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Inhibition of α-toxin production by subinhibitory concentrations of naringenin controls *Staphylococcus aureus* pneumonia

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ABSTRACT

Staphylococcal pneumonia provoked by methicillin-resistant *Staphylococcus aureus* (MRSA) is a life-threatening infection in which α-toxin is an essential virulence factor. In this study, we investigate the influence of naringenin on α-toxin production and further assess its therapeutic performance in the treatment of staphylococcal pneumonia. Remarkably, the expression of α-toxin was significantly inhibited when the organism was treated with 16 μg/ml of naringenin. When studied in a mouse model of *S. aureus* pneumonia, naringenin could attenuate the symptoms of lung injury and inflammation in infected mice. These results suggest that naringenin is a promising agent for treatment of *S. aureus* infection.

1. Introduction

Staphylococcus aureus is a commensal pathogen that evokes human and animal diseases ranging from atopic dermatitis to soft-tissue infections, and even more invasive illnesses such as bacteremia, pneumonia and osteomyelitis, which have high morbidity and mortality [1,2]. In 1961, only 1 year after the introduction of methicillin into clinical practice, methicillin-resistant *S. aureus* (MRSA) was first described in the United Kingdom as a hospital-acquired pathogen [3]. Over 50% of staphylococcal pneumonia isolates are classified as MRSA, and more than one-half of the patients who contract this pathogenic bacteria struggle to get well, even in this day and age. In recent years, due to the limited therapeutic options available for the treatment of MRSA infections as well as the decline in efficiency of development of new antibiotics with novel mechanisms of action, there is a clear need for novel therapeutic strategies against staphylococcal infections.

The ability of *S. aureus* to cause diseases depends on its production of a series of surface-related and secreted virulence factors. The 33.2-kDa α-toxin, one of the major exotoxins of *S. aureus*, is a member of the β-barrel pore-forming toxin (PFT) family, members of which are secreted as water-soluble monomeric proteins [4]. The monomer assembles into a stable homoheptameric transmembrane pore that creates a 2-nm internal diameter hole through the membranes of susceptible cells (e.g., lymphocytes, macrophages, alveolar epithelial cells, pulmonary endothelium, and erythrocytes) [5,6]. This new pore leads to the rapid egress of vital molecules, thereby causing cell lysis and death. Bubeck Wardenburg et al. [7] reported that α-toxin is essential for the pathogenesis of *S. aureus* infections, especially in staphylococcal pneumonia. *S. aureus* mutant strains lacking *hla* (the gene encoding α-toxin) cannot induce

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neutrophil-mediated inflammatory lung injury in the murine model of this disease [8].

Naringenin (Fig. 1) is one of the flavonoids present in grapefruits and tomatoes, and it has a wide range of pharmacological properties, including anti-oxidant, anti-fibration, anti-cancer, anti-atherogenic and anti-proliferative activities [9]. Kanno S. et al. have shown that naringenin can induce cytotoxicity in various human cancer cell lines as well as apoptosis in human colon cancer Caco-2 cells and promyelocytic leukemia HL-60 cells [10]. Naringenin has also been shown to dose-dependently inhibit the assembly and long-term production of infectious hepatitis C virus particles [11]. Additionally, Bodet C. has employed two different models (both in vitro and ex vivo experiments) to investigate the capacity of naringenin to inhibit the LPS-induced inflammatory response [12]. In our current research, we elucidate the influence of naringenin on the α-toxin expression of *S. aureus* in vitro and further examine the in vivo performance of naringenin in the treatment of *S. aureus* pneumonia.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and reagents

S. aureus strains involved in this study are described in Table 1. For haemolysis, western blot and real-time RT-PCR assays, the strains were grown in tryptic soy broth (TSB) at 37 °C, supplemented with naringenin as required, and harvested at the post-exponential phase with $OD_{600\ nm}$ of 2.5, 2.0, 2.7, 2.5 and 2.5 for strains ATCC 29213, wood 46, USA 300, 8325-4 and DU 1090 respectively. For cytotoxicity and in vivo studies, 8325-4 and DU1090 were grown in TSB at 37 °C to an $OD_{600\ nm}$ of 0.5. Five-milliliters of the culture described above was resuspended in 10 ml of DMEM medium (Invitrogen, CA) for cytotoxicity studies, and fifty-milliliters of culture aliquots was centrifuged, washed with PBS and resuspended in 1 ml PBS (2×10^8 CFU per 30 μl) for in vivo assay. Naringenin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and it was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) to make a stock solution for in vitro studies. Naringenin was suspended in PBS for in vivo assay.

2.2. Minimal inhibitory concentrations (MICs) determination

The minimal inhibitory concentrations of naringenin for *S. aureus* were determined using the broth microdilution method described by the CLSI (2005) [13]. Oxacillin was used as a positive control.

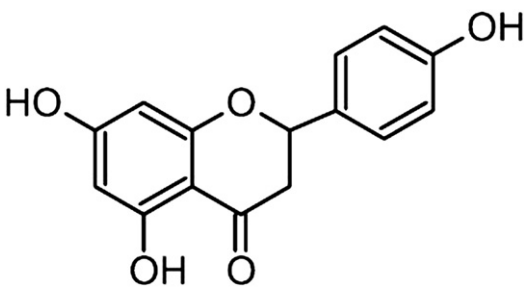

Fig. 1. Chemical structure of naringenin.

Table 1
Bacterial strains used in the study and their MICs to naringenin.

S. aureus strains	Description	Source	MIC (μg/ml)	
			Oxacillin	Naringenin
ATCC 29213	MSSA, α-toxin and β-lactamase producing strain	ATCC	0.25	256
ATCC 10832	Wood 46, a natural isolate that produces high levels of α-toxin	ATCC	0.125	256
BAA-1717	USA 300, isolated from adolescent patient with severe sepsis syndrome in Texas Children's Hospital, α-toxin-producing strain	ATCC	128	512
8325-4	A high-level α-toxin-producing strain derived from NCTC 8325	Timothy J. Foster	0.125	256
DU 1090	8325-4 defective in α-toxin, prepared by insertion of a transposon in the *hla* gene	Timothy J. Foster	0.125	256

2.3. Haemolysis assay

Haemolytic activity was measured as described previously [14]. Briefly, culture supernatants were collected by centrifugation (5,500 ×*g* at 4 °C for 1 min) and filter sterilized with a 0.22 μm (pore-size) acetate syringe filter. The reaction mixture was incubated at 37 °C including 100 μl supernatant described above, 25 μl defibrinated rabbit erythrocytes (v/v 2.5%) and 875 μl phosphate-buffered saline (PBS) for 30 min. The supernatants were collected by centrifugation (5,500 ×*g* at room temperature for 1 min). Then the haemolytic activity was evaluated with the values of cell-free supernatants at the OD_{543nm}.

2.4. Western blot assay

A 20 μl volume of boiled culture supernatants were applied to SDS–PAGE. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After blocked in 5% bovine serum albumin (Wako) for 2 h, the membranes were incubated with the rabbit polyclonal antibody to α-toxin (Sigma-Aldrich) diluted 1:4000 overnight at 4 °C. Then the membranes were stained with horseradish peroxidase-conjugated anti-rabbit antiserum (1:5000 dilution; Sigma–Aldrich) used as the secondary antibody. The blot was then developed using Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

2.5. Real-time RT-PCR

S. aureus 8325-4 was cultured in TSB with or without various dose of naringenin to the post-exponential growth phase (OD600nm of 2.5). Bacterial cells were pelleted (5,000×*g* 5 min, 4 °C) and immediately resuspended in TES buffer containing 100 μg/ml lysostaphin (Sigma-Aldrich) and