Inhibition of S. aureus biofilm formation by linezolid alleviates sepsis-induced lung injury caused by S. aureus infection through direct inhibition of icaA activity

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SUMMARY

Antibiotic-resistant S. aureus infections can be life-threatening. Linezolid is known to hinder S. aureus biofilm formation, but the underlying molecular mechanism remains unclear. Molecular docking revealed that linezolid can bind to icaA, and this was confirmed by thermal drift assays. Linezolid demonstrated a dose-dependent inhibition of icaA enzyme activity. Mutating Trp267, a key residue identified through molecular docking, significantly decreased linezolid binding and inhibitory effects on mutant icaA activity. However, the mutant icaA Trp267Ala showed only slight activity reduction compared to icaA. Linezolid had minimal impact on icaB's thermal stability and activity. The 50S ribosomal L3Ser145 mutant S. aureus exhibited similar growth and biofilm formation to the wild-type strain. Linezolid effectively suppressed the growth and biofilm formation of wild-type S. aureus. Although linezolid lost its ability to inhibit the growth of the mutant strain, it still effectively hindered its biofilm formation. Linezolid exhibited weaker attenuation of sepsis-induced lung injury caused by 50S ribosomal L3Ser145 mutant S. aureus compared to wild-type S. aureus. These findings indicate that linezolid hampers S. aureus biofilm formation by directly inhibiting icaA activity, independently of its impact on bacterial growth.

INTRODUCTION

Staphylococcus aureus (S. aureus) is a Gram-positive spherically shaped bacterium. Under normal conditions, about 30% of people carry S. aureus. In most cases, S. aureus does not affect health, but under the following conditions S. aureus infection can cause serious illness or even death. For example, S. aureus entering the blood can cause bacteremia or sepsis, entering the lungs can cause pneumonia, entering the heart valves can cause endocarditis, and entering bone tissue can cause osteomyelitis (Turner et al., 2019). At the same time, in patients with tumors, diabetes or ICU condition, S. aureus infection will increase the risk of disease and mortality. To make matters worse, S. aureus is resistant to multiple antibiotics. According to the 2014 WHO Global Antibiotic Resistance Report, as much as 60%-90% of S. aureus infections are MRSA (methicillin-resistant S. aureus) infections, and the mortality rate of MRSA infections was 64% higher than that of non-resistant S. aureus infections (https://apps.who.int/iris/bitstream/handle/10665/112642/9789241564748_eng.pdf?sequence=1&isAllowed=y). It is of great significance to study the mechanism of antibiotic resistance of S. aureus and develop therapeutic drugs to overcome the drug resistance of S. aureus.

A biofilm generally refers to an aggregate composed of extracellular matrix and bacteria (Periasamy et al., 2012). S. aureus is a typical biofilm-forming bacterium, and biofilm formation is associated with S. aureus infection, pathogenicity, and antibiotic sensitivity or resistance (Ciofu et al., 2022; Parastan et al., 2020). Specifically, bacteria in biofilms easily evade immune surveillance and are not easily cleared by the immune system. Antibiotics do not easily penetrate into bacteria in biofilms, so that killing bacteria in biofilms requires a higher concentration (usually 100-1000 times higher) of antibiotics than is needed to kill free bacteria. In addition, bacteria in biofilms can transfer resistance factors to each other. It can be seen that biofilm formation can easily lead to antibiotic resistance, so inhibiting biofilm formation may be an effective way to treat diseases caused by antibiotic-resistant bacterial infections. Linezolid, the first oxazolidinone antibiotic, is commonly used to treat a variety of diseases associated with S. aureus infection, including pneumonia and respiratory infections in ICU patients (Hashemian et al., 2018; Pin-
tado et al., 2020; Wunderink et al., 2012). Linezolid can bind to 50S ribosomal subunits and inhibit the initiation of protein synthesis (Belousoff et al., 2017; Crowe-McAuliffe & Wilson, 2022; Wilson et al., 2008). Linezolid has also been reported to inhibit S. aureus from forming a biofilm (Bayston et al., 2012; Fernandez-Barat et al., 2019; Zhang et al., 2021), but the molecular mechanism of such inhibition has not yet been reported. The current study is the first to confirm that linezolid can directly bind to icaA to inhibit its enzyme activity and reduce the biofilm formation of S. aureus. The use of genetic modification to establish linezolid tolerance in S. aureus confirmed that linezolid can alleviate diseases caused by S. aureus infection by inhibiting biofilm formation in vivo.

MATERIALS AND METHODS

Bacteria and mice
Staphylococcus aureus (S. aureus) was purchased from ATCC (ATCC 29213), and grown in Luria Broth (LB) media. Mice were purchased from JAX and housed in a specific pathogen-free rodent room with 12-h day and night alternation. Mice had free access to food and drinking water.

Establishment of S. aureus-associated sepsis lung injury model
Mice were inoculated intraperitoneally with S. aureus (2x10⁸ CFU per mouse). Two hours after inoculation, mice were treated intraperitoneally with appropriate doses of linezolid, twice a day for two days. Two days later, the mice were anesthetized and sacrificed by dislocation of the cervical vertebrae. After the blood was perfused, the lungs were removed for various experiments.

Genome modification with CRISPR
The rpsl promoter initiates Gas9 transcription, and the Cap1a promoter initiates gRNA transcription for bacterial genomic editing in CRISPR plasmid (Ran et al., 2013). ChopChop (https://chopchop.cbu.uib.no/) was used to design gRNA (Labun et al., 2019) and commercially synthesized gRNA strands containing overhang CACC at the 5’ end and complementary strands containing overhang AAAC at the 5’ end. After phosphorylation with PNK and annealing, the gRNA was cloned into the CRISPR/Cas9 vector which was linearized with BbsI. The PAM mutant sequence was used as the repair template and the template was cloned into the CRISPR/Cas9 vector through the XbaI/KpnI restriction site. All target sequences were confirmed to be correct by Sanger sequencing. All sequences are listed in Table 1.

Deletion of icaA, icaB or icaA and icaB by homologous recombination
The 400bp homologous recombination arm was amplified from the upstream and downstream CDS of icaA or icaB by PCR with high-fidelity DNA polymerase. The homology arm and erythromycin resistance gene sequence ermB were cloned into the homologous recombination vector. After electroporation, single clones resistant to erythromycin and sensitive to chloramphenicol were chosen. PCR and Sanger sequencing confirmed that the positive clones were successfully knocked out. PCR amplification and validation primers are listed in Table 1.

Protein Expression and Purification
HEK293 cells (about 70% confluence) were transfected with secretory expression plasmids. After 24 hours of transfection, the medium was replaced with a serum-free medium. Every 24 hours, the supernatant containing the target protein was collected and fresh medium was added. After four-time collections, the media containing the target protein was combined for subsequent purification. The supernatant was concentrated by centrifugal ultrafiltration with an ultrafiltration tube of appropriate pore size. The concentrated protein solution was added to the activated nickel column, and then the column was washed with washing solution. The protein was eluted using elution buffer, and was then cleaved by TEV protease. The cleaved protein was passed through a nickel column again to obtain the target protein, which was further desalted and used for corresponding experiments.

Construction of protein secretory expression vector
The icaA, icaB and their mutants were cloned into secretory expression vectors. The icaA and icaB were obtained by PCR with high-fidelity DNA polymerase. Mutants of the icaA and icaB were obtained by overlapping PCR. The coding gene sequence was inserted into the secretory expression vectors through the NotI/XhoI restriction site, and it maintained the correct reading frame including secretory and His tag. Positive clones were confirmed to have the correct sequences by Sanger sequencing. All clone primer sequences are listed in Table 1.

RT-qPCR
An appropriate volume of Trizol reagent (600 µl/20 mg) was added to the lung tissue and then homogenized. One third of the volume of the supernatant was used as the template, and the template was cloned into the CRISPR/Cas9 vector through the XbaI/KpnI restriction site. All target sequences were confirmed to be correct by Sanger sequencing. All sequences are listed in Table 1.
(1 µg) was synthesized using a commercial reverse transcription kit. Gene-specific qPCR primers were designed with NCBI's Primer-blast tool. PCR was performed with specific primers, cDNA, SYBR green, enzyme and buffer. The relative expression of specific genes was quantified using the ∆∆Ct method. All detective primer sequences are listed in Table 1.

The icaA and icaB activity analysis
At 37°C, 500 ng of purified icaA was co-incubated with different concentrations of N-acetylglucosamine, UDP-GlcNAc (40 mM) and MnCl2 (20 mM) in 50 µl reaction solution for 2 hours (Siala et al., 2016). Then 15 µl of free UDP capture reagent was added and incubated at room temperature for 1 hour. Polarization fluorescence (mP) was measured according to kit instructions (UDP2 FP Kit, BellBrook Labs, Madison, WI, USA). The amount of free UDP was calculated according to the standard curve.

Purified icaB (500 ng) was co-incubated with different concentrations of PNAG and CoCl2 (30 µM) in 50 µl reaction solution for 10 hours at 37°C (Pokrovskaya et al., 2013). Then 10 µl of 0.5 M borax solution was added to adjust the pH of the sample to 9, after

Table 1 - The sequences of PCR or qPCR primers, gRNA, and repair template.

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<th>Name</th>
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which 10 µl of fluorescamine solution (20 mg/ml) was added. The mixture was incubated continuously for 10 minutes at room temperature and 30 µl of water was added. Under excitation at 360 nm, the emission fluorescence at 460 nm was measured. Enzyme activity was indicated by fluorescence intensity.

Measuring bacterial growth
A bacterial clone was inoculated in 3 ml medium and cultured overnight. After measurement of OD600, OD600 of the bacterial liquid was adjusted to 0.5. The 10 µl of bacterial liquid was inoculated into 3 ml of LB medium and was co-cultivated with the different concentrations of linezolid for 12 or 24 hours, after which OD600 of the bacterial liquid was measured. The OD600 value indicated the growth of bacteria.

In vitro assays for biofilm formation
The 190 µl of blank medium or 190 µl of medium containing the various concentrations of linezolid was added to a 96-well culture plate, and then 10 µl of bacterial solution with an OD600 of 0.05 was inoculated in each well. The bacteria were incubated at 30°C for 24 hours and washed twice with PBS. After incubation at 60°C for 1 hour in PBS to fix the biofilm, the biofilm of bacteria was stained with 50 µl 2% crystal violet for 30 min and washed twice with PBS. 100 µl of 33% glacial acetic acid was added and incubated at room temperature for 30 min to completely dissolve the stained biofilm. The OD570 value was determined to indicate biofilm formation.

Thermal shift analysis
A 20 µl reaction solution containing 1 µg of purified icaA, icaB or mutant protein, linezolid, and a dye which binds with hydrophobic residues of proteins was added into a 96-well qPCR plate well. A melting curve was determined in a qPCR instrument. The raw data of the melting curve were obtained and used to prepare shift curve graphs in GraphPad Prism software.

Lung leakage analysis
Mice were injected intravenously with 0.8 mg/ml EBA (20 µl/g body weight) (Huang et al., 2016). 45 minutes after injection, the mice were anesthetized, and the lungs were perfused with PBS. After weighing the lungs, the lung tissue was homogenized in 0.5 ml PBS. After homogenization, 1 ml formamide was added and co-incubated at 60°C for 18 hours to extract EBA. After centrifugation at 20,000 g for 10 min, the supernatant was taken and OD620 was measured. EBA was calculated based on the standard curve.

MPO activity assay
The mice were anesthetized. The lungs were perfused with PBS and then the lung tissues were removed and weighed. Lung tissues were homogenized in 0.5 ml PB solution. After homogenization, 50 µl 5% HTBS was added to the homogenates and mixed well. After repeated freezing and thawing, the supernatant was collected by centrifugation at 20,000 g for 10 min and used for MPO activity assay. MPO activity was determined according to the instructions of commercial kits.

Molecular docking
The 3D structures of IcaA and IcaB were downloaded from the AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/entry/A0A830Z2G3,
Mechanism of linezolid inhibited S. aureus

IcaA; https://alphafold.ebi.ac.uk/entry/A0A830YSS5, IcaB). The water molecules in both 3D structures were removed and the corresponding hydrogen atoms were added to both 3D structures using PyMol software. The 3D structure of the compound linezolid was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/#query=linezolid). Molecular docking was performed using AutoDock Vina (https://vina.scripps.edu, 1.1.2) with the default parameters (Eberhardt et al., 2021). After docking, the conformations with the lowest binding energy were analyzed with PyMol software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The results are expressed as the mean ± standard error (SE). The data of two groups were compared by Student t test, and multiple groups were compared by one-way analysis of variance (ANOVA) followed by Tukey’s test. P<0.05 was considered statistically significant.

RESULTS

Linezolid inhibits the growth and biofilm formation of S. aureus

Linezolid is very effective against bacterial infections, especially colds caused by infections of antibiotic-resistant bacteria. Thus, the effect of linezolid on the growth and biofilm formation of S. aureus was investigated, and it was found that linezolid effectively inhibited the growth and biofilm formation of S. aureus (Figure 1), with IC50 of 814.7 µM and 273.5 µM, respectively. This suggests that linezolid is more effective in inhibiting S. aureus biofilm formation, but inhibition of S. aureus growth may also contribute to the inhibition of S. aureus biofilm formation by linezolid since its IC50 is in the same order of magnitude.

Genome modification of S. aureus isolates the inhibitory effect of linezolid on S. aureus biofilm formation

S. aureus 50S ribosomal L3∆Ser145 mutation causes L3 structure rearrangement, which significantly re-
duces the affinity between linezolid and L3 (Belousoff et al., 2017). The L3ΔSer145 mutant S. aureus was generated by using CRISPR technology. The growth of mutant S. aureus was similar to that of wild-type S. aureus (Figure 2A), but the mutant S. aureus was significantly resistant to linezolid, and the IC50 increased from 814.7 µM to 92.08 mM, about 113.9 times higher (Figure 2B). The L3ΔSer145 mutation did not alter the biofilm-forming ability of S. aureus (Figure 3A). Linezolid effectively inhibited the biofilm formation of L3ΔSer145 mutant S. aureus (Figure 3B), with an IC50 of 927.2 µM, which was higher than that of the wild type (273.5 µM). The above results indicated that in addition to the inhibition of S. aureus growth contributing to the inhibition of S. aureus biofilm formation by linezolid, there were other mechanisms that might be involved in the inhibition of S. aureus biofilm formation.

**icaA or icaB knockout inhibits S. aureus biofilm formation**

icaA and icaB are proteases necessary for the synthesis of mature biofilms by S. aureus. The homologous recombination method was used to knock out icaA, icaB, or icaA and icaB, and it was found that knockout of icaA or icaB significantly inhibited the biofilm formation of S. aureus, although there was no superimposed effect of both icaA and icaB knockout (Figure 4B). Knockout of icaA, icaB, or both icaA and icaB had no effect on the growth of S. aureus (Figure 4A). Linezolid inhibited wild-type S. aureus biofilm formation dose-dependently. At high concentration, the inhibitory effect of linezolid was almost the same as the icaA knockout or both icaA and icaB knockout (Figure 4C). Linezolid can also inhibit the biofilm formation of icaB knockout S. aureus and make it decrease to the similar level of icaA knockout S. aureus at high dosage (Figure 4C). Linezolid no longer affected S. aureus biofilm formation in icaA or both icaA and icaB knockout S. aureus (Figure 4C). The above results suggest that inhibition of icaA activity may be the main reason for linezolid’s inhibition of S. aureus biofilm formation.

**Linezolid binds to icaA and inhibits its activity**

Molecular docking (DOCK) showed that linezolid may bind to icaA, and multiple residues including Tyr184, Asn199, Ser202, Asp227, Trp267, Asn380, etc. may be involved in the interaction between linezolid and icaA (Figure 5A). Thermal shift analysis revealed that linezolid significantly enhanced the thermal stability of wild-type icaA, while linezolid did not change the thermal stability of Trp267Ala mutant icaA (Figure 5B), indicating that linezolid directly binds to icaA, and that residue Trp267 plays a key role in the interaction between icaA and linezolid. The Trp267Ala mutation weakened icaA enzyme activity, increasing its EC50 for the substrate N-acetylglucosamine from 67.4 µM to 194.2 µM (Figure 5C). Linezolid dose-dependently inhibited the activity of wild-type icaA (Figure 5D), with an IC50 of 0.72 mM, but had little effect on the activity of Trp267Ala mutant icaA (Figure 5D). This was consistent with the results of thermal drift.

![Figure 3 - 50S ribosomal L3ΔSer145 mutant S. aureus is not resistant to linezolid in biofilm formation. 50S ribosomal L3ΔSer145 mutant of S. aureus was generated by using CRISPR technology. A. 50S ribosomal L3ΔSer145 mutant S. aureus has the same biofilm formation as wild-type S. aureus. Biofilm formation of different clones of mutant S. aureus was measured after co-culture with linezolid for 24 hours. B. linezolid inhibited the biofilm formation of 50S ribosomal L3ΔSer145 mutant S. aureus as wild-type S. aureus in a dose-dependent pattern. Biofilm formation was measured after 24 hours of co-culture of wild-type or mutant S. aureus with linezolid. Data are mean ± SE from three experiments.](image-url)
Mechanism of linezolid inhibited S. aureus

Figure 4 - Knockout of icaA, icaB or double knockout of icaA and icaB in S. aureus inhibits its biofilm formation. The icaA, icaB or icaA and icaB were knocked out by homologous recombination. A. Knockout of icaA, icaB or icaA and icaB has no effect on the growth of S. aureus. B. Knockout of ica, icaB or icaA and icaB inhibits the biofilm formation of S. aureus. C. Linezolid no longer affected the biofilm formation of icaA or icaA and icaB double knockout S. aureus, but linezolid could further inhibit the biofilm formation of icaB knockout S. aureus to icaA or double icaA and icaB knockout S. aureus biofilm formation levels. Growth and biofilm formation of icaA, icaB or double knockout S. aureus were measured as described in methods. Data are mean ± SE from three or four experiments.

Figure 5 - Linezolid directly binds to icaA and inhibits its activity. A. Molecular docking suggests that linezolid can embed into the pocket formed by icaA and bind to it. Residues such as Trp267 may be involved in the interaction between icaA and linezolid. Molecular docking of icaA and linezolid was performed using AutoDock Vina. B. Thermal shift analysis shows that linezolid binds directly to icaA, and residue Trp267 plays a key role in the interaction between icaA and linezolid. Purified icaA or Trp267Ala icaA were mixed with linezolid respectively, and then analyzed for thermal stability in qPCR instrument. Data are representative of three experiments. C. Mutation of Trp267 to Ala only partially affects the activity of icaA compared with wild-type icaA. D. Linezolid dose-dependently inhibited the activity of wild-type icaA, but no longer affected the activity of Trp267Ala icaA. Purified wild-type or mutant icaA were used for activity assays. Data are mean ± SE from three experiments.
The above results show that linezolid directly binds to icaA to inhibit its activity.

**Weak binding of Linezolid to icaB and its modest effect on its activity**

Molecular docking showed that linezolid may bind to icaB, and residues Asn74, Tyr75, Tyr230, Glu253, etc. may be involved in the interaction between linezolid and icaB (Figure 6A). Thermal drift analysis showed that linezolid weakly enhanced the thermal stability of icaB, but had no effect on the thermal stability of Tyr230Ala mutant icaB (Figure 6B). The Tyr230Ala mutation slightly reduced the enzymatic activity of icaB, increasing its EC50 for the substrate PNAG from 8.8 mM to 17.3 mM (Figure 6C). Linezolid has a weak effect on the activity of icaB, with an IC50 of 205.8 mM, and has a very minor effect on the activity of Tyr230Ala mutant icaB, with an IC50 greater than 1000 mM (Figure 6D). The above results indicated that linezolid had only a very minor effect on icaB. Compared with linezolid's very significant inhibition of icaA activity, it can be confirmed that linezolid inhibits the biofilm formation of S. aureus mainly by affecting icaA activity.

**Linezolid alleviates S. aureus-induced sepsis-caused acute lung injury by inhibiting biofilm formation**

The 50S ribosomal L3∆Ser145 mutant S. aureus was resistant to linezolid but had normal growth and biofilm formation capabilities. Sepsis was induced by intraperitoneal injection of mutant or wild-type S. aureus, and hematoxylin and eosin (HE) staining revealed that mutant S. aureus induced acute lung injury similar to wild-type S. aureus, including inflammatory cell invasion, lung leakage, and alveolar wall thickening (Figure 7A). Linezolid not only almost completely cured the lung injury induced by wild-type S. aureus infection, but also significantly improved the linezolid-resistant S. aureus infection-induced lung injury (Figure 7A and B). The lung leakage and neutrophil invasion were quantitated. It was found that linezolid not only almost blocked lung leakage induced by wild-type S. aureus infection (Figure 8A) and neutrophil invasion (Figure 8B), it also significantly reduced lung leakage and neutrophil invasion caused by 50S ribosomal L3∆Ser145 mutant S. aureus infection (Figure 8A and B). At the
same time, quantitative analysis of inflammatory factors revealed that linezolid inhibited pro-inflammatory factors increased by wild-type S. aureus infection to almost normal levels, including IL1β, IL6 and TNFα (Figure 9A, B and C). Linezolid also significantly inhibited the production of pro-inflammatory cytokines induced by 50S ribosomal L3ΔSer145 mutant S. aureus infection, including IL1β, IL6 and TNFα, although they were still significantly higher than normal levels (Figure 9). The above results indicate that biofilm formation of S. aureus is involved in diseases caused by S. aureus infection, and that the inhibition of biofilm formation is sufficient to alleviate the lung injury caused by S. aureus infection. They also indicate that linezolid can treat diseases caused by s. aureus infection by inhibiting S. aureus growth and biofilm formation.

DISCUSSION

Biofilm formation is one of the main causes of antibiotic resistance in S. aureus. Intercellular polysaccharide (PIA) plays a key role in biofilm formation (Nguyen et al., 2020). The ica operon of S. aureus encodes five proteins: icaR, icaA, icaD, icaB, and icaC (de Silva et al., 2002; Diemond-Hernandez et al., 2010). The above five proteins control the synthesis of PIA. IcaR is the repressor protein of ica operon, and knocking
Figure 9 - Linezolid reduces pro-inflammatory factors, including IL1β (A), IL6 (B), and TNFα (C), in lungs of S. aureus-infected mice, although linezolid inhibits pro-inflammatory factors induced by wild-type S. aureus infection more than the pro-inflammatory factors induced by 50S ribosomal L3ΔSer145 mutant S. aureus infection. The expression of pro-inflammatory factors in S. aureus-infected lung tissue was detected by qPCR. Data are mean ± SE from five mice.

out icaR can increase the expression of PIA. IcaA encodes a transmembrane Poly-beta-1,6-N-acetyl-D-glucosamine synthase, which is responsible for catalyzing the polymerization of glucosamine into oligomeric PNAG (poly-N-acetylglucosamine production) of about 10-20 residues. IcaD, the chaperone protein of icaA, enhances the activity of icaA approximately 20-fold. IcaB encodes a de-N-acetylation of Poly-β-1,6-N-acetyl-d-glucosamine, which partially deacetylates PNAG (about 15%-20%) and regulates some physical properties of PNAG, including charge. IcaC is involved in the polymerization of short-chain PNAG into long-chain PNAG and in the extracellular secretion of PNAG. IcaD has enzymatic activity similar to that of icaA, but it mainly synthesizes short-chain PNAGs and provides them to icaA to further synthesize long-chain PNAG (Arciola et al., 2015). It can be seen that icaA plays a vital role in biofilm formation. In fact, icaA expression was positively correlated with PIA production. Vancomycin treatment of S. aureus induced an upregulation of icaA and a significant increase in PIA. In contrast, the combination of clindamycin and gentamicin downregulated the expression of icaA in S. aureus, which also reduced biofilm formation. It can be seen that the synthesis of PIA depends on the expression of icaA, so icaA is a potential drug target. icaA expression was positively correlated with PIA production. Vancomycin treatment of S. aureus induced an upregulation of icaA and a significant increase in PIA. In contrast, the combination of clindamycin and gentamicin downregulated the expression of icaA in S. aureus, which also reduced biofilm formation. It can be seen that the synthesis of PIA depends on the expression of icaA, so icaA is a potential drug target. icaA expression was positively correlated with PIA production. Vancomycin treatment of S. aureus induced an upregulation of icaA and a significant increase in PIA. In contrast, the combination of clindamycin and gentamicin downregulated the expression of icaA in S. aureus, which also reduced biofilm formation. It can be seen that the synthesis of PIA depends on the expression of icaA, so icaA is a potential drug target (Abdel-Shafi et al., 2022; Cramton et al., 1999; Cue et al., 2012). For example, caspofungin promotes the anti-S. aureus activity of fluoroquinolone by inhibiting N-acetylglucosamine transferase (Siala et al., 2016).

The present research showed that linezolid directly bound to icaA, inhibited the activity of icaA, reduced the biofilm formation of S. aureus, and alleviated the lung injury caused by S. aureus infection. Linezolid bound to icaB weakly and slightly inhibited icaB activity. Compared with icaA, linezolid had a much weaker effect on icaB and required a much higher concentration. It can be seen that icaA is linezolid’s main target for inhibition of S. aureus biofilm formation. Linezolid can bind to 50S and inhibit the initiation of protein synthesis (Belousoff et al., 2017; Crowe-McAuliffe & Wilson, 2022; Wilson et al., 2008). The S. aureus 50S ribosomal L3ΔSer145 mutation caused a structural rearrangement of L3 such that the affinity of linezolid to ribosomes was significantly reduced (Belousoff et al., 2017). To rule out that linezolid reduces icaA expression by inhibiting protein synthesis and thus reduces biofilm formation, ribosomal L3ΔSer145 mutant S. aureus was generated by CRISPR. We found that L3ΔSer145 mutant S. aureus had growth and biofilm formation similar to wild-type S. aureus. However, linezolid no longer affected the growth of L3ΔSer145 S. aureus, but inhibited the biofilm formation of L3ΔSer145 mutant as well as of wild-type S. aureus. It has been reported that S. aureus infection can cause lung injury and even pneumonia (Rubinstein et al., 2008; Wang et al., 2022). Linezolid almost completely blocked lung injury induced by wild-type S. aureus infection by inhibiting the invasion of inflammatory cells into the lung, reducing inflammatory factors in the lung and preventing thickening of the lung wall after S. aureus infection. More importantly, linezolid can also alleviate the lung injury caused by the mutant strain infection. It can be seen that linezolid can bind directly to icaA to inhibit the activity of icaA, thereby reducing the biofilm formation of S. aureus. Therefore, inhibition of biofilm formation is beneficial to the treatment of diseases caused by S. aureus infection.
Mechanism of linezolid inhibited S. aureus

Through computer-based molecular docking screening of small molecule compound libraries, it was found that multiple oxadiazole compounds can bind to the penicillin-binding protein 2a (PBP2a) of MRSA and have anti-MRSA activity (O’Daniel et al., 2014). Recently, the crystal structure of the main protease of SARS-CoV-2 was docked with a library of small molecule compounds with anti-HIV activity to identify potential inhibitors that can bind to the main protease of CoV2 (Arthur et al., 2022). In the current study, mutating potential key residues confirmed the correctness of molecular docking between linezolid and icaA, indicating that the 3-D structure of the binding site between icaA and linezolid was suitable for structure-based computer-aided drug design (Yu & MacKerell, 2017). The present study provided a right molecular model suitable for computer-aided drug screening of small molecule inhibitors of icaA. In conclusion, our current research shows that linezolid can bind directly to icaA, inhibit its activity, reduce biofilm formation of S. aureus, and alleviate diseases caused by S. aureus infection. The 3-D model of the interaction between icaA and linezolid, and genetically modified S. aureus can be used for the development of highly effective icaA inhibitors and the investigation of S. aureus infection pathology.

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**References**


