Apigenin alleviates the symptoms of *Staphylococcus aureus* pneumonia by inhibiting the production of alpha-hemolysin

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**Abstract**

*Staphylococcus aureus* is a common human pathogenic bacteria that can cause serious infections, including lethal staphylococcal pneumonia. The development of antimicrobial resistance has limited treatment options for this pathogen; consequently, novel antibiotics and strategies are urgently desired to combat these infections. In recent years, virulence factors secreted by pathogenic microorganisms have been developed as targets for drug discovery. Alpha-hemolysin, a pore-forming cytotoxin that is secreted by most *S. aureus* strains, is essential for the pathogenesis of *S. aureus* pneumonia. In this study, we report that apigenin, a compound extracted from parsley that has no antimicrobial activity vs. *S. aureus* in vitro, can remarkably decrease the production of α-hemolysin at low concentrations. When added to the A549 cells and *S. aureus* co-culture system, apigenin protected A549 cells from α-hemolysin-mediated injury. Furthermore, *in vivo* tests indicated that apigenin alleviated injury of the lung tissue and decreased cytokine levels in the bronchoalveolar lavage fluid in the mouse model of *S. aureus* pneumonia.

**Introduction**

*Staphylococcus aureus* is one of the most important Gram-positive pathogens, causing extensive skin and soft tissue infections as well as life-threatening septicemia, pneumonia, and endocarditis. Pneumonia is among the most important disease caused by *S. aureus*, which occurs in c. 13.3% of all invasive staphylococcal infections (Klevens *et al.*, 2007). The emergence and spread of methicillin-resistant *S. aureus* (MRSA) has become a worldwide challenge. Therefore, new antimicrobial strategies for treating MRSA infections urgently need to be developed.

*Staphylococcus aureus* cause the diseases described above (Foster, 2005). There are over 40 secreted proteins and enzymes that are known to cause or associate with *S. aureus* diseases (Diep *et al.*, 2006). Alpha-hemolysin is a water-soluble monomer of 33.2 kDa that is produced by most *S. aureus* strains. It is a pore-forming exotoxin that can lyse a variety of mammalian cells, including erythrocytes, keratinocytes, fibroblasts, endothelial, and epithelial cells. Alpha-hemolysin is encoded by the *hla* gene in the staphylococcal genome, and it is strongly expressed in the postexponential phase of growth. Many global regulators have been found to contribute to the expression of α-hemolysin, such as Agr, Sar, Sae, Rot, and sigma B (Xiong *et al.*, 2006). Among these regulators, the Agr two-component system is the most important and best-characterized (Novick, 2003). The role of α-hemolysin in *S. aureus* infections has been well studied. Notably, recent studies have shown that α-hemolysin plays an essential role in the pathogenesis of *S. aureus* pneumonia in a mouse model of the disease, as strains lacking the pore-forming cytotoxin were shown to be avirulent (Bubeck Wardenburg *et al.*, 2007a).

Apigenin (Fig. 1) is a common flavonoid that can be extracted from a variety of fruits and vegetables, including parsley, onions, oranges, chamomile tea, wheat sprouts, and certain seasonings (Duthie & Crozier, 2000). Apigenin has been shown to possess a number of pharmacological effects, such as anticarcinogenic and free
radical-scavenging activities (Liu et al., 2005; Yoon et al., 2006), which have potential uses in cancer prevention and therapy. In this study, the impact of apigenin on the production of α-hemolysin in S. aureus was investigated, and the therapeutic effect of apigenin on S. aureus-related pneumonia was further evaluated.

Materials and methods

Microorganism and reagents

Staphylococcus aureus strains used in the study were presented in Table 1.

Apigenin (purity > 98%) was purchased from National Institutes for Food and Drug Control (Beijing, China). For vitro assays, apigenin stock solutions of various concentrations were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). For vivo studies, apigenin was dissolved in sterile PBS.

Preparation of cultures

For hemolysis, Western blot, and real-time RT-PCR assays, S. aureus strains were grown at 37 °C in tryptic soy broth (TSB) with graded concentrations of apigenin to the postexponential phase (OD600 nm of 2.5, 2.0, 2.0, 2.5, and 2.5 for strains ATCC 29213, wood 46, BAA-1717, 8325-4, and DU 1090, respectively). For cytoxicity studies and lung infection, S. aureus 8325-4 and DU 1090 were cultured in TSB at 37 °C to an optical density at 600 nm of 0.5. Fifty-milliliter culture aliquots were centrifuged, washed with PBS, and resuspended in 1 mL PBS (2 × 10^8 CFU per 30 µL) for histopathology experiments. For cytotoxicity studies, 5 mL of culture described above was resuspended in 10 mL of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, CA).

Determination of minimal inhibitory concentrations

The minimal inhibitory concentrations (MICs) of apigenin for S. aureus were evaluated using the broth microdilution method according to CLSI guidelines (CLSI, 2005). Briefly, apigenin was diluted in a 96-well plate over the concentration range of 4-1024 µg mL^-1 using double dilution method. Following inoculation with 5 × 10^5 CFU mL^-1 of overnight broth cultures in each well, the plate was inoculated at 37 °C for 24 h. The MIC was defined as the lowest concentration at which the growth of S. aureus was inhibited.

Growth curves

Staphylococcus aureus strain 8325-4 was cultured in TSB medium at 37 °C, shaken at 200 r.p.m. to an optical density (OD600 nm) of 0.3, and aliquoted into five 250-mL flasks in a volume of 100 mL. Apigenin dissolved in DMSO was added to the four cultures to obtain final concentrations of 1, 4, 16, and 64 µg mL^-1. 1% DMSO was added to the control culture. The bacteria were cultured at 37 °C with constant shaking, and cell growth was measured by reading the OD600 nm values every 30 min.

Hemolysis assay

Hemolytic activity was measured as described previously (Worlitzsch et al., 2001; Qiu et al., 2010a) using rabbit erythrocytes. Briefly, S. aureus cultures with different concentrations of apigenin were harvested when grown to the postexponential growth phase by centrifugation (5500 g, 8000 × g, 20 min). Table 1. Staphylococcus aureus strains used in this study and their MICs to apigenin

<table>
<thead>
<tr>
<th>S. aureus strains</th>
<th>Properties</th>
<th>Source</th>
<th>MIC (µg mL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 29213</td>
<td>MSSA, α-hemolysin-producing strain, β-Lactamase-producing strain</td>
<td>ATCC</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>ATCC 10832</td>
<td>Wood 46, a natural isolate with high-level production of α-hemolysin</td>
<td>ATCC</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>BAA 1717</td>
<td>USA 300, isolated from adolescent patient with severe sepsis syndrome in Texas Children’s Hospital, α-hemolysin-producing strain</td>
<td>ATCC</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>8325-4</td>
<td>A high-level alpha-hemolysin-producing strain derived from NCTC 8325</td>
<td>Timothy J. Foster</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>DU 1090</td>
<td>8325-4 defective in α-hemolysin, prepared by insertion of a transposon in the hla gene</td>
<td>Timothy J. Foster</td>
<td>&gt; 1024</td>
</tr>
</tbody>
</table>
fugation (5500 g, room temperature, 1 min). Following centrifugation, the hemolytic activity of the supernatants was detected by measuring the optical density at 543 nm. Medicine-free culture supernatant served as the 100% hemolysis control. The percent of hemolysis was calculated by comparison with the control culture supernatant.

Western blot assay

The culture supernatants collected previously were used in Western blot analysis. Samples were boiled with Laemmli sample buffer for 10 min, and then 25 μL of the sample was fractionated by SDS-PAGE (12% polyacrylamide gels; Laemmli, 1970). The Western blot protocol was performed as described previously (Qiu et al., 2010a, b). Proteins were transferred onto polyvinylidene fluoride membranes (Roche, Basel, Switzerland) using a semi-dry transfer cell (Bio-Rad, Munich, Germany). The membrane was blocked for 2 h with 5% bovine serum albumin (Amresco) at room temperature. Subsequently, a primary antihemolysin polyclonal antibody (Sigma-Aldrich) was added at a 1:5000 dilution and then incubated overnight at 4 °C, followed by 2 h of incubation with HRP-conjugated secondary goat anti-rabbit antiserum (Sigma-Aldrich) diluted to 1:4000. The blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare, UK).

Real-time RT-PCR

Staphylococcus aureus strain 8325-4 was grown in TSB with or without apigenin to OD600 nm of 2.5. Total bacterial RNA was extracted as described previously (Leng et al., 2011). Cells were harvested by centrifugation (5000 g for 5 min at 4 °C) and resuspended into TES buffer (10 mM Tris-Cl, 1 mM EDTA, 0.5% SDS) containing 100 μg mL⁻¹ of lysostaphin (Sigma-Aldrich) for 10 min, and then the samples were applied to a Qiagen RNeasy Maxi column (Qiagen, Hilden, Germany) to isolate total RNA following the manufacturer’s instructions. The DNA contained in total RNA was digested by RNase-free DNase I (Qiagen). cDNA was synthesized from total RNA using the Takara RNA PCR kit (AMV) version 3.0 (Takara, Kyoto, Japan) according to the manufacturer’s instructions. cDNA was stored at −20 °C until used. The sequences of hla primers were forward: 5′-TTGGTGCAAAATTTTC-3′ and reverse: 5′-TTACCTTCCAGCTACT-3′. The sequences of agrA primers were forward: 5′-CTGTAATGCCAATCC-3′ and reverse: 5′-GGAGGTGATCCCACTGG-3′. The sequences of 16s RNA gene primers were forward: 5′-TTATGGTGCTGGCATAAC-3′ and reverse: 5′-CAC CATGTAACCCGAC-3′.

The PCRs were carried out in a 25-μL total volume and contained SYBR Premix Ex Taq (Takara) according to the manufacturer. The PCRs were performed using the 7000 Sequence Detection System (Applied Biosystems, Courtabœuf, France). The reaction cycles were performed as followed: 95 °C for 30 s; 30 cycles at 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 40 s; and one dissociation step of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Triplet samples were performed as concurrent control. The housekeeping gene 16s rRNA was served as an internal control to normalize the expression levels between samples.

Live/dead and cytotoxicity assays

Human lung epithelial cells (A549) were obtained from the American Tissue Culture Collection (ATCC CCL 185) and propagated in DMEM supplemented with 10% fetal bovine serum (Invitrogen). Cells were seeded in 96-well dishes at a density of c. 2 × 10⁴ cells each well. Cells were incubated in triplicate with the presence of 100 μL of staphyloccocal suspension prepared as described previously with indicated concentrations of apigenin for 6 h at 37 °C.

Cell viability was measured either using live/dead (green/red) reagent (Invitrogen) or by measuring lactate dehydrogenase (LDH) release using a Cytotoxicity Detection kit (LDH; Roche) according to the manufacturer’s directions. Microscopic images of stained cells were obtained using a confocal laser scanning microscope (Nikon, Japan). LDH activity was measured on a microplate reader (Tecan, Austria).

Mouse model of S. aureus pneumonia

All animal studies were performed according to the experimental practices and standards developed by the Animal Welfare and Research Ethics Committee of Jilin University, and the experimental protocols were approved and supervised by the animal care committee. Eight-week-old C57BL/6J mice were obtained from the Experimental Animal Center of Jilin University (Changchun, China).

For lung infection, 50 μL of rodent III anesthetic was injected intraperitoneally into each mouse. Then, mice were infected intranasally with 30 L of rodent III anesthetic and then at 12-h intervals. Mice were euthanized by anesthesia followed by cervical dislocation 24 h postinfection. Each group contains 10 mice. Lungs were weighed and homogenized for calculation of bacteria.
burden using serial dilution and plating method. Lungs were removed and placed in 1% formalin. Formalin-fixed tissues were processed, stained with hematoxylin and eosin, and visualized by light microscopy.

Bronchoalveolar lavage fluid collection and analysis

Bronchoalveolar lavage fluid collection was performed twice by intratracheal instillation of 500 μL of PBS. After centrifugation, the supernatants were used for cytokine measurements. Cytokine levels were measured using an enzyme-linked immunosorbent assay (ELISA) by specific mouse ELISA kits (BioLegend, CA).

Statistical analysis

The experimental data were assessed using independent Student’s t-test with spss 13.0 statistical software (SPSS Inc., Chicago, IL), and a P value < 0.05 was considered to be statistically significant.

Results

Effect of apigenin on S. aureus growth

The MICs of apigenin against different S. aureus strains are shown in Table 1. All the values were > 1024 μg mL⁻¹. Growth curves with increasing concentrations of apigenin were shown in Fig. 2a, and apigenin cannot inhibit the growth of S. aureus from the concentration from 1 to 128 μg mL⁻¹. Furthermore, we investigated the effect of apigenin on the growth of S. aureus strains ATCC 29213, wood 46, and BAA-1717. No inhibition was found in all these strains (data not shown).

Apigenin reduces the level of α-hemolysin in S. aureus culture supernatants

To investigate the hemolytic activity of S. aureus culture supernatants in the presence of apigenin, hemolysis assays were performed using rabbit erythrocytes. As shown in Table 2, the hemolytic activity of S. aureus culture supernatants was decreased in a dose-dependent manner by the addition of apigenin. Following treatment with 4 μg mL⁻¹ of apigenin, the hemolytic activities were reduced to 12.64%, 14.77%, 10.64%, and 12.06% for S. aureus strains ATCC 29213, wood 46, BAA-1717, and 8325-4, respectively. When incubated with 8 μg mL⁻¹ of apigenin, no detectable hemolytic activity was found in any of the tested strains.

Of the exotoxins secreted by S. aureus that causes hemolysis of rabbit erythrocytes, α-hemolysin is the most important. Based on the data from the hemolysis assay, it was reasonable to infer that the production of α-hemolysin could be influenced by apigenin. To test this hypothesis, a Western blot assay was performed with the culture supernatant of S. aureus strain 8325-4. As expected, the addition of apigenin decreased the content of α-hemolysin in the culture supernatants in a concentration-dependent manner (Fig. 2b). At 8 μg mL⁻¹ apigenin, the α-hemolysin could not be detected in the culture supernatant.
Alpha-hemolysin is encoded by the \textit{hla} gene, which is regulated by the Agr two-component system. Consequently, a real-time RT-PCR assay was performed to examine whether apigenin can affect the transcription of the \textit{hla} and \textit{agrA} genes. As shown in Fig. 2c and d, the transcription of \textit{hla} and \textit{agrA} was remarkably inhibited when increasing concentrations of apigenin were added. When cells were co-cultured with 8 \( \mu \text{g mL}^{-1} \) apigenin, the transcriptional levels of the \textit{hla} and \textit{agrA} genes were reduced 14.03- and 9.13-fold, respectively.

Apigenin protects A549 cells from \( \alpha \)-hemolysin-mediated cell injury

Human A549 alveolar epithelial cells are widely used in pulmonary disease models (Nizet \textit{et al.}, 1996; Hirst \textit{et al.}, 2002). Previous studies have demonstrated that \( \alpha \)-hemolysin can cause A549 cell injury in a dose-dependent manner (Liang \textit{et al.}, 2009). Therefore, apigenin was assayed for its ability to protect A549 cells from \( \alpha \)-hemolysin-mediated cell injury. In this study, A549 cells were co-cultured with \textit{S. aureus} and different concentrations of apigenin. Cells were strained with a live/dead (green/red) reagent. As shown in Fig. 3a, uninfected cells retained a green fluorophore, while dead cells were red (Fig. 3b). As shown in Fig. 3c, apigenin conferred significant protection from cell injury at the concentration of 8 \( \mu \text{g mL}^{-1} \). Furthermore, a LDH release assay was performed to quantify cell injury, and as shown in Fig 3e, apigenin provided a dose-dependent protection to co-cultures of A549 cells with concentrations from 1 to 8 \( \mu \text{g mL}^{-1} \).

Apigenin protects mice from \textit{S. aureus} pneumonia

Alpha-hemolysin has been established as the main virulence factor in mouse models of \textit{S. aureus} pneumonia (McElroy \textit{et al.}, 2002; Gomez \textit{et al.}, 2004). Alpha-hemolysin has also been shown to damage the air–blood barrier in a rat model of \textit{S. aureus} lung infection (McElroy \textit{et al.}, 1999). On the foundation of \textit{in vitro} research that apigenin can

Table 2. Hemolytic activities of the \textit{Staphylococcus aureus} supernatants co-cultured with increasing concentrations of apigenin

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hemolysis (%) of rabbit erythrocytes by culture supernatant&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ATCC 29213</td>
<td>100</td>
</tr>
<tr>
<td>ATCC 10832</td>
<td>100</td>
</tr>
<tr>
<td>BAA-1717</td>
<td>100</td>
</tr>
<tr>
<td>8325-4</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup>The apigenin-free \textit{Staphylococcus aureus} culture supernatants were set as 100% hemolytic activity.

<sup>†</sup>No hemolytic activity was detected.

All data shown in the table are the mean and standard error of three independent experiments. *\( P < 0.05 \) and **\( P < 0.01 \) compared with the drug-free culture.
reduce the expression of α-hemolysin at very low concentrations, a *S. aureus*-mediated mouse pneumonia model was used to investigate the *in vivo* protective effects of apigenin. Mice were infected intranasally with a 30-μL *S. aureus* 8325-4 suspension as described in the Materials and methods. Next, mice were subcutaneously administered either PBS or 50 mg kg⁻¹ apigenin. The hla⁻ strain DU1090 was used as a negative control. The bacteria burden was quantified to evaluate the influence of apigenin on the survival in the lungs. As shown in Fig. 4a, the CFUs of lungs from infected mice treated with 50 mg kg⁻¹ were remarkably lower than those treated with PBS. The lung tissues of *S. aureus* 8325-4-infected mice that had been treated with apigenin were pink and spongy. However, the lung tissues of mice that were treated with PBS were kermesinus and had a firm texture (Fig. 4b). Further histopathological study indicated that more severe alveolar destruction and inflammatory cell aggregation occurred in infected mice treated with PBS than in those treated with apigenin (Fig. 4c).

To investigate the extent of the inflammation, we analyzed the level of cytokines in the bronchoalveolar lavage fluid from infected mice. As shown in Fig. 4d, mice that had received 50 mg kg⁻¹ of apigenin showed significantly decreased concentrations of IL-1β, IL-6, and TNF-α in bronchoalveolar lavage fluid when they were tested 24 h postinfection.

**Discussion**

*Staphylococcus aureus* is an important pathogen that causes a variety of human diseases. *Staphylococcus aureus*
pneumonia is one of the most common invasive diseases caused by the pathogen (Klevens et al., 2007). In the past 20 years, nosocomial pneumonia infections have been reported with increasing frequency as a result of the emergence of MRSA. However, community-acquired MRSA pneumonia has been associated with more severe and difficult-to-treat infections (Koomanachai et al., 2009). It leads to a necrotizing S. aureus pneumonia, which can emerge as one of the most lethal forms of this disease (Lina et al., 1999; Francis et al., 2005). For these reasons, S. aureus pneumonia is often serious and difficult to treat with antibiotics (Locksley et al., 1982). Vancomycin and linezolid are recommended empirically for the treatment of infections caused by MRSA (Mandell et al., 2007). Only two-thirds of patients, however, obtain a clinical cure after treatment with the appropriate doses of antibiotics. To improve patient outcomes, novel drugs for treating S. aureus pneumonia are urgently required (Rubinstein et al., 2001).

Staphylococcus aureus secretes a wide range of virulence factors that are involved in its pathogenicity. Alpha-hemolysin is known as the most critical factor for the induction of lung injury in S. aureus pneumonia. Previous studies have shown that S. aureus strains lacking α-hemolysin display significantly reduced levels of toxicity in a murine model of pneumonia (Patel et al., 1987); however, β-lactam therapy may induce the expression of α-hemolysin production and increase both pneumonia symptoms and lethality in a murine model. On the basis of these results (Kernodle et al., 1995), it is essential to design and investigate new strategies to treat diseases caused by S. aureus. A popular idea is to use antivirulence strategies, in which the expression or activity of virulence factor production is decreased without killing or inhibiting the growth of targeted bacteria. It is a more compelling approach than traditional strategies because it reduces selective pressure, which may otherwise lead to a rapid development of bacterial resistance (Cegelski et al., 2008; Rasko & Sperandio, 2010). For these reasons, α-hemolysin can be recommended as a potential target for this novel approach of developing new therapies against S. aureus infection. We have previously demonstrated that several compounds (such as licochalcone A, allicin, and eugenol) can decrease α-hemolysin production at a subinhibitory concentration (Qiu et al., 2010a, b; Leng et al., 2011).

In this study, we found a new natural compound, apigenin, which inhibits the expression of α-hemolysin both in vitro and in vivo at a low concentration. Apigenin has only slight antimicrobial activity against S. aureus, which is thought to reduce selective pressure against the growth of this species. Moreover, it can significantly protect the alveolar epithelial cells against α-hemolysin-mediated cell injury at 4 μg mL⁻¹, and it can release the pulmonary infection in a murine model. Because of the decrease in levels of α-hemolysin, the quantity of cytokines found in the alveolar lavage fluid is also greatly reduced. From our study of the quantitative RT-PCR, we can conclude in general that all the effects we observed may be related to the apigenin-induced inhibition of the agr two-component system, which occurs in a dose-dependent manner. Consequently, we can infer from the data shown in this study that apigenin, combined with β-lactam antibiotics, is a promising candidate for use in the treatment of S. aureus pneumonia.

Acknowledgements

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Authors’ contribution

J.D. and J.Q. contributed equally to this work.

Reference


Apigenin protects against S. aureus pneumonia


