. The MIC was defined as the lowest concentration of the agent that inhibited bacterial growth. The MIC experiment was repeated three times.

Growth Curve Assay

S. aureus USA300 was grown in 500 ml of TSB at 37°C to OD600nm = 0.3. The cell culture was divided into five flasks (250 ml), and sclareol was added to a final concentration of 0, 1, 2, 4, or 8 μg/ml. DMSO (0.04%) was used for the solvent control group. The flasks were incubated at 37°C with constant shaking (200 rpm) and bacterial growth was determined by measuring the absorbance of the cultures at OD600nm at intervals ranging from 0 to 390 min after treatment.

Hemolysis Assay

Sclareol was investigated for its ability to inhibit the hemolytic activity of S. aureus culture supernatants. Bacterial cultures (1 ml) were centrifuged (5,000 × g, 20°C, 2 min) and sterilized by filtration through a 0.2 μm filter. One-hundred microliters of culture supernatant were transferred to a sterile tube, to which 875 μl of sterile PBS and 25 μl of defibrinated rabbit red cells were added and then mixed. The mixture was incubated at 37°C for 15 min. All samples were then centrifuged (10,000 × g, 20°C, 1 min). Hemolytic activity was determined by measuring the OD600nm values of the supernatant of the mixtures. A negative control (without sclareol) served as 100% hemolysis, and all the percentage hemolysis of the test groups were compared with that of the drug-free control.

Western Blotting

Secretion of Hla in the bacterial cultures was detected by western blotting. Bacterial cultures (1 ml) were centrifuged (5,000 × g, 20°C, 2 min) and filtered (0.2 μm) to remove residual bacteria. Samples (25 μl) were boiled with Laemmli sodium dodecyl sulfate (SDS)
sample buffer for 5 min and loaded onto a 12% SDS-polyacrylamide gel. Western blotting was performed as previously described [19]. In short, proteins were transferred to polyvinylidene fluoride membranes after SDS-PAGE. An anti-\textit{S. aureus} Hla antibody (Sigma-Aldrich) was diluted 1:8,000 and used as the primary antibody. An anti-rabbit antiserum conjugated to horseradish peroxidase (Sigma-Aldrich) was diluted 1:5,000 and used as the secondary antibody. Proteins of interest were detected using ECL western blotting detection reagents (Chemidoc MP; Bio-Rad, USA).

RNA Isolation and Real-Time Reverse Transcriptase PCR

The expression levels of hla and RNAIII were determined using real-time reverse transcriptase-PCR. Bacterial cultures, prepared in the same manner as for the hemolysis assay, were harvested by centrifugation (5,000 \textit{x}g, 4°C, 5 min) and the pellet was resuspended in TES containing 100 \mu g/ml lysostaphin (Sigma-Aldrich). Total RNA was isolated from the bacterial cultures according to the manufacturer’s instructions. RNA quantity and purity were determined by measuring the optical density at 260 nm with a UV spectrophotometer (Agilent Technologies, USA). RNA was reverse transcribed into cDNA (Takara, Japan) following the manufacturer’s directions. PCR amplification was assessed by a Real-Time System (CFX Connect; Bio-Rad). All results were repeated three times. The housekeeping gene 16S rRNA was selected as an internal control to determine changes in transcription levels between samples. The results were analyzed with ABI Prism 7000 SDS software. The DNA sequences of the PCR primers were as follows: hla, 5'-TTGGTGCCAAATGTTC-3' (forward) and 5'-TCA CTTTCCAGGCTACT-3' (reverse); RNAIII, 5'-TTCACTGGTGG ATAAACCAA-3' (forward) and 5'-CGGAGGGATGTATATCC-3' (reverse); 16S rRNA, 5'-GCTGCCCTTTGTATTGTC-3' (forward) and 5'-AGATG TTGGGTTAAGTCCC-3' (reverse).

Live/Dead and Cytotoxicity Assay

Human alveolar epithelial A549 cells were grown in minimal essential medium (MEM; Invitrogen, USA) containing d-glucose (4.5 mg/ml; Merck, USA), heat-inactivated fetal calf serum (10%; Bioind, USA), and penicillin/streptomycin (100 U/ml; Sigma) at 37°C in a CO\textsubscript{2} incubator. The cells were washed with sterile PBS and resuspended in DMEM. A 100 \mu l volume of cells (2.0 × 10\textsuperscript{4} cells) was seeded in each well and incubated at 37°C for 12–24 h in CO\textsubscript{2} incubator. For live/dead staining and cytotoxicity assays, 100 \mu l of bacterial suspension was added to A549 cells per assay well (96-well plate) in DMEM with different concentrations of sclareol and incubated at 37°C. After incubation for 6 h, live/dead (green/red) staining (Invitrogen) was used to determine cell viability. The production of lactate dehydrogenase (LDH) in the cell culture medium was measured using the Cytotoxicity Detection kit (LDH) (Roche, Switzerland). A confocal laser scanning microscope (Nikon, Japan) was used to scan stained cells and acquire microscopic images. The amount of released LDH was measured by a microplate reader (Tecan, Austria) at an absorbance of 490 nm.

Fig. 2. Growth curves of \textit{Staphylococcus aureus} strain BAA-1717 (USA300) in TSB with or without sclareol. Symbol ●, ■, ▲, ◆, and x represent \textit{Staphylococcus aureus} grown in TSB with 0, 1, 2, 4, and 8 \mu g/ml of sclareol, respectively.

Statistical Analysis

Statistical analyses were performed with SPSS 13.0 software. The results were analyzed for significance using an independent Student’s \textit{t}-test, and a \textit{p}-value of <0.05 was considered to be statistically significant.

Results

Effect of Sclareol on \textit{S. aureus} Growth

The MIC of sclareol against USA300 was 32 \mu g/ml. A growth curve assay was performed on USA300 with different concentrations of sclareol, ranging from 1 to 8 \mu g/ml. No inhibition was observed in the drug-treated groups compared with the drug-free group (Fig. 2). These data indicate that sclareol has no effect on the growth of \textit{S. aureus} at the tested concentrations.

Sclareol Inhibits Hemolytic Activity of \textit{S. aureus} Culture Supernatants

To examine the effect of sclareol on the hemolytic activity of bacterial culture supernatants, hemolysis assays were performed. The hemolytic activity of the bacterial culture medium was dose dependent and decreased after treatment with sub-inhibitory concentrations of sclareol (Fig. 3). A significant reduction was observed when bacteria were cultured with 2 \mu g/ml of sclareol. The percentage hemolysis of \textit{S. aureus} USA300 when cultured with 4 \mu g/ml of sclareol was 40.83\%, compared with the negative group. Almost no hemolytic activity (2.71\%) was seen at a sclareol concentration of 8 \mu g/ml. Additionally, no further inhibitory effect on hemolysis was seen even with sclareol concentrations up to 64 \mu g/ml (data not shown). The results showed that sclareol...
did not cause hemolysis of rabbit red blood cells at the tested concentrations. Sclareol inhibited the hemolytic activity of the culture supernatants in a dose-dependent manner in this study.

Sclareol Decreases the Secretion of Hla in Culture Supernatant

Hla is the major extracellular secreted protein of *S. aureus* and is responsible for the hemolysis of rabbit erythrocytes [20]. Western blotting analysis was used to detect changes in the amount of Hla in spent bacterial culture medium. The results showed that the amount of Hla in the culture supernatants decreased with increasing sclareol concentrations (Fig. 4). At 2 μg/ml of sclareol, Hla production was visibly decreased. However, Hla could not be detected in supernatants from cultures that had been incubated with a dose of 8 μg/ml sclareol. These results showed that sclareol reduced Hla production in *S. aureus* in a concentration-dependent manner, which coincided with a decrease in the hemolytic activity of the bacterial supernatants.

Sclareol Attenuates *hla* and *argA* Transcription in *S. aureus*

Based on the results of hemolysis assay and western blotting, the transcription of *hla* (the Hla protein-encoding gene) in USA300 was quantified using RT-PCR after treatment with various concentrations of sclareol. In *S. aureus*, transcription of *hla* is positively controlled by the *agr* two-component regulatory system. The transcription of RNAIII was investigated, as RNAIII is an effector molecule of the *agr* system. As shown in Fig. 5, a concentration-dependent decrease in the transcription of *hla* and RNAIII was observed in *S. aureus* USA300 when cultured with sclareol. At a dose of 8 μg/ml sclareol, the relative transcription levels of *hla* and RNAIII in *S. aureus* USA300 were 9.0 and 11.5%, respectively, compared with the drug-free group.

Sclareol Antagonizes *S. aureus*-Mediated A549 Cell Injury

A549 alveolar epithelial cells are commonly used as a cell model in physiological and biological studies on pulmonary diseases [21]. Previous studies have demonstrated that Hla plays a crucial role in *S. aureus*-induced human alveolar epithelial cell injury [22, 23]. In the co-culture system, A549 cells were cultured with USA300 and were used to study the protective effect of sclareol on Hla-mediated cell injury. Live/dead (green/red) staining was used to determine the amount of cell death, where dead cells fluoresced red and

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**Fig. 3.** Hemolytic activity of Hla produced in supernatant from *Staphylococcus aureus* strain BAA-1717 (USA300) cultured with sub-inhibitory concentrations of sclareol. The data shown are representative of three independent experiments. *indicates $p < 0.05$ and ** indicates $p < 0.01$, compared with the sclareol-free culture.

**Fig. 4.** Western blotting assays of Hla secreted by *Staphylococcus aureus* strain BAA-1717 (USA300) grown with or without sclareol. Equivalent volumes of supernatant from cells grown to the same optical density were loaded.

**Fig. 5.** Relative expression of *hla* and RNAIII in *Staphylococcus aureus* strain BAA-1717 (USA300) after growth with various concentrations of sclareol. Results are presented as the mean ± SD for three independent experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$, compared with the sclareol-free culture.
live cells fluoresced green under confocal laser scanning microscopy. As presented in Fig. 6A, uninfected A549 cells fluoresced green with almost no red fluorescence. When co-cultured with \emph{S. aureus} USA300, cell death was apparent and there were more red fluorescent cells, and cells with unusual cellular morphology were seen (Fig. 6B). However, treatment with 2 and 8 $\mu$g/ml (Figs. 6C and 6D) of sclareol caused a notable reduction in cell injury.

We also quantitated the amount of LDH in the cell culture medium to examine the protective effect of sclareol on A549 cellular injury, using an LDH release assay kit. The results are shown as percentage of cell death. A concentration-dependent decrease in cell death was seen at a level of 1 to 8 $\mu$g/ml of sclareol in the co-culture system (Fig. 6E). In the sclareol-free group, 97.0\% cells were damaged; however, when 8 $\mu$g/ml of sclareol was used, only 9.5\% cells died. Previous experiments showed that sclareol has no effect on the growth of \emph{S. aureus} in this concentration range. Therefore, sclareol did not appear to protect A549 cells by killing \emph{S. aureus}.

**Discussion**

MRSA is an important cause of hospital-acquired and community-associated infections globally and is resistant to several antibiotics. The Center for Disease Control and Prevention has reported that more than 2\% of people worldwide carry MRSA. MRSA causes serious diseases, including lethal pneumonia, severe sepsis, and septic shock. Obviously, MRSA infections have become an important problem for clinicians worldwide and have increased the burden of diseases. In Europe, approximately EUR 380 million is spent on extra hospitalization expenses caused by MRSA every year [24]. Antibiotics are still the mainstay of treatment for MRSA infections. Consequently, the antibiotic resistance of MRSA becomes more complicated with extensive antibiotic use. Therefore, novel therapeutic methods that do not exert selective pressure on the growth of \emph{S. aureus} are needed.
of bacteria are urgently required. Antivirulence therapies are approaches that interfere with bacterial virulence factors and/or pathways that regulate the production of virulence factors without affecting bacterial growth [25–29].

Previous reports have shown that *S. aureus* pathogenesis depends on the production of virulence factors that mediate adherence to host tissues, damage the host immune defense, and destroy host cells and tissues [1]. Bacterial virulence factors, such as extracellular toxins, surface proteins, and various enzymes, destroy tissues and cells. Alpha-hemolysin is the most prominent cytolytic extracellular toxins secreted by *S. aureus*. *S. aureus* Hla, which consists of water-soluble monomers, is the prototype for small β-barrel pore-forming toxins. *S. aureus* Hla binding to sensitive host cells forms a β-hairpin-lined amphipathic pore through the lipid bilayer, from which the cytoplasm leaks through the barrel of the pore, causing cytolysis. Many studies have suggested that Hla plays a significant role in the pathogenesis of *S. aureus* infections [10]. Antibodies against Hla were detected in the serum of humans who carried or were infected with *S. aureus* [30–32]. A Hla null strain showed reduced invasiveness and virulence in animal and cell models of pneumonia [33], peritonitis [34], and skin infection [35]. Hla has been considered as a potential antivirulence target for *S. aureus* infections, particularly for *S. aureus* pneumonia. Some studies have shown that USA300 can cause disease by increasing Hla production and virulence in animal models [7]. The accessory gene regulator (agr) quorum-sensing system is a major regulatory system that controls Hla production in *S. aureus*. The expression of Hla is controlled via a regulatory RNA molecule, RNAIII [36, 37], which is the major effector of agr system. The expression of RNAIII was also investigated in this study.

Miski et al. [15] reported that sclareol has little anti-*S. aureus* activity. However, in this study, sub-inhibitory concentrations of sclareol were found to significantly reduce Hla secretion in a co-culture system in a concentration-dependent manner. The effectiveness of sclareol as an antivirulence agent targeting Hla for *S. aureus* infection was tested. In this study, sclareol protected A549 cells from injury by *S. aureus* in the co-culture system (Fig. 6). Our results suggested that sclareol has the potential to be developed into an antivirulence drug targeting Hla for *S. aureus* infection, or to be a lead compound in further studies.

Previous studies have demonstrated that *S. aureus* increases Hla production and up-regulates hla expression when exposed to sub-inhibitory concentrations of β-lactams and fluoroquinolones [38, 39]. Consequently, this may complicate *S. aureus* infections treated with these antibiotics. Our study has shown that sclareol can reduce Hla expression in vitro through down-regulation of hla and RNAIII transcription. Depending on the conditions, sclareol could also be applied for the treatment of *S. aureus* infections in combination with β-lactams and fluoroquinolones to improve the therapeutic effects and extend the usefulness of these drugs.

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**References**


