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

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Introduction

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 IC₅₀ 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.

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covalently anchored to the peptidoglycan layer of the gram-positive cell wall by means of a universal sorting mechanism mediated by the sortase 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 were reported to possess broad-spectrum antimicrobial activity [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, anti-inflammatory, anticancer, cardiovascular system protection and neuroprotection effects [18–21]. In the present study, we found that isovitexin could inhibit S. aureus SrtA activity with 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 (ΔsrtA) was used throughout this study. The ΔsrtA 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)-NH₂ (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 amplified by PCR using the primers 5’-GGAGATTCATATGCAGCTAACCCTCCTAAATTC CG-3’ (forward) and 5’-CGGATCTTATTGATTACCTCTAGCG TACAAAAGA-3’ (reverse). The resulting amplified fragment was digested with BamHI and XhoI and ligated into plasmid pET28a digested 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 SrtAexo was purified by the 6× His/NiNTA 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 OD600 (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 (OD600) 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)-NH₂ 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 CaCl₂, 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 ΔsrtA strains were grown overnight, back-diluted 1:1000 in sterile BHI medium and cultured to an OD600 of 0.5 in the presence of isovitexin or DMSO with shaking at 37°C. The ΔsrtA 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 OD600 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 ΔsrtA 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 ΔsrtA strains were grown overnight and then back-diluted 1:1000 in fresh BHI medium and cultured to OD₆₀₀ between 0.8–1.0 with isovitexin or DMSO at 37°C. The untreated ΔsrtA 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)-NH₂, 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 SrtAΔN₅₉ 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 (IC₅₀) value of isovitexin was 28.98 μg/ml (Fig. 1A).

![Figure 1](image-url)

**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)-NH₂, 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 ΔsrtA 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* ΔsrtA 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* ΔsrtA 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
comparison with the distributions visible on the *S. aureus ΔsrtA* 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 factor A 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.
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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

**References**


