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Isovitexin, a Potential Candidate Inhibitor of Sortase A of *Staphylococcus aureus* USA300

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Staphylococcus aureus causes a broad variety of diseases. The spread of multidrug-resistant *S. aureus* highlights the need to develop new ways to combat *S. aureus* infections. Sortase A (SrtA) can anchor proteins containing LPXTG binding motifs to the bacteria surface and plays a key role in *S. aureus* infections, making it a promising antivirulence target. In the present study, we used a SrtA activity inhibition assay to discover that isovitexin, a Chinese herbal product, can inhibit SrtA activity with an IC_{50} of 28.98 μg/ml. Using a fibrinogen-binding assay and a biofilm formation assay, we indirectly proved the SrtA inhibitory activity of isovitexin. Additionally, isovitexin treatment decreased the amount of staphylococcal protein A (SpA) on the surface of the cells. These data suggest that isovitexin has the potential to be an anti-infective drug against *S. aureus* via the inhibition of sortase activity.

Keywords: *Staphylococcus aureus*, antivirulence, isovitexin, inhibitor, sortase A

Introduction

Staphylococcus aureus is a major human commensal and opportunistic pathogen and causes a broad spectrum of human diseases, ranging from moderate soft tissue infections to more severe infections such as endocarditis, pneumonia, septic shock, necrotizing fasciitis, osteomyelitis and blood infections [1–3]. This pathogen is also one of the leading reasons of both hospital- and community-acquired infectious diseases [4]. The importance of *S. aureus* has been amplified owing to the rise and spread of multidrug-resistant (MDR) *S. aureus*, such as vancomycin-resistant *S. aureus* (VRSA) and methicillin-resistant *S. aureus* (MRSA), which cause increased morbidity and mortality and present a clinical challenge [5].

Traditional approaches for treating bacterial infection rely primarily on the application of antibiotics, which disrupt the growth cycle by inhibiting the synthesis or activity of essential proteins or by preventing the assembly of key constituents such as cell wall [6]. Although antibiotics can eliminate most of the target bacteria, they also exert remarkable selective pressure on bacterial evolutionary adaptation, resulting in the development of bacterial resistance [7]. Importantly, broad-spectrum antibiotics also induce disordered host intestinal microbiota and result in diarrhoea and intestinal infection of the host [8]. Hence, alternative therapies involving antivirulence agents have aroused great attention. *S. aureus* uses two types of virulence factors, adhesins and toxins, to establish infection or cause pathological changes in the human host [9]. Adhesins are covalently anchored cell surface proteins that are primarily responsible for mediating infection processes such as attachment, colonization, cell-cell interactions, the invasion of host endothelial tissues, the evasion of host immunity and iron acquisition [10]. Interference with virulence-associated surface protein anchoring has emerged as a promising strategy to combat *S. aureus* diseases since those virulence factors are not essential for the survival of bacteria [11, 12]. The cell surface proteins containing sorting LPXTG motifs are secreted as precursors and are

covalently anchored to the peptidoglycan layer of the gram-positive cell wall by means of a universal sorting mechanism mediated by thesortase transpeptidase [13]. Sortase A (SrtA) plays a pivotal role in pathogenesis of *S. aureus* by modulating the sorting of the virulence-associated protein to the cell surface [14]. It is an appealing target for drug development for *S. aureus* infections.

Natural products have been considered a major source of pharmaceutical leads and therapeutic drugs. They have functional diversity and are being exploited for a variety of novel bactericidal or antivirulence agents against bacterial infections [15]. Among them, naturally existing flavonoids are worthy of special attention because they possess not only a wide range of pharmacological effects but also the potential to inhibit microbial virulence [16, 17]. Isovitexin, apigenin-6-C-D-glucopyranoside, is a naturally derived flavonoid. It has been proven to possess various pharmacological effects such as antioxidant, antineoplastic, anticancer, anti-inflammatory, antihyperalgesic, cardiovascular system protection and neuroprotection effects [18–21]. In the present study, we found that isovitexin could inhibit *S. aureus* SrtA activity without affecting bacterial growth and survival, indicating that isovitexin could be employed as a prospective lead compound for further development of drugs against *S. aureus*.

Materials and Methods

Bacteria, Chemicals and Growth Conditions

S. aureus USA300 strain BAA-1717 (American Type Culture Collection) as well as its *srtA* mutant strain (Δ*srtA*) was used throughout this study. The Δ*srtA* strain was constructed by a method described previously [22]. Cells of *S. aureus* were subcultured in brain heart infusion (BHI) broth at 37°C. Isovitexin was purchased from ChemBest Research Laboratories Ltd (China). All other compounds used were obtained from Sigma-Aldrich (China). The peptide substrate Abz-LPATG-Dap(Dnp)-NH_2 (Abz: ortho-aminobenzoic acid; Dnp: 2,4-dinitrophenyl) was synthesized by GL Biochem (China).

Cloning, Overexpression, and Purification of SrtA

The genomic DNA of *S. aureus* USA300 was isolated and used as the template DNA in PCR reactions. The *srtA* gene lacking the transmembrane domain (N1 – 59) was amplifiedby PCR using the primers 5′-GGGAATTCCATATGCAAGCTAAACCTCAAATTC CG-3′ (forward) and 5′-CGCGGATCCTTATTTGACTTCTGTAGC TACAAAGA-3′ (reverse). The resulting amplified fragment was digested with BamHI and XhoI and ligated into plasmid pET28adigested with the same restriction enzyme. The recombinant plasmid was transformed into the *Escherichia coli* strain BL21 (Novagen) for the overexpression of the SrtA. Production of recombinant SrtA protein was induced at mid-log phase with 1 mM isopropylthio-β-D-galactoside (IPTG) for 4 h at 16°C. Recombinant His-tagged $SrtA_{\Delta N59}$ was purified by the 6× His/Ni-NTA system as described previously [23].

Analysis of Anti-*S. aureus* Activity of Isovitexin

The minimum inhibitory concentration (MIC) for the isovitexin was determined through broth dilution methods as suggested by Kim ES [24]. In brief, overnight bacterial cultures were diluted to an OD_{600} (optical density at 600 nm) of 0.3 with BHI broth containing different concentrations of isovitexin (0, 16, 32, 64, 128, 256, 512, and 1,024 μg/ml) and were cultured for 16 h at 37°C. Absorbance values (OD_{600}) were measured using a multimode microplate reader (Infinite F500, Tecan, China). For growth curve plotting, *S. aureus* from overnight cultures was diluted back 1:100 in sterile BHI broth with or without 256 μg/ml isovitexin and were grown at 37°C with shaking for 24 h. Then absorbance at 600 nm was measured at different time intervals.

SrtA Activity Assay

Sortase activity was determined by the fluorescence resonance energy transfer (FRET) method according to a published protocol described previously [25, 26]. The synthetic peptide Abz-LPATG-Dap(Dnp)-NH_2 was used as the fluorescent internally quenched substrate. The assay was conducted in the wells of a 96-well black plate (PerkinElmer). Wells contained 10 μM purified SrtA and different concentrations of isovitexin in a final volume of 300 μl of the reaction buffer (50 mM Tris·HCl, 150 mM NaCl and 5 mM $CaCl_2$, pH 7.5). Wells contained all the above, except for the test sample, which was used as a blank control. The reaction was carried out for 30 min at 37°C. Then 50 μM substrate peptide was added and incubated for a further hour, and fluorescence was read at 495 nm emission wavelengths with an excitation wavelength of 350 nm. Inhibitory rates of isovitexin on SrtA activities were determined by the formula: 100% × (C − T)/C, where C is the fluorescent value of the untreated group, and T is the fluorescent value of the experimental group.

Adherence of *S. aureus* to Immobilized Fibrinogen

S. aureus wild-type (WT) and Δ*srtA* strains were grown overnight, back-diluted 1:1000 in sterile BHI medium and cultured to an OD_{600} of 0.5 in the presence of isovitexin or DMSO with shaking at 37°C. The Δ*srtA* strain grown in BHI medium supplemented with 256 μg/ml isovitexin served as the positive control. The untreated group served as the negative control. The cultures were centrifuged at 3,000 g for 5 min, washed three times with phosphate-buffered saline (PBS) and diluted to an OD_{600} of 1.0. Wells of Costar flat-bottomed microtiter plate were coated with 20 μg/ml bovine fibrinogen (Fg) overnight at 4°C. The plates were rinsed two times and blocked for 2 h with 5% bovine serum albumin (BSA). The plates were washed three times with PBS, and 100 μl of the cell suspensions was added to the fibrinogen-coated plates. Following incubation for 2 h at 37°C, the suspension

was discarded, and the wells were washed three times with PBS. Then the bound bacteria were fixed with 2% (v/v) glutaraldehyde for 30 min. After washing once, the bacteria were stained with 12.5 g/l crystal violet for 15 min. Then the plates were washed again with PBS and allowed to dry overnight. The bound dye was eluted with 95% ethanol and the amount of dye was then determined using a microplate reader. The percent inhibition of isovitexin on the adhesion of *S. aureus* is determined by the formula: 100% × (C – T)/C, where C is the absorbance value of the non-treatment group, and T is the absorbance value of the drug treatment group.

Crystal Violet Biofilm Assay

A static biofilm assay of *S. aureus* was carried out in flat-bottomed, microtiter plates (BD, Falcon) based on a method described previously [27]. Briefly, bacteria cultures were suspended into 200 µl of BHI medium supplemented with 3% (w/v) sucrose to an absorbance of 0.5 at 600 nm and grown statically with or without isovitexin at 37°C for 24 h. The medium was then discarded, and the plates were washed thrice with PBS to remove residual planktonic cells. To quantify the biofilm formation, the adherent cells were stained with 0.1% crystal violet for 20 min. The wells were thoroughly rinsed with sterile deionized water to remove unbound dye. The bound dye was eluted in 300 µl of 33% acetic acid. The absorbances at 595 nm were determined using a microplate reader. The Δ*srtA* strain grown in BHI medium served as a positive control. The untreated group served as a negative control.

Staphylococcal Protein A (SpA) Display Analysis

S. aureus WT and Δ*srtA* strains were grown overnight and then back-diluted 1:1000 in fresh BHI medium and cultured to OD_{600} between 0.8–1.0 with isovitexin or DMSO at 37°C. The untreated Δ*srtA* strain served as a positive control for SpA inhibition. The WT group served as a negative control. The cells from each culture were collected by centrifugation at 3,000 g for 5 min, and the bacteria were washed twice with PBS and fixed in a 4% formaldehyde solution for 20 min. After washing twice in PBS, the bacteria were stained with a 1:100 dilution of polyclonal FITC-conjugated goat anti-rabbit immunoglobulin G (IgG) (eBioscience) for 2.5 h at 25°C. At last the bacteria were washed twice and loaded on poly-L-lysine-coated glass slides. The images were obtained by a confocal microscope (Olympus, China).

Results

Isovitexin Inhibits the Activity of SrtA

To determine if isovitexin had an inhibitory effect on SrtA activity, a FRET assay was performed with the self-quenched fluorescent peptide Abz-Leu-Pro-Glu-Thr-Gly-Lys-Dap(Dnp)-NH_2, which contains the LPXTG-motif of the SrtA substrate protein. After cleavage by SrtA, the fluorophore Abz within the peptide is separated from the quencher Dnp, which causes an increase in fluorescence. We incubated purified $SrtA_{\Delta N59}$ with the fluorescent peptide substrate at various concentrations of isovitexin in the reaction buffer, and the results showed that the addition of isovitexin decreased the fluorescence signal in a dose-dependent manner, indicating that isovitexin possesses potent SrtA inhibitory activity. The half-maximal inhibitory concentration (IC_{50}) value of isovitexin was 28.98 µg/ml (Fig. 1A).

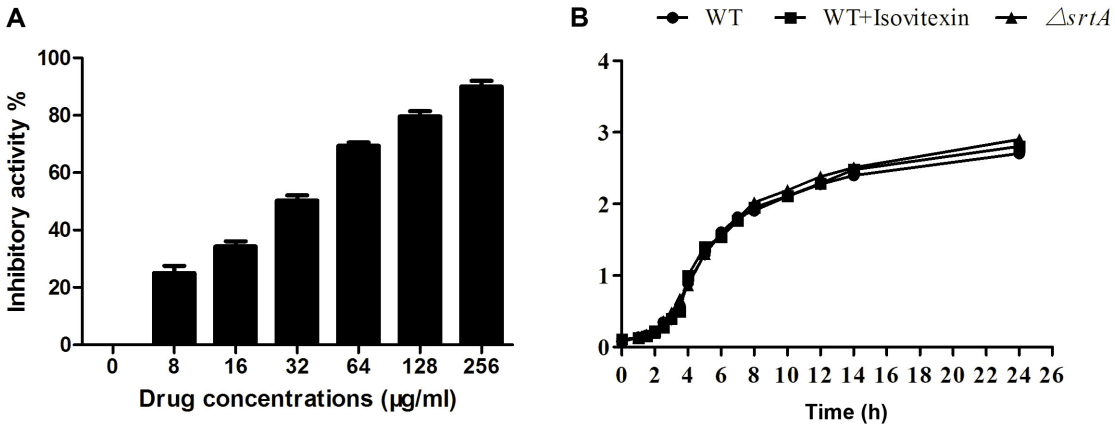

Fig. 1. Inhibitory effect of isovitexin against SrtA in vitro.

(**A**) The reaction solutions contained SrtA, the synthetic fluorescent peptide substrate Abz-LPATG-Dap(Dnp)-NH_2 and various concentrations of isovitexin. (**B**) Effect of isovitexin on the growth of *S. aureus*. The WT strain was cultured in BHI broth with 256 µg/ml isovitexin (square) or without isovitexin (circle). The Δ*srtA* strain served as a positive control (triangle). The graphs indicate the averages of three independent experiments.

Isovitexin Has No Influence on *S. aureus* Growth

To test whether isovitexin can inhibit the growth of *S. aureus*, the MIC of isovitexin against *S. aureus* was measured. The MIC value of isovitexin against *S. aureus* WT strain was > 1,024 μg/ml. The growth curves of the *S. aureus* showed that the growth rates of the *S. aureus* WT strain treated with isovitexin and the *S. aureus* Δ*srtA* strain were similar to that of the *S. aureus* WT strain (Fig. 1B). These results suggest that isovitexin doesn't affect *S. aureus* proliferation.

Isovitexin Reduces *S. aureus* Adhesion to Fibrinogen

An earlier study has shown that the inhibitors of SrtA can inhibit *S. aureus* cell adhesion to fibrinogen (Fg)- or fibronectin-coated plates [28]. Therefore, we used the *S. aureus* adhesion assay to test whether isovitexin inhibits the activity of the SrtA enzyme. As expected, the result showed that the *S. aureus* adhesion to Fg-coated plates was inhibited by isovitexin in a dose-dependent manner (Fig. 2). The *S. aureus* Δ*srtA* strain exhibited a minimum binding capacity to Fg-coated surfaces of 9.3 ± 1.7%, and the fibrinogen binding capacity of the *S. aureus* WT strain treated with 32, 64, 128, or 256 μg/ml of isovitexin was measured, and the adhesion rates were 86.3 ± 5.8%, 54.3 ± 4.0%, 34.7 ± 4.0%, and 25.3 ± 4.6%, respectively.

Isovitexin Reduces Biofilm Formation

S. aureus is capable of forming biofilms on many tissues or implanted medical devices. *S. aureus* biofilms often cause chronic infections since that bacteria in biofilms can evade antibiotic eradication and host immune responses [29]. A previous study revealed that the SrtA knockout strain showed significant reduction of biofilm formation of MRSA strains [30]. As expected, the results of biofilm formation assay showed *S. aureus* biofilm formation was inhibited by isovitexin. Further quantitative analysis showed that 256 μg/ml isovitexin inhibited biofilm formation by ≥ 61% (Fig. 3).

Isovitexin Reduces SpA Display on the Surface of *S. aureus*

SpA, a major virulence factor, is expressed by almost all clinical isolates of *S. aureus* [31]. It is secreted by the general secretory pathway and is displayed on the surface of bacteria by SrtA during secretion [32, 33]. In this assay, *S. aureus* cultured with or without isovitexin was stained with goat anti-rabbit IgG labelled with FITC to detect SpA on the surface of the bacteria. The result showed that the *S. aureus* WT strain cultured in the presence of 256 μg/ml isovitexin revealed a very faint distribution of SpA, in

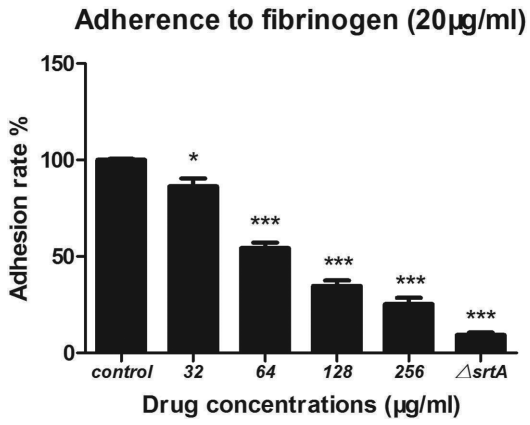

Fig. 2. Inhibition of *S. aureus* binding to immobilized Fg by isovitexin.

The *S. aureus* WT strain was cultured in BHI medium supplemented with DMSO or various concentrations of isovitexin. The *S. aureus* Δ*srtA* strain treated with 256 μg/ml isovitexin served as a positive control. The control group was the non-treatment group. The bacteria were added into Fg-coated microtiter wells and incubated for 2 h at 37°C. The attached bacteria in microtiter plates were stained with crystal violet. Data are the means ± SD of three replicates. The symbol "*" represents $p < 0.05$, and the symbol "***" represents $p < 0.0001$.

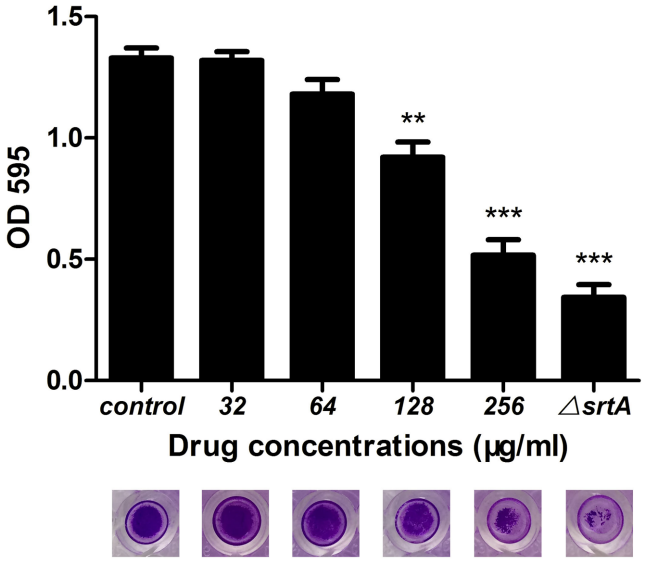

Fig. 3. Inhibition of the biofilm formation by isovitexin.

The *S. aureus* WT strain was incubated in BHI broth supplemented with various concentrations of isovitexin for 24 h. The biofilm grown in microtiter plates was stained with crystal violet. The data were obtained from three replicates and were presented as the means ± SD. The symbol "**" represents $p < 0.05$, and the symbol "***" represents $p < 0.0001$.

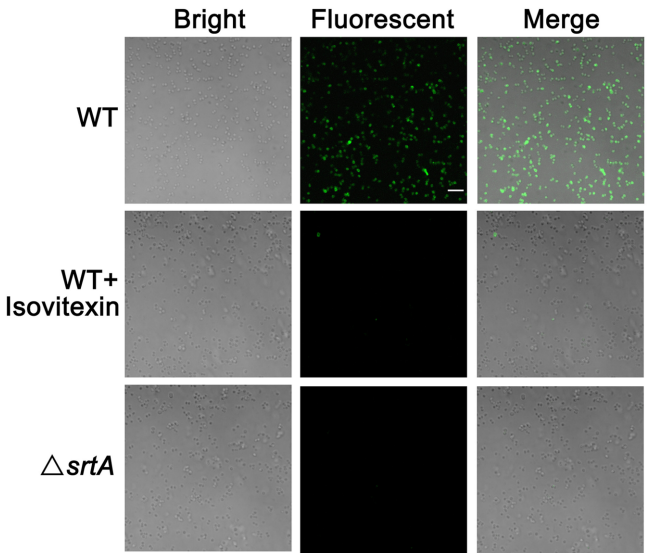

Fig. 4. Decrease of SpA display on the *S. aureus* surface by isovitexin.

The *S. aureus* WT strain treated with 256 μg/ml isovitexin was cultured in BHI broth. The untreated *S. aureus* WT strain and the *S. aureus* Δ*srtA* strain were used as a negative control and a positive control for SpA inhibition respectively. The cells were stained with FITC-labeled IgG. Fluorescence images were collected by a confocal laser-scanning microscopy. Scale bar indicates 5 μm.

comparison with the distributions visible on the *S. aureus* Δ*srtA* strain and the untreated *S. aureus* WT strain (Fig. 4). These consequences indicate that isovitexin can disturb the display of SpA on the bacterial surface by inhibiting SrtA.

Discussion

The emergence and spread of *S. aureus* strains such as MRSA, VRSA, and multiple-drug-resistant *S. aureus* (MDRSA) pose a considerable challenge to the treatment of clinical *S. aureus* infections [34]. The conventional antibiotics have targets associated with bacterial growth and survival, which exert substantial selective pressure on the adaptive evolution of the bacteria and promote the development of antibiotic resistance [35]. Therefore, it is imperative to develop alternative strategies to combat *S. aureus* infections.

S. aureus can anchor at least 25 LPXTG proteins on its surface by SrtA, such as coagulation factorA and B (ClfA and ClfB), fibronectin-binding protein A and B (FnbpA and FnbpB), and SpA [36]. These cell wall-anchored (CWA) surface proteins are essential virulence factors and possess various functions, including the adhesion to host tissues, the invasion of non-phagocytic cells, the evasion of immune responses, nutrient acquisition, and biofilm formation [37]. SrtA, an intensively studied virulence factor, can anchor CWA proteins to its surface and plays a vital role in *S. aureus* infections. Research showed that *srtA* gene knockout or inhibition of SrtA in *S. aureus* by inhibitors resulted in the defective display of LPXTG proteins and severe virulence defects in the establishment of infection [38]. Therefore, screening SrtA inhibitors is an especially appealing strategy for treating *S. aureus* infections. Chemical extracts from natural products are of great variety and have chemical diversity. They are a major source of therapeutic agents in some bacteria infectious diseases. Early studies have shown that flavonoids are a group of promising natural compounds against SrtA [39, 40]. Recently, our research work has primarily focused on discovering potent flavonoid compounds from Chinese herbal products against important virulence factors in *S. aureus* [41, 42]. As a continuation of that work, in this study, we found that isovitexin, a natural flavonoid active ingredient without growth inhibitory activity against *S. aureus*, can significantly inhibit the activity of SrtA. The Fg-binding assay indicates that isovitexin can interfere with SrtA activity and can affect the adhesion of *S. aureus* cells to Fg-coated surface (Fig. 2). LPXTG-containing surface proteins such as biofilm-associated protein (Bap) and/*S. aureus* surface protein (SasG), which are identified mediators of *S. aureus* biofilm development [43, 44]. Further research showed that the *srtA* gene mutation had an impact on biofilm accumulation by clinical MRSA strains [30]. The results of our biofilm formation assay show that isovitexin can inhibit the biofilm formation of *S. aureus* USA300, indirectly reflecting the inhibition of sortase activity (Fig. 3). SpA, as a kind of multifunctional surface protein of *S. aureus*, binds to the Fc portion of IgG and to the von Willebrand factor (vWF) during infection [45]. It is synthesized in the cytoplasm and then translocated onto the staphylococcal surface by SrtA via transpeptidation and transglycosylation reactions [46]. An earlier study has shown that *S. aureus srtA* mutants are incapable of anchoring SpA to cell surfaces [47]. As expected, the SpA-related fluorescence analysis showed that isovitexin reduced the amount of SpA on the bacterial surface by inhibiting SrtA activity (Fig. 4).

In summary, our results show that isovitexin has potent inhibitory activity against SrtA in vitro and has no anti-*S. aureus* growth activity. It will be meaningful to develop antivirulence drugs based on isovitexin.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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