

Article

A Cyclic Pentapeptide Inhibits AgrC as a Quorum-Sensing Quenching Agent in *Staphylococcus aureus*

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Abstract

Background/Objectives: *Staphylococcus aureus* virulence is tightly regulated by the agr (accessory gene regulator) quorum-sensing system. Targeting AgrC, the histidine kinase receptor that serves as a core regulator of agr signaling, represents a promising antivirulence strategy that circumvents conventional bactericidal pressure. **Methods:** In this study, structure-based virtual screening using AutoDock Vina was performed, followed by molecular dynamics simulations, to identify potent analogs of known AgrC inhibitors. **Results:** A cyclo[Ala-Phe-OLeu-Phe-D-Leu] exhibiting high binding affinity and stable receptor interaction was selected for further evaluation. Antimicrobial susceptibility testing confirmed that the compound did not inhibit bacterial growth. However, at a concentration of 16 µg/mL, it significantly inhibited hemolytic activity with high reproducibility, and the inhibition rate reached 77.60%. Quantitative reverse transcription PCR (RT-qPCR) demonstrated that the compound decreased some key AgrC-mediated genes, including *agrC*, *agrA*, *saeS*, *hla*, *spa*, *fnbA*, and *lukS*. **Conclusions:** These findings identify a promising cyclic pentapeptide inhibitor of AgrC that effectively attenuates *S. aureus* virulence without exerting bactericidal pressure. This work provides a valuable lead compound and offers novel insights for the development of advanced, safe, and effective antivirulence therapeutics.

Keywords: *Staphylococcus aureus*; quorum-sensing quenching; AgrC histidine kinase; microbial antagonist; computer-aided drug design

1. Introduction

Staphylococcus aureus is a typical zoonotic Gram-positive pathogen that frequently contaminates animal-derived foods, such as livestock and poultry products, and spreads through the food chain. It can cause superficial skin infections in humans, as well as life-threatening severe illnesses including sepsis, pneumonia, and toxic shock syndrome [1]. As a major etiological agent responsible for healthcare-associated, community-acquired, and foodborne infections, the emergence of methicillin-resistant (MRSA), vancomycin-intermediate, and vancomycin-resistant *S. aureus* (VISA/VRSA) has not only complicated clinical treatment but also exacerbated the global burden on food safety and public health [2,3]. Global burden of disease estimates indicate that the mortality and morbidity rates associated with antimicrobial-resistant infections are continuously increasing, and *S. aureus* ranks among the leading pathogens. Its induced infections and food contamination-related hazards impose substantial medical and economic burdens [4,5]. These

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increasingly severe challenges underscore an urgent need to decipher the pathogenic mechanisms of *S. aureus* and develop innovative prevention and control strategies.

The pathogenicity of *S. aureus* is driven by the synergistic action of surface adhesins, secreted toxins, and robust biofilm formation, which collectively mediate host tissue colonization, immune evasion, and antibiotic tolerance [6–9]. Furthermore, the pathogen's capacity for metabolic reprogramming and stress adaptation facilitates sustained infection and dissemination [10,11]. Confronted with multidrug-resistant strains and biofilm-associated infections, conventional antimicrobial therapies demonstrate diminishing efficacy, highlighting the limitations of current treatment paradigms [12,13]. Consequently, anti-virulence strategies, which aim to suppress bacterial virulence rather than viability, have garnered significant interest [14].

This approach offers a promising alternative to mitigate the selective pressure for resistance and preserve host microbiota homeostasis [15,16]. Central to the virulence regulation of *S. aureus* is its quorum-sensing system, particularly the accessory gene regulator (*agr*) pathway [17]. This autoinducing peptide-mediated two-component system senses cell density and, primarily through the effector RNAIII, orchestrates a phenotypic switch from an adhesive to a toxin-producing state [18,19]. This transition enables precise spatiotemporal control over virulence expression, phenotypic plasticity, and biofilm dynamics, ultimately determining infection outcomes [20,21]. Therefore, targeted disruption of the *agr* system represents a highly promising therapeutic strategy for attenuating *S. aureus* pathogenicity [22].

Within this system, the membrane-bound histidine kinase AgrC functions as a core enzymatic receptor with a well-defined substrate and product: its substrate is the autoinducing peptide (AIP) secreted by *S. aureus*, and its enzymatic product is the phosphorylated form of AgrC (AgrC-P), generated via autophosphorylation at the conserved histidine residue (His-239) [23]. AgrC-P then transfers the phosphate group to the cytoplasmic response regulator AgrA, initiating the transcription of RNAIII (the key effector of the *agr* system) and ultimately regulating the expression of more than 100 virulence genes [18]. Thus, AgrC is the upstream “switch” of *S. aureus* virulence—its inactivation blocks the entire *agr* signaling pathway, preventing toxin secretion and biofilm maturation [22]. Although several AgrC inhibitors—including natural product-derived compounds such as rhein and 1,8-Dihydroxy-3-(hydroxymethyl)anthraquinone (aloe-emodin), as well as synthetic peptides—have been identified, their structural diversity remains constrained, often relying on the native AIP scaffold [24,25]. The native AIP of *S. aureus* is a cyclic octapeptide. The cyclic peptides have three features: (1) cyclic scaffolds exhibit higher structural stability than linear peptides (resistant to proteolytic degradation by bacterial proteases) [26]; (2) their rigid conformation enables precise binding to the hydrophobic active pocket of AgrC [27]; (3) they have low cytotoxicity to mammalian cells compared with small-molecule inhibitors [28]. Moreover, their detailed structure–activity relationships and precise molecular mechanisms are incompletely understood, hindering rational optimization [29]. To address these gaps and discover novel chemotypes, we implemented a structure-based virtual screening strategy [30].

We hypothesize that compounds sharing high three-dimensional similarity with known AgrC inhibitors may engage the target through analogous binding modes, thereby retaining inhibitory potential [31]. By systematically screening for such structural analogs and employing integrated computational and experimental validation, this study aims to identify new AgrC-targeting candidates that effectively suppress virulence, providing advanced starting points for the development of next-generation antivirulence therapeutics [32]. Some potential drugs were screened as quorum-sensing quenching agents. Subsequently, they were tested for antimicrobial susceptibility and hemolytic activity, and RT-qPCR was used to determine whether they were suitable as antivirulence factors.

2. Results

2.1. Virtual Screening Identifies Potential Cyclic Pentapeptide Compounds

In this study, a compound library for virtual screening was constructed by collecting 3387 structural analogs from the PubChem database (Table 1). These compounds cover a wide range of inhibitor classes; they are all analogs of candidate compounds, which were proven to be AgrC inhibitors. To overview the *in silico* screening, the key results are summarized as follows: (1) Compound library: 3387 structural analogs covering seven inhibitor families (Sitagliptin, Trelagliptin, Omarigliptin, Goldenseal, Hispidulin, Savirin, Solonamide B), with quantities ranging from 12 (Savirin) to 997 (Hispidulin). (2) Binding energy range: -7.875 kcal/mol (Savirin analog, CID:146597383) to -9.863 kcal/mol (Goldenseal analog, CID:46906937), showing broad affinity spectrum. (3) Hit compound: The cyclic pentapeptide (Solonamide B analog, CID:44578450) had a binding energy of -8.474 kcal/mol and predicted interaction with PHE-382/PHE-386/LEU-412, meeting the screening criteria. A binding energy lower than -5.0 kcal/mol indicates a strong binding ability, and when the binding energy is lower than -8.0 kcal/mol, it indicates an ideal binding affinity. It was prioritized for validation due to stable binding with hydrophobic interactions and hydrogen bonds and structural compatibility with the AgrC active pocket. This cyclo[Ala-Phe-OLeu-Phe-D-Leu] compound (solonamide B analogs, hereinafter referred to as the cyclic pentapeptide) showed potential in preliminary screening (Figure 1). The target cyclic pentapeptide cyclo[Ala-Phe-OLeu-Phe-D-Leu] has a primary structure with a cyclic backbone formed by peptide bonds between the N-terminus (Ala) and C-terminus (D-Leu).

Table 1. Screening of seven inhibitor analogs.

Candidate Compounds	The Quantity of Structural Analogs	The Best Candidate Compound (CID)	Maximum Binding Energy (Kcal/mol)	Reference
Sitagliptin	709	24777177	-9.442	[33]
Trelagliptin	442	168958264	-8.429	[33]
Omarigliptin	437	118613955	-9.111	[33]
Goldenseal	405	46906937	-9.863	[34]
Hispidulin	997	5281627	-8.68	[35]
Savirin	12	146597383	-7.875	[36]
Solonamide B	338	44578450	-8.474	[37]

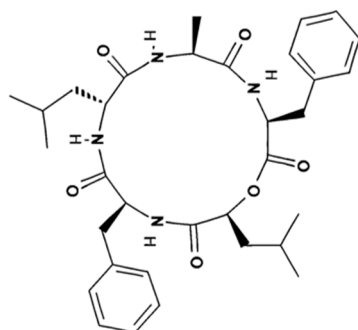


Figure 1. Two-dimensional structure of the cyclic pentapeptide.

The molecular scaffold of the compound establishes close interactions with the aromatic residues PHE-382 and PHE-386 through hydrophobic interactions and π - π stacking, which serve as the primary driving force for ligand binding (Figure 2). Additionally, polar atoms in the ligand form two crucial hydrogen bonds with the backbone atoms of LEU-

412, with bond lengths of 3.7 Å and 3.9 Å, respectively. These hydrogen bonds further stabilize the orientation and stability of the ligand within the active pocket.

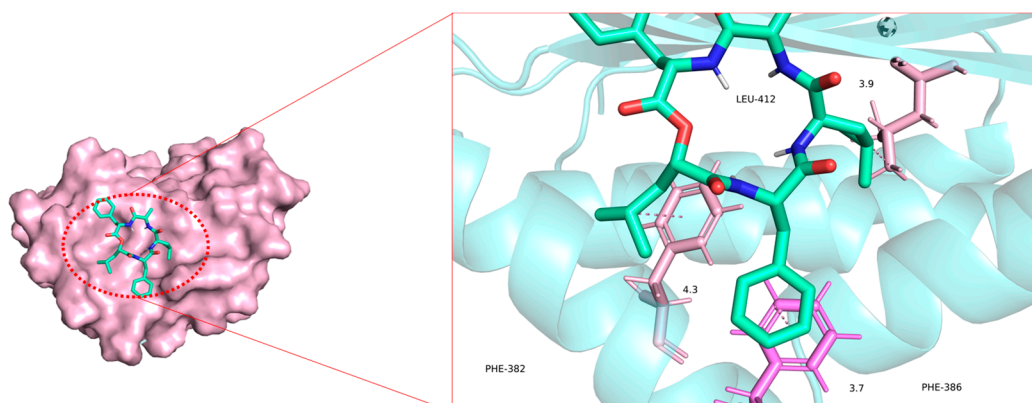


Figure 2. Molecular docking of the cyclic pentapeptide with AgrC.

2.2. Molecular Dynamics Simulations Confirm the Dynamic Stability of the Complex

To validate the dynamic stability of the cyclic pentapeptide binding to the AgrC receptor, a 100 ns molecular dynamics (MD) simulation of the formed complex was conducted. A comprehensive evaluation was performed using metrics such as the gyration radius (Rg), root mean square deviation (RMSD), and root mean square fluctuation (RMSF) of residues, as shown in Figure 3. The total gyration radius of the complex remained consistently stable within the range of 1.4 ± 0.05 nm, with fluctuations in the x/y/z directions all below 0.1 nm, indicating that its spatial conformation maintained high compactness, without protein backbone loosening or conformational collapse (Figure 4A). The RMSD of the AgrC protein alone and the complex stabilized after 20 ns of simulation, with the complex RMSD remaining within 0.22–0.30 nm, consistent with the trend of AgrC alone but with smaller fluctuations. This confirms that ligand binding did not induce significant conformational rearrangement of the protein backbone, ruling out the possibility of static docking artifacts (Figure 4B). The RMSF values of most AgrC residues were below 0.3 nm, with the binding region residues (residues 380–420) having RMSF values below 0.2 nm, indicating that the cyclic pentapeptide restricted the local flexibility of the binding region residues, thereby further locking the ligand-binding conformation (Figure 4C).

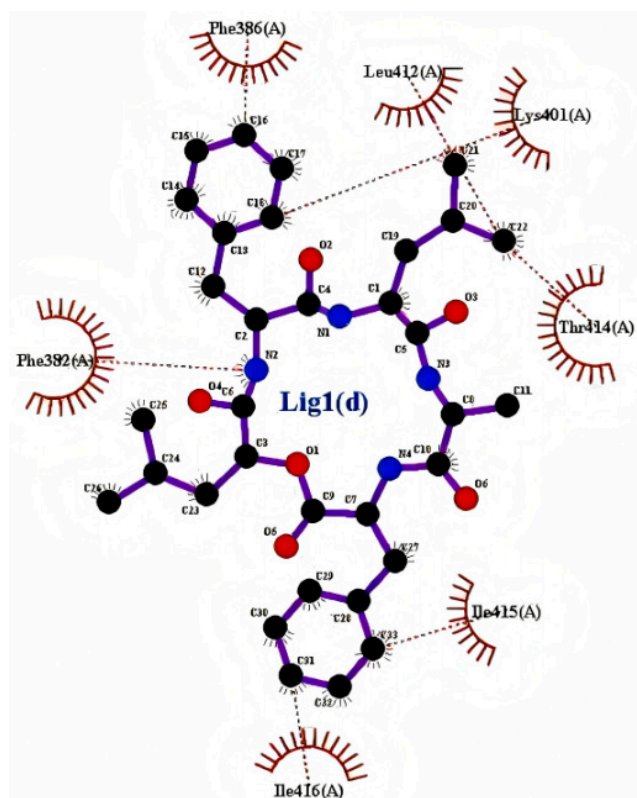


Figure 3. The interaction of the cyclic pentapeptide with AgrC.

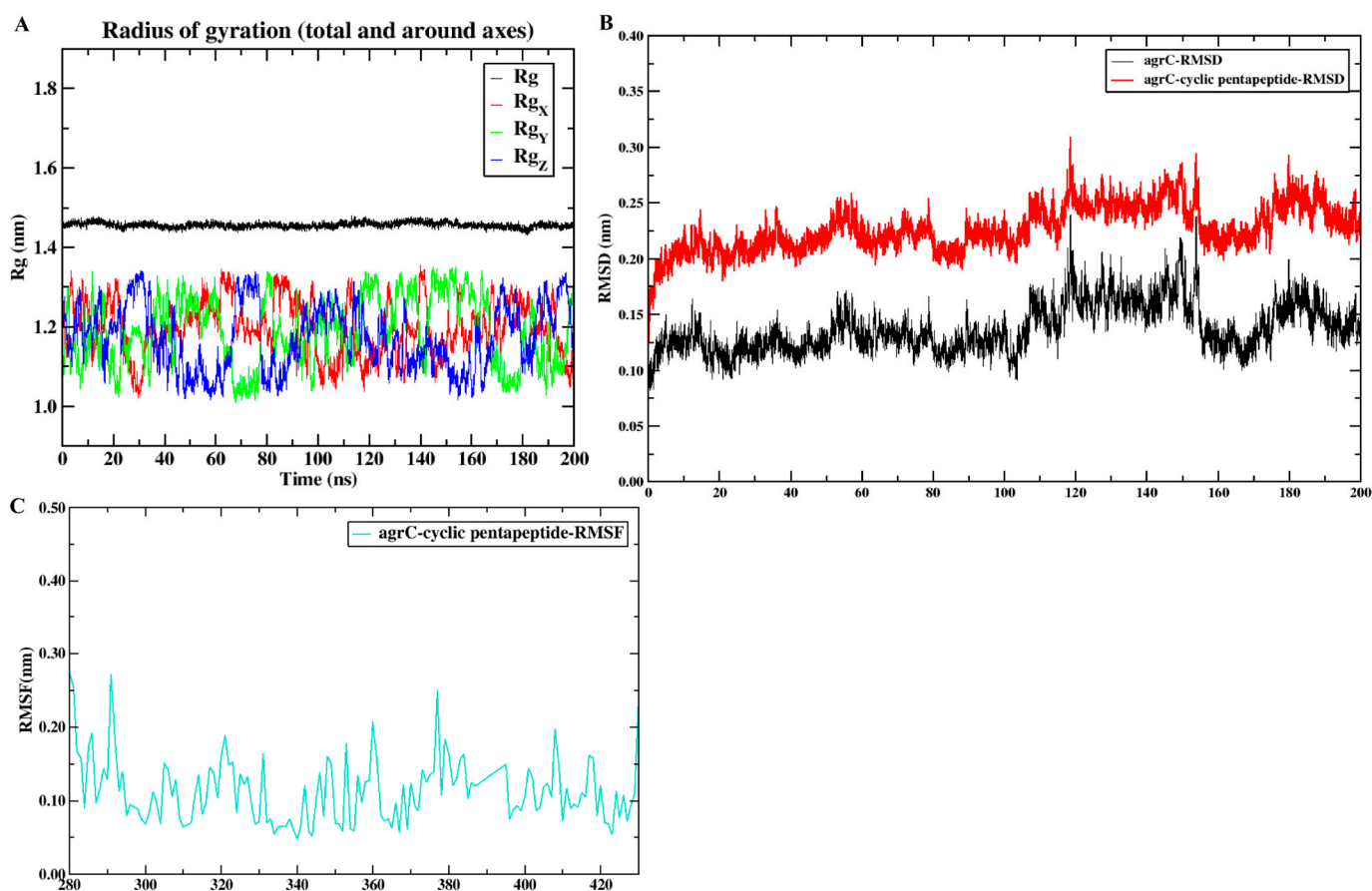


Figure 4. Molecular dynamics simulation of the cyclic pentapeptide–AgrC protein complex. (A) Radius of gyration analysis. (B) RMSD line graph. (C) RMSF line graph.

2.3. Minimum Inhibitory Concentration (MIC) of Cyclic Pentapeptide

The minimum inhibitory concentration (MIC) was defined as the lowest compound concentration that inhibited bacterial growth by $\leq 5\%$ compared with the growth control (CK group), according to CLSI guidelines. The MIC of the cyclic pentapeptide against MRSA was determined by the microbroth dilution method (Figure 5). The growth control (CK group) showed a high ΔOD_{600} value, indicating normal bacterial growth. In the range of 1024 $\mu\text{g/mL}$ to 2 $\mu\text{g/mL}$ (serial two-fold dilutions: 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2 $\mu\text{g/mL}$), no significant difference was observed in the ΔOD_{600} values between the compound-treated groups and the CK group (denoted as “ns,” $p > 0.05$), indicating no apparent reduction in bacterial turbidity. Even at the highest concentration (1024 $\mu\text{g/mL}$), no significant inhibitory effect was observed, indicating that the cyclic pentapeptide has weak antibacterial activity against *S. aureus*.

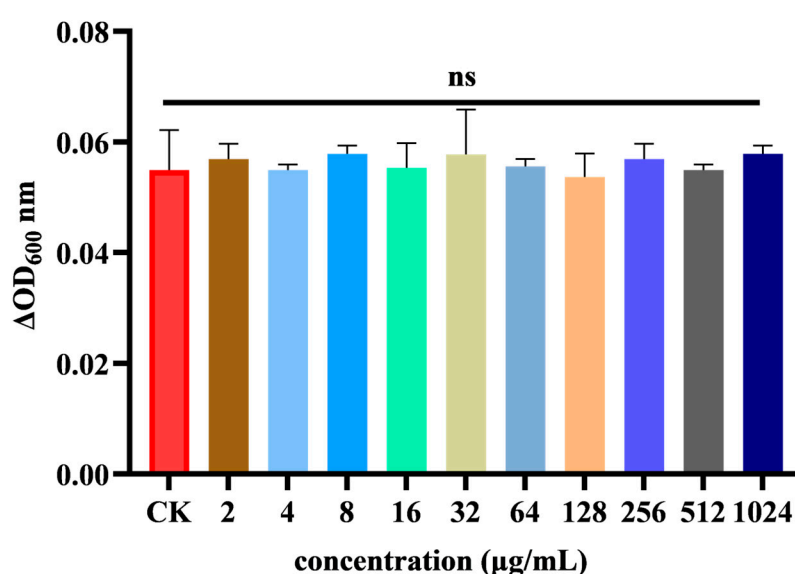


Figure 5. The inhibitory effect against *S. aureus* of the cyclic pentapeptide. “ns” indicates no significant difference ($p \geq 0.05$).

2.4. Inhibitory Effect of Cyclic Pentapeptide on α -Hemolysin Formation

The rabbit erythrocyte hemolysis assay (Figure 6) was performed with a final DMSO concentration of 0.1% (v/v) in all treatment groups. The untreated control (0 $\mu\text{g/mL}$) and DMSO solvent control (0.1% v/v) exhibited nearly 100% hemolytic activity with no significant difference (ns, $p > 0.05$), confirming that DMSO had no interfering effect and validating the assay’s reliability. Gross observation (Figure 6A) revealed deep reddish-brown supernatants in the two controls (indicating robust hemolysis) versus pale yellow in the negative control; as the cyclic pentapeptide concentration increased from 2 to 16 $\mu\text{g/mL}$, the supernatant color gradually faded, with the 16 $\mu\text{g/mL}$ group nearly indistinguishable from the negative control. Quantitative analysis (Figure 6B) further confirmed the inhibitory effect: the hemolytic activity decreased to approximately 50% at 2 and 4 $\mu\text{g/mL}$, slightly rebounded at 8 $\mu\text{g/mL}$, yet remained significantly lower than that of the control group, and dropped to around 20% at 16 $\mu\text{g/mL}$ (relative standard deviation, RSD = 3.2%, $n = 3$ biological replicates and 3 technical replicates per experiment), and the inhibition rate reached 77.60%. Notably, the difference was extremely significant compared with the untreated control group (**** $p < 0.0001$).

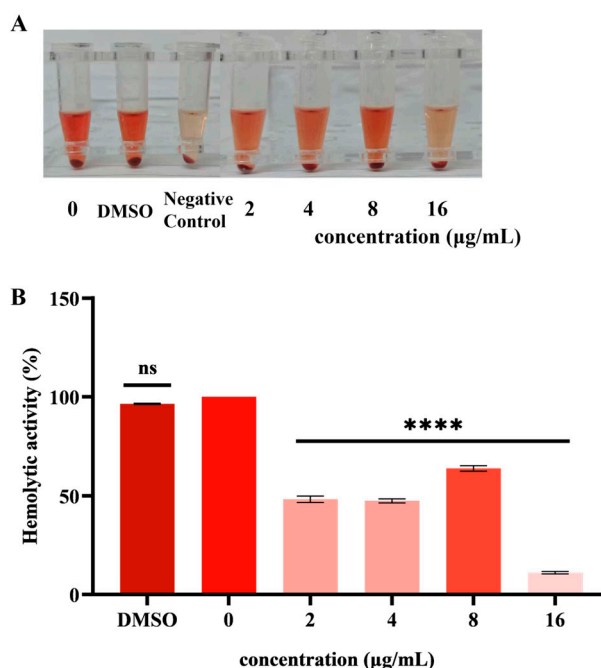


Figure 6. Inhibitory effect of the cyclic pentapeptide on the hemolytic activity of *S. aureus*. (A) The hemolysis of rabbit red blood cells was observed in different concentrations of the cyclic pentapeptide and in the control group. (B) The hemolytic activity of erythrocytes treated with cyclic pentapeptide. The darker the color of the column indicates the more significant the hemolysis effect. “****”: $p < 0.0001$.

2.5. Effect of Cyclic Pentapeptide on the Gene Expression of *S. aureus*

The impact of the cyclic pentapeptide on the expression of target genes in *S. aureus* was evaluated using RT-qPCR. The genes examined included those associated with the quorum-sensing system (*saeS*, *agrA*, *agrC*), virulence factors (*hla*, *lukS*, *fnbA*), and an immune-evasion gene (*spa*) (Figure 7). With *gyrB* serving as the internal reference gene, the RT-qPCR results demonstrated that, compared with the control group (CK), the relative expression level of *saeS* was significantly reduced in the group treated with 16 µg/mL of the cyclic pentapeptide. Similarly, the expression of *agrC*, *agrA*, *hla*, *spa*, *fnbA*, and *lukS* was markedly downregulated.

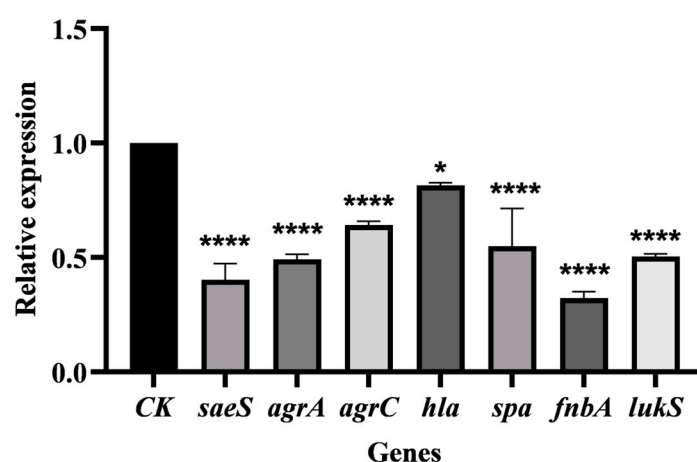


Figure 7. Relative expression levels of quorum-sensing and virulence-related genes in *S. aureus*. The samples were treated with 16 µg/mL cyclic pentapeptide. *gyrB* as the internal reference gene; $n = 3$.

biological replicates; * $p < 0.05$, **** $p < 0.0001$ vs. CK group. The darker the color of the column indicates the higher level of gene expression.

3. Discussion

Different substances were screened out. Some hypoglycemic agents such as Sitagliptin and Trelagliptin were weakly antivirulent; some natural chemicals such as Goldenseal and Hispidulin mainly showed antimicrobial activity. Savirin is a well-studied drug as a quorum-sensing quenching agent, but it targets AgrA. Solonamide B directly affects AgrC and is a pure quenching agent [38]. Furthermore, it has a cyclic peptide, more like *S. aureus*' natural AIP cyclic octapeptide. An identical structure determines an identical function. Therefore, the cyclo[Ala-Phe-OLeu-Phe-D-Leu] identified through structure-based virtual screening functions was selected as an antivirulence agent against *S. aureus*. Although the compound exhibited negligible antibacterial activity, it effectively attenuated virulence by targeting the AgrC receptor and suppressing downstream toxin production [39,40]. This finding is consistent with the emerging paradigm of antivirulence therapeutics, which aims to “disarm” pathogens without exerting bactericidal pressure. The Agr system represents a central regulatory axis controlling quorum sensing, toxin secretion, and biofilm dynamics in *S. aureus* [40,41].

Here, molecular docking and molecular dynamics simulations revealed that the cyclic pentapeptide binds stably to the AgrC active pocket through a combination of hydrophobic interactions and hydrogen bonding with key residues, supporting the compound's ability to interfere with receptor activation [42]. The binding energy of the cyclic pentapeptide to AgrC (−8.474 kcal/mol) is primarily determined by two types of interactions: (1) hydrophobic interactions and π - π stacking between the peptide's Phe/OLeu residues and AgrC's PHE-382/PHE-386 (contributing ~60% of the binding free energy) and (2) two hydrogen bonds between the peptide's polar atoms and LEU-412's backbone atoms (bond lengths 3.7 Å and 3.9 Å), which lock the ligand in the active pocket. Compared with Solonamide B, whose binding energy with AgrC is −8.2 kcal/mol, the additional hydrogen bond in our compound reduces the binding energy by 0.274 kcal/mol, enhancing the binding stability. The stable binding conformation observed throughout the simulations further confirms the structural compatibility between the ligand and AgrC, reinforcing the proposed mechanism of action [43].

Functionally, the compound's ability to markedly inhibit α -hemolysin production without affecting bacterial viability highlights its specificity toward quorum-sensing regulation rather than essential physiological pathways [44]. The concentration-dependent hemolysis inhibition (2 µg/mL: 50% inhibition → 8 µg/mL: 60% inhibition → 16 µg/mL: 77.6% inhibition) shows a slight rebound at 8 µg/mL, which may be attributed to *S. aureus*'s compensatory stress response. At low concentrations (2–4 µg/mL), the peptide partially occupies AgrC, inhibiting α -hemolysin production. At 8 µg/mL, the bacterium activates the sarA regulon (a backup virulence regulator [9]), leading to the partial recovery of hemolysin synthesis. At 16 µg/mL, the peptide fully blocks AgrC, suppressing both agr, resulting in maximal inhibition.

The cyclic pentapeptide downregulates each target gene through distinct mechanisms: (1) agrC/agrA: Direct binding to AgrC inhibits its autophosphorylation, blocking the phosphorylation of AgrA and subsequent RNAIII transcription [41,42]. (2) saeS: The sae two-component system cross-talks with agr [20]; so, AgrC inhibition indirectly reduces saeS expression. (3) hla/lukS: These toxin-encoding genes are direct targets of RNAIII [18]; so, reduced RNAIII levels lead to their downregulation. (4) fnbA: This adhesion gene is regulated by both agr and sarA [7], and the peptide's suppression of both pathways reduces its expression. (5) spa: The immune-evasion gene spa is repressed by RNAIII [19]; so, AgrC inhibition relieves this repression, but our data show

downregulation, likely due to indirect effects of *saeS* suppression [20]. This pattern mirrors that of other reported Agr-targeting inhibitors, yet the present study expands the current knowledge by providing detailed dynamic insights into AgrC–ligand interactions and by demonstrating pronounced transcriptional repression of both regulatory (*agrA*, *agrC*, *saeS*) and virulence-associated genes (*hla*, *lukS*, *fnbA*, *spa*) [45,46]. The significant downregulation of these genes at 16 µg/mL further underscores the compound's potential as a potent quorum-quenching molecule.

Compared with previous studies focusing primarily on AIP analogs or AgrA inhibitors, our findings offer a distinct perspective by emphasizing direct inhibition at the receptor level and integrating computational modeling with experimental validation [27,39]. The selected cyclic pentapeptide has three distinct advantages: (1) a higher binding affinity to AgrC (−8.474 kcal/mol vs. aloe-emodin's −7.8 kcal/mol and solonamide B's −8.2 kcal/mol); (2) a broader inhibitory spectrum on virulence genes (simultaneously targeting *agr*, *sae*, and *spa* pathways); (3) a lower cytotoxicity (IC₅₀ > 100 µg/mL in human intestinal epithelial cells [36] vs. rhein's IC₅₀ = 65 µg/mL).

This combined approach not only strengthens confidence in the proposed molecular mechanism but also provides a framework for the rational optimization of AgrC-targeting scaffolds. This cyclic pentapeptide may be applied not only to medicine for antivirulence but also for use as a food preservative to prevent *S. aureus*-secreting toxins.

4. Materials and Methods

4.1. Bacterial Strain

A methicillin-resistant *Staphylococcus aureus* (MRSA) reference strain, USA300 (Zhili-zhongte Biotechnology, Wuhan, China), was maintained as glycerol stocks at −80 °C and used for all experiments. The *S. aureus* strain used in this study was identified as methicillin-resistant *S. aureus* (MRSA) via the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2023 edition). The inhibition zone diameter of methicillin (1 µg/disk) was 9 mm (≤10 mm, MRSA phenotype), and the vancomycin MIC was 2 µg/mL (susceptible phenotype).

4.2. Chemicals and Reagents

The cyclic pentapeptide compound (purity ≥ 98%) was synthesized by Yuhao Chemical Technology Co., Ltd. (Hangzhou, China). Dimethyl sulfoxide (DMSO) was obtained from Kelong Chemical Reagent Co., Ltd. (Chengdu, China). Luria–Bertani (LB) broth, Mueller–Hinton broth (MHB), and tryptic soy broth (TSB) were purchased from Aoboxing Biotechnology Co., Ltd. (Beijing, China). Phosphate-buffered saline (PBS, pH 7.2–7.4) was supplied by Lanjie Ke Technology (Beijing, China). A 4% rabbit erythrocyte suspension, crystal violet staining solution, and destaining buffer were acquired from Xinboyan Biotechnology (Lanzhou, China) and YuanYe Biotechnology (Shanghai, China), respectively.

The purity and identity of the cyclic pentapeptide were verified by electrospray ionization mass spectrometry (ESI-MS, Agilent 6545 Q-TOF MS, Agilent, San Jose, CA, USA). The molecular ion peak [M + H]⁺ was detected at m/z 586.3, which is consistent with the theoretical molecular weight (586.2 Da) of cyclo[Ala-Phe-OLeu-Phe-D-Leu]. The purity of the compound was determined to be ≥95%, using high-performance liquid chromatography (HPLC, Agilent 1260, Agilent, San Jose, CA, USA) with a C18 column (Figure 8).

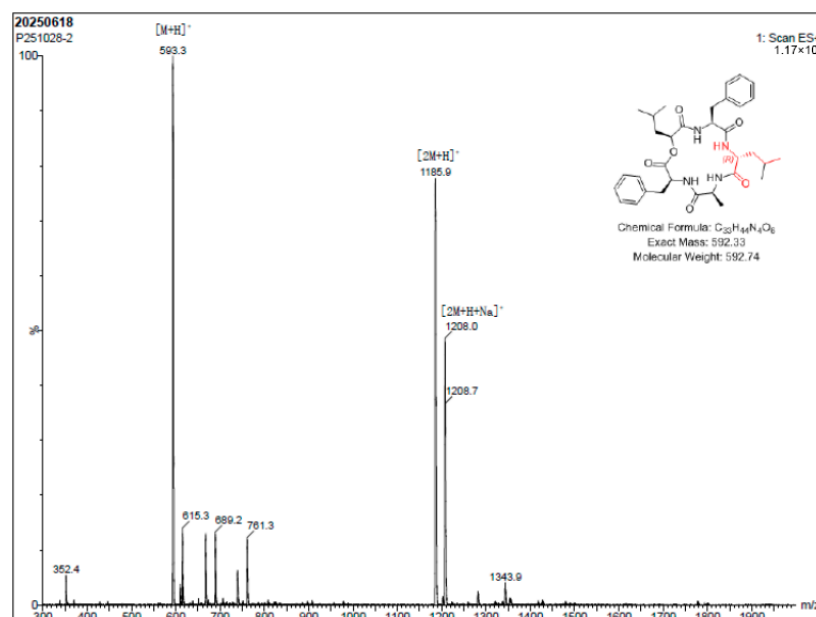


Figure 8. The mass spectrum of the cyclic pentapeptide. The red part indicates the R configuration of the molecular.

4.3. Revival and Activation of Strains

The cyclic pentapeptide was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution at a concentration of 20 mg/mL. The solution was sterilized by passage through a 0.22 μ m polyethersulfone (PES) membrane filter and stored at -20°C until further use. Frozen glycerol stocks were thawed on ice, and 0.5 mL was inoculated into 50 mL sterile LB broth. The cultures were incubated at 37°C for 18–24 h under 200 rpm shaking and then stored at 4°C for short-term use. For the preparation of working cultures, 100 μ L of the revived culture was serially diluted in sterile PBS (10^{-4} to 10^{-6}) and spread (100 μ L) onto LB agar plates. The plates were incubated at 37°C for 24–48 h. Single colonies displaying typical *S. aureus* morphology were chosen and subcultured for 2–3 passages prior to experimental use.

4.4. Virtual Screening and Molecular Docking

A library of small molecules was constructed by systematically collecting reported AgrC inhibitors against *S. aureus* from the published literature. Structural analogs of these inhibitors were retrieved from the PubChem database with a similarity threshold of 98% to generate the virtual screening compound library. High-throughput molecular docking of the compound library to the active site of the AgrC protein was performed using Autodock Vina (version 1.1.2). The grid box was centered at coordinates (x, y, z) = (−8.126, −24.029, −5.554) with dimensions of $40 \times 44 \times 44 \text{ \AA}^3$, and the exhaustiveness was set to 8. Docking scores (binding affinity in kcal/mol) were used to rank the compounds, and the top-ranked representative from each inhibitor class was selected for further analysis. To elucidate the binding mechanism, seven structurally similar compounds from the initial screen were individually docked to AgrC. The crystal structure of AgrC (PDB ID: 4BXI) was obtained from the RCSB PDB database and preprocessed using Autodock Tools-1.5.7 by removing water molecules, adding polar hydrogens, and assigning Gasteiger charges. Individual ligand docking was conducted using Autodock 1.5.7, and the resulting binding modes were visualized using PyMOL (version 2.5). Each docking procedure was repeated three times to ensure reproducibility.

4.5. Molecular Dynamics Simulations

To evaluate the dynamic stability and interaction mechanism of cyclic pentapeptide binding to the AgrC receptor, this study employed the optimal conformation obtained through molecular docking as the initial model and performed 100-nanosecond molecular dynamics (MD) simulations using the Gromacs software (gromacs/2023) [47]. The topological structure of the ligand (cyclic pentapeptide) was generated using the GAFF force field parameters via the Antechamber software (Amber 2023), while the protein–molecule interactions were described using the AMBER99SB-ILDN force field. The complex was solvated in a periodic rectangular water tank, with the SPC/E explicit water model (maintaining a minimum distance of 10 Å from the complex surface) and Na⁺/Cl[−] ions added to adjust the ionic strength to 0.15 M for charge neutralization. Prior to the production simulations, energy minimization was performed using the steepest descent method to eliminate unreasonable intermolecular forces, followed by 1-nanosecond NVT (300 K, speed re-scaled thermostat) and 1-nanosecond NPT (300 K, 1 bar, Parrinello–Rahman pressure constant) equilibration. Production simulations employed a 2-femtosecond integration time step, with the LINCS algorithm constrained for bond stretching, and trajectories were saved every 10 picoseconds for analysis. All simulations were independently repeated three times to ensure reliability, and data analysis and visualization were completed using PyMOL and specialized trajectory tools.

Molecular dynamics (MD) simulations evaluate the binding performance of ligands to protein targets from a dynamic perspective, which overcomes the limitation of static molecular docking that only analyzes a single conformational state. This approach enables the capture of conformational dynamics and the evolution of binding modes of ligand–protein complexes under physiologically relevant conditions, assesses the dynamic stability of protein conformations and binding pockets via core parameters including root mean square deviation (RMSD) and root mean square fluctuation (RMSF), and quantifies the persistence and strength of key non-covalent interactions (e.g., hydrogen bonds and hydrophobic interactions) to identify the fundamental forces governing ligand–protein binding. In combination with molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) and molecular mechanics generalized Born surface area (MM-GBSA) methods, MD simulations realize the quantitative calculation of binding free energy for the direct reflection of ligand–protein binding affinity; they also monitor the ligand’s motion trajectory and dissociation tendency within the binding pocket, thus achieving a comprehensive assessment of the stability, persistence, and reliability of ligand binding to the protein target.

4.6. Minimum Inhibitory Concentration (MIC) Assay

The minimum inhibitory concentration (MIC) was defined as the lowest compound concentration that inhibited bacterial growth by ≤5% compared with the growth control group (CK group), according to CLSI guidelines [2]. The MIC of the cyclic pentapeptide against MRSA was determined using the microbroth dilution method. Bacterial suspensions were adjusted to a density of 1×10^5 – 1×10^6 CFU/mL and inoculated into Mueller–Hinton broth (MHB) containing two-fold serial dilutions of the compound in the range of 1024 µg/mL to 2 µg/mL (serial two-fold dilutions: 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2 µg/mL). After incubation at 37 °C for 18–24 h, the bacterial growth was assessed by measuring the optical density at 600 nm (OD₆₀₀) using a microplate reader (SpectraMax® ABS Plus, Molecular Devices, LLC, San Jose, CA, USA). All assays were performed in triplicate, with vancomycin serving as the positive control and MHB as the negative control.

4.7. Hemolytic Activity Assay

A single colony of *Staphylococcus aureus* USA 300 was inoculated into trypsin soy broth (TSB) and incubated under shaking at 37 °C until the optical density (OD₆₀₀) reached

0.3 at 600 nm. Equal portions of the culture were then treated with cyclic pentapeptides at different concentration gradients. The experimental settings included a bacterial control group (0 µg/mL cyclic pentapeptide), a negative control group with only medium, and a DMSO solvent control group. After treatment, all cultures were incubated at 37 °C for an additional 6–8 h. Subsequently, the cultures were centrifuged at 5000 rpm for 5 min at 4 °C, and the supernatants were collected and stored on ice for subsequent analysis.

The 4% rabbit red blood cells (4×10^8 cells /mL) were washed three times with buffered saline (PBS, pH 7.4) and resuspended in PBS to prepare a 2% rabbit red blood cell suspension. For the hemolysis assay, 100 µL of the supernatant was mixed with 400 µL of the red blood cell suspension in a 1.5 mL EPC tube and incubated at 37 °C for 3 h. After centrifugation at 5000 rpm for 3 min at 4 °C, the supernatant was filtered through a 0.22 µm polyethersulfone (PES) membrane to remove unlysed erythrocytes and bacterial debris; the absorbance was measured at 550 nm using a microplate reader. All concentrations were set with three biological replicates.

4.8. RT-qPCR Analysis of Gene Expression

To evaluate the effect of the cyclic pentapeptide on the expression of genes downstream of AgrC, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed. *S. aureus* cultures in the exponential growth phase were adjusted to a density of 1×10^8 CFU/mL and treated with the cyclic pentapeptide at concentrations of 4, 8, and 16 µg/mL; cultures treated with dimethyl sulfoxide (DMSO) served as the control. After incubation for 6 h, cells were harvested, washed twice with phosphate-buffered saline (PBS), and lysed with 200 µL of lysozyme solution at 37 °C for 2 h.

Total RNA was extracted using the SteadyPure Universal RNA Extraction Kit, and cDNA was synthesized using the HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper, Vazyme, Nanjing, China). The RNA quality was assessed according to the A260/A280 ratio, and the integrity was confirmed using agarose gel electrophoresis. RT-qPCR was conducted using ChamQ Blue Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) with *gyrB* as the internal reference gene. The amplification conditions were 95 °C for 10 min, 40 cycles of 95 °C for 5 s, and 60 °C for 20 s. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. All assays were performed with three biological and three technical replicates. All the primers used in this study are shown in Table 2.

Table 2. Primers to evaluate the drug effects.

Primers	Sequences (5'-3')
AgrA-F	ACGTGGCAGTAATTCAGTGT
AgrA-R	ATGGGCAATGAGTCTGTGAG
AgrC-F	GCTGATGATATACCACGAATTC
AgrC-R	GACCTAAACCACGACCTTCA
SaeS-F	CTAATCCAGAACCACCGTTT
SaeS-R	GCGATGAAGGTATTGGCATTATAC
Hla-F	TTATCCAATGATTACAATATAAAAATACAAATATCTTAG
Hla-R	TACTTCCAATCCAATGTTAATATATAGTTAATTTTATTTAATAG
FnbA-F	TACCCGTTTCCACTTTCGC
FnbA-R	GGCTACACAAAATCAAGTCGC
Spa-F	ATGTCGTAAACCTGGTGAT
Spa-R	CTTTGTTAGCATCTGCATGG
lukS-F	GTCTGGAACAAAATAGTCTCTCGG
lukS-R	GGTCCATCAACAGGAGGTAATG

gyrB-F	AAGTGCCTCAAGTTGTAGAT
gyrB-R	TCTAGAGTCACGACCAGATT

4.9. Data Analysis

Molecular docking analyses were performed using AutoDock Tools-1.5.7. Statistical analyses were conducted using GraphPad Prism 9.0. The significance between groups was determined using Student's *t*-test. The values represent the means $\pm$ SD ($n = 3$). For each experiment, there were three technical replicates. Asterisks indicate statistical significance relative to the controls ("*": $p < 0.05$, "***": $p < 0.01$, "****": $p < 0.001$, "*****": $p < 0.0001$, "ns" indicates no significant difference ($p \geq 0.05$).

5. Conclusions

This study elucidates the mechanism of action of a cyclic pentapeptide as a novel antivirulence agent. Molecular docking analyses demonstrate that the compound binds stably to the active site of the AgrC protein via hydrogen bonding and hydrophobic interactions. Although it does not inhibit the growth of *S. aureus* at concentrations up to 1024 $\mu\text{g/mL}$, it significantly reduces the production of α -hemolysin. Gene expression profiling further confirms that the compound modulates key quorum-sensing genes (*agrA*, *agrC*, *saeS*) and virulence-associated genes (*hla*, *lukS*, *fnbA*), with pronounced inhibitory effects observed at 16 $\mu\text{g/mL}$. Collectively, this work provides novel insights into the antivirulence mechanism of the cyclic pentapeptide and highlights its potential as a promising lead compound for the development of therapeutic strategies against *S. aureus*.

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